

Plasma miRNAs as Biomarkers for Amnestic Mild Cognitive Impairment and Modulation on BACE1

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Research

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Abstract

Background: Amnesic mild cognitive impairment (aMCI) is a prodromal stage of Alzheimer's disease with the beta-site amyloid precursor protein cleaving enzyme 1 (BACE1) imbalance. Altered microRNAs (miRNA) associated with BACE1 may be potential biomarkers for aMCI diagnosis. The aims of this study were to find an effective diagnostic model for aMCI, and to explore the modulation of candidate miRNAs on BACE1 expression.

Methods: Plasma RNA was extracted from participants enrolled in China longitudinal aging study (CLAS). MiRNA profiling was performed using microarray sequencing on a discovery set, comprising five aMCI subjects that confirmed by ^{18}F -Flutemetamol PET scan and five normal controls (NC). Quantitative reverse transcription PCR was used to validate the expression levels of differently expressed miRNAs on an analysis set (20 aMCI subjects and 10 NC). Diagnostic capability of candidate miRNAs was assessed in a validation set (40 aMCI subjects and 40 NC). Modulation of candidate miRNAs on BACE1 was explored in rat and human hippocampal neurons *in vitro* through transfection of miRNA mimics or inhibitor lentivirus.

Results: In discovery set, we verified 46 significantly differentially expressed miRNAs between aMCI and NC groups ($P < 0.05$). Among these, 33 miRNAs were down-regulated and 13 miRNAs were up-regulated. In analysis set, miR-1185-2-3p, miR-1909-3p, miR-22-5p, and miR-134-3p also significantly decreased in aMCI group. Four miRNAs above and miR-107 which was found to decline in previous study were selected as potential biomarkers. A diagnostic model consists of five miRNAs above had an outstanding diagnostic accuracy (81.25%) to diagnose aMCI. Except for miR-134-3p, other four miRNAs modulated BACE1 expression and C-terminal fragments-beta production effectively in rat hippocampal neurons *in vitro*. Similar modulation of miR-1185-2-3p and miR-1909-3p were confirmed in human hippocampal neurons *in vitro*.

Conclusion: A diagnostic model consists of five plasma miRNAs could be novel biomarker for aMCI diagnosis. These miRNAs might be involved in pathogenesis of AD through regulating expression of BACE1.

Background

Alzheimer's disease (AD) is the most common form of dementia worldwide. With a growing aging population worldwide, AD has become a major health concern. The amyloid hypothesis posits that progressive accumulation of aggregates of β -amyloid ($\text{A}\beta$) triggers a pathological cascade that accelerates tau pathological accumulation and leads to synapses and neurons degeneration¹⁻³. Deposition of $\text{A}\beta$ is reported to start 15-20 years before the onset of clinical symptoms⁴. In past two decades, all high-profile drugs targeting $\text{A}\beta$ peptide or deposited amyloid plaques have failed in phase III clinical trials or have been prematurely discontinued for futility⁵⁻⁷. It has been proposed that agents which

bind to A β would have only marginal effects on preexisting amyloid⁸. Thus, many failures remind us that the gene regulation on the production of A β in prodromal stage of AD should be concern.

Recent studies advanced the view that cognitive decline in AD is a continuum, while mild cognitive impairment (MCI) is defined as an intermediate phase between normal cognition and dementia⁹. Amnestic MCI (aMCI) may have a higher risk of developing AD^{10,11}. Clinical characteristics, neurocognitive tests, positron emission tomography (PET) and magnetic resonance imaging (MRI) can diagnose aMCI but they are time consuming¹². Amyloid PET with different probes (¹⁸F-flutemetamol and ¹⁸F-florbetapir, et al.) are available to precisely detect and monitor brain amyloid deposition in MCI patients but costliness^{13,14}. Longitudinal studies showed that amyloid-positive MCI subjects have greater cognitive deterioration than amyloid-negative subjects^{15,16}. In addition to technologies, body fluids biomarkers should also be used for accurate diagnosis of MCI. However, CSF assay requires a lumbar puncture which is an invasive procedure, and neuroimaging requires high costs and sophisticated equipment, making their universal utility impractical. Thus, blood biomarker could be an attractive option, since getting blood sample is an economical, non-invasive and simple procedure.

MicroRNA (miRNA) is a class of noncoding RNA, fine-tuning the protein-encoding genes at a post-transcriptional level^{17,18}. Several miRNAs have been shown to play significant role in brain and neuronal development¹⁹. Aberrant expression of miRNAs may predict onset of cognitive dysfunctions²⁰. Recently, the role of altered miRNAs in the diagnosis for AD has been extensively studied. Several miRNAs have been shown to regulate AD-related genes, such as amyloid precursor protein (APP), beta-site APP cleaving enzyme 1 (BACE1), and brain-derived neurotrophic factor (BDNF)²¹⁻²³. This ability of selected miRNAs to target mRNAs which are altered in disease conditions makes them potential candidate as therapeutics or as targets of therapeutics. However, few studies have distinguished different subtypes of MCI. Since not every MCI-affected subject will develop AD, it is important to improve the accurate diagnostic criteria for aMCI. In this study, we aimed to find an effective diagnostic model composed of plasma miRNAs for early diagnosis of aMCI, and to investigate the modulation mechanism of miRNAs on BACE1 expression *in vitro*.

Methods

Study Population

The workflow of this study is shown in Fig 1a. All plasma samples were collected from subjects enrolled in the China longitudinal aging study (CLAS, ClinicalTrials.gov Identifier: NCT03672448)²⁴. The CLAS study was approved by Institution's Ethical Committee of Shanghai Mental Health Center, Shanghai Jiao Tong University School of Medicine. Written informed consent was obtained from each study participant and/or his/her legal guardians.

Petersen's criteria²⁵ was used to diagnose aMCI: i) memory complaint, preferably corroborated by a spouse or relative; ii) objective memory impairment; iii) normal general cognitive function; iv) intact

activities of daily living; v) absence of dementia. In discovery set, clinical diagnosis of aMCI was confirmed by ¹⁸F-Flutemetamol PET scan according to the 2011 criteria of National Institute on Aging and Alzheimer's Association. Controls were age-, gender-, and educational level-matched elderly. Subjects with other mental disorders, nervous system diseases, and history of psychotropic medicines were excluded.

Peripheral blood was collected in every subject after fasting for 12 hours. Then plasma was separated by centrifugation at 3,000 rpm for 20 minutes at 4°C and stored at -80°C until required.

miRNA microarray sequencing in discovery set

RNA was isolated using TRIzol reagent (Invitrogen life technologies) and purified with RNeasy mini kit (Qiagen, Germany) according to manufacturer's instructions. RNA quality and quantity were determined by 260/280nm absorbance using a Nanodrop ND-1000 spectrophotometer (Nanodrop Technologies). MiRNA expression profiles were carried out by applying the miRCURYTMTM LNA Array (v.19.0) (Exiqon, Denmark). Replicated miRNAs were averaged and miRNAs that intensities ≥ 30 in all samples were chosen for calculating normalization factor. Expressed data were normalized using the Median normalization. After normalization, significant differentially expressed miRNAs between two groups were identified through fold-change and *P* value of two-sample independent t-test.

Replication of results in an analysis set

The expression levels of miRNAs were confirmed by real-time quantitative reverse transcription PCR (qRT-PCR) in an analysis set. RNA was extracted with TRIzol LS Reagent (Invitrogen, USA). MiRNA quality and quantity were determined by 260/280nm absorbance using a Nanodrop ND-1000 spectrophotometer (Nanodrop Technologies). Total of 300ng RNA was used to prepare cDNA samples on Gene Amp PCR System 9700 (Applied Biosystems, USA). PCR reaction was set in the QuantStudio5 Real-time PCR System (Applied Biosystems, USA). Primers were designed by Primer 5.0 (Supplementary Table 1). The relative levels of miRNAs were determined in terms of their fold change, using the formula $(2^{-\Delta\Delta CT})^{26}$. Hsa-miR-93 was used as endogenous control. Real time qRT-PCR was performed in triplicate.

Construction of Diagnostic Model

To identify independent predictive parameters of aMCI, univariable and multivariable logistic regression analyses were performed in each miRNA plasma levels. $P < 0.05$ was considered statistically significant. Parameters with $P < 0.05$ based on the univariate analysis were further included in the multivariable logistic regression analysis. According to the multivariable logistic regression results, we performed a diagnostic model. The diagnostic accuracy of this model was examined using a receiver operating characteristic (ROC) curve analysis. The best cut-off value was selected according to Youden-index.

Cell Culture

SH-SY5Y cells were purchased from American Type Culture Collection (ATCC) (USA) and were cultured in Dulbecco's Modified Eagle Medium (DMEM, Gibco, USA) containing 10% fetal bovine serum (FBS) (Gibco, USA).

Rat primary hippocampal neurons were isolated from E18 embryos according to the following protocol. The protocol has received approval by the Institution's Ethical Committee of Shanghai Mental Health Center, Shanghai Jiao Tong University School of Medicine. Euthanize the pregnant rat by inhalation of CO₂. Remove the uterine and place it in cold phosphate buffer saline (PBS) as soon as possible. Then remove the embryos from uterine segments and place in a new petri dish containing cold PBS. Remove the brain from head and dissect the embryonic rat hippocampal tissues under surgical microscope after decapitating embryos. Digest the tissues with 0.15% trypsin for 20 min at 37°C. To filter the hippocampal neuronal suspensions with 70-µm cell strainers. Neurons were plated on poly-L-lysine coated 6-well plates at ~100,000 cells/cm², then cultured in neurobasal media supplemented with 2% B27, 0.25% Glutamax, 0.25% Glutamate, and 0.5% Penicillin/Streptomycin. The media were replaced with another neurobasal media supplemented with 2% B27, 0.25% Glutamax, and 0.5% Penicillin/Streptomycin next day.

Human hippocampal neurons were purchased from Sciencell (Californian, USA), and were plated on poly-L-lysine (2µg/cm², Sigma-Aldrich, USA) coated 6-well plates at ~50,000 cells/cm². Cultured medium, comprising of Neuronal Medium (Sciencell, USA), 1% Neuronal Growth Supplement (Sciencell, USA), and 1% Penicillin/Streptomycin, were replaced every two days.

All cells were cultured at 37°C in humidified incubator with 5% CO₂ environment.

Lentivirus transduction

Lentiviral particles containing the GV358 expression vector encoding BACE1 were purchased from GeneChem (Shanghai, China) and were transduced into SH-SY5Y cells following the manufacturer's instruction. The virus-infected SH-SY5Y^{BACE1} cells, stably overexpressing BACE1, were filtrated by puromycin.

Lentiviral particles containing expression vectors encoding miRNA mimics/inhibitor or flanking sequence control were purchased from GenePharma (Shanghai, China). MiRNA mimics or inhibitors (miRNA antisense oligonucleotides) were transfected to SH-SY5Y^{BACE1} cells at optimal concentration (mimics: 50nM, inhibitor: 100nM) with Lipofectamine miRNA iMax (Invitrogen, USA). Cells were harvested 48 hours after transfection. Rat hippocampal neurons were transfected with control or miRNA mimics/inhibitor lentivirus at optimal concentration (miR-107 and miR-134-3p: MOI=5; miR-1185-2-3p, miR-1909-3p, and miR-22-5p: MOI=10, Supplementary Fig. 1) on the first day *in vitro* (DIV1) and harvested on DIV7 then processed for protein and total RNA. Human primary hippocampal neurons were transfected at optimal concentration (MOI=10, Supplementary Fig. 2) on DIV4 and harvested on DIV10.

Real-time qRT-PCR

For cultured cells and neurons, total RNAs were extracted with TRIzol LS Reagent (Invitrogen, USA). RNA quality and quantity were measured by Nanodrop-2000 (Thermo Fisher Scientific, USA). The cDNAs were synthesized using HiScript II 1st Strand cDNA Synthesis Kit (Vazyme, China) according to manufacturer's instructions. PCR reactions were set in the Fast-7000 Real-time PCR system (Thermo Fisher Scientific). Threshold cycles (C_T value) were generated automatically, and the relative expressions were shown as $(2^{-\Delta\Delta C_T})^{26}$. Real-time qRT-PCR was performed in triplicate. Relative mRNA levels of target genes were normalized to the indicated reference genes. Primers were synthesized by Sangon Biotech (Shanghai, China. Supplementary Table 1).

Western blotting analysis

Cells or neurons were lysed in RIPA Lysis Buffer (Beyotime, Shanghai) which contained 1mM of Phenylmethanesulfonyl fluoride (Beyotime, Shanghai) according to manufacturer's instructions. Cell lysate samples were electrophoresed on SDS-polyacrylamide gels in Tris-glycine buffer containing SDS. Proteins were transferred to 0.22 μ m NC-membranes. Then membranes were blocked in 3% Bovine Serum Albumin (BSA) diluted in TBST buffer at room temperature, and were incubated overnight at 4°C with primary antibodies (BACE1 [Abcam, UK, 1:2000], β -actin [Cell Signaling Technology, USA, 1:2000], APP [Thermo Fisher, USA, 1:500]). The membranes were then washed in TBST and incubated with secondary antibody for 1 hour at room temperature. The immunoreactivity of the proteins was detected using Chemiluminescent Substrate. All experiments were performed in triplicate.

Results

Screening of miRNAs as potential biomarkers

Total of 3,100 mature miRNAs were detected in a discovery set, which consists of five aMCI subjects confirmed by 18 F-Flumetamol PET (amyloid positive) and five normal controls (NC, Table 1). There were 46 differently expressed miRNAs between aMCI group and NC group ($P < 0.05$, Fig. 1b). As compared with normal controls, 13 miRNAs were up-regulated and 33 were down-regulated in aMCI group (Fig. 1b). Among down-regulated miRNAs, 31 miRNAs are human miRNAs. Besides miRNAs above, we were also interested in miR-134-3p, the mature sequence of miR-134. Reason are as followed: i) an outlier value in miR-134-3p was found in microarray sequencing data (Data are openly available in GEO database, reference number [GSE147232]), which may cause a low fold-change value with high P value (Fold-change=0.099, $P=0.362$); ii) miR-134 is abundantly expressed in brain and was demonstrated to regulate synaptic plasticity and memory formation^{27, 28}; iii) few previous studies have focused on miR-134-3p. Thus, miR-134-3p together with thirty-one significantly decreased miRNAs above were selected for secondary screening.

Table 1. Demographic characteristics of all participants in study.

	aMCI group	NC group	<i>P</i> value
Discovery set			
n	5	5	
Age (years) ^a	68.00 (±2.83)	70 (±2.12)	0.24
Gender (female, %) ^b	2 (40%)	2 (40%)	1.00
Education (years) ^c	11.0 (±3.08)	11.6 (±2.88)	0.76
MoCA ^c	24.40 (±2.19)	26.4 (±0.55)	0.07
Brain A β deposition [†]	Positive	-	
Analysis set			
n	20	10	
Age (years) ^a	71.8 (±7.38)	70 (±6.65)	0.54
Gender (female, %) ^b	14 (70%)	6 (60%)	0.69
Education (years) ^c	10.65 (±2.55)	10.2 (±2.64)	0.16
MoCA ^c	20.95 (±2.42)	24.9 (±1.97)	<0.01 ^{**}
Validation set			
n	40	40	
Age (years) ^a	78.25±5.48	78.23±5.62	0.98
Gender (female, %) ^b	34(85%)	34(85%)	1.00
Education (years) ^c	2.65±2.94	3.65±3.36	0.16
MoCA ^c	11.98±3.35	21.22±4.40	<0.01 ^{**}
Note: ^a Data were analyzed by two sample independent t-test; ^b Data were analyzed by χ^2 test; ^c Data were analyzed by Mann-Whitney U test; [†] Brain A β position was detected by ¹⁸ F-Flumetomal PET. * <i>P</i> <0.05, ** <i>P</i> <0.01. Abbreviation: aMCI=amnesic mild cognitive impairment; NC=normal controls.			

Target genes of these thirty-two miRNAs were predicted by Targetscan7.2 database²⁹ and miRDB database^{30,31}. Combining results from both databases, thirteen miRNAs (Supplementary Table 2) and miR-134-3p were deemed to have target sites on BACE1 mRNA 3'UTR. Among these fourteen, five miRNAs (miR-1185-2-3p, miR-1909-3p, miR-22-5p, miR-134-3p, and miR-5691) were selected advanced validation, and their potential target sites on BACE1 mRNA 3'UTR are shown in Figure 2. Mature sequence of miR-

5691 has four putative binding sites on BACE1 mRNA with complementary seed match, while miR-1185-2-3p has three putative binding sites. The remaining three miRNAs has one target site on BACE1 mRNA.

To validate microarray results, an analysis set consists of 20 aMCI subjects and 10 normal controls (Table 1), was utilized to quantitate the above selected miRNAs through real-time qRT-PCR. Except miR-5691, the remaining four downregulated miRNAs in aMCI subjects which were consistent with microarray results (Fig. 3a). Our previous study demonstrated a high capability of plasma miR-107 to discriminate aMCI subjects from normal controls³². Furthermore, miR-107 has a potential target site in the BACE1 mRNA^{21, 32, 33}. Hence, combining the results above, miR-107 together with miR-1185-2-3p, miR-1909-3p, miR-22-5p, and miR-134-3p were selected as candidates for potential biomarkers.

Diagnostic capability of selected miRNAs

Next, we investigate diagnostic capability of these potential biomarkers in a validation set, which was composed of 40 aMCI subjects and 40 normal controls (Table 1). All five miRNAs levels were each significantly positively correlated with Mini-Mental State Examination (MMSE) scores and Montreal Cognitive Assessment (MoCA) scores in all participants (Fig. 3b). Surprisingly, miR-1909 was significantly positively correlated with MMSE and MoCA scores, and negatively correlated with Activities of Daily Living (ADL) (Fig. 3b), indicating the strongest correlation with clinical symptoms. In parallel, miR-1909-3p had the highest discrimination accuracy, followed by miR-107 (miR-1909-3p: AUC=0.872, miR-107: AUC=0.773, Fig. 3c). While miR-134-3p had the weakest discrimination accuracy (AUC=0.631, Fig. 3c).

The predictive effects of five altered miRNAs were performed using logistic regression. That lower levels of each miRNA increased the risk of aMCI according to univariate logistic regression (all $P < 0.05$, Table 2). All factors were subsequently included in the multivariable logistic regression (Enter) analysis. The results showed that miR-1909-3p ($P=0.001$) and miR-107 ($P=0.023$) might be independent factors for diagnosis of aMCI (Table 2). According to multivariable logistic regression results, we performed a diagnostic model: $7.341 - 0.029 \times (\text{expression level of miR-134-3p}) - 0.150 \times (\text{expression level of miR-22-5p}) - 0.604 \times (\text{expression level of miR-1185-2-3p}) - 5.321 \times (\text{expression level of miR-1909-3p}) - 1.372 \times (\text{expression level of miR-107})$. This combinative model improved the individual performance of altered miRNAs, performed well in discriminating aMCI subjects from normal controls (AUC: 0.901, Fig. 3d). According to Youden-index, the optimal cut-off value for this model was 0.174, the sensitivity and specificity were 80.0% and 82.5% separately.

Table 2. The results of logistic regression analyses of five miRNAs in predicting aMCI in validation set.

miRNAs	Univariable Logistic		Multivariable Logistic (Enter)			
	OR	95%CI	<i>P</i> value	OR	95%CI	<i>P</i> value
miR-1185-2-3p	0.334	0.176-0.634	<0.01	0.547	0.220-1.360	0.19
miR-1909-3p	0.002	0.001-0.033	<0.01	0.005	0.001-0.127	<0.01
miR-22-5p	0.054	0.010-0.301	<0.01	0.861	0.066-11.257	0.91
miR-107	0.175	0.065-0.470	<0.01	0.254	0.078-0.827	0.02
miR-134-3p	0.447	0.208-0.958	0.04	0.972	0.304-3.105	0.96

Note: Abbreviation: OR=odds ratio; CI=confidence interval

Modulation on BACE1 expression *in vitro*

To determine the modulation of miRNAs on BACE1 expression *in vitro*, we transfected miRNA mimics/inhibitor separately to SH-SY5Y^{BACE1} cells. Except miR-1185-2-3p, other four miRNAs decreased BACE1 mRNA levels as compared with control (Fig. 4a). All five miRNAs significantly decreased BACE1 protein levels in SH-SY5Y^{BACE1} cells (Fig. 4b and c). It demonstrated that these miRNAs suppress BACE1 expression by both inhibiting translation and destabilizing mRNA. Inhibition of endogenous miR-107 and miR-1185-2-3p significantly increased BACE1 protein levels separately (Fig. 4e and f). Nevertheless, inhibition of other three miRNAs did not increase BACE1 protein levels, which might be due to the competition with other miRNAs for the same targeting site. One of the potential multiple reasons that miR-1185-2-3p regulated the target gene more strongly than other selected miRNAs is that it has more binding positions than the others (Fig. 2).

Modulation on BACE1 expression in hippocampal neurons

The elevated expression of BACE1 may exacerbate endoproteolysis of APP and promote subsequent production of CTF- β and toxic soluble A β peptide, thereby contributing to the pathophysiology of AD. Therefore, we next investigated effects of selected miRNAs on modulating BACE1 expression and CTF- β production.

In rat hippocampal neurons, overexpression of miR-1185-2-3p, miR-1909-3p, miR-22-5p, and miR-107 separately suppressed BACE1 expression as compared with control (Fig. 5c and d). Downregulation of subsequent CTF- β production was observed in parallel with BACE1 protein levels (Fig. 5c to e). Conversely, inhibition of these four miRNAs significantly increased the BACE1 protein levels (Fig. 5f and g), indicating that BACE1 levels are regulated by these miRNAs in hippocampal neurons. Upregulation of BACE1 levels led to the significant increase in production of CTF- β (Fig. 5f to h).

In human hippocampal neurons, inhibition of miR-1185-2-3p/miR-1909-3p significantly increased the BACE1 protein levels and promoted subsequent CTF- β production (Fig. 6), in line with the results found in rat hippocampal neurons. Interestingly, downregulation of miR-134-3p only elevated BACE1 protein and

CTF- β protein levels in human hippocampal neurons, but not in rat. It may be due to the low conservativeness of miR-134-3p between human and rat. Meanwhile, overexpression of BACE1 levels increased APP expression may through positive regulation mechanism.

Discussion

In the face of continuous failure of new drugs, searching for effective diagnostic biomarkers for prodromal AD or aMCI is currently urgent. Although existing neuroimaging techniques are available to detect A β deposition very early, the high costs make it unavailable for screening. Tau level in plasma is too low to detection, while A β peptides themselves may not serve as effective biomarkers. Circulating miRNAs offer a rapid, convenient, non-invasive and cost-effective method for early diagnosis. Currently, many efforts have been made to discover altered circulating miRNAs in AD patients, which providing many potential biomarkers. However, few studies have focused on aMCI subjects. In this study, we used microarray sequencing to provide altered miRNA profiles in plasma of aMCI subjects and narrow the field of potential peripheral biomarkers.

Altered miRNA profiles between aMCI and controls

Using microarray sequencing, we detected 3,100 mature miRNAs in plasma of aMCI and normal controls. In a discovery set, 46 differentially expressed miRNAs were found between aMCI and normal controls, 13 miRNAs were up-regulated and 33 miRNAs were down-regulated. In an analysis set, applying bioinformatics methods and real-time qRT-PCR technique, four of the original 31 down-regulated human miRNAs were replicated (miR-1185-2-3p, miR-1909-3p, miR-22-5p, and miR-134-3p). As compared with previous study, current study is the first to report dysregulation of miR-1185-2-3p, miR-1909-3p, miR-22-5p, and miR-134-3p in plasma of aMCI subjects. We hypothesized these four miRNAs above and miR-107³² as candidate biomarkers for aMCI for further validation. In a validation set, we verified that miR-1909-3p and miR-107 are potent biomarkers separately. The high diagnostic accuracy of miR-107 observed here is consistent with our previous study³². Several studies have revealed that miR-107 is decreased in brain and biofluids in AD patients, and indicated that miR-107 may be involved in AD pathogenesis through regulating BACE1 and CDK5 activity^{21, 32-34}. This consistency in different tissues and different stages of cognitive decline makes it a stable biomarker. Combination of all five altered miRNAs in a diagnostic model had high diagnostic accuracy with 81.25%, which considered to be promising biomarkers. This model was calculated as follows: $7.341 - 0.029 \times (\text{expression level of miR-134-3p}) - 0.150 \times (\text{expression level of miR-22-5p}) - 0.604 \times (\text{expression level of miR-1185-2-3p}) - 5.321 \times (\text{expression level of miR-1909-3p}) - 1.372 \times (\text{expression level of miR-107})$.

Modulation on BACE1 expression *in vitro*

We demonstrated that four miRNAs (miR-1185-2-3p, miR-1909-3p, miR-22-5p, and miR-107) modulate BACE1 expression and downstream production of CTF- β *in vitro*, which may be important in the initiation

of AD pathology. In addition, the modulate function of miR-1185-2-3p and miR-1909-3p were replicated in human hippocampal neurons.

BACE1 is the rate-limiting enzyme in APP process. A β production is owing to sequentially cleaved by β -secretase (BACE1) and γ -secretase from APP³⁵. Therapeutic strategies targeting BACE1 may be useful to normalize BACE1 and reduce A β production. However, recent clinical studies have shown that cognitive decline is worsen in participants who received BACE1 inhibitor than those received placebo³⁶⁻³⁸. BACE1 has multiple substrates that are involved in various processed in brain³⁹⁻⁴¹. These clinical trials indicated that BACE1 inhibitor lower CSF A β levels up to ~90%⁴². The strongly inhibition might cause risk mechanism-based side effects³⁶⁻³⁸. Furthermore, several BACE1 inhibitor even elevated BACE1 level by extending the protein's half-life⁴³. Thus, how to decrease BACE1 effectively and moderately remains a challenge. Our study demonstrated that over-expression of five altered miRNAs decrease BACE1 expression approximately 20-50% separately. These miRNAs may be potential treatment target to modulate BACE1. Several miRNAs have been identified as regulators of BACE1, suggesting that an extensive network of multiple miRNAs may regulate BACE1 expression in a coordinated manner. The ability of five selected miRNAs to target BACE1 which are associated with sporadic AD makes these molecules interesting candidates as therapeutics (in the form of miRNA mimics) to reduce brain A β *in vivo*. Several miRNAs mimics are currently under investigation in clinical trials in some cancers^{44, 45}. Since miRNAs are important in neuronal biological functions, their role may extend to AD as potential therapeutic targets. Analysis of sequencing data enables us to understand miRNA-target networks and to identify key miRNAs involved in cognitive decline processes.

Inhibition of four miRNAs (miR-1185-2-3p, miR-1909-3p, miR-22-5p, and miR-107) markedly increased expression of BACE1 and CTF- β , which suggesting a tantalizing possibility that certain lower miRNAs levels may initiate A β deposition *in vivo* through weakened inhibition on BACE1 expression. Cells likely have homeostatic modulation mechanism for regulating gene expression, reducing the effects of dysregulation of a single gene regulator dysregulation. Therefore, it seems that multiple altered miRNAs as well as other gene regulators synergize to regulate BACE1 expression *in vivo*. Our results supported that these four decreased miRNAs play a role in regulation network and may promote AD pathogenesis.

Limitations of study and future direction

There are some limitations in current study. We believe that current study provides preliminary clinical and pathology evidence for the inclusion of plasma-based biomarkers toward aMCI. However, this plasma miRNAs battery may have somewhat limited value in differentiating Alzheimer's disease from other types of dementia. This plasma miRNAs battery may be not specific to Alzheimer's disease, as one of the miRNAs in the battery, miR-107, is also hypothesized to regulate progranulin/granulin, a molecule known to be important in non-tau frontotemporal dementia⁴⁶. The possibility of differentiating aMCI from other types of MCI (MCI caused by frontotemporal dementia or Lewy body dementia) by this plasma miRNAs battery should be examined in separate future studies. Secondly, effects of miRNAs on

modulation of BACE1 were only explored in hippocampal neurons *in vitro*. Thus, we need more extensive research requires validation *in vivo* to demonstrate the particular regulatory mechanism of these miRNAs.

Conclusion

Overall, we uncovered a differential expression profile of both previously identified and novel miRNAs battery altered in aMCI subjects. The plasma levels of all five miRNAs effectively discriminate aMCI from normal controls, suggesting the potential diagnostic value of combinatorial miRNAs. Furthermore, to discover the miRNAs regulation process may help us to deeply understand the fundamental cause of BACE1 imbalance. Last, since these miRNAs are relevant to molecular processes underlying AD by targeting BACE1, further functional characterization of these miRNAs may offer new therapeutic avenues for patients with AD.

Abbreviations

aMCI: amnesic mild cognitive impairment; BACE1: beta-site amyloid precursor protein cleaving enzyme 1; miRNA: microRNAs; CLAS: China longitudinal aging study; NC: normal controls; AD: Alzheimer's disease; A β : β -amyloid; MCI: mild cognitive impairment; PET: positron emission tomography; MRI: magnetic resonance imaging; APP: amyloid precursor protein; BDNF: brain-derived neurotrophic factor; ROC: receiver operating characteristic.

Declaration

Ethics approval and consent to participate

The study was approved by Institution's Ethical Committee of Shanghai Mental Health Center, Shanghai Jiao Tong University School of Medicine. Written informed consent was obtained from each study participant and/or his/her legal guardians.

Consent for publication

Not applicable.

Availability of data and materials

The microarray sequencing data generated during the current study was available in GEO dataset (reference number: GSE147232), <https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE147232>.

Other datasets generated during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

Tao W and Shifu X contributed to the conception and design of the study; Haining H conducted the analysis of data, and contributed to drafting the text and preparing the figures. Haining H, Huanqing Y, Wei Z, Zuoquan X, and Minmin Z conducted the experiments *in vitro*. Yuanyuan L, Na A, and Bo H contributed to collecting and proofreading the basic information, and performing psychological and psychosocial tests in all participants. Hua X, Feng Y, Ling Y, and Jinghua W conducted physical examinations, evaluated functioning and determined the diagnosis in all participants.

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Figures

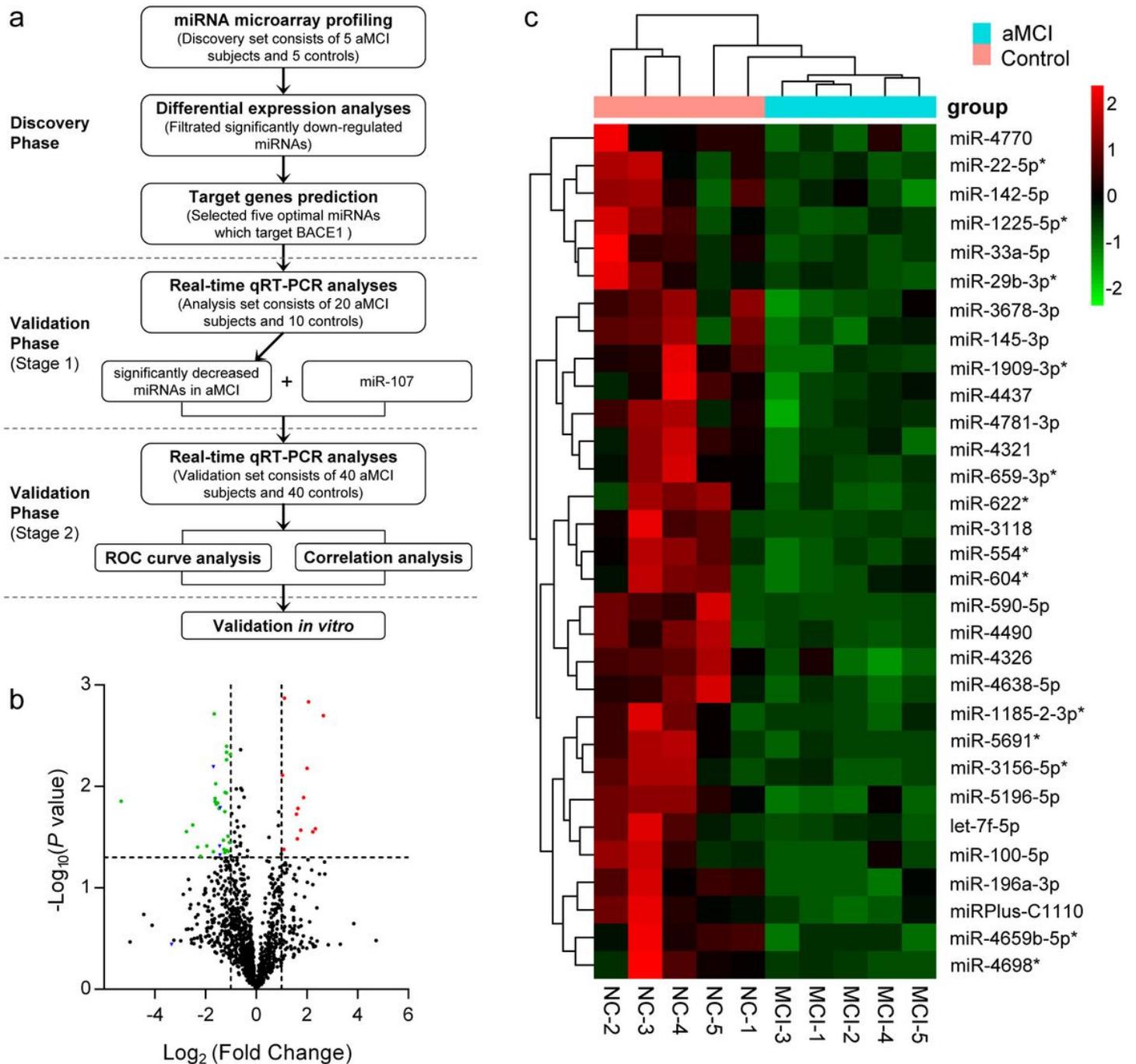


Figure 1

Identification of significantly down-regulated miRNAs in aMCI. (a) Workflow of this study. (b) Volcano plots of all miRNAs detected by microarray in discovery set. Red and green color indicate significantly up- and down-regulated miRNAs respectively. Triangle plot with blue are the selected miRNAs validated in analysis set. (c) Heatmap of thirty-one significantly down-regulated human miRNAs. detected by microarray. *miRNA which has targets sites on BACE1 mRNA predicted by TargetsCan7.2 database or miRDB database. Abbreviation: aMCI: amnesic mild cognitive impairment.

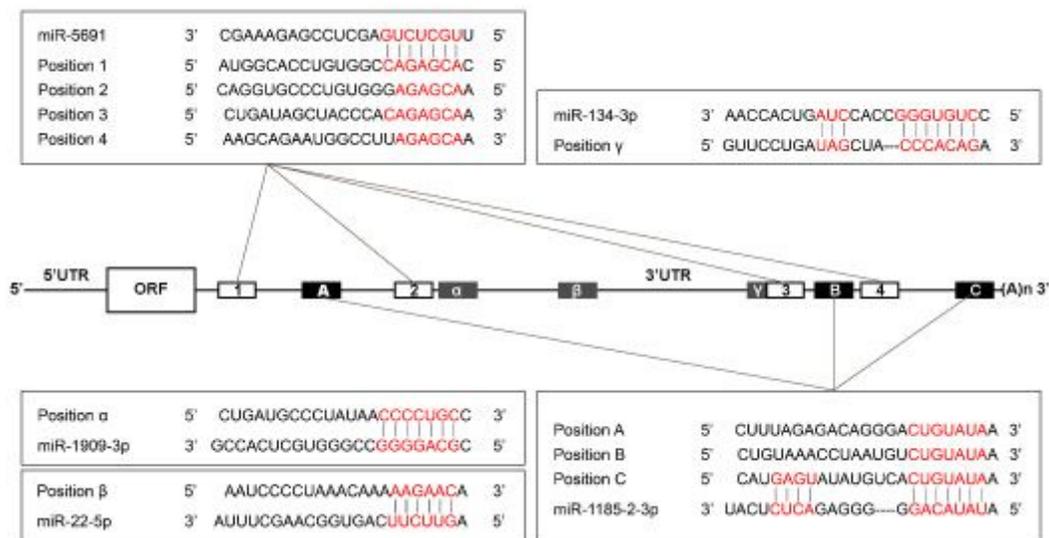


Figure 2

Schematic diagram of target sites of miRNAs in BACE1 mRNA 3'UTR.

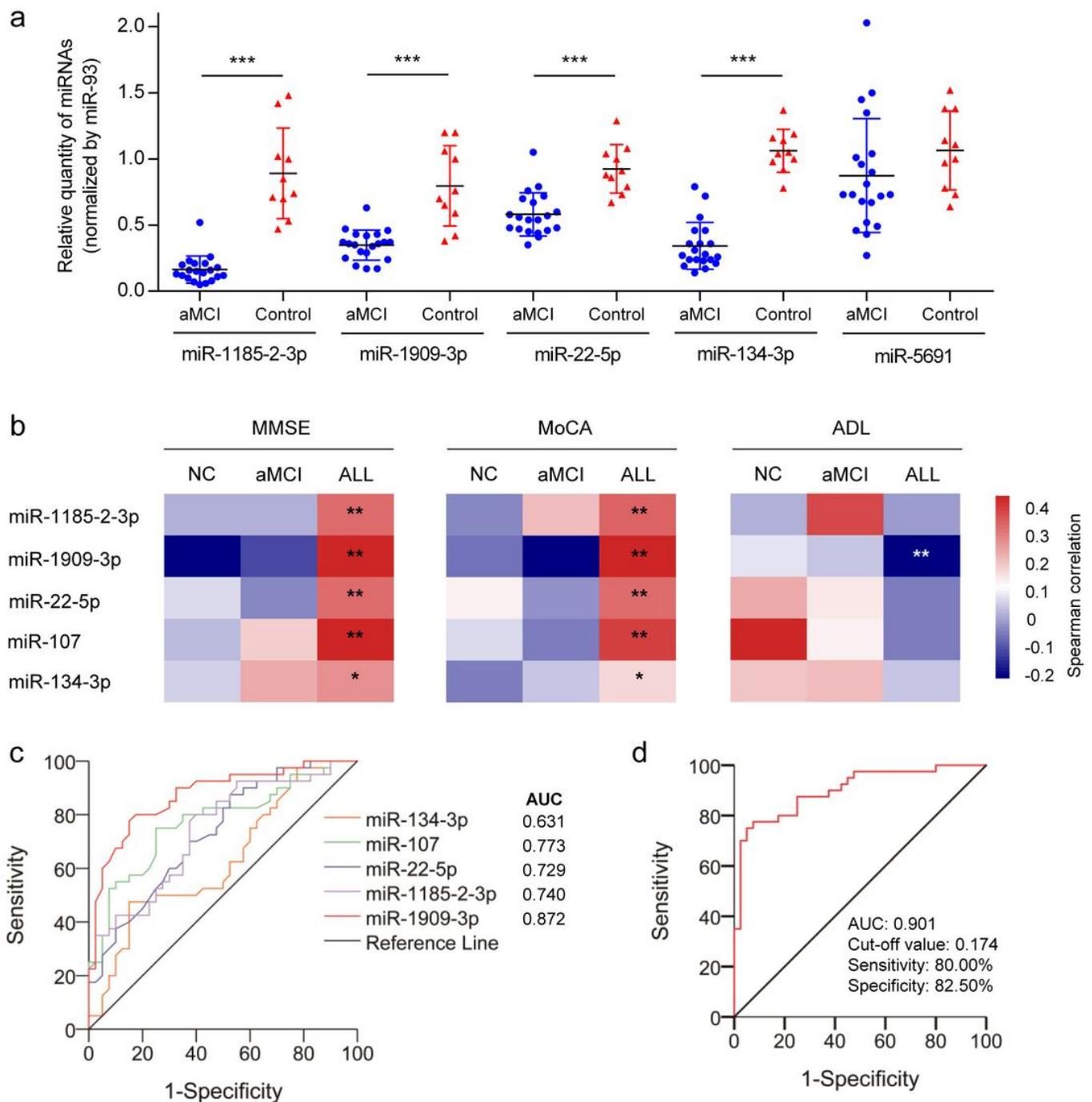


Figure 3

Identification of candidate miRNAs as potential biomarkers. (a) The relative expression levels of miR-1185-2-3p, miR-1909-3p, miR-22-5p, miR-134-3p, and miR-5691 in plasma samples in analysis set, which were calculated by $2^{-\Delta\Delta CT}$ method. Significant difference between two groups were assessed by two sample independent t test. (b) Correlation of candidate biomarkers (miR-1185-2-3p, miR-1909-3p, miR-22-5p, miR-107, and miR-134-3p) with MMSE, MoCA, and ADL scores in validation set. (c) Receiver operating characteristic (ROC) curve results of each candidate biomarker (miR-1185-2-3p, miR-1909-3p, miR-22-5p, miR-107, and miR-134-3p) in discriminating aMCI subjects from normal controls in validation set. (d) ROC

curve results of diagnostic model for aMCI diagnosis. Abbreviation: aMCI: amnesic mild cognitive impairment; NC: normal controls; MMSE: Mini-Mental State Examination; MoCA: Montreal Cognitive Assessment; ADL: Activities of Daily Living. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

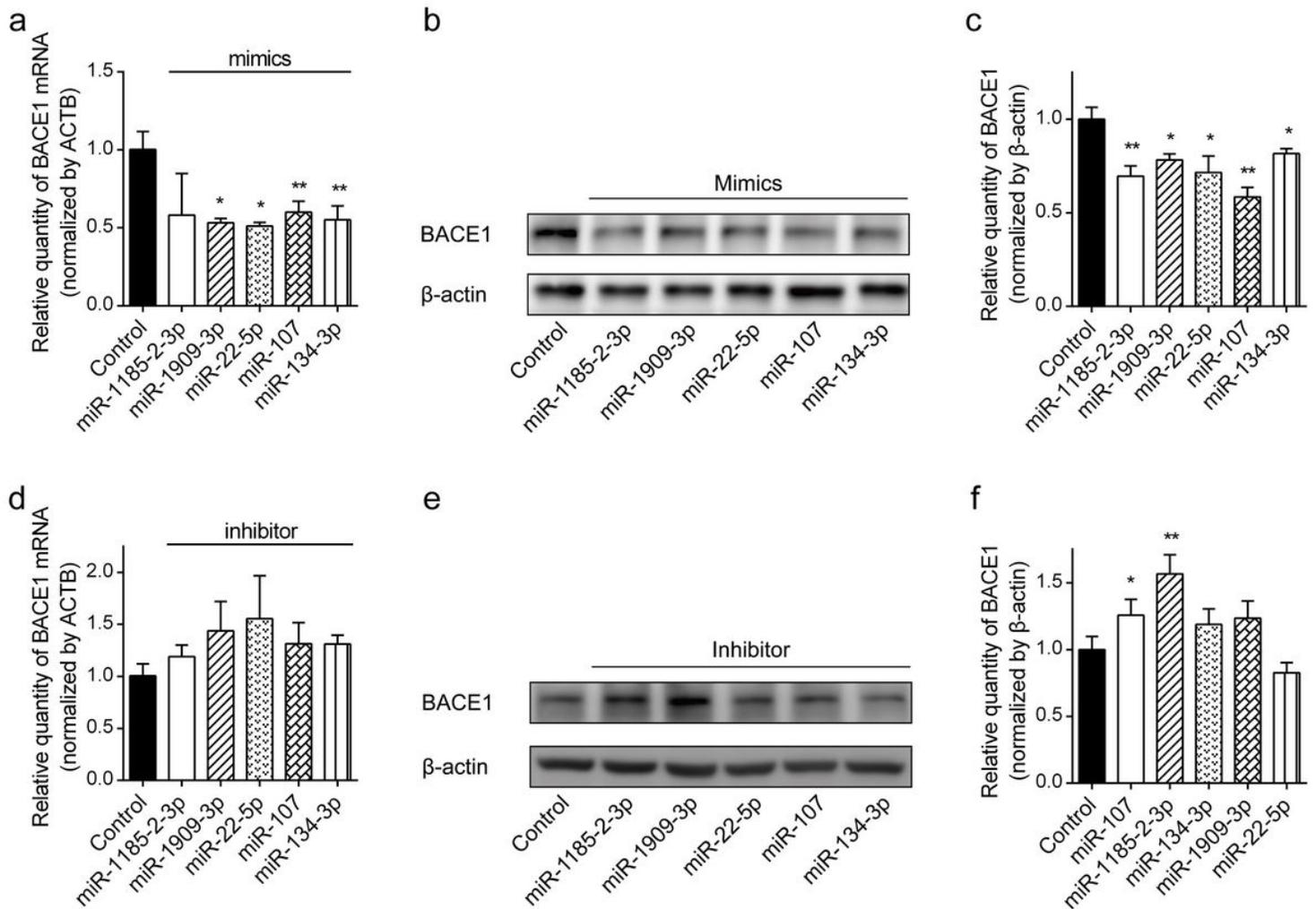


Figure 4

Five selected miRNAs suppressed BACE1 expression in SH-SY5YBACE1 cells. (a) BACE1 mRNA levels, (b) representative western blot images, and (c) relative BACE1 protein levels of 48h post-transfection with 50 nM of control or miRNA mimics separately. (d) BACE1 mRNA levels, (e) representative western blot images, and (f) relative BACE1 protein levels of 48 h post-transfection with 100 nM of control or miRNA inhibitor separately. Error bar presents the standard error. (* $P < 0.05$, ** $P < 0.01$, student's t test as compared to the control).

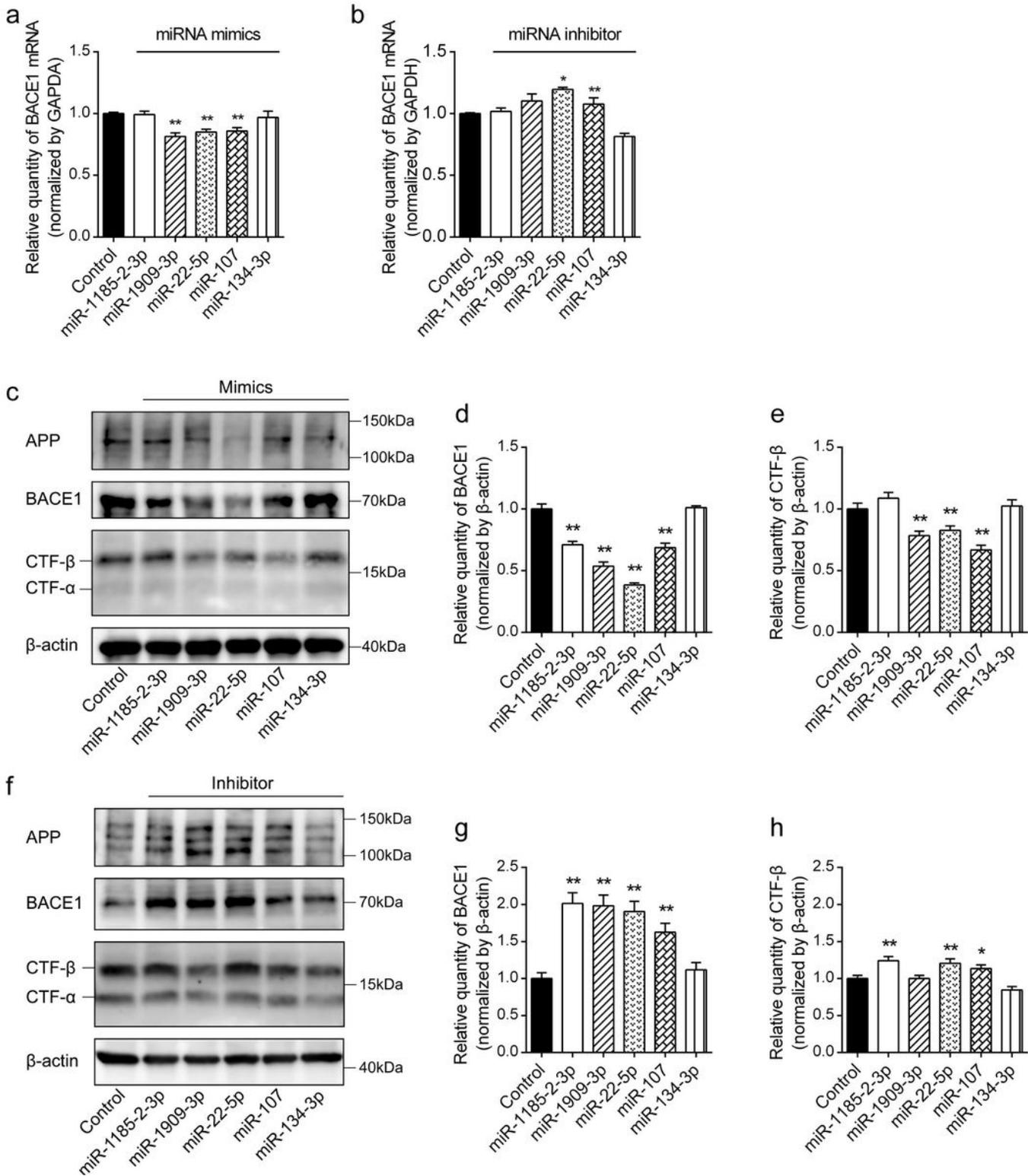


Figure 5

Five selected miRNAs modulate proteins which participated in A β cascade hypothesis in rat hippocampal neurons. BACE1 mRNA levels of 7 days post-transfection with (a) miRNA mimics lentivirus, or (b) miRNA inhibitor lentivirus in rat hippocampal neurons. (c) Represent western blot image, (d) relative BACE1 protein levels, and (e) relative CTF- β protein levels of 7 days post-transfection with control or miRNA mimics lentivirus separately in rat hippocampal neurons. (f) Representative western blot images, (g)

relative BACE1 protein levels, (h) CTF- β protein levels of 7 days post-transfection with control or miRNA inhibitor lentivirus separately in rat hippocampal neurons. Error bar presents the standard error. (* $P < 0.05$, ** $P < 0.01$, student's t test as compared with control).

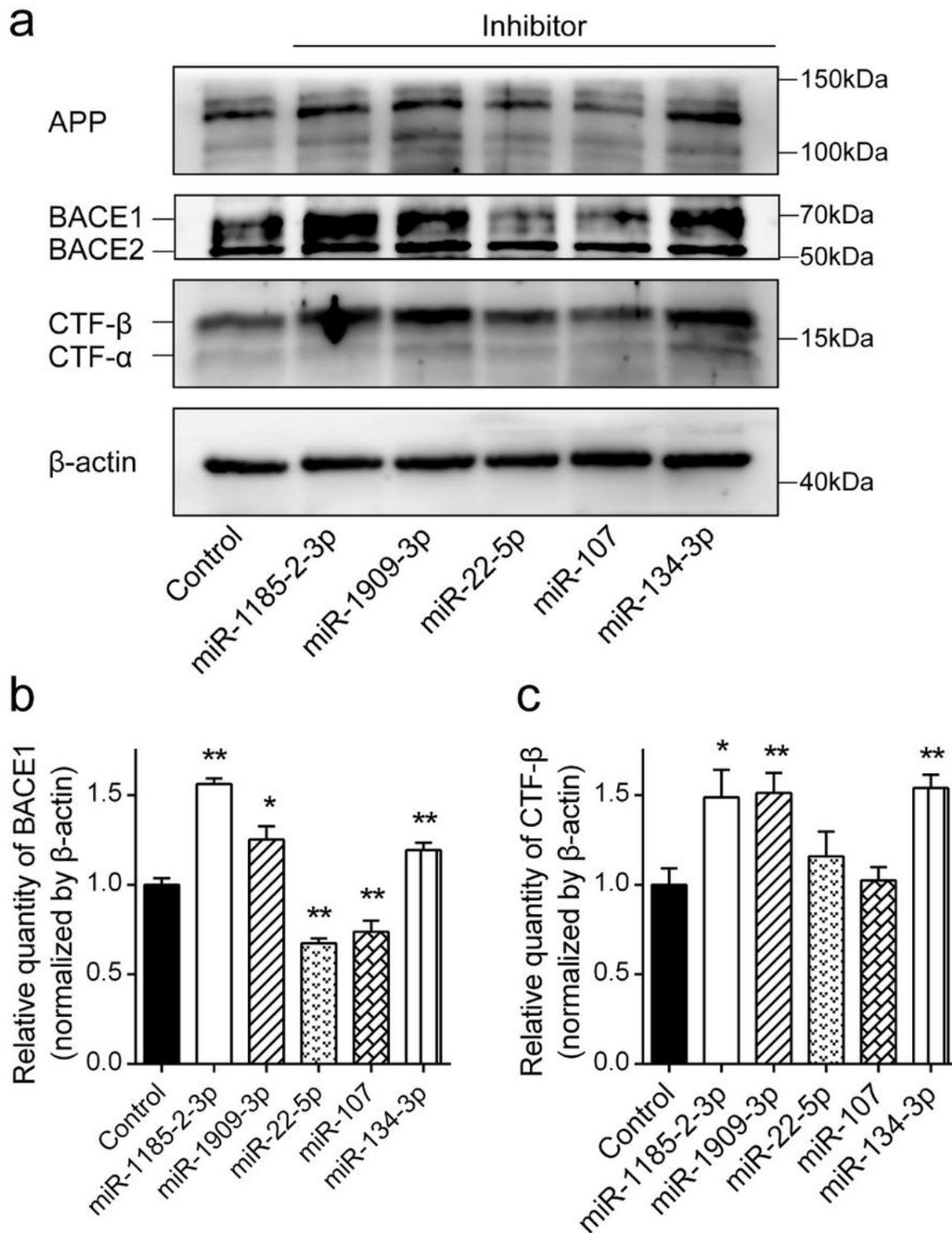


Figure 6

Five selected miRNAs modulate proteins which participated in A β cascade hypothesis in human hippocampal neurons. (a) Representative western blot image, (b) relative BACE1 protein levels, and (c)

relative CTF- β protein levels in human hippocampal neurons after transfected with miRNA inhibitor lentivirus separately. Error bar presents the standard error. (*P<0.05, **P<0.01, student's t test as compared with control).

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