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ORIGINAL ARTICLE

# Reduced TRPC6 mRNA levels in the blood cells of patients with Alzheimer's disease and mild cognitive impairment

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Transient receptor potential canonical 6 (TRPC6) inhibits β-amyloid (Aβ) production. Hyperforin, the TRPC6 agonist, reduces Aβ levels and improves cognitive performance in Alzheimer's disease (AD) models. However, it's unknown whether TRPC6 expression is changed in AD patients. In this case–control study, we measured TRPC6 expression levels in the peripheral blood cells of four independent AD sets from five hospitals and one mild cognitive impairment (MCI) set from a local community (229 AD, 70 MCI, 40 Parkinson disease and 359 controls from China, total $n = 698$) using quantitative real-time PCR assay. We found a specific reduction of TRPC6 mRNA levels in four AD sets and one MCI set. The median TRPC6 mRNA levels were lower in the following: (1) combined AD patients than in age-matched controls (0.78 vs 1.73, $P < 0.001$); (2) mild-to-moderate AD patients than in age-matched controls (0.81 vs 1.73, $P < 0.001$); and (3) MCI patients than in age-matched controls (0.76 vs 1.72, $P < 0.001$). In the receiver-operating characteristic curve analysis, the area under curve was 0.85 for combined AD, 0.84 for mild-to-moderate AD and 0.79 for MCI. In a subgroup of AD patients with brain Aβ examination, TRPC6 was associated with standardized uptake value ratio of Pittsburgh Compound B (Spearman's $r = -0.49$, $P = 0.04$) and cerebrospinal fluid Aβ42 (Spearman's $r = 0.43$, $P = 0.04$). The TRPC6 reduction in AD patients was further confirmed in blood RNA samples from The Australian Imaging, Biomarkers and Lifestyle Flagship Study of Aging, in post-mortem brain tissues from The Netherlands Brain Bank and in induced pluripotent stem cells-derived neurons from Chinese donors. We conclude that TRPC6 mRNA levels in the blood cells are specifically reduced in AD and MCI patients, and TRPC6 might be a biomarker for the early diagnosis of AD.

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## INTRODUCTION

Alzheimer's disease (AD) is the most common type of dementia and the number of AD patients would double every two decades in the future.[1] However, no effective disease-modifying therapy is available so far. Recent clinical trials for AD treatment have shown that therapy might be effective in early-stage patients.[2] Early diagnosis of AD is thus critical and also challenging. Objective and quantitative biomarker tests will help to identify AD patients before the onset of obvious cognitive impairment. Cerebrospinal fluid (CSF) Aβ42, Tau and brain β-amyloid (Aβ) imaging represent the most well-accepted biomarkers in AD diagnosis,[3] but their clinical applications in large populations might be compromised because of the invasiveness and high cost. In contrast, blood biomarkers of AD may offer unique advantages for easy access and cost-effectiveness.[4–6]

The Omics technologies have been used to search blood biomarkers[7] and the top hits usually turn out to be inflammation-related cytokines.[4,5,8] These results suggest that periphery blood, although away from the brain, could be a valuable source for AD biomarkers. However, inflammation is a common and systemic response to diverse diseases so that the specific biological relevance of most hits to AD remains ambiguous. As AD patients are pathologically characterized by Aβ accumulation in the brain, insights from the mechanistic studies of Aβ production might reveal novel biomarkers with a clear biological relevance to AD pathology.

Our recent work shows that transient receptor potential canonical 6 (TRPC6), known as a non-selective cation channel,[9] specifically inhibits γ-secretase cleavage of amyloid precursor protein and reduces Aβ production in AD models.[10] Consistently, independent studies have demonstrated that hyperforin, a drug which activates TRPC6 (refs 11–13) and increases TRPC6 expression,[14,15] could reduce Aβ levels and improve cognitive performance in AD models.[12,16–18] These results suggest that

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TRPC6 is a potential therapeutic target for AD. However, it is unknown whether TRPC6 expression is different between AD patients and normal controls. As gene expression profiles in the peripheral blood cells could be informative for brain diseases,[19–21] we reason that TRPC6 expression levels in the periphery white blood cells of sporadic AD patients might provide new insight into the pathogenesis of AD and a novel candidate for AD biomarkers. Here we report a specific reduction of TRPC6 mRNA levels in four independent AD sets and one mild cognitive impairment (MCI) set from the Chinese population, and evaluate its potential diagnostic accuracy for AD and MCI patients. Moreover, the TRPC6 reduction in AD patients is also found in blood RNA samples from The Australian Imaging, Biomarkers and Lifestyle Flagship Study of Aging (AIBL), in post-mortem brain tissues from The Netherlands Brain Bank (NBB) and in induced pluripotent stem cell (iPSC)-derived neurons from Chinese donors.

## MATERIALS AND METHODS

### Participants

This study was approved by the Research Ethics Committees of all the participating hospitals and institutes, and conducted according to the principles of the Declaration of Helsinki. Written informed consent was obtained from all participants or their primary caregivers. A total of 698 Chinese participants (229 AD, 70 MCI, 40 Parkinson's disease (PD) and 359 controls) were included in this study by consecutive sample from 2012 to 2015, consisting of four independent AD sets (pilot, training, test and Aβ-positive sets) from five hospitals and one MCI set from a community in Shanghai. Clinical evaluations by experienced clinicians and biomarker measurements by laboratory team were performed in a double-blind manner. In the external validation set, blood RNA samples of 36 AD and 33 controls were from AIBL, brain tissues of 15 AD and 13 controls from NBB, and iPSCs of 3 AD and 3 controls from the Chinese participants.

### AD diagnosis

The clinical evaluations were performed by clinicians with experience in diagnosing dementia. All AD patients underwent the following: (1) clinical examination including personal medical history and family history, neurological, internal examinations and neuropsychological testing with the Mini-Mental State Examination (MMSE);[22] and (2) routine laboratory testing including electrocardiogram, blood tests and computed tomography scan or magnetic resonance imaging of the brain. Diagnosis of AD was based on the following: (1) the criteria of probable AD according to ICD-10 (International Classification of Diseases) and NINCDS-ADRDA (National Institute of Neurological and Communicative Disorders and Stroke—Alzheimer's disease and Related Disorders Association);[23] (2) MMSE score $\leqslant 26$; and (3) exclusion by known causes of cognitive impairment, such as brain tumor, cerebrovascular diseases, vascular dementia, subdural hematoma and alcohol abuse. Cognitively normal participants underwent clinical examination and neuropsychological testing with MMSE. Inclusion criteria were based on the following: (1) MMSE score $\geqslant 27$; (2) no history of dementia, absence of memory complaints or any other cognitive symptoms; and (3) preservation of general cognitive functioning and no signs of active neurological or psychiatric diseases. Participants with other medical conditions that did not affect cognition were not excluded.

### MCI diagnosis

All participants were community-dwelling, old adults recruited from a community in Shanghai. They underwent clinical evaluation including personal medical history, family history, neurological and internal examinations, routine electrocardiogram and blood tests, and cognitive testing with MMSE and Clinical Dementia Rating.[24] Participants with Clinical Dementia Rating 0 are cognitively normal controls. Clinical Dementia Ratings of 0.5, 1, 2 or 3 indicate very mild, mild, moderate or severe cognitive impairments, respectively. MCI diagnosis was based on Petersen criteria,[25] including (1) memory complaint, preferably corroborated by an informant; (2) Clinical Dementia Rating 0.5 and $22 \leqslant$ MMSE $\leqslant 28$; (3) preservation of general cognitive functioning and no or minimum impairment of daily life activities; and (4) not meeting criteria for dementia.

### PD diagnosis

PD patients were diagnosed by specialists on movement disorders according to the United Kingdom Parkinson's Disease Society Brain Bank clinical diagnostic criteria.[26]

### PiB imaging

In the Aβ-positive set, the *in-vivo* amyloid burden in the brains of 18 AD patients was assessed by Pittsburgh Compound B (PiB)-positron emission tomography.[27] Images of PiB-positron emission tomography were co-registered with computed tomography images to determine region-of-interest. The cerebellum was chosen as a reference region. Standardized uptake value (SUV) of PiB was calculated for each region-of-interest. Then, for each region-of-interest the SUV ratio (SUVR) was obtained by dividing its SUV by the cerebellar SUV. Finally, amyloid burden was presented as the average SUVR of frontal, parietal and temporal SUVRs. Positive amyloid deposition was defined as the average SUVR $\geqslant 1.5$.

### RNA extraction

In the pilot set, replicate whole blood samples of each participant were collected in PAXgene Blood RNA Tubes (Qiagen, Hilden, Germany) and BD vacutainer EDTA tubes (Becton Dickinson, Franklin Lakes, NJ, USA) and the total RNA of white blood cells was extracted using the PAXgene Blood RNA Kit (Qiagen) and QIAamp RNA Blood Mini Kit (Qiagen), respectively. For all other participants, fresh whole blood was collected in BD vacutainer EDTA tubes and the total RNA of white blood cells was extracted using QIAamp RNA Blood Mini Kit (Qiagen) according to the manufacturer's instructions.

For blood samples from AIBL, peripheral white blood cells were collected between 2009 and 2011, and stored in liquid nitrogen using RNAlater (Ambion, Austin, TX, USA)[28] since then. The total RNA was extracted using TRIzol reagent (Life Technologies, Carlsbad, CA, USA) according to the manufacturer's instructions. For cell lines, iPSCs, iPSC-derived neurons and animal tissues, total RNA was extracted using TRIzol reagent.

### Animal experiments

All animal experiments were in accordance with the Institutional Animal Care and Use Committee of the Institute of Neuroscience, Shanghai, China. APP/PS1 mice were from Jackson Laboratory (Bar Harbor, ME, USA) (004462). TRPC6 and TRPC5 expression levels were determined by western blotting and quantitative real-time PCR (qRT-PCR) in 13- to 18–month-old mice.

### Cell cultures

HEK293 and Jurkat cell lines from American Type Culture Collection (Manassas, VA, USA) were maintained in Dulbecco's modified Eagle's medium with 10% fetal bovine serum and RIPM1640 with 10% fetal bovine serum, respectively. Transfection in HEK293 cells was performed with Lipofectamin 2000 (Invitrogen, Carlsbad, CA, USA).

### Drugs and antibodies

Memantine (S2043), galantamine (S1339), donepezil (S2462) and rivastigmine (S2087) were from Selleck Chemicals (Houston, TX, USA). Hyperforin was from Abcam (Cambridge, MA, USA) (ab144276). TRPC6, TRPC5 and APP antibodies were from SIGMA (St. Louis, MO, USA) (SAB4300572), SIGMA (SAB4301827) and Invitrogen (13-0200, clone LN27), respectively.

### Western blottings

Proteins were extracted from white blood cells, cell lines, iPSCs, iPSC-derived neurons or tissues using SDS lysis buffer (2% SDS, 10% glycerol, 0.1 mM dithiothreitol and 0.2 M Tris-HCl, pH 6.8). Protein samples were resolved by SDS–polyacrylamide gel electrophoresis, transferred to polyvinylidene difluoride membrane and blotted with antibodies. The specificity of TRPC6 antibody was verified by expressed TRPC6-HA and antigenic peptide blocking (Supplementary Figure 1A and B).

### Immunohistology of paraffin-embedded brain tissues

The post-mortem human brain material was obtained from The NBB, Netherlands Institute for Neuroscience, Amsterdam. All materials have been collected from donors or from whom a written informed consent for a brain autopsy and the use of the material and clinical information for

research purposes had been obtained by the NBB. The brain tissues include frontal and temporal cortex for 15 AD patients and 13 controls. The paraffin-embedded brain tissues were cut into 5 μm sections. The immunohistological staining of brain tissues was performed in a double-blinded manner. Briefly, sections were sequentially processed by deparaffinization, hydrogen peroxide quenching, antigen retrieval, blocking and stained with TRPC6 (1:70 dilution) or TRPC5 (1:70 dilution) antibodies overnight. Then, sections were further processed by the POLINK-2 Plus HRP DAB Detection System (ORIGENE, Rockville, MD, USA). Sections were counter-stained with hematoxylin, dehydrated, cleared and mounted. For negative controls, TRPC6 antibody was replaced by PBS. To verify the antibody specificity, TRPC6 antibody and its antigenic peptide were mixed at 1:10 ratio for 2 h at room temperature before staining the sections (Supplementary Figure 1C). The photographs were taken by NIKON E600FN microscope (Tokyo, Japan) with $\times 40$ objective.

The analysis of images was performed by the quickscore method[29] blindly to the clinical information. In brief, for each area (frontal or temporal cortex), 20 visual fields were chosen at random for each individual and the positive neurons were counted. For each individual, the percentage of positive neurons was expressed as the total number of positive-neurons divided by the total number of all neuron in 20 fields. In each field, the percentage of positive neurons (P) was further scored as: 1, $< 25\%$; 2, $< 50\%$; and 3, $> 50\%$. The stain intensity (I) was scored as 1 for weak, 2 for moderate and 3 for strong (Supplementary Figure 1D). The quickscore for each field was $P \times I$. For each individual, the quickscore of each area was expressed as the average quickscore of 20 fields.

## Enzyme-linked immunosorbent assay tests of CSF Aβ42 and total Tau

The CSF samples were collected by lumbar puncture and aliquoted into polypropylene tubes before storing at − 80 °C. Levels of Aβ42 and total Tau in CSF were assayed by enzyme-linked immunosorbant assays (Invitrogen KHB3544: Aβ42 Human Ultrasensitive ELISA Kit and Invitrogen KHB0042: Total Tau Human ELISA Kit) according to the manufacturer's instructions.

## Quantitative real-time PCR

For each RNA sample from EDTA tube or PAXgene tube, an equal amount of total RNA (1 μg) was reverse-transcribed into cDNA with random-hexamer primer mix using M-MLV Reverse Transcriptase (Promega, Madison, WI, USA) according to the manufacturer's instructions. qRT-PCR was performed on a Rotor-Gene Q instrument (Qiagen) with cDNA (equivalent to 50 ng RNA per 20 μl PCR assay), gene-specific primers and SYBR Premix Ex Taq (TaKaRa, Shiga, Japan). Primers are shown in Supplementary Table 1. The specificity of primers was confirmed by NCBI Primer-BLAST, melting curve analysis, gel electrophoresis (Supplementary Figure 2) and DNA sequencing of amplicons. The possibility of nonspecific amplification was ruled out by DNA sequencing and the presence of only one amplicon for each target gene. The detection limitation is 1ng total RNA per 20 μl PCR assay. The linear range is 1–128 ng RNA per 20 μl PCR assay.

For each RNA sample from AIBL, 1 μg total RNA was reverse-transcribed into cDNA with High Capacity cDNA Reverse Transcription Kit with RNase inhibitor (Applied Biosystems, Foster City, CA, USA). The qRT-PCR was performed on Applied Biosystems ViiA 7 Real-Time PCR System with SYBR Select Master Mix (Applied Biosystems).

## Establishment of iPSCs

Briefly, mononuclear cells were isolated from peripheral blood of AD patients and age-matched healthy controls by density-based centrifugal separation. After an 8–10-day expansion with a defined condition, highly proliferative erythroblasts were specifically enriched.[30,31] The expanded cells from mononuclear cells were then transduced with EBNA1/OriP-based episomal vectors EV SFFV-OS, EV SFFV-MK and EV SFFV-BCL-XL.[32] The iPSC clones were isolated 2–3 weeks after transduction and maintained on mouse embryonic fibroblasts with the human embryonic stem cell medium supplemented with 5 ng ml$^{-1}$ basic fibroblast growth factor (Pufei, Shanghai, China).

## Neural differentiation of iPSCs

The mouse embryonic fibroblasts were removed from iPSC cultures before neural differentiation. The iPSCs were dissociated into clumps, collected and suspended to form embryoid bodies. The embryoid bodies were cultured with human embryonic stem cell medium without basic fibroblast growth factor and then with N2 medium. Both medium were supplemented with 10 μM SB431542 (Selleck) and 2 μM dorsomorphin (SIGMA). Six days later, embryoid bodies were transferred into Matrigel-coated culture plates and cultured in N2B27 medium for 10 days. Then, neural stem/progenitor cells derived from embryoid bodies were separated by collagenase digestion and cultured for another 10 days to allow neural sphere formation. At last, neural spheres were separated into single cells and replated in B27 medium supplemented with 50 ng ml$^{-1}$ NGF (Peprotech), 10 ng ml$^{-1}$ brain-derived neurotrophic factor (Peprotech), 10 ng ml$^{-1}$ NT-3 (Peprotech), 10 ng ml$^{-1}$ IGF-1 (Peprotech) and 1 μM cAMP (SIGMA) for neuronal differentiation and maturation.

## Characterization of iPSCs and iPSC-derived neurons

Markers of iPSCs and iPSC-derived neurons were validated by immunofluorescence staining. Cells were fixed with 4% paraformaldehyde for 1 h at room temperature and rinsed with PBS. The fixed cells were permeabilized and blocked with PBS containing 5% bovine serum albumin and 0.3% Triton X-100 for 1 h at room temperature. Then, cells were incubated overnight at 4 °C with primary antibodies, including anti-OCT4 (1:200; Santa Cruz, Santa Cruz, CA, USA), anti-TRA-1-60 (1:50; Millipore, Bedford, MA, USA), anti-Tuj1 (1:1000; Covance, Princeton, NJ, USA) or anti-MAP2 (1:200; SIGMA). The secondary antibodies were then applied for 1.5 h at room temperature. The images were captured on Olympus IX71 (Olympus, Tokyo, Japan) or Leica TCS SP5 (Leica, Heidelberg, Germany) confocol laser microscopy.

Karyotyping of iPSCs was performed by the International Peace Maternity and Child Health Hospital using standard G banding. The pluripotency of iPSCs was validated using the teratoma assay. The iPSCs were dissociated into clumps and injected into hind limbs of nude mice subcutaneously. Six to 8 weeks after injection, teratomas were excised, fixed and embedded in paraffin. Teratoma sections were analyzed by hematoxylin/eosin staining.

To verify the iPSC-derived neurons, whole-cell patch-clamp recordings were performed on iPSC-derived neurons using Multiclamp 700B Amplifier (Molecular Devices, Foster City, CA, USA). The resistance of the recording micropipettes was 5–7 MΩ. The recording micropipettes were filled with pipette solution containing 143 mM K-gluconate, 3 mM KCl, 2 mM $MgCl_2$, 10 mM HEPES, 2 mM $Na_2ATP$ and 0.025 mM BAPTA, pH 7.25–7.30, adjusted with KOH. Cells were maintained at room temperature in the external solution consisting of 150 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 2 mM $CaCl_2$, 10 mM D-glucose and 10 mM HEPES, adjusted to pH 7.4 with Tris base. Action potentials were recorded under the current-clamp configuration. Neurons were depolarized by injecting step currents to elicit action potential. To record spontaneous synaptic currents, cells were held at − 70 mV and recorded in voltage-clamp mode. Data were analyzed by Clampfit (Axon Instruments, Foster City, CA, USA) and OriginPro 8 (OriginLab, Northampton, MA, USA).

## Statistics

For qRT-PCR analysis, β-actin was used as a reference gene and the relative levels of target genes were calculated by the comparative Ct method.[33] The intra-assay and inter-assay variation coefficients for the Ct value were 0.69% and 0.67%, respectively. One blood sample was randomly chosen as the reference sample and it was amplified in each PCR run. The levels of target genes in the reference sample were set as 1 and the relative levels of all samples were normalized to the reference sample. Data were presented as median (range). All the analyses were performed on actual data, although the mRNA levels in the figures are shown on a log scale. The normality of data distribution was evaluated by Kolmogorov–Smirnov test. Difference in the target gene levels was analyzed by non-parametric Mann–Whitney *U*-test for two groups or Kruskal–Wallis test followed by *post hoc* Dunn's multiple comparison test for more than two groups.

For associations between biomarkers and baseline characteristics, correlation coefficient was used for continuous variables. Receiver operating characteristic (ROC) curve analysis was used to analyze the diagnostic performance of TRPC6 mRNA levels and determine the cutoff that maximized the sum of specificity and sensitivity. The $P < 0.05$ was considered as statistically significant. All statistical analysis was performed using SPSS statistical package 15.0 (SPSS, Chicago, IL, USA).

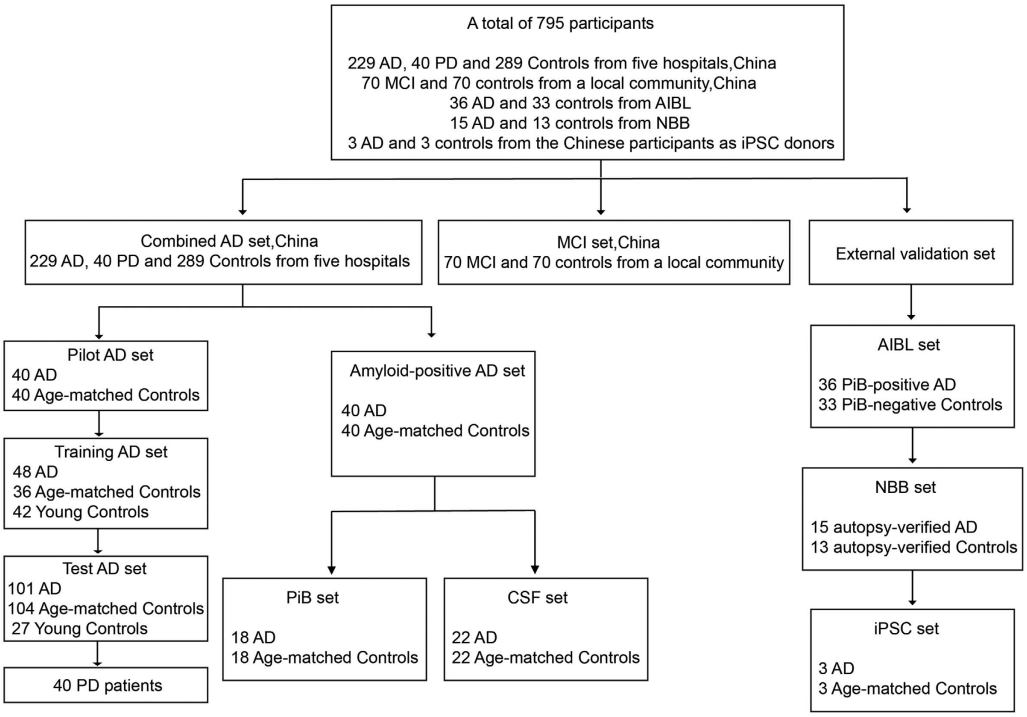


**Figure 1.** Study Design. AD, Alzheimer's disease; AIBL, The Australian Imaging, Biomarkers and Lifestyle Flagship Study of Aging; CSF, cerebrospinal fluid; iPSC, induced pluripotent stem cell; MCI, mild cognitive impairment; NBB, The Netherlands Brain Bank; PD, Parkinson's disease; PiB, Pittsburgh Compound B.

## RESULTS

The study consists of four independent AD sets (pilot, training, test and Aβ-positive sets) and one MCI set in the Chinese population, and one external validation set including non-Chinese population (Figure 1). Baseline characteristics of the Chinese participants (229 AD, 70 MCI, 40 PD and 359 controls, total $n = 698$) are displayed in Supplementary Table 2. Demographic information of AIBL subjects, NBB subjects and iPSC donors is displayed in Supplementary Table 3–5, respectively.

### TRPC6 mRNA levels in pilot AD set

The pilot AD set consists of 40 medication-naive mild AD patients and 40 age-matched controls. Their fresh replicate blood samples were collected in routine EDTA tubes and in PAXgene tubes, respectively. There was no difference in the RNA purity (260/280 ratio, 2.05 vs 2.06, $P = 0.78$) or the RNA amount (μg RNA per ml blood, 3.23 vs 3.02, $P = 0.49$) between the EDTA tubes and PAXgene tubes. For the analysis of the RNA isolated from the EDTA tubes, we found that TRPC6 mRNA levels in the periphery white blood cells of AD patients were significantly reduced compared to those in age-matched controls ($P < 0.001$, Figure 2a). Consistently, TRPC6 protein levels estimated by western blotting were also reduced in the periphery white blood cells of these AD patients ($P < 0.001$, Figure 2b). In contrast, the mRNA levels of several other $Ca^{2+}$ channels, including TRPC5, L-type $Ca_V1.3$, P/Q-type $Ca_V2.1$ and T-type $Ca_V3.2$, were not different (*P*-values by Mann–Whitney test before Bonferroni correction were 0.40, 0.74, 0.76 and 0.63, respectively; Figure 2c). There was no correlation between TRPC6 mRNA levels and white blood cell counts ($P = 0.47$), hemoglobin ($P = 0.20$), blood glucose ($P = 0.19$), serum creatinine ($P = 0.63$) or body mass index ($P = 0.32$) in the AD patients, suggesting that TRPC6 reduction is unlikely a secondary consequence of inflammation, renal dysfunction or malnutrition. It is important to note that previous study has shown that AD drugs could affect gene expression in the periphery white blood cells of AD patients.[34] However, AD patients in this pilot AD set are medication-naive, excluding potential interference from AD drug exposure. To directly investigate whether current AD drugs could affect TRPC6 mRNA levels in peripheral blood cells, we examined the effects of memantine, galantamine, donepezil and rivastigmine, drugs commonly used for the treatment of AD patients,[35–38] on TRPC6 mRNA levels in Jurkat cell, a human leukemia cell line. With oral administration, the steady-state plasma concentrations of memantine, galantamine, donepezil and rivastigmine range from 70 to 150 ng ml$^{-1}$, 29 to 137 ng ml$^{-1}$, 10 to 50 ng ml$^{-1}$ and 1 to 40 ng ml$^{-1}$, respectively. We incubated Jurkat cells with these drugs at the concentrations including their maximal plasma concentrations for 24 h. The results showed that these four drugs had no effect on TRPC6 mRNA levels (Supplementary Figure 3A–D). In contrast, hyperforin increased TRPC6 mRNA levels dose dependently at the concentrations including its maximal plasma concentrations (150–300 ng ml$^{-1}$)[39] (Supplementary Figure 3E). These results provided initial evidence of a specific TRPC6 reduction in AD patients.

The diagnostic accuracy of TRPC6 mRNA levels was evaluated by ROC curve analysis (Figure 2d). AUC was $0.81 \pm 0.05$ (95% confidence interval, 0.72–0.90, $P < 0.001$), sensitivity was 72.50% (56.11–85.40%) and specificity was 80.00% (64.35–90.95%). We then validated the reduction of TRPC6 mRNA levels in the replicate blood samples collected in PAXgene tubes ($P < 0.001$, Figure 2a). The AUC was $0.82 \pm 0.05$ (0.73–0.91, $P < 0.001$, Figure 2d), sensitivity was 80.00% (64.35–90.95%) and specificity was 72.50% (56.11–85.40%). Correlation analysis was used for the direct comparison of TRPC6 mRNA levels between these two methods (Pearson's $r = 0.85$, $P < 0.001$, Figure 2e). These results suggest that, for the RNA extraction and measurement of TRPC6 mRNA in the periphery blood cells, the quality of the blood samples collected in EDTA tubes or PAXgene tubes were not

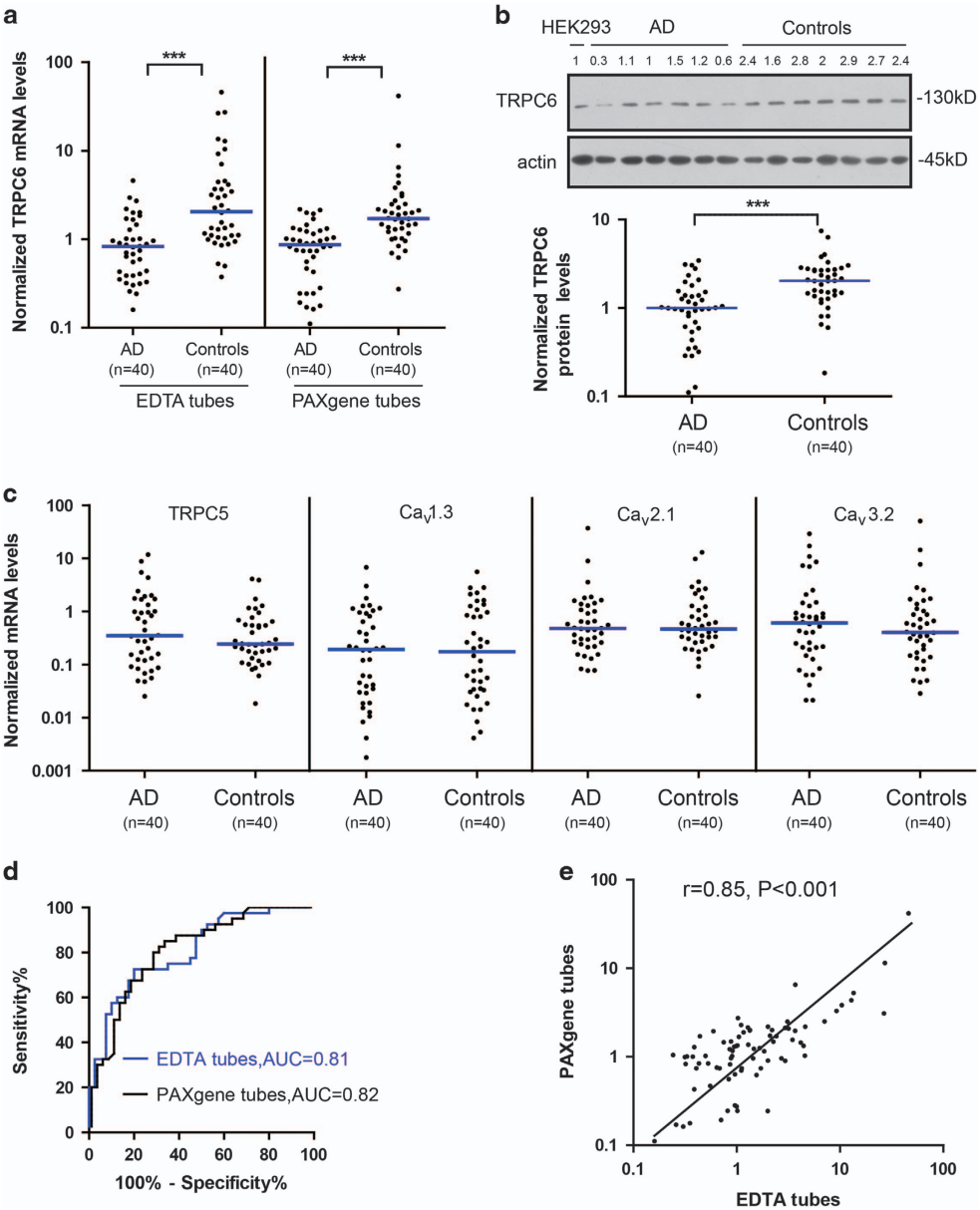


**Figure 2.** TRPC6 mRNA levels in pilot AD set. (**a**) TRPC6 mRNA levels of replicate blood samples collected in EDTA or PAXgene tubes. TRPC6 protein levels (**b**) and the mRNA levels of other calcium channels (**c**) in the blood samples collected in EDTA tubes. HEK293 cells were used as a control (**b**). The comparison of TRPC6 mRNA levels of replicate blood samples collected in EDTA or PAXgene tubes by ROC curve analysis (**d**) and correlation analysis (**e**). Horizontal line indicates the median. ***$P < 0.001$. AD, Alzheimer's disease.

different. Thus, the EDTA tubes were used for our subsequent studies because of their high cost-effectiveness and convenience.

### TRPC6 mRNA levels in training and test AD sets

Next, TRPC6 mRNA levels were measured in moderate-to-severe AD patients. In the training (48 severe AD, 36 age-matched controls and 42 young controls) and test (101 moderate AD, 104 age-matched controls and 27 young controls) sets, TRPC6 mRNA levels in AD patients were greatly reduced compared with those in age-matched controls ($P < 0.001$, Figure 3a). In the training set, AUC of AD vs age-matched controls was $0.87 \pm 0.04$ (0.79–0.94, $P < 0.001$, Figure 3d), sensitivity was 83.33% (69.78–92.52%) and specificity was 80.56% (63.98–91.81%). In the test set, AUC was $0.88 \pm 0.02$ (0.84–0.92, $P < 0.001$, Figure 3d), sensitivity was 78.22% (68.90–85.82%) and specificity was 78.85% (69.74–86.24%).

### TRPC6 mRNA levels in amyloid-positive AD sets

In addition to the cognitive assessment, the definitive diagnosis of AD requires the confirmation of Aβ plaque in the brain. To provide the clear evidence that TRPC6 reduction is associated with changes in brain Aβ levels, we investigated an independent Aβ-positive AD set (40 AD and 40 age-matched controls). A total of 18 AD patients underwent PiB-positron emission tomography scan (mean SUVR = 2.01, Supplementary Figure 4A and B) and their TRPC6 mRNA levels were greatly reduced compared to those in 18 age-matched controls ($P < 0.001$, Figure 3a). There is a negative association between TRPC6 mRNA levels and SUVR (Spearman's $r = -0.49$, $P = 0.04$, Figure 3b). Moreover, in additional 22 AD patients with decreased CSF Aβ42 levels (434 pg ml$^{-1}$ vs 926 pg ml$^{-1}$, $P < 0.001$, Supplementary Figure 4C), increased total Tau levels (1175 pg ml$^{-1}$ vs 363 pg ml$^{-1}$, $P < 0.001$,

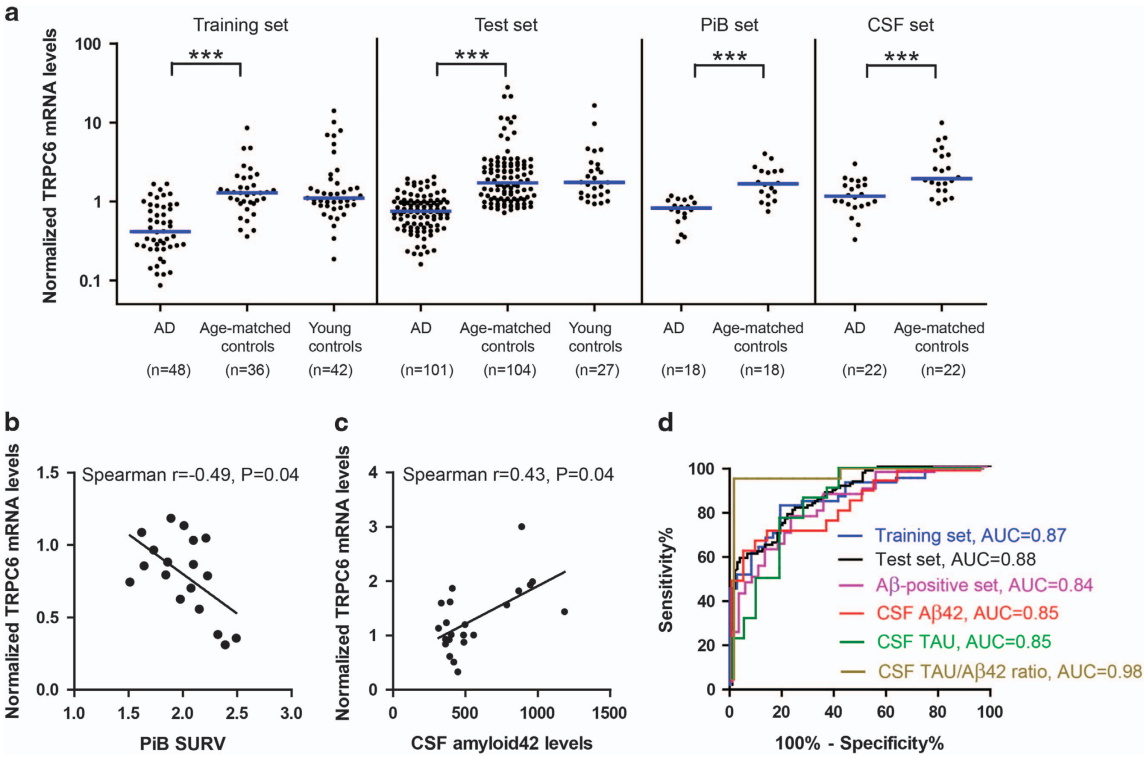


**Figure 3.** TRPC6 mRNA levels in three independent AD sets. (**a**) TRPC6 mRNA levels in the training, test and amyloid-positive AD sets. Correlation analysis of TRPC6 with PiB SUVR (**b**) and CSF Aβ42 (**c**). (**d**) ROC curve analysis of TRPC6 and CSF biomarkers. Horizontal line indicates the median. ***$P < 0.001$. AD, Alzheimer's disease; CSF, cerebrospinal fluid; PiB, Pittsburgh Compound B; SUVR, standardized uptake value ratio.

Supplementary Figure 4D) and increased total Tau/Aβ42 ratio (2.10 vs 0.37, $P < 0.001$, Supplementary Figure 4E), their TRPC6 mRNA levels were markedly reduced compared with those in 22 age-matched controls ($P < 0.001$, Figure 3a). There is a positive correlation between TRPC6 and CSF Aβ42 (Spearman's $r = 0.43$, $P = 0.04$, Figure 3c) in AD patients. The AUC of CSF Aβ42, total Tau, total Tau/Aβ42 ratio and TRPC6 was $0.85 \pm 0.06$ (0.74–0.96, $P < 0.001$), $0.85 \pm 0.06$ (0.74–0.97, $P < 0.001$), $0.98 \pm 0.02$ (0.94–1, $P < 0.001$) and $0.84 \pm 0.04$ (0.75–0.92, $P < 0.001$), respectively (Figure 3d). Together, these results suggest that TRPC6 reduction was correlated with the strong Aβ deposition in AD brains, and that the AUC of TRPC6 was close to that of CSF Aβ42.

### TRPC6 mRNA levels in combined AD sets

To avoid bias from small sample size, we pooled four AD sets into a combined AD set (229 AD, 220 age-matched controls and 69 young controls) for a planned re-analysis. TRPC6 mRNA levels in AD patients were significantly reduced compared to those in age-matched controls (Figures 4a, $P < 0.001$). AUC was $0.85 \pm 0.02$ (0.82–0.88, $P < 0.001$, Figure 4g), sensitivity was 74.24% (68.06–79.77%) and specificity was 79.55% (73.60–84.67%). The TRPC5 mRNA levels in AD patients ($n = 148$), age-matched controls ($n = 155$) or young controls ($n = 52$) were not different (Figures 4b, $P = 0.96$). To investigate whether TRPC6 mRNA levels are also reduced in other neurodegenerative diseases, we examined its levels in 40 PD patients and found no difference between the PD patients and the controls (Figures 4c, $P = 0.36$). Collectively, these results further confirm the specific TRPC6 reduction in AD patients. The TRPC6 mRNA levels of the combined study are summarized in Supplementary Table 2.

### TRPC6 mRNA levels in mild-to-moderate AD patients and MCI set

Recent clinical trials take the mild-to-moderate AD patients ($15 \leqslant \text{MMSE} \leqslant 26$) as a target population with the expectation that these early-stage patients are likely to respond to the intervention. Thus, we performed a planned analysis of mild-to-moderate AD patients ($15 \leqslant \text{MMSE} \leqslant 26$, median MMSE score = 18, $n = 138$) in our study and found that their TRPC6 mRNA levels were significantly reduced compared to those in age-matched controls ($n = 220$) ($P < 0.001$, Figure 4d). AUC was $0.84 \pm 0.02$ (0.80–0.88, $P < 0.001$, Figure 4g), sensitivity was 72.46% (64.22–79.72%) and specificity was 80.91% (75.08–85.88%). In the MCI set, TRPC6 mRNA levels in 70 MCI patients were greatly reduced compared with those in 70 age-matched controls (Figures 4e, $P < 0.001$). AUC was $0.79 \pm 0.04$ (0.72–0.87, $P < 0.001$, Figure 4g), sensitivity was 78.57% (67.13–87.48%) and specificity was 71.43% (59.38–81.60%). In contrast, TRPC5 mRNA levels in these patients ($n = 43$) or age-matched controls ($n = 43$) were not significantly different (Figures 4f, $P = 0.70$). Together, these results suggest that the TRPC6 reduction might be an early molecular event in AD pathogenesis before the appearance of obvious cognitive symptoms.

### External validation of TRPC6 reduction in AIBL blood RNA, NBB brain tissues and iPSC-derived neurons

To investigate whether TRPC6 expression is also reduced in AD patients from other ethnic group beyond Chinese, we examined TRPC6 mRNA levels in the blood RNA from AIBL. In the blood RNA samples of 36 PiB-positive AD and 33 PiB-negative age-matched controls collected almost 10 years ago, the reduction of TRPC6 mRNA levels in AD patients was independently confirmed ($P < 0.05$, Figure 5a). Moreover, there was a negative association between PiB SUVR and blood TRPC6 mRNA levels (Spearman's

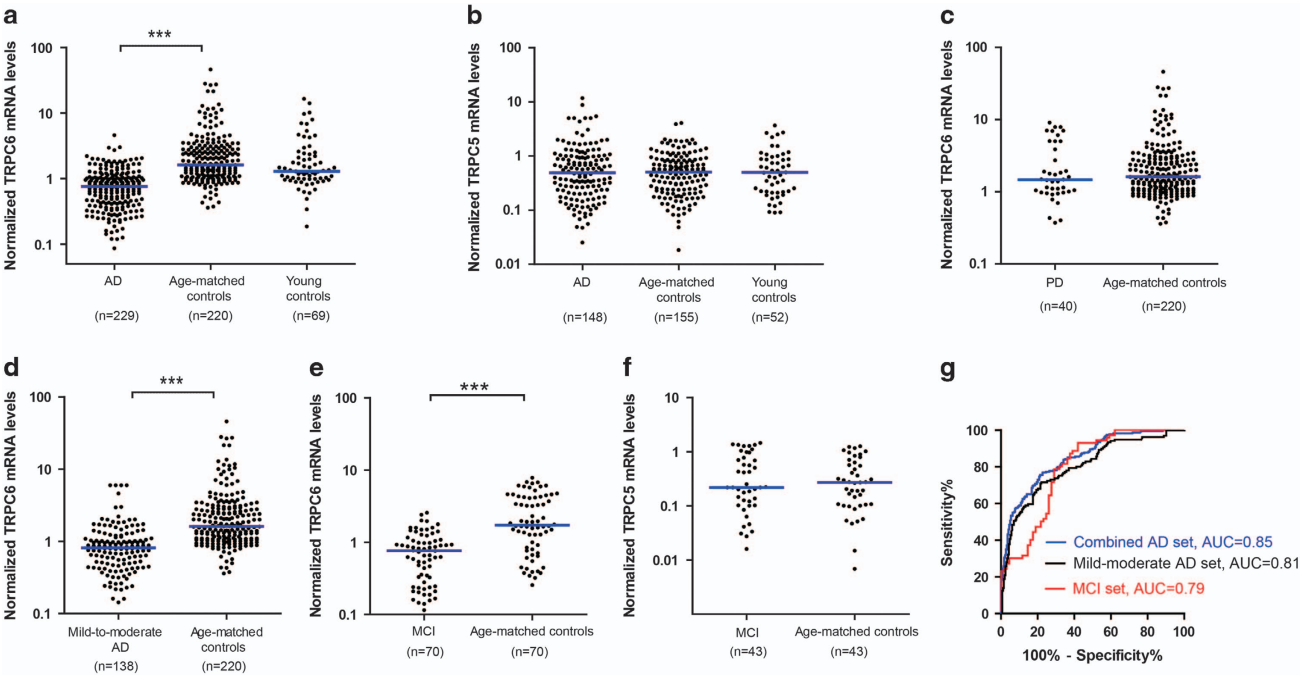


**Figure 4.** TRPC6 mRNA levels in combined AD set and MCI set. TRPC6 mRNA levels in the combined AD set (**a**), PD patients (**c**), mild-to-moderate AD patients (**d**) and MCI set (**e**). TRPC5 mRNA levels in the combined AD set (**b**) and MCI set (**f**). (**g**) ROC curve analysis of TRPC6. Horizontal line indicates the median. ***$P < 0.001$. AD, Alzheimer's disease; MCI, mild cognitive impairment; PD, Parkinson's disease.

$r = -0.36$, $P = 0.04$; Figure 5b) in 30 PiB-positive AD patients (the other 6 AD patients did not undergo PiB scan at the time when their blood samples were collected). Thus, the TRPC6 reduction in AD blood cells and the negative association between TRPC6 and PiB SUVR are fully replicated in the Australian population.

To investigate whether TRPC6 expression is also reduced in AD brain neurons, we conducted immunohistological staining of post-mortem brain tissues from NBB. The results showed that TRPC6 expression was reduced in the temporal (Figure 5c) and frontal cortex (Figure 5d) of AD patients. In semiquantitative analysis, the percentage of TRPC6-positive neurons (Figure 5e) and the quickscore (Figure 5f), which combined positive percentage and intensity, were reduced in the temporal and frontal cortex of AD patients. In contrast, there was no difference in TRPC5 staining between AD and controls in these areas (Supplementary Figure 5). Thus, TRPC6 expression is specifically reduced in AD brains.

To provide additional evidence that TRPC6 in AD neurons is indeed reduced, we examined the TRPC6 expression in the neurons derived from the iPSCs established from the peripheral mononuclear cells of Chinese donors (three AD and three age-matched controls). The TRPC6 mRNA levels in the peripheral blood cells of these three AD patients were lower than those of controls (Figure 5g). The characterization of iPSCs and iPSC-derived neurons is shown in Supplementary Figure 6. As shown in Figure 5h, both TRPC6 mRNA and protein levels in AD iPSCs were lower than those in control iPSCs. Moreover, when these iPSCs were differentiated into neurons after induction for 50 days, both TRPC6 mRNA and protein levels in the neurons derived from AD iPSCs were lower than those in the neurons derived from control iPSCs (Figure 5i). Consistently, the Aβ40 and Aβ42 levels in the culture medium of these AD neurons were higher than those in the culture medium of these control neurons (Supplementary Figure 6G). Taken together, TRPC6 expression is reduced in AD blood cells and AD brain neurons, and TRPC6 reduction in AD patients is also found in non-Chinese population.

## DISCUSSION

Early diagnosis of AD by non-invasive ways is critical and challenging. Gene expression profiles in blood cells have been proposed as biomarkers for brain diseases.[40–42] Here we report a specific reduction of TRPC6 mRNA levels in the peripheral blood cells from sporadic patients at different stages of AD (MCI and mild-to-severe AD patients). Blood TRPC6 is inversely associated with Aβ deposition in AD brains and ROC curve analysis shows the diagnostic accuracy of TRPC6 close to that of CSF Aβ42. In addition, TRPC6 reduction in AD blood cells and the negative association between TRPC6 and PiB SUVR are independently confirmed in AIBL samples from the Australian population. The direct relevance between TRPC6 and synaptogenesis,[43,44] neuronal survival[45] and Aβ homeostasis may make TRPC6 a more specific biomarker for the early diagnosis of AD.

In addition, TRPC6 expression in the neurons derived from AD iPSCs is reduced than that in the neurons derived from control iPSCs. Moreover, neuronal TRPC6 expression in the post-mortem AD brains is reduced in the temporal and frontal cortex, brain areas vulnerable for amyloid deposition in MCI[46,47] and early-stage AD patients.[48,49] It is thus possible that TRPC6 reduction might enhance Aβ production to increase the possibility of conversion from MCI to AD or to develop severe symptoms from mild AD. Therefore, TRPC6-based modulation of Aβ levels is a plausible intervention for MCI/AD therapy. Indeed, hyperforin, which is a specific agonist of TRPC6, shows therapeutic potential in AD models.[50] However, its effectiveness in AD patients remains to be validated. In that case, blood TRPC6 test might help to identify the 'low-TRPC6' AD patients who are most likely to respond to hyperforin treatment. Thus, blood TRPC6 test might be beneficial for the early diagnosis and treatment of AD.

It should be pointed out that TRPC6 reduction is also found in MCI patients. MCI represents a heterogeneous group and about 50% of MCI patients will convert into dementia in several years.[51] Although AD accounts for majority converters (above 80%), the remaining converters will mainly develop into vascular dementia

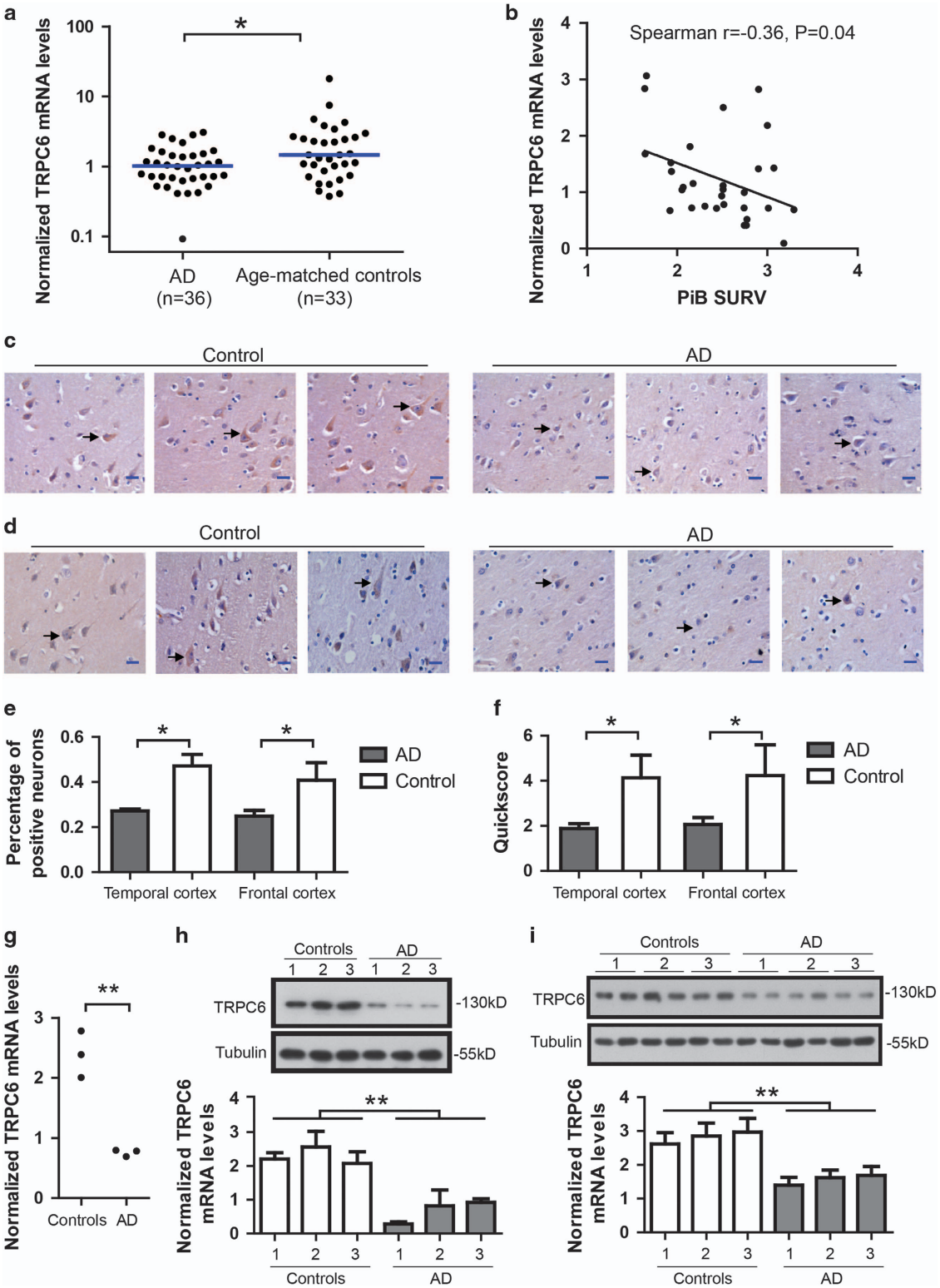


**Figure 5.** External validation of TRPC6 reduction in AIBL blood RNA, NBB brain tissues and iPSC-derived neurons. (**a**) TRPC6 mRNA levels in the blood RNA from AIBL. Horizontal line indicates the median. (**b**) Correlation analysis of TRPC6 with PiB SUVR in AD patients from AIBL. Representative immunohistological stainining images of temporal cortex (**c**) and frontal cortex (**d**) of the brain tissues obtained from NBB probed with the antibody against TRPC6. Arrows indicate neurons. Scale bar = 60 μm. Percentage of positive neurons (**e**) and quickscores (**f**) of TRPC6 staining. (**g**) TRPC6 mRNA levels in the peripheral blood cells of the indicated iPSC donors. (**h**) TRPC6 mRNA (lower) and protein (upper) levels in iPSCs established from the AD patients and controls. (**i**) TRPC6 mRNA (lower) and protein (upper, duplicate wells) levels in the neurons derived from AD or control iPSCs. *$P < 0.05$ and **$P < 0.01$. AD, Alzheimer's disease; NBB, The Netherlands Brain Bank; PiB, Pittsburgh Compound B; SUVR, standardized uptake value ratio.

(VaD).[51] It is possible that TRPC6 reduction is a common characteristic of dementia beyond AD. However, autopsy studies show that in many VaD patients, the brain Aβ plaques are evident.[52,53] This relatively high prevalence of mixed dementia suggests that the definite conclusion has to be obtained from longitudinal study of pure AD and pure VaD patients with autopsy confirmation. Nevertheless, two pieces of evidence support a specific TRPC6 reduction in AD. First, TRPC6 expression was not changed in PD patients. Second, the expression of other $Ca^{2+}$ channels, important for learning and memory, was not changed in AD patients.

The TRPC6 reduction may result from complex interactions between environmental and genetic factors. It is also possible that TRPC6 reduction may simply result from Aβ accumulation or AD drug treatments in AD patients. However, we found that Aβ did not change TRPC6 expression in cell and mouse AD models with high Aβ levels (Supplementary Figure 7), and that AD drug exposures did not change its expression. Consistently, a familiar AD-linked presenilin2 mutant could influence TRPC6 channel activity without affecting its expression level in cultured cells.[43] Therefore, TRPC6 reduction might be an important and early molecular event in the amyloid cascade,[54] instead of a late secondary consequence of Aβ accumulation or AD drug exposures in AD patients. It has been reported that TRPC6 expression is inhibited by TNF-α[55] or reactive oxygen species[55,56] in kidney cells and that increased TNF-α levels[57] and excessive oxidative stress[58] are found in AD patients. Investigation of the effects of these factors on TRPC6 expression in AD patients may be helpful for understanding the mechanism by which TRPC6 expression is regulated in AD pathogenesis.

In summary, TRPC6 mRNA levels in the periphery blood cells of AD and MCI patients are specifically reduced and its value as a biomarker in early AD diagnosis deserves further validation. Thus, prospective follow-up studies to investigate the longitudinal change of TRPC6 over time and disease progression could be informative.

## CONFLICT OF INTEREST

A Chinese patent application entitled 'Methods for early diagnosis of Alzheimer's disease by the measurement of TRPC6 mRNA levels in the peripheral blood cells' (201410035943.8) was filed on 24 January 2014. RL, JW and YW are the inventors. The applicant is Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences. A PCT patent application entitled 'Methods for early diagnosis of Alzheimer's disease by the measurement of TRPC6 mRNA levels in the peripheral blood cells' (PCT/CN2014/071325) was filed on 24 January 2014. RL, JW and YW are the inventors. The applicant is Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences. All other authors declare that there are no conflicts of interest.

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Supplementary Information accompanies the paper on the Molecular Psychiatry website (http://www.nature.com/mp)