Abstract. Alzheimer’s disease (AD), the most common form of dementia in the aged population, presents an increasing clinical challenge in terms of diagnosis and treatment. Neurodegeneration is one of the hallmarks of AD, which consequently induces cognitive impairment. Brain-derived neurotrophic factor (BDNF), a neuroprotective factor, has been implicated in neuronal survival and proliferation. The epigenetic mechanism of BDNF methylation may be responsible for the reduced expression of BDNF in patients with AD. DNA methyltransferase may contribute to the methylation of BDNF, which is involved in neuroprotection in AD. In addition, epigenetic modifications, including a combination of microRNAs (miRNAs/miRs) and DNA methylation, have been suggested as regulatory mechanisms in the control of neuronal survival. In the present study, the expression of miR-29c was determined in the cerebrospinal fluid (CSF) of patients with AD and of healthy control individuals. A marked decrease in the expression of miR-29c was observed in the AD group compared with the normal control group, accompanied by a decrease in the expression of BDNF. Additionally, a significant increase in the expression of DNA methyltransferase 3 (DNMT3) was observed in the CSF from the patients with AD. Correlation analysis revealed that the expression of miR-29c was positively correlated with BDNF and negatively correlated with DNMT3 protein in the CSF of patients with AD. In addition, the regulatory association between miR-29c, DNMT3 and BDNF were also examined in vitro. It was demonstrated that miR-29c directly targeted DNMT3 and contributed to neuronal proliferation by regulating the expression of BDNF, at least partially, through enhancing the activity of the tyrosine receptor kinase B/extracellular signal-regulated kinase signaling pathway. In conclusion, the present study suggested that miR-29c may be a promising potential therapeutic target in the treatment of AD.

Introduction

Alzheimer’s disease (AD), the most common form of dementia in the aged population, presents an increasing clinical challenge in terms of diagnosis and treatment (1). The molecular and cellular mechanisms triggering AD remain to be elucidated. Neurodegeneration is one of the hallmarks of AD, which consequently induces cognitive impairment (2). The effects of several potential disease-modifying drugs for AD on neuroprotection have been investigated in clinical trials (3), suggesting that neuroprotection is important in AD therapeutics.

Brain-derived neurotrophic factor (BDNF), a neuroprotective factor, has been implicated in neuronal survival and proliferation (4). Lower levels of BDNF have been reported in the cerebrospinal fluid (CSF) of patients with AD, compared with age-matched healthy individuals (5). However, the underlying mechanism causing the altered BDNF levels in the CSF of patients with AD remains to be elucidated.

It has been suggested that epigenetic mechanisms affect gene expression in AD, as DNA methylation levels are associated with protein expression in the postmortem AD brain (6). The methylation of histone H3 lysine 9 (H3K9) has been demonstrated to increase with age in a triple transgenic mouse model of AD, in combination with a reduced gene expression of BDNF (7). Thus, BDNF methylation is a possible mechanism responsible for the low expression of BDNF in patients with AD; however, further investigation is required to confirm this hypothesis. DNA methyltransferases (DNMTs) are responsible for the establishment of de novo genomic DNA methylation patterns, involved in normal development and age-associated diseases, including AD (8). Altered expression levels of DNMT have been observed in patients with AD (9,10). Thus, DNMT
may contribute to the BDNF methylation, which is involved in neuroprotection in AD. In addition, epigenetic modifications, including a combination of microRNAs (miRNAs/miRs) and DNA methylation, have been suggested as regulatory mechanisms in the control of neuronal survival (11). Several lines of evidence have also suggested a role for miRNAs, including miR-29, in AD (12-14).

miRNAs, act as regulators of gene expression at a post-transcriptional level and are important in governing DNMTs (15). Specific miRNAs targeting DNMT transcripts lead to the demethylation and transcriptional activation of numerous protein coding gene sequences, thereby contributing to gene expression. In addition, DNMTs are important in controlling the expression of specific miRNAs (16). This cooperative action among DNMTs, miRNAs and DNA methylation indicates that miRNAs and DNMTs may be involved in the pathogenesis of AD.

The present study aimed to investigate the role of miR-29c in AD and to further examine the possible epigenetic mechanisms underlying AD. The expression of miR-29c, DNMT3, and BDNF were detected in CSF samples collected from patients with AD and from age-matched normal control individuals. DNMT3, an inhibitor of BDNF, was identified as a target of miR-29c using a dual luciferase reporter assay. Gain and loss of function experiments were used to examine the role of miR-29c in hippocampal neurons.

Materials and methods

CSF samples. The present study was approved by the ethics committee of Southern Medical University (Guangzhou, China). A total of 60 CSF samples (3 ml) were obtained by lumbar puncture from patients with AD and from age-matched normal control individuals from the Department of Neurology, Haikou People's Hospital (Haikou, China). All samples were collected according to the legislation and ethical boards of Haikou People's Hospital. Written informed consent was obtained from all of the patients. The samples were stored at -80°C until use.

Cell culture and treatment. Primary hippocampal neurons were prepared from rat embryos (Laboratory Animal Centre, Southern Medical University) at embryonic day 18. The hippocampi were mechanically dissociated from the brains of the embryos and treated with trypsin (Sigma-Aldrich, St. Louis, MO, USA) for 15 min at 37°C in phosphate-buffered saline (PBS). The cell suspensions were maintained in glial-conditioned medium (Invitrogen Life Technologies, Carlsbad, CA, USA) in 100 mm dishes at 37°C under 5% CO₂ until use. The experimental protocol was approved by the Animal Care and Use Committee of Haikou People's Hospital, and was in compliance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (Bethesda, MD, USA). Ectopic expression of miR-29c was achieved in the cells via transfection with miR-29c mimics or inhibitors using Lipofectamine 2000 (Invitrogen Life Technologies), according to the manufacturer's instructions.

Treatment with the DNMT inhibitor (DNMTI). DNMTI, 5-azacytidine (AZC), was purchased from Sigma-Aldrich. A quantity of 30 mg/l DNMTI was added to the primary hippocampal neurons and the culture continued for 3 days at 37°C in an atmosphere containing 5% CO₂ and 95% air, followed by three washes with PBS. The culture was then continued for a further 2 days in Dulbecco's modified Eagle's medium, prior to experimentation.

Reverse transcription-quantitative-polymerase chain reaction (RT-qPCR). Total RNA was extracted from the tissues and cells, according to the manufacturer's instructions (CWBio, Beijing, China). RNA was reverse transcribed using the ReverTra Aid first strand cDNA synthesis kit (Thermo Scientific, Waltham, MA, USA). A total of 2 µl cDNA was analyzed by PCR. The mRNA expression of DNMT3 was detected using a SYBR green qPCR assay (CWBio). The expression of β-actin was used as an endogenous control. The specific primers used in the present study were as follows: DNMT3, forward 5'-CTG GGT CAT GTG TCG G-3' and reverse 5'-TCTA-ATA A CT A C TC G GTG TG T-3'; and β-actin, forward 5'-CAT TAA GGA GAG C CGT TG T-3' and reverse 5'-GTT GA GG GAT TT GC GTG A-3' (Sangon Biotech Co., Ltd., Shanghai, China). An miScript SYBR-Green PCR kit (RiboBio, Guangzhou, China) was used for RT-qPCR to detect the expression of miR-29c. The specific primer sets for miRNA-29c and U6 were purchased from GeneCopoeia (Rockville, MD, USA). The sequences of the miRNA-29c and U6 primers were as follows: miRNA-29c; forward 5'-ACACTC-GAC TCT GG GTAC GGA TTT CTTC CTGG-3', reverse 5'-CTCAACTGGTGTCGAGTCGGCAATTACATGGTGA GAGGGATTC-3'; and U6, forward 5'-CTCGCTTCGGGACGCACA-3' and reverse 5'-AACGCTTCAGATTTGCCTG-3'. The expression of U6 was used as an endogenous control. RT-qPCR was performed using the following conditions: 95°C for 5 min; 40 cycles of 95°C for 30 sec, 58°C for 30 sec and 72°C for 30 sec and 72°C for 10 min using the CFX96 Real-Time system thermal cycler (Bio-Rad Laboratories, Inc., Hercules, CA, USA). Data were analyzed using the 2^ΔΔCT method (17).

ELISA determination of the expression levels of BDNF, DNMT1 and DNMT3. The Epigentek human BDNF, DNMT1 and DNMT3 immunoassay kits (Epigentek, Brooklyn, NY, USA) were used to determine the expression levels of BDNF, DNMT1 and DNMT3 in the CSF samples and in the primary hippocampal neurons. The CSF samples and primary hippocampal neurons were centrifuged at 3,000 x g for 15 min at room temperature. According to the manufacturer's instructions, the supernatants of the CSF samples or the primary hippocampal neurons were used to measure the total protein quantity of each sample. Briefly, the samples and the BDNF, DNMT1 or DNMT3 antibody were incubated overnight at 4°C. Subsequently, they were incubated with horseradish peroxidase-labeled anti-rabbit antibody for 30 min at room temperature. The samples were then developed using tetramethylbenzidine reagent (100 µl) in the dark and the absorbance was measured at 450 nm (Synergy™; Mx; BioTek, Winooski, VT, USA).

Measurement of the BDNF promoter CpG island methylation status using bisulfite genomic sequencing PCR (BSP) and methylation-specific PCR (MSP). Genomic DNA was
extracted using a genomic DNA isolation kit (BioVision, Milpitas, CA, USA). The genomic DNA (1 μg per sample) was modified with bisulfite using an Epitect Bisulfite kit (Epigenetek), according to the manufacturer's instructions, and the modified DNA was amplified using the following primers: BDNF, forward 5'-CTGTATCAAAGGCCACTGGA-3' and reverse 5'-GTGTCATCCTTTAGTAGCATGCCA-3'. The PCR products were gel extracted (Qiagen, Hilden, Germany) to confirm that a single band had been obtained. The DNA band was excised from the gel and then dissolved in buffer QXI and QIAEX II at 50°C for 10 min. The sample was then centrifuged at 12,000 x g for 30 sec. The pellet was washed and air-dried, then dissolved in 20 μl sterile water. The supernatant containing the purified DNA was then sequenced by Invitrogen Life Technologies.

MSP was performed on the bisulfite-treated DNA. The unmethylated and methylated BDNF primers were designed and synthesized by Sangon Biotech Co., Ltd. The annealing temperature was 60°C for the methylated-PCR and 55°C for the unmethylated-PCR, with 27 cycles used for each.

Western blotting. The total protein was extracted from the cells using cold radioimmunoprecipitation assay lysis buffer. A Bicinchoninic Acid Protein Assay kit (Thermo Fisher Scientific, Rockford, IL, USA) was used to determine the protein concentration. The proteins were then separated with 10% SDS-PAGE and transferred onto a nitrocellulose membrane (Wuhan Boster Biological Technology Ltd., Wuhan, China). Following blocking in 5% nonfat dried milk in PBS for 4 h, the membrane was incubated with the indicated primary antibodies (TrkB, mouse; cat. no. 4603s; 1:2,000; Cell Signaling Technology, Danvers, MA, USA; phosphorylated (p) Erk and Erk; rabbit; cat. nos. 9101S and 9102S, respectively; 1:1,000; Cell Signaling Technology) overnight at 4°C. The membrane was subsequently washed with tris-buffered saline containing Tween and incubated with horseradish peroxidase-conjugated goat anti-rabbit (1:5,000; cat. no. A12004-1) and goat anti-mouse (1:5,000; cat. no. A12003-1) immunoglobulin G secondary antibodies (Epigenetek, Farmingdale, NY, USA) for 2 h at room temperature. Enhanced chemiluminescence reagent (Wuhan Boster Biological Technology Ltd.) was used to detect the signal on the membrane. The data were analyzed via densitometry using Image-Pro plus software 6.0 (Media Cybernetics, Rockville, MD, USA) and normalized to the expression of the internal control.

Dual luciferase reporter assay. The wild type (wt) and mutant (mut) 3'-untranslated region (UTR) of DNMT3 were constructed and inserted into the dual luciferase reporter vector. To generate the wt-DNMT3-3'-UTR plasmid the DNMT3-3'-UTR was cloned into the XbaI (cat. no. ER0683; Thermo Scientific) site of the pGL3-control vector (Promega Corporation, Madison, WI, USA) downstream of the luciferase gene. The mut-DNMT3-3'-UTR plasmid was generated from the wt-DNMT3-3'-UTR by mutating the binding site for miR-29c via site-directed mutagenesis of the DNMT3-3'-UTR performed by Genecopeoia (Genecopeoia, Guangzhou, China). For the luciferase assay, 100,000 cells were cultured, at 37°C in an atmosphere containing 5% CO₂ and 95% air, to reach ~70% confluence in 24-well plates. Subsequently, the cells were co-transfected with the miR-29c mimic and the wt or mut 3'-UTR of DNMT3 dual luciferase reporter vector, respectively. Following a 5 h incubation with a Lipofectamine 2000 (Invitrogen Life Technologies; 2 μl)DNA complex at 37°C in an atmosphere containing 5% CO₂ and 95% air, the medium were replaced with fresh medium containing 10% fetal bovine serum (FBS). At 48 h after transfection, a dual luciferase reporter gene assay kit (BioVision) was used to determine the luciferase activities in each group, using a luminometer (Elecsys 2010; Roche Diagnostics, Basel, Germany). The activity of Renilla luciferase was normalized to that of firefly luciferase.

MTT assay. For all groups, 3,000 cells per well were seeded into a 96-well plate. Following treatment, the plates were incubated for 0, 12, 24, 48 or 72 h at 37°C in 5% CO₂. To assess cell proliferation, an MTT assay was performed, according to the manufacturer's instructions. MTT reagent (10 μl; 5 mg/ml) in 100 μl FBS-free medium was added to each well and incubated for 4 h at 37°C. Subsequently, the medium was removed and 150 μl dimethyl sulfoxide was added. The absorbance was detected at 490 nm using a microplate reader (Elecsys 2010; Roche Diagnostics). The assay was repeated three times in triplicate wells.

Statistical analysis. Statistical analyses were performed using GraphPad Prism 5 software (Graphpad Software, Inc., La Jolla, CA, USA) and the data are presented as the mean ± standard deviation. An unpaired two-tailed Student's t-test was used to analyze the data. P<0.05 was considered to indicate a statistically significant difference.

Results

Levels of miR-29c are positively correlated with the protein expression of BDNF and negatively correlated with the protein expression of DNMT3 in the CSF of patients with AD. The expression level of miR-29c was detected using SYBR green RT-qPCR analysis. In the sample of 30 patients with AD and control individuals, the results revealed that miR-29c was significantly decreased in the CSF of patients with AD, compared with that in the paired normal control individuals (Fig. 1A). In addition, a significant decrease was observed in the protein expression of BDNF in the CSF of patients with AD compared with the control (Fig. 1B). Subsequently, an ELISA assay was used to measure the protein expression levels of DNMT1 and DNMT3. As shown in Fig. 1D and E, compared with the normal control individuals, the protein expression of DNMT3, but not DNMT1 protein, was significantly increased in the CSF of patients with AD compared with the normal controls. Furthermore, the expression of miR-29c was positively correlated with the protein expression of BDNF and negatively correlated with the protein expression of DNMT3 in the CSF of patients with AD (Fig. 1C and F).

Methylation of the BDNF promoter is increased in the CSF of patients with AD. The methylation status of the miR-196bp CpG island in the CSF of patients with AD and normal control individuals was examined using BSP. The results indicated that methylation was significantly increased in the CSF of patients with AD, compared with the normal control group...
Figure 1. Correlation of miR-29c and BDNF/DNMT3 in patients with AD. (A) Relative expression of miR-29c in CSF samples of normal control individuals and patients with AD. (B) Relative expression of BDNF in CSF samples of normal control individuals or patients with AD. (C) Correlation analysis for the expression of miR-29c and BDNF. (D) Relative protein expression of DNMT1 in CSF samples of normal control individuals or patients with AD. (E) Relative protein expression of DNMT3 in CSF samples of normal control individuals or patients with AD. (F) Correlation analysis for the expression of miR-29c and DNMT3. Data are presented as the mean ± standard deviation. AD, Alzheimer's disease; DNMT3, DNA methyltransferase 3; BDNF, brain-derived neurotrophic factor; miR, microRNA; CSF, cerebrospinal fluid.

Figure 2. BDNF Cpg island methylation. (A) BSP detection method demonstrated increased methylation in CSF samples of patients with AD compared with normal control individuals. Closed circles represent methylation, open circles represent unmethylation. (B) MSP assessment results for five patients with AD and five normal control individuals. N represents the normal control individuals, A represents the patients with AD. AD, Alzheimer's disease; BDNF, brain-derived neurotrophic factor; CSF, cerebrospinal fluid; BSP, bisulfite genomic sequencing polymerase chain reaction; MSP, methylation specific polymerase chain reaction; U, unmethylated; M, methylated.
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(Fig. 2A). MSP was used to detect the methylation status of BDNF in five patients with AD and five normal control individuals (Fig. 2B). A significantly higher level of methylation was observed in the patients with AD compared with the normal control individuals.

miR-29c regulates the expression of DNMT3 by directly targeting its 3’UTR. To investigate whether miR-29c targets the 3’UTR of DNMT3, the 3’UTR of DNMT3 was cloned downstream of a luciferase reporter gene (wt-DNMT3). A mutant version (mut-DNMT3), with the binding site mutagenesis, was also constructed (Fig. 3A). The wt-DNMT3 vector and pre-miR-29c mimics or scramble control were co-transfected into primary hippocampal neurons. The luciferase activities of the pre-miR-29c and wt-DNMT3 co-transfected cells were significantly reduced compared with the scramble
control cells (Fig. 3B). Additionally, to assess whether miR-29c regulated the expression of DNMT3 at a transcriptional or translational level, the pre-miR-29c or anti-miR-29c were transfected into primary hippocampal neurons. As shown in Fig. 3C, the transfection efficiency was satisfactory for further analysis. The present results revealed that the overexpression of miR-29c significantly reduced the expression of DNMT3 at the mRNA and protein levels, whereas downregulation of miR-29c increased the expression of DNMT3 at the mRNA and protein levels (Fig. 3D and E). In addition, the protein expression of BDNF was detected in the cells treated with pre-miR-29c or anti-miR-29c alone, or combined with DNMTI. The protein expression of BDNF was decreased by upregulation of miR-29c and increased by downregulation of miR-29c, and was also induced by treatment with DNMTI alone. Higher levels of BDNF were observed in the pre-miR-29c + DNMTI treatment group compared with the pre-miR-29c or the DNMTI alone treatment groups. In addition, a lower level of BDNF was observed in the anti-miR-29c + DNMTI treatment group compared with the DNMTI alone group, but was marginally higher than that of the group treated with anti-miR-29c alone (Fig. 3F). These results suggested that miR-29c regulated the expression of DNMT3 at the transcriptional level by directly targeting its 3'UTR, consequently affecting the expression of BDNF.

**miR-29c promotes cell proliferation via TrkB/Erk signaling.** To assess whether miR-29c regulated neuronal proliferation, an MTT assay was performed by transfecting pre-miR-29c or anti-miR-29c into primary hippocampal neurons. Compared with the control group, pre-miR-29c transfection significantly promoted cell proliferation, whereas anti-miR-29c transfection significantly inhibited cell proliferation (Fig. 4A). To further
investigate the molecular mechanisms of miR-29c on the promotion of cell growth, the expression levels of molecules associated with TrkB signaling were detected. As shown in Fig. 4B, the protein expression of TrkB was increased by upregulation of miR-29c, but decreased by downregulation of miR-29c. In addition, the expression of pErk was induced markedly by pre-miR-29c transfection, and reduced by anti-miR-29c transfection (Fig. 4C). These results suggested that miR-29c promoted neuronal proliferation by activating TrkB/Erk signaling.

Discussion

The clinical assessment of AD is key in patient management and clinical trials, however, this evaluation has several limitations, such as the inability to clearly predict cortical changes to cognitive functioning (18). Thus, CSF biomarkers are useful tools to diagnose AD promptly and accurately, enabling effective prognosis and treating patients with AD accordingly (19). Improved understanding of the system biomarkers may assist in identifying the most efficient drug targets for developing optimal therapeutic strategies for AD. Levels of BDNF are reduced in specific brain regions, including the cortex and hippocampus, in AD, and BDNF gene polymorphisms have been suggested to affect the risk of developing AD and memory (20). Low serum levels of BDNF are associated, in patients with AD, with rapid cognitive decline and exhibit a significant correlation with the rate of cognitive decline (21). The BDNF level in the CSF is also lower in patients with AD compared with age-matched normal control individuals (5). Therefore, the levels of BDNF in the serum and CSF are consistent with that of the brain in patients with AD, suggesting that the level of BDNF in the CSF may reflect the status of the brain in patients with AD. In the present study, the levels of BDNF in the CSF were lower in patients with AD compared with age-matched normal control individuals, consistent with previous findings (22).

In addition to the MSP and BSP investigations, the present study demonstrated that BDNF methylation was higher in patients with AD compared with age-matched normal control individuals. In addition, levels of DNMT3 in the CSF were increased significantly in patients with AD compared with the age-matched normal control individuals. In an AD animal model, lower gene expression levels of Bdnf are associated with increased H3K9 methylation (7). The hypermethylation of BDNF has been observed to correlate with elevated levels of neuroinflammation and altered pro-apoptotic factors in the postmortem frontal cortex of patients with AD (6). DNMT1 is the major enzyme responsible for maintenance of the DNA methylation pattern, and the levels of DNMT3 are often increased in various cancer tissues and cell lines (23). Thus, DNMT3 may, in part, account for the hypermethylation of the CpG-rich promoter regions of genes, including BDNF. It has been reported that prenatal stress induces a decrease in the expression of BDNF, which may be mediated by increased levels of DNMTs levels, in rats (24). In the present study, the inhibition of DNMT activity with AZC increased the potential therapeutic target in the treatment of AD.

References


