Original Article

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MicroRNA-613 regulates the expression of brain-derived neurotrophic factor in Alzheimer's disease

Wei Li, Xu Li, Xin Xin, Peng-Cheng Kan, Yan Yan*

Department of Clinical Laboratory, Tianjin Huan Hu Hospital, Tianjin, China.

Summary **Alzheimer's disease (AD) is a neurodegenerative disorder characterized by progressive loss of memory and other cognitive functions and presents an increasing clinical challenge in terms of diagnosis and treatment. Brain-derived neurotrophic factor (BDNF) plays an important role in neuronal survival and proliferation. In the present study, the mRNA and protein expression level of BDNF was detected in serum, and cerebrospinal fluid (CSF) of patients with mild cognitive impairment (MCI), dementia of Alzheimer's type (DAT), and hippocampus in APP/PS1 mice. A significant decrease of BDNF mRNA and protein expression was observed in serum and CSF of patients and hippocampus in APP/PS1 mice compared with the corresponding controls. miR-613, which is predicted to target the 3'-UTR of BDNF, was also detected in patients and the mouse model. Opposite to the alteration of BDNF, miR-613 expression in serum, CSF and hippocampus were obviously increased compared to the controls. In conclusion, these findings showed that miR-613 may function in the development of AD and may provide new insights in diagnosis and treatment of AD.**

Keywords: miRNAs, mild cognitive impairment, dementia of Alzheimer's type, APP/PS1 mice

1. Introduction

Alzheimer's disease (AD) is a progressive neurodegenerative disease leading to deteriorating cognitive and memory function, immobility, and eventually death in affected patients (*1*). It is predicted that AD will affect 1 in 85 people globally by 2050 (*2*), thus AD has become a global health problem, and will bring a heavy burden to society. Until now, the etiology and pathogenesis of AD have not been elucidated.

Brain-derived neurotrophic factor (BDNF) is a member of the neurotrophic factor family and is known to protect against neurotoxicity of the Aβ peptide and neural cell death by aggregation of Aβ and tau proteins (*3,4*). Previous research suggested that BDNF was decreased in the frontal cortex and hippocampus of patients with AD (*5*), and DNA methylation of BDNF promoter is associated with the manifestation and clinical presentation of AD (*6*).

MicroRNAs (miRNAs) are small non-coding RNA molecules that regulate gene expression at the post transcriptional level. Recently, several miRNAs have been found to be related to AD pathogenesis by affecting the expression of function of AD-relevant molecules such as amyloid precursor protein (APP) (*7*), β-site amyloid precursor protein cleaving enzyme 1 (BACE1) (*8*) or Tau (*9,10*). Measurements of miRNAs in blood and cerebrospinal fluid (CSF) have become a novel diagnostic tool for various neurological diseases, including AD. miR-613, which was predicted to target BDNF in our study has neither been detected in patients with AD, nor been reported to be associated with AD.

In this study, we found that BDNF was significantly decreased in serum and CSF of patients with mild cognitive impairment (MCI) and dementia of Alzheimer's type (DAT), and also in hippocampus in APP/PS1 transgenic mice compared to the controls. Conversly, the expression of miR-613, which was predicted and confirmed to target 3'-UTR of BDNF, was increased in patients and APP/PS1 mice. This finding demonstrates that miR-613 may be a new biomarker for diagnosis of AD and helpful to explore new treatment strategies.

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**Address correspondence to:*
Dr. Yan Yan, Department of Clinical Laboratory, Tianjin Huan Hu Hospital, No. 6, Ji-Zhao Road, Jin-Nan District, Tianjin 300051, China.
E-mail: yanyandoctor1982@163.com

2. Materials and Methods

2.1. *Study population*

The present study was approved by the ethics committee of Huan Hu Hospital (Tianjin, China), and written informed consent was received from all the patients. Patients were diagnosed and characterized based on the National Institute of Neurological and Communicative Disorders and Stroke and the Alzheimer's Disease and Related Disorders Association (NINCDS-ADRDA) diagnostic criteria amendment, which was published by the National Institute on Aging and the Alzheimer's Association (NIA-AA) in April 2011 (*11*). A total of 32 MCI (22 females, 20 males, mean age 64.8 ± 7.2) and 48 DAT patients (26 females, 22 males, mean age 65.5 ± 6.8) were selected for the following study. 40 healthy individuals (22 females, 18 males, mean age 63.2 ± 6.3) were obtained from Physical examination center of Huan Hu Hospital and informed consent was also received from the participants. The serum and CSF samples were extracted from the patients, centrifuged at 12,000× g for 5 min at 4°C. Serum was stored at -80°C, and CSF samples were stored at -20°C for further analysis.

2.2. *Animals*

The 3-, 6- and 9-month-old APP/PS1 double-transgenic mice with C57BL/6J genetic background were purchased from Zhongke Biotechnology Co., Ltd (Beijing, China). The study protocols of animals were approved by Ethics Committee of Huan Hu Hospital of Tianjin in China. Non-transgenic mice (wild-type mice) were used as controls. Mice were anesthetized with ether and blood was taken by removing the eyes. After the mice were sacrificed, their brains were moved into a 35-mm dish. The cranial cavity and cerebral ventricles, which include lateral, third and fourth ventricles, were rinsed with PBS, and CSF was harvested with PBS, the washing solution was CSF-like fluid.

The hippocampus was isolated for further detection. There were 6 mice in each group. All the samples were stored in liquid nitrogen until required.

2.3. *Cell culture and transfection*

SH-SY5Y cells were cultured in Dulbecco's Modified Eagle Medium (DMEM, Gibco, USA) which contains 10% fetal bovine serum (FBS) at 37°C with 5% CO_2. Oligonucleotides and plasmids were transfected using LipofectamineTM 2000 reagent (Invitrogen, USA) according to the manufacturer's instructions.

2.4. *Plasmid construction and oligonucleotides*

The miR-613 sequence was amplified and inserted into pcDNA3 vector. miR-613 antisense oligonucleotides (ASO-miR-613) was used as the inhibitor of miR-613 and ASO-NC was used as control. EGFP coding region from the pEGFP-N2 vector was cloned into pcDNA3. The fragment of the 3'-UTR of BDNF (wild-type or mutant-type) was amplified and cloned into the pcDNA3-EGFP vector.

2.5. *Reverse transcription-quantitative polymerase chain reaction (RT-qPCR)*

Total RNA and miRNAs were isolated using Trizol reagent (Invitrogen) and mirVana miRNA isolation kit (Ambion, Austin, TX, USA). Then cDNA was obtained by using oligo-dT primers or stem-loop reverse transcriptase (RT) primers, respectively. β-actin and U6 were used as controls for BDNF and miR-613, respectively. PCR was performed under the following conditions: 94°C for 4 min followed by 40 cycles at 94°C for 1 min, 56°C for 1 min and 72°C for 1 min. Relative expression levels of the genes were calculated using the $2^{-\Delta\Delta Ct}$ method.

2.6. *Western blot analysis*

Total proteins in serum and CSF samples were extracted with protein lysis solution (Tiangen Biotech). Cell lysates were obtained with RIPA lysis buffer after transfection. Protein samples were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), transferred to nitrocellulose membranes and blocked with 5% skimmed milk for 1 h at room temperature. The membranes were incubated with rabbit anti-human monoclonal anti-BDNF antibody (1:500, Abcam, Cambridge, MA, USA) or antibody against glyceraldehyde phosphate dehydrogenase (GAPDH) overnight at 4°C. A goat anti-rabbit polyclonal IgG secondary antibody (1:1,000, Abcam) was added for incubation with the membrane at room temperature for 2 h. Protein expression level was assessed by enhanced chemiluminescence and exposure to film (Fujifilm, Tokyo, Japan). The relative expression was determined as the ratio to the GAPDH.

2.7. *EGFP reporter assay*

SH-SY5Y cells were plated in 24-well plates. Cells were transiently transfected with pcDNA3-pri-613, pcDNA, ASO-miR-613, ASO-NC and reporter vectors bearing either BDNF 3'-UTR wild-type or BDNF 3'-UTR mutant-type. The RFP expression vector was used as the corresponding control. The intensity of fluorescence was detected with an F-4500 fluorescence spectrophotometer (Hitachi, Tokyo, Japan). EGFP fluorescence intensity was normalized to the RFP fluorescence intensity.

2.8. *Statistical analysis*

Data are expressed as the mean ± standard deviation. A two-tailed Student's *t*-test was performed for group comparisons and $p < 0.05$ was considered to be statistically significant.

3. Results

3.1. *BDNF mRNA and protein expression levels were decreased in the serum and CSF of patients with MCI and DAT*

BDNF was reported to be associated with cognitive impairment, especially immediate memory (*12*). To explore the role of BDNF in the pathogenesis of AD, RT-qPCR and Western blot analysis were performed to detect the mRNA and protein expression levels of BDNF. The experimental groups were divided into the MCI ($n = 32$) and DAT ($n = 48$) groups, and healthy participants ($n = 40$), respectively. Data demonstrated that BDNF mRNA expression in the serum and CSF of MCI and DAT groups were significantly reduced compared to the control group (Figure 1A and 1B). Relatively, the expression levels of BDNF were much lower in the DAT group compared to the MCI group. Similarly, the protein expression of BDNF in serum and CSF were significantly decreased in the MCI and DAT groups compared to that in the control group. Moreover, the expression levels of BDNF protein were lower in the DAT group than in the MCI group (Figure 1C and 1D). All of these findings suggest that BDNF mRNA and protein expression are reduced in the serum and CSF of patients with MCI and DAT and BDNF is maybe related to the development of AD.

3.2. *Expression levels of BDNF mRNA and protein were reduced in the serum, CSF-like fluid and hippocampus of transgenic mice*

To further determine the crucial role of BDNF, we used APP/PS1 transgenic mice and also detected mRNA and protein levels of BDNF in the serum, CSF-like fluid and hippocampus of the animal model. The results suggested that the relative mRNA and protein expression of BDNF were significantly decreased in 3-, 6- and 9-month transgenic mice compared to the wild-type (WT) mice, respectively. Furthermore, the expression level of BDNF in the serum, CSF-like fluid and hippocampus of 6- and 9-month transgenic mice were much lower than that of the 3-month mice (Figure 2).

3.3. *Bioinformatics prediction*

We used miRanda, Targetscan and PicTar to predict the miRNAs which may target BDNF. Combining the results from these three bioinformatics softwares, we

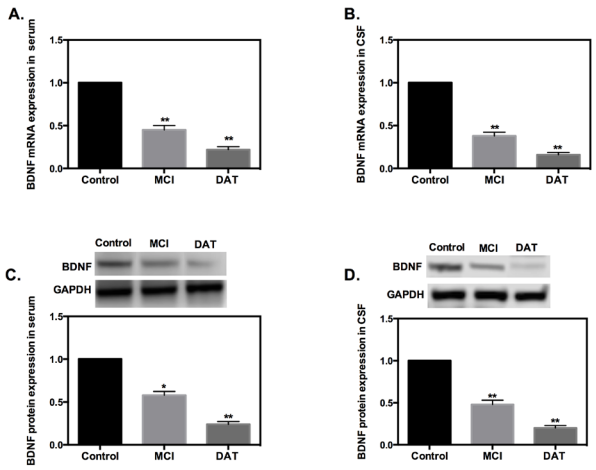

Figure 1. Alterations of BDNF mRNA and protein levels in the serum and CSF of patients with MCI and DAT. RT-qPCR was used to detect the mRNA expression of BDNF in the serum and CSF **(A** and **B)** and Western blot was used to detect the protein levels of BDNF **(C** and **D)**. Compared to the control group, $^{*}p < 0.05$ and $^{**}p < 0.01$. The expression levels of control group were normalized to 1. MCI, mild cognitive impairment; DAT, dementia of Alzheimer's type.

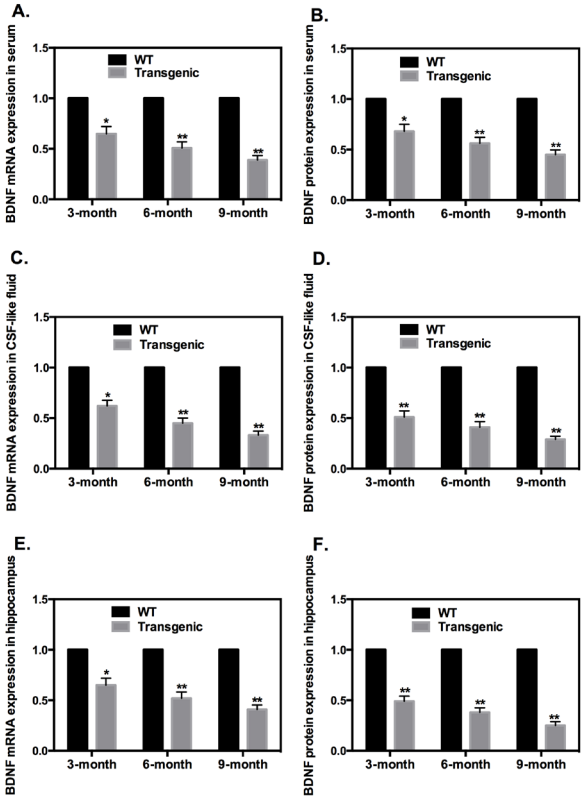

Figure 2. BDNF mRNA and protein expression were reduced in the serum, CSF-like fluid, and hippocampus tissues of APP/PS1 transgenic mice. Expression levels of BDNF in the serum **(A** and **B)**, CSF-like fluid **(C** and **D)**, and hippocampus tissues **(E and F)** of mice. Compared to the WT group, $^{*}p < 0.05$ and $^{**}p < 0.01$. The expression levels of WT group were normalized to 1. WT, wild type mice; transgenic, transgenic mice.

selected 12 miRNAs. Considering that miR-613 has never been determined as a regulator in AD, then miR-613 was finally chosen for further study (Figure 3).

3.4. *BMiR-613 directly targets the 3'-UTR of BDNF*

To further determine whether or not miR-613 directly targets BDNF, an EGFP reporter analysis was performed. EGFP vectors bearing wild-type or mutant-type BDNF 3'-UTR were transfected into SH-SY5Y cells when miR-613 was over-expressed or inhibited. As shown in Figure 4, over-expression of miR-613

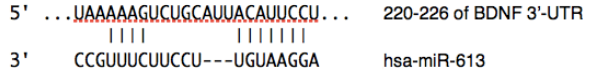

Figure 3. miR-613 could target the 3'-UTR of BDNF. The predicted sites of miR-613 binding to the 3'-UTR of BDNF are shown.

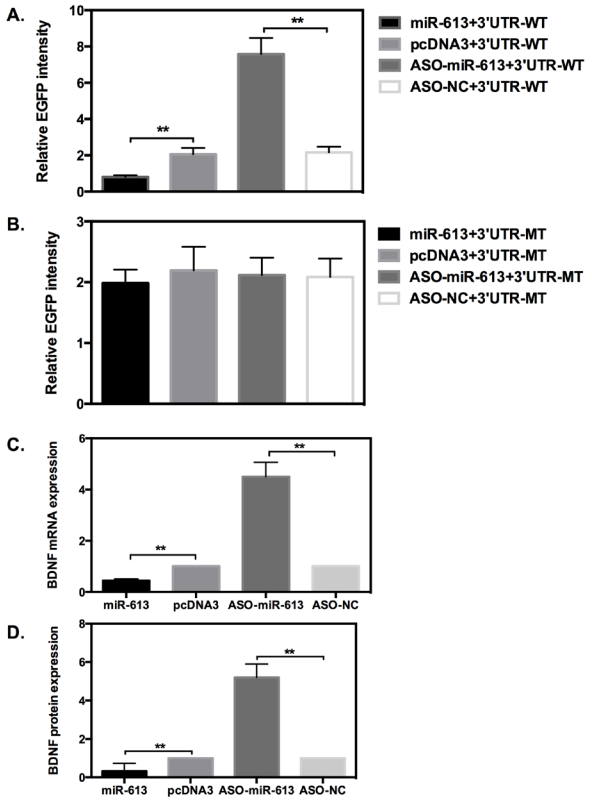

Figure 4. miR-613 could directly target BDNF and down-regulate its mRNA and protein expression in SH-SY5Y cells. (A) EGFP analysis was performed to detect the intensity of the wild-type 3'-UTR of BDNF when miR-613 was over-expressed or inhibited. **(B)** The intensity of the mutant-type 3'-UTR of BDNF when the expression level of miR-613 was changed. **(C)** RT-qPCR analysis was used to detect the mRNA expression of BDNF when miR-613 expression was altered. **(D)** Western blot analysis was used to detect the protein expression level of BDNF. $^{**}p < 0.01$. miR-613, pcDNA3-pri-miR-613; ASO-miR-613, miR-613 inhibitor; WT, wild-type; MT, mutant-type. pcDNA3 and ASO-NC groups are the corresponding controls. The mRNA and protein expression of BDNF in control groups were normalized to 1.

decreased the intensity with wild-type 3'-UTR by almost 61%. While the EGFP intensity with mutant-type 3'-UTR was increased approximately 2.5-fold when miR-613 was inhibited (Figure 4A). However, the EGFP intensity with the mutant-type 3'-UTR was not affected when altering the expression level of miR-613 (Figure 4B). These data indicate that miR-613 can directly target BDNF by binding to the 3'-UTR.

We then explored whether or not miR-613 affects the expression of BDNF. RT-qPCR and Western blot analysis were examined and showed that miR-613 significantly reduced the mRNA and protein expression by 55% and 67%, respectively. However, the expression levels of BDNF were increased by 3.5- and 4.2-fold when miR-613 was inhibited (Figure 4C and 4D). This finding suggests that miR-613 negatively regulates the expression of BDNF in SH-SY5Y cells.

3.5. *Expression levels of miR-613 were increased in patients with MCI and DAT, and also in APP/PS1 transgenic mice*

Since miR-613 can directly target BDNF and down-regulates its expression both at the mRNA and

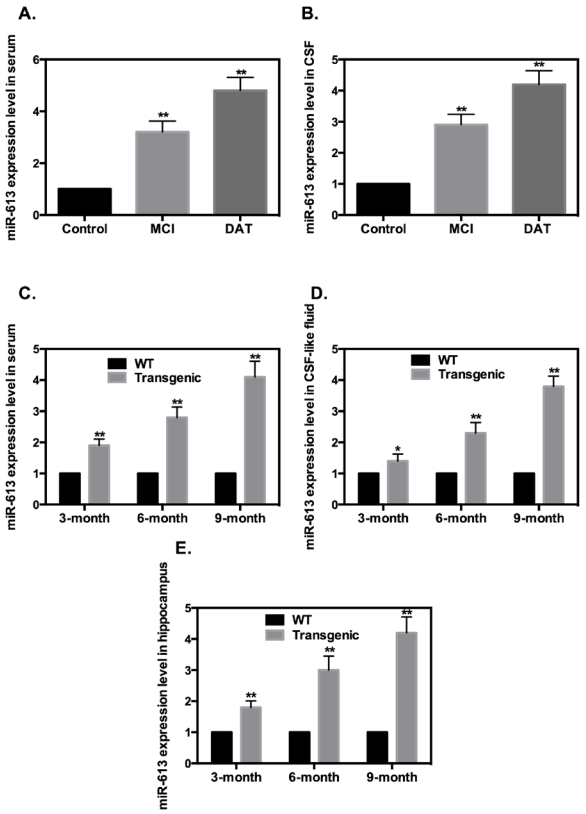

Figure 5. The expression level of miR-613 was significantly increased in the patients and animal models. (A and **B)** miR-613 expression level in the serum and CSF of patients with MCI and DAT. **(C)** miR-613 expression in the serum of transgenic mice and wild type mice. **(D)** The expression of miR-613 in the CSF-like fluid in animal models. **(E)** miR-613 expression level in the hippocampus tissues in transgenic and wild-type mice. Compared to the corresponding controls, $^{*}p < 0.05$ and $^{**}p < 0.01$.

protein level, we then investigated whether or not the expression level of miR-613 was changed in patients or transgenic mice. Accordingly, RT-qPCR was used and showed that miR-613 expression levels were significantly increased in serum and CSF of patients with MCI and DAT compared to healthy individuals, respectively (Figure 5A and 5B). Moreover, miR-613 mRNA expression was also remarkably increased in serum, CSF-like fluid, and hippocampus of APP/PS1 transgenic mice compared to wild-type mice (Figure 5C, 5D, and 5E).

4. Discussion

Accumulated evidence has shown that miRNAs are related to various diseases, including cancer, immune diseases, inflammation, and neurodegenerative diseases (*13-16*). Furthermore, numerous miRNAs have been determined to be associated with AD, such as miR-512 (*17*), miR-29c (*18*), and miR-155 (*19*). miR-613 is also reported to play a role in the pathogenesis and development of various cancers (*20-22*). However, there has been no data to suggest a link between miR-613 and AD. In the present study, we first detected a significant increase of miR-613 in body fluid of patients with MCI and AD and also in APP/PS1 transgenic mice. We also determined that miR-613 directly targets the 3'-UTR of BDNF and down-regulates its mRNA and protein expression.

BDNF is one of the neurotrophic factors that support differentiation (*23*), maturation (*24*), and survival of neurons in the nervous system (*25*) and shows a neuroprotective effect. Previous studies have indicated that the level of BDNF was reduced in many neurodegenerative diseases, such as Huntington's disease (*26*), multiple sclerosis (*27*), and Parkinson's disease (*28*). In this research, we found that BDNF is also significantly decreased in the body fluid of patients and an animal model. Biomarkers, which are the objective indicators of biological and pathological processes, were utilized to assess the risk or prognosis of disease, to guide clinical diagnosis or to monitor the intervention effect. CSF is in direct contact with the extracellular space of the brain and can directly reflect biochemical changes. Therefore, CSF is the preferred source of AD biomarkers. Because the process of obtaining CSF is traumatic, serum is also suitable for study. We detected BDNF and miR-613 expression levels in serum and CSF both in patients with MCI and DAT and transgenic mice, and also in hippocampus of an animal model. Results showed significant alterations of BDNF and miR-613 expression.

Furthermore, we identified that miR-613 directly targets BDNF. This conclusion is based on several experimental results. First, EGFP reporter assay showed that miR-613 significantly decreased the fluorescence intensity of wild-type 3'-UTR of BDNF, while miR-613 inhibitor increased the relative intensity of the 3'-UTR. However, the intensity of mutant-type 3'-UTR was not affected by alteration of miR-613 expression. Second, miR-613 decreased the expression of BDNF both at the mRNA and protein levels in SH-SY5Y cells. Third, both in body fluid of patients and the animal model, the expression of BDNF was negatively correlated to that of miR-613.

Moreover, we also found that the changes of BDNF and miR-613 were more obvious in patients with DAT than those with MCI. Meanwhile, the alteration of BDNF and miR-613 expression levels was more significant in 6- and 9-month transgenic mice than that in 3-month mice. These data suggested that BDNF and miR-613 may be related to the severity and progression of AD.

In conclusion, the present results indicated that BDNF mRNA and protein expression levels were decreased in patients with AD and transgenic mice. miR-613, which could directly target the 3'-UTR of BDNF, was inversely increased in patients and animal models. These findings demonstrated that miR-613 may be a new biomarker of AD pathogenesis and development, and may provide novel insights into the diagnosis and therapy of AD.

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