MicroRNA-384 regulates both amyloid precursor protein and β-secretase expression and is a potential biomarker for Alzheimer's disease

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Abstract. Amyloid precursor protein (APP) and β-site APP cleaving enzyme (BACE-1) play important roles in the pathogenesis of Alzheimer's disease (AD). In this study, using bioinformatics analysis, we demonstrate that miR-384 is a microRNA (miRNA or miR) predicted to potentially target the 3' untranslated regions (3'-UTRs) of both APP and BACE-1. SH-SY5Y cells were transfected with miR-384 mimic oligonucleotide, miR-384 inhibitor oligonucleotide, or a non-specific control siRNA. We found that the overexpression of miR-384 suppressed the mRNA and protein expression of both APP and BACE-1. The miR-384 inhibitor oligonucleotide induced the upregulation of APP and BACE-1. The activity of BACE-1 was altered following the change in its protein expression. The binding sites of miR-384 on the 3'-UTRs of APP and BACE-1 were identified by luciferase assay. Furthermore, cells were treasted with amyloid-β (Aβ)42. Aβ42 downregulated miR-384 expression, leading to the continuous reduction in miR-384 expression. In addition, using a mouse model of AD, as well as patients with mild cognitive impairment (MCI) and dementia of Alzheimer's type (DAT), we examined the levels of miR-384 in cerebral spinal fluid (CSF) and serum. Patients with MCI and DAT had lower blood miR-384 levels compared with the controls. In addition, patients with DAT had lower blood miR-384 levels in blood compared with the MCI group. We also found decreased miR-384 expression in the several cerebral spinal fluid (CSF) of the patients with DAT. Negative correlations were observed between miR-384 and Aβ42 in the serum and CSF from patients with AD. In conclusion, these findings demonstrate that miR-384 may plays a role in the development of AD and may be a potential non-invasive biomarker for the diagnosis of AD.

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Introduction

Alzheimer's disease (AD) is a prominent neurodegenerative disorder characterized by the progressive loss of memory and other cognitive functions. Despite considerable progress in genetics and cell biology, there are still a number of unresolved issues regarding the mechanisms responsible for neurodegeneration, as well as the molecular and pathological components. Extracellular amyloid-β (Aβ), which is derived from a larger protein known as amyloid precursor protein (APP), is believed to be responsible for the death of neurons and dementia in AD. The increased APP expression may increase the risk of developing AD (1,2). APP levels can be regulated at the genomic, transcriptional or translational level and participate in the degradation of neurons. Genetic variants in the APP promoter increase APP transcription by 2 to 3-fold and have been reported to increase the risk of developing AD. APP can be processed by a group of secretases, where α-secretase produces soluble fragments, and β- and γ-secretase generate Aβ from APP (2). Several lines of evidence have suggested that Aβ regulates neuronal and synaptic activities and that the accumulation of Aβ in the brain causes an intriguing combination of aberrant network activity and synaptic depression (3). The β-secretase, β-site APP cleaving enzyme (BACE-1), is a rate-limiting enzyme of Aβ generation and a key target of drugs for AD (1,4,5).

MicroRNAs (miRNAs or miRs) are endogenous, short, non-coding RNAs, which act as important post-transcriptional regulators of gene expression by binding with their target mRNAs and are essential for neuronal function and survival (5,6). Several miRs have been shown to be important in neuropathology by downregulating AD-related proteins, such as APP and BACE-1. It has been demonstrated that miR-16, -101, -106a/b, -147 and -160a function as APP suppressors; another study using an APP/presenilin 1 (PS1) mutant mouse model of AD revealed a negative correlation between BACE-1 protein levels and 2 miRs (miR-298 and -328); miR-298 and -328 downregulated BACE-1 expression through direct interaction (5-7).

Several cerebral spinal fluid (CSF)- or blood-based markers, such as Aβ, soluble APP (sAPP)α/sAPPβ, tau, phosphorylated tau (p-tau) and BACE-1, have been proposed as biomarkers for predicting future cognitive decline in healthy

individuals and the progression to dementia in patients who are cognitively impaired (8,9). However, there is still an urgent need for the availability of more biomarkers that can detect AD in the predementia phase (1,6,8,9). The expression of AD-related proteins, such as APP and BACE-1, is controlled by a number of regulatory factors. The levels of these regulatory factors may be altered before changes occur in the levels of AD-related proteins. Thus, regulatory factors, such as miRs, may serve as potential biomarkers for AD, particulary in the diagnosis of mild cognitive impairment (MCI). In the present study, miRs that potentially target the 3' untranslated region (3'-UTR) of APP or BACE-1 were predicted using retrieve engines, as previously described (10). Bioinformatics analysis revealed that miR-384 was a miR that can potentially target both the 3'-UTRs of APP and BACE-1. The effects of miR-384 on APP and BACE-1 expression were examined, and the levels of miR-384 in samples from mice with AD, as well as in patients with MCI and dementia of Alzheimer's type (DAT) were measured.

Materials and methods

Study population. This study was approved by the Ethics Committee of Xuanwu Hospital of Capital Medical University, Beijing, China and the written informed consent was obtained from all participants. Thirty-two patients with MCI (13 females, 19 males; mean age, 63.2±6.1 years), 45 patients with DAT (18 females, 27 males; mean age, 64.2±5.8 years) were included in this study. A total of 50 control subjects, including 28 females and 22 males were also included (mean age, 63.9±5.7 years). Matched CSF and blood samples were drawn from 7 patients with DAT (2 females, 5 males; mean age, 65.1±3.5 years) and 7 control subjects (2 females, 5 males; mean age, 66.2±4.1 years). Venous blood was collected using a vacuum tube within 2 h after CSF collection. Serum was isolated within 1 h after blood collection. The samples were stored at -80˚C until analysis. Homocysteine (HCY) and apolipoprotein E (ApoE) levels were determined using the Hitachi 7600 biochemical analyzer (Hitachi, Ltd., Tokyo, Japan). The Aβ, tau and p-tau levels were determined using an ELISA kit (Cusabio Biotech Co., Ltd., Suffolk, UK).

APP/PS1 double-transgenic and wild-type (WT) mice. For our study purposes, 3-, 6- and 9-month-old APP/PS1 double-transgenic mice with a C57BL/6J genetic background were purchased from the Institute of Laboratory Animal Science, Chinese Academy of Medical Sciences and Comparative Medical Center, Beijing, China. All the animal protocols were approved by the Ethics Committee of Xuanwu Hospital of Capital Medical University. Non-transgenic mice were used as the WT controls. The mice were anesthetized with ether and blood was taken by removing the eyeballs, and then CSF-like fluid was collected as previously described (11). Briefly, the mice were sacrificed and their brains were removed into a 35-mm dish. The cranial cavity and cerebral ventricles (lateral, third and fourth ventricles) were rinsed with 1 ml PBS, and CSF was thus harvested with PBS, the washing solution being CSF-like fluid. The hippocampi were then isolated for miR-384 qPCR detection. The samples were placed in liquid nitrogen until analysis. There were 5 mice examined in each group.

Cell culture. The SH-SY5Y and HEK293 cell lines were purchased from the Shanghai Institute of Cell Biology, Shanghai, China. The cells were grown in antibiotic-free DMEM supplemented with 10% FBS at 37˚C with 5% CO_2. Primary mouse hippocampal neurons were isolated from mouse fetuses. Sixteen-day pregnant WT mice were sacrificed by CO_2 inhalation, and the primary mouse hippocampal neurons were then isolated as previously described (12).

Transfection. The cells were transfected with 100 nM (final concentration) miR-384 mimic oligonucleotide, miR-384 inhibitor oligonucleotide or a non-specific control siRNA (GenePharma, Shanghai, China) using Lipofectamine™ 2000 reagent (Invitrogen Life Technologies, Carsbad, CA, USA) following the manufacturer's instructions. Cells treated with Lipofectamine 2000 reagent only served as the negative control There were 5 samples tested in each group.

Aβ42 treatment. Synthetic Aβ42 peptides (Bachem, Heidelberg, Germany) dissolved in PBS were aged by incubation at 37˚C for 24 h with shaking at 1,000 rpm to allow fibril formation, as previously described (13). The cells were treated for 0, 12, 24, 36, 48 and 72 h with either a mock treatment containing PBS or 5 μM aged Aβ42, followed by miR-384 detection. There were 5 samples tested in each group.

5-aza-deoxycytidine (5-Aza-dC) treatment. The SH-SY5Y cells were exposed to 0.2 μM 5-Aza-dC (Sigma, St. Louis, MO, USA) dissolved in dimethyl sulfoxide for 96 h. The medium and the 5-Aza-dC were replaced every day, as previously described (14). The levels of miR-384 in the harvested cells were detected by qPCR as follows: The 0 μM 5-Aza-dC group served as the control. The CpG islands upstream of miR-384 were analyzed by the CpG Island Searcher. There were 5 samples tested in each group.

Isolation of mRNA and qPCR analysis. Total RNA from the harvested cells was isolated using TRIzol Reagent (Invitrogen Life Technologies). The isolated RNA was reverse transcribed using PrimeScript™ RT reagent (Takara Bio, Inc., Shiga, Japan). The mRNA expression of APP and BACE-1 was determined using SYBR®-Green qPCR (Takara Bio, Inc.) in the Light Cycler 480 System (Roche Diagnostics GmbH, Mannheim, Germany). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used to normalize the target genes. The primers used for PCR were as follows: APP forward, 5'-TTGCGAAACTCATC TTCACTGG-3' and reverse, 5'-CAGTGGGCAACACACAAA CTCTAC-3'; BACE-1 forward, 5'-AGGCAGTCTCTGGTAT ACACCCATC-3' and reverse, 5'-TGCCACTGTCCACAATG CTC-3'; and GAPDH forward, 5'-GCACCGTCAAGGCTGAG AAC-3' and reverse, 5'-TGGTGAAGACG CCAGTGGA-3'.

Isolation of miRs and qPCR analysis. Total RNA from the CSF, plasma and serum samples was extracted by a spin column method using the miRNeasy Serum/Plasma kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Total RNA in the hippocampal tissues from the animals and cultured cells was extracted by a spin column method using the miRNeasy kit (Qiagen). The miRs were reverse transcribed into cDNA using the miScript II RT kit

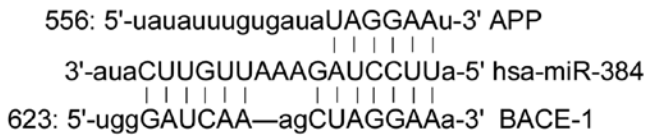

Figure 1. The seed regions of miR-384 in the 3'-UTRs of amyloid precursor protein (APP) and β-site APP cleaving enzyme (BACE-1).

(Qiagen) in 10-μl reaction system. miR-384 was detected by the TaqMan qPCR method (Qiagen), using U6 snRNA as an endogenous control.

Reporter vectors and DNA constructs. Reporter vectors containing the putative miRNA target sites from the APP and BACE-1 3'-UTRs were synthesized with double-stranded oligonucleotides perfectly complementary to putative miRNA target sites and oligonucleotides in which the seed regions were mutated. The APP oligonucleotides had following the sequence (seed region shown in bold): 5'-CCCAAGCTTTATATTTGT GATA**TAGGAA**TAAGCTTGGG-3' and 3'-GGGTTCGAAA TATAAACACTAT**ATCCTT**ATTCGAACCC-5'. The mutant APP target oligonucleotides had nucleotides 3 through 6 of the seed region mutated (italicized): 5'-CCCAAGCTTTATATTT GTGATA***TCGTAG***TAAGCTTGGG-3' and 3'-GGGTTCG AAATATAAACACTAT***AGCATC***ATTCGAACCC-5'. The BACE-1 oligonucleotides had the sequence (seed region shown in bold): 5'-CCC AAGCTTTGG**GATCAA**AG**CTAGGAA**A AAGCTTGGG- 3' and 3'-GGGTTCGAAACC**CTAGTT**TC **GATCCTT**TTTCGAACCC-5'. The mutant BACE-1 target oligonucleotides had nucleotides 7 through 13 of the seed region mutated (italicized): 5'-CCCAAGCTTTGG***TACCCA***CGC***C A TGCG***AAAGCTTGGG-3' and 3'-GGGTTCGAAACC**AT** ***GGGT***GC**G*GTACGC***TTTCGAACCC-5'. We utilized established methods to clone these synthetic versions of the putative miRNA target sites into a luciferase reporter gene (pMIR-REPORT; Ambion Inc., Austin, TX, USA) (11). A total of 10,000 HEK-293 cells were plated in 24 well plates. The following day, the cells were transfected with a miRNA mimic oligonucleotide, reporter vectors bearing either the miRNA target sequence or the miRNA seed region mutant target sequence, and one tenth of the molar volume of pRL-SV40, a Renilla luciferase control vector. We utilized Arrest-In transfection reagent (Open Biosystems Inc., Lafayette, CO, USA); any differences in transfection efficiency were accounted for by measuring Renilla luciferase activity. At 48 h post-transfection, the cells were lysed using 100 μl of GLB (Glo Lysis Buffer; Promega, Madison, WI, USA). Firefly and Renilla luciferase activities were measured using a dual luciferase reporter assay kit (Promega), as per the manufacturer's instructions. Firefly luciferase activity was normalized to Renilla luciferase activity. There were 5 samples examined in each group.

Western blot analysis. Western blot analysis was performed as previously described (15). Briefly, proteins (30 μg/well) were separated by 10% SDS-polyacrylamide gel electrophoresis (SDS-PAGE; Bio-Rad, Hercules, CA, USA). Proteins in the gel were transferred to nitrocellulose membranes (Pall Life Sciences, Port Washington, NY, USA). In the sequence, the membranes were incubated with anti-APP antibody (diluted 1:400; Abcam, London, UK), anti-BACE-1 antibody (diluted 1:1,000, Abcam), or anti-GAPDH antibody (diluted 1:400, Abcam) at room temperature for 1.5 h. The membranes were washed and incubated with anti-IgG antibody conjugated to horseradish peroxidase at room temperature for 1 h. Subsequently, the membranes were incubated with substrate for peroxidase and chemiluminescence enhancer (KPL Inc., Gaithersburg, MD, USA) for 1 min and exposed immediately to X-ray film for 1-5 min. The films were then revealed in the conventional manner. The amount of each protein was measured by densitometric analysis and standard relative to the GAPDH. There were 5 samples examined in each group.

In vitro BACE-1 activity assay. BACE-1 activity was determined by a fluorescence resonance energy transfer method using a kit from Sigma. The BACE-1 activity in [fluorescence units (FU)] was quantified through the standard curve. Negative control (no enzyme) reaction and standard curve blank were contained in the assay. There were 5 samples examined in each group. All reactions were performed in duplicate.

Statistical analysis and ROC curve analysis. Statistical analyses were performed using SPSS 13.0 software for Windows. For normally distributed data, the results are expressed as the means ± standard deviation (SD); differences between 2 groups were assessed by t-tests. Differences between multiple groups were analyzed using the Mann-Whitney U test, while correlations were determined by computing Spearman rank correlation coefficients. P-values <0.05 were considered to indicate significantly different differences.

Results

Bioinformatics analysis. A total of 62 miRs were found to be putatively target on the 3'-UTR of BACE-1, and 34 miRs were found to be putatively target on the 3'-UTR of APP. miR-384 was an miR that may target the 3'-UTRs of BACE-1 and APP (Fig. 1).

MiR-384 suppresses the expression of BACE-1 and APP. As illustrated in Figs. 2 and 3A-D, both the mRNA and protein expression of BACE-1 and APP was markedly decreased following transfection with miRNA-384 mimic oligonucleotide in primary mouse hippocampal neurons and SH-SY5Y cells (P<0.05). The miR-384 inhibitor oligonucleotide induced a significant upregulation in the mRNA and protein expression of APP and BACE-1 compared with the groups transfected with the non-specific control siRNA or the negative control (P<0.05). We observed a 57% downregulation in endogenous miR-384 under this condition (Fig. 3E). The activity of BACE-1 in the primary mouse hippocampal neurons and SH-SY5Y cells was also significantly suppressed by miR-384 and was significantly increased by the miR-384 inhibitor oligonucleotide (P<0.05) (Fig. 3F).

The UTRs of BACE-1 and APP 3' are both targets of miR-384. The overexpression of miR-384 significantly reduced fluorescence from the APP and BACE-1 reporter vectors in the HEK293 cells (P<0.05). These reductions were not observed

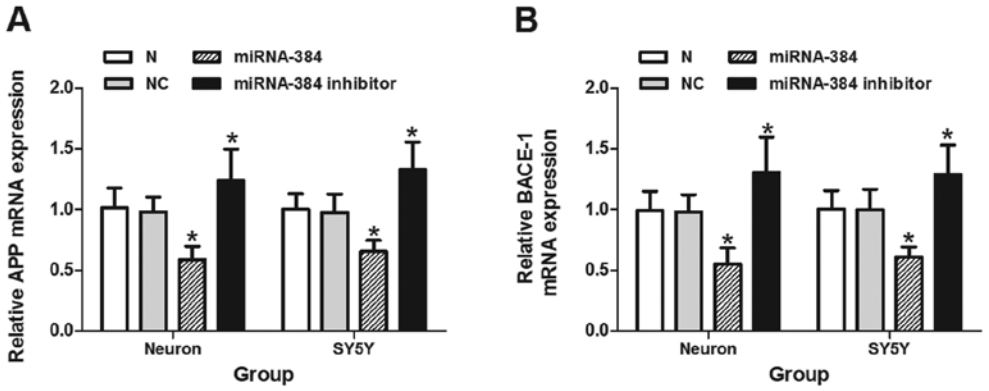

Figure 2. The effects of miR-384 and miR-384 inhibitor on the mRNA expression of (A) amyloid precursor protein (APP) and (B) β-site APP cleaving enzyme (BACE-1) were examined using SH-SY5Y cells (SY5Y). N, negative controls. NC, non-specific control. *P<0.05 compared with NC group.

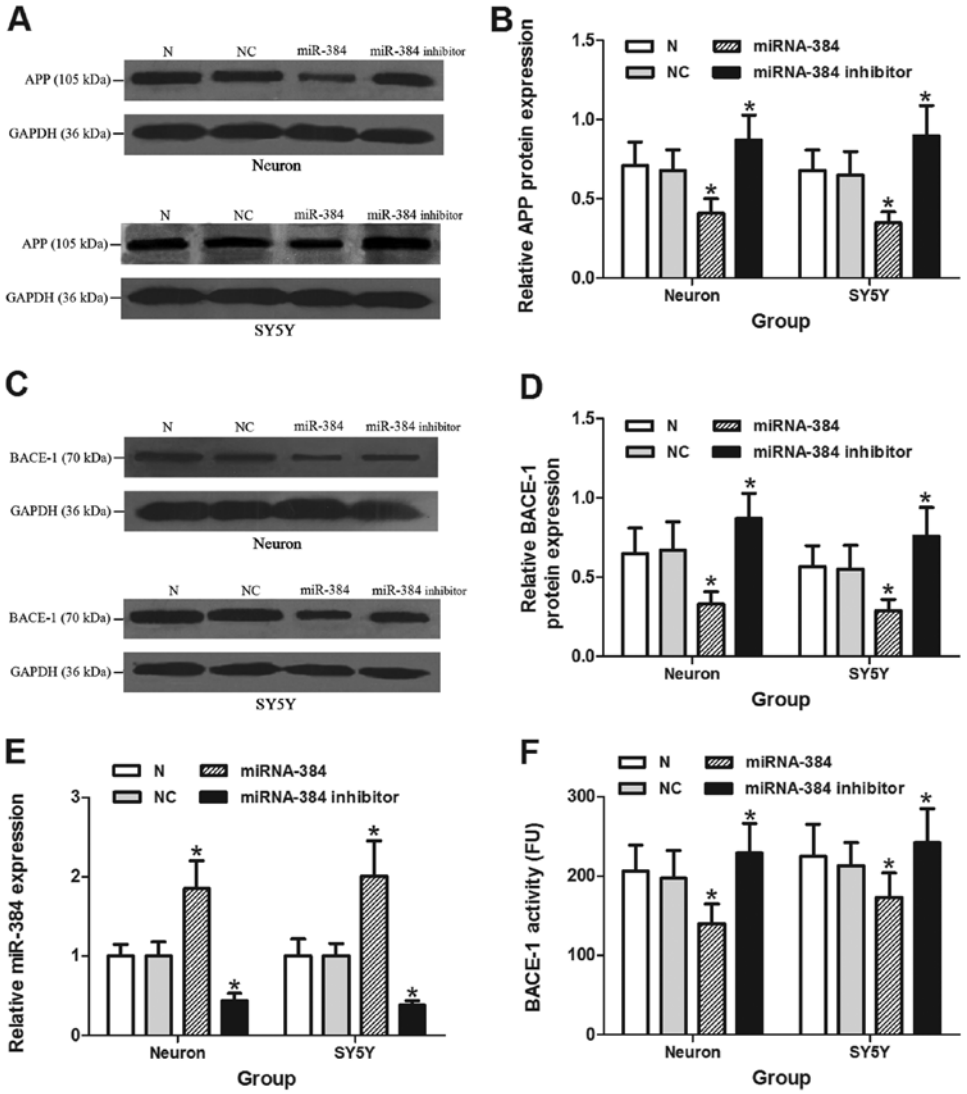

Figure 3. The effects of miR-384 and miR-384 inhibitor on the protein expression of (A and B) amyloid precursor protein (APP), (C and D) β-site APP cleaving enzyme (BACE-1), (E) intracellular miR-384 levels and (F) activity of BACE-1 were examined using neurons and SH-SY5Y cells (SY5Y). N, negative controls. NC, non-specific control. *P<0.05 compared with NC group.

when the seed regions mutants of the APP or BACE-1 3'-UTRs were utilized (Fig. 4).

Aβ42 downregulates miR-384 expression in vitro. 5-Aza-dC at a concentration of 0.2 μM did not affect miR-384 expression

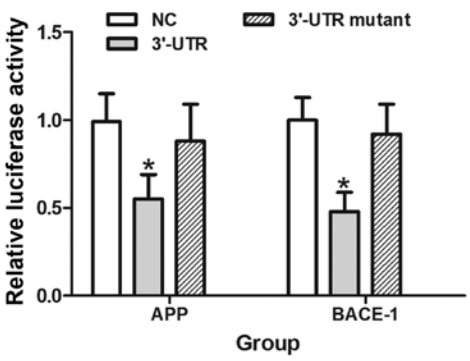

Figure 4. Binding sites of miR-384 on the 3'-UTRs of amyloid precursor protein (APP) and β-site APP cleaving enzyme (BACE-1) were identified. NC, non-specific control. *P<0.05 compared with the NC group.

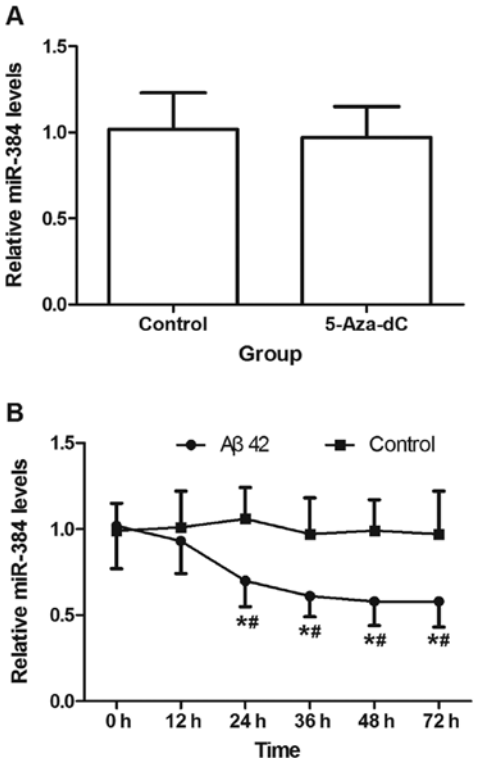

Figure 5. Effects of (A) 5-aza-deoxycytidine (5-Aza-dC) and (B) amyloid-β (Aβ)42 on the expression of miR-384 in SH-SY5Y cells. *P<0.05 compared with the control group; #P<0.05 compared with the 0- and 12-h groups (B).

in the SH-SY5Y cells (P>0.05) (Fig. 5A). miR-384 expression decreased after 12 h of incubation with 5 μM aged Aβ42, and continued to decline until 48 h (Fig. 5B). The results of bioinformatics analysis suggested that there was no CpG island upstream of miR-384.

Expression of miR-384 is decreased in the hippocampus, CSF-like fluid and serum of transgenic mice. The levels of miR-384 were significantly downregulated in the hippocampi, CSF-like fluid and serum obtained from the 3-, 6- and 9-month-old APP/PS1 transgenic mice compared with the WT mice (P<0.05). The levels of miR-384 in the hippocampi, CSF-like fluid and serum of the 6- and 9-month-old transgenic mice were significantly lower than those in the hippocampi, CSF-like fluid and serum from the 3-month-old transgenic mice (P<0.05) (Fig. 6A-C).

Expression of miR-384 is decreased in the CSF, serum and plasma of patients with AD. Compared with the control groups, patients with MCI and DAT had lower levels of miR-384 in the serum and plasma (P<0.05). The patients with DAT had lower miR-384 levels in their serum and plasma compared with the MCI groups (P<0.05). We also found decreased a miR-384 expression in the CSF of patients with DAT compared with the control group (n=7) (P<0.05) (Fig. 6D and E).

The levels of miR-384 obtained from plasma or serum strongly correlated with each other (r=0.957, P<0.05), indicating that both serum and plasma samples are suitable for investigations of miR-384 as blood-based biomarkers (Fig. 6F). The levels of miR-384 were lower in the CSF than those in the serum from a given individual (P<0.05) (data not shown). There was no correlation observed in miR-384 expression in the CSF and serum from a given individual (data not shown). When the cut-off values were set as 0.771 and 0.526 according to the ROC curve analysis, the positive rates of serum miR-384 were 53.1% (17/32) and 66.7% (30/45) of the MCI and DAT subjects, respectively.

miR-384 negatively correlates with Aβ42 in serum and CSF. A weak but significant negative correlation was observed between the levels of miR-384 and Aβ42 in the serum obtained from patients with MCI (r=-0.372, P<0.05) and DAT (r=-0.437, P<0.05), as well as the control group (r=-0.463, P<0.05). A stronger negative correlation was observed between the levels of miR-384 and Aβ42 in the CSF obtained from patients with DAT (r=-0.571, P<0.05) and the control group (r=-0.577, P<0.05). miR-384 expression showed no correlation with HCY, ApoE, tau and p-tau (data not shown).

Discussion

miRs are integral components of biological networks with fundamental roles in regulating gene expression (1,2,5). miR profiles are known to be altered in several regions of the brain in AD; however, the cause or consequence of the disease remains unknown. There are no data suggesting a direct genetic link between miRs or miR recognition elements and neurodegenerative disease (6-8). The increased expression of APP correlates with the accelerated accumulation of Aβ in the brain in AD. As the rate-limiting enzyme of Aβ generation, BACE-1 is a key drug target for AD. In the present study, we found that miR-384 suppressed the expression of APP and BACE-1 by binding their 3'-UTRs. The activity of BACE-1 was also suppressed by miR-384, which coincided with the the change in its protein expression. The suppressive effects of miR-384 on BACE-1 and APP expression may provide a new direction for targeted therapy for AD. This may allow researchers to suppress two key proteins using one miR, which may prove to be more effective with fewer side-effects.

The increase in Aβ42 expression is caused by the aberrant processing of APP, which is the major constituent of senile plaques in the brain in AD (16). It has been demonstrated that Aβ42 affects miR profiles (17). miR downregulation in

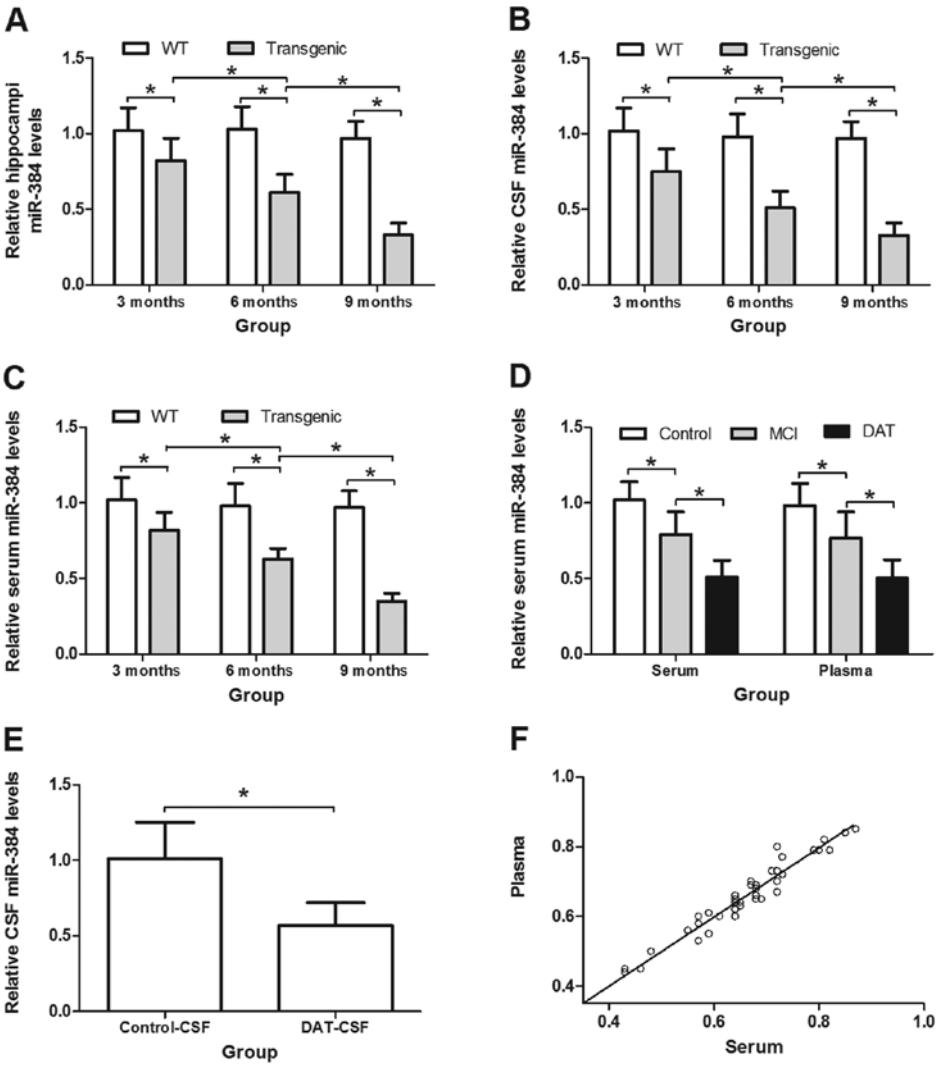

Figure 6. (A-C) The levels of miR-384 in the hippocampus, blood and several cerebral spinal fluid (CSF) of amyloid precursor protein (APP)/presenilin 1 (PS1) transgenic mice (at 3, 6 and 9 months of age) and (D and E) patients with mild cognitive impairment (MCI) or dementia of Alzheimer's type (DAT) were determined by TaqMan qPCR. (F) The correlation of miR-384 levels in matched samples of serum or plasma collected from a given individual was analyzed. *P<0.05. WT, wild-type mice.

Aβ-treated hippocampal neurons was observed in the hippocampus of Aβ-plaque forming APP23 mice at the onset of plaque formation (13). In this study, the downregulation of miR-384 expression by Aβ42 *in vitro* indicates that excessive Aβ42 generated from transgenic APP may play a role in the downregulation of miR-384 in APP/PS1 double-transgenic mice, and the decreased expression of miR-384 may lead to the upregulation of APP. In this manner, a vicious circle is generated; this vicious circle may play a role in the progression of AD.

For the majority of diseases, multiple biomarkers rather than a particular one are required for diagnosis. CSF is in direct contact with the extracellular space of the brain and can reflect biochemical changes that occur in the latter (17,18). For these reasons, CSF is the optimal source of AD biomarkers. However, CSF is not an appropriate sample for the screening and routine test as requires an invasive process of sample collection. Previous studies have demonstrated that miRNAs are stably expressed in animal serum/plasma, and that their unique expression patterns can serve as 'fingerprints' of various diseases (18,19). Thus, blood-based biomarkers for AD would be ideal. APP/PS1 double-transgenic mice contain insoluble amyloid peptides at the age of 6-9 months, concomitant with the formation of amyloid plaques (19). In this study, we found that the level of miR-384 was decreased in the hippocampi of 3-month-old transgenic mice, which suggests that the change in miR-384 expression occurs earlier than the formation of amyloid plaques. The detection of miR-384 in the CSF-like fluid and serum of 3-, 6- and 9-month-old transgenic mice demonstrates that miR-384 is a potential AD biomarker, particularly durin the earlier stages. This conjecture was further confirmed by clinical detection, which showed that the miR-384 level in patients with MCI was higher than the control group and lower than that in patients with DAT in the CSF and blood samples. This indicates that the level of miR-384 may change with the progression of AD and may serve as a biomarker for MCI and DAT.

There is no direct evidence that miRs can move across the blood-brain barrier (BBB) freely. However, some miRs can be selectively packaged into microvesicles, exosomes and actively secreted, and a microvesicle-mediated secretion pathway exists

in BBB (18,20). Although circulating miRs can be derived from many sources, the decreased secretion of miR-384 may lead to the decreased level of miR-384 in the blood.

In conclusion, these findings demonstrate that miR-384 may play a role in the development of AD, and shows great potential as a novel, non-invasive and easily detected blood-based biomarker for MCI and DAT.

Acknowledgements

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