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 treated 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 withDAT. 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 play a role in the development of AD and may be a potential non-invasive biomarker for the diagnosis of AD.

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, particularly 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 instructions. Total RNA from the harvested cells was isolated using TRIzol 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.

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’-TTGGCAAACCTATCATC TTACCTGG-3’ and reverse, 5’-CAGTGGGCAACACACAAA CTCTAC-3’; BACE-1 forward, 5’-AGGCAGTCTCTGTTGAT ACACCCATC-3’ and reverse, 5’-TGGCCACTTGCC AAATG CTC-3’; and GAPDH forward, 5’-GCACGGTCAAGGCTGAG AAC-3’ and reverse, 5’-TGGTGAAGACG CCAATGGA-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.
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 mimics 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 in the groups transfected with the non-specific control siRNA or the negative control (P<0.05). The miR-384 inhibitor oligonucleotide induced a significant upregulation in the fluorescent resonance energy transfer (FRET) signal from the APP and BACE-1 reporter vectors in the HEK293 cells (P<0.05).
when the seed regions mutants of the APP or BACE-1 3'-UTRs were utilized (Fig. 4).

\[ A\beta42 \text{ downregulates miR-384 expression in vitro. } 5\text{-Aza-dC at a concentration of } 0.2 \mu\text{M did not affect miR-384 expression} \]
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 of the 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 serum than those in the serum from a given individual (P<0.05) (data not shown). There was no correlation observed 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 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
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 during 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.

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