Original Article

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Comparison between the Plasma Levels of Long Noncoding RNA BDNF-AS in Patients with Alzheimer's disease and Healthy Subjects

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ABSTRACT

BACKGROUND AND OBJECTIVE: Diagnosis of Alzheimer's disease usually occurs when serious damages have occurred in the brain and common treatments are ineffective in preventing it. One of the RNAs involved in Alzheimer's disease is a long noncoding RNA, called BDNF antisense (BDNF-AS). The aim of this study is to determine the presence and compare the BDNF-AS levels in plasma of Alzheimer's patients and healthy subjects, and to evaluate its potential as a plasma marker for Alzheimer's disease.

METHODS: In this case-control study, 30 patients with late-stage Alzheimer's disease and 30 healthy subjects without neurological disease who matched the patients in terms of age were selected by a specialist according to the criteria for clinical diagnosis of Alzheimer's disease and their intravenous blood samples were collected. The plasma of the blood samples was isolated and total plasma RNA was extracted. After cDNA synthesis, the presence of BDNF-AS in plasma was examined by PCR. Finally, the relative level of BDNF-AS transcripts in plasma samples of patients with Alzheimer's disease and healthy subjects was evaluated using Real Time PCR.

FINDINGS: The results of this study showed that long noncoding RNA BDNF-AS was present in the plasma of patients and controls. Comparison of Real Time PCR data showed that BDNF-AS levels in the plasma of patients (0.107±0.021) showed significant increase compared to healthy subjects (0.039 ± 0.006).

CONCLUSION: The results of this preliminary study indicate that the levels of long noncoding RNA BDNF-AS in plasma can be used as a blood/plasma marker for the diagnosis of Alzheimer's disease.

KEY WORD: *Alzheimer's disease, BDNF-AS, Biomarker*.

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Introduction

Alzheimer's disease is a common neurological disorder worldwide and often causes dementia in older people (1). However, a small number of middle-aged people develop the disease, according to which, this disease is categorized into two types of early-stage and late-stage (2).

Late-stage Alzheimer's disease occurs at ages 65 and above, and various studies have shown that complex interactions between genetic, and epigenetic factors and the environment are the cause of it (3). However, the main role in the development of Alzheimer's disease is played by the β – amyloid protein (βA), which is derived from the proteolytic cleavage of the amyloid precursor protein (APP) protein precursor (4,5).

The imbalance between production and purification and thus, the accumulation of Aβ peptides leads to an increase in Aβ, which can be a starting point for Alzheimer's disease (4). Another factor in the development of Alzheimer's disease is Tau protein. Tau protein is abnormally hyper-phosphorylated in the brain of patients with Alzheimer's disease and with a stoichiometry of at least three times more than normal Tau protein, it is a histological characteristic of the disease (6).

Evolving evidence suggests that the reduction in the levels of the brain-derived neurotrophic factor (BDNF) can be associated with the pathogenesis of Alzheimer's disease (7, 8). BDNF is one of the most important factors in neurogenesis and synaptic plasticity, and its reduction in the brain, especially the hippocampus, causes damage to memory and learning, a process that has been proven to occur in Alzheimer's disease (8). Examining the serum levels of BDNF in patients with advanced and mild Alzheimer's disease has shown that its serum level decreases in these patients (8, 9).

These studies suggest that impairment in regulation of BDNF transcription and translation can help the process of cognitive impairment in Alzheimer's disease by reducing its expression. Therefore, much effort has been made to understand how to regulate BDNF transcription and translation. Over the past few years, natural antisense transcripts (NATs) have been reported to be potentially involved in most human disorders including Alzheimer's disease, Parkinson's disease, fragile X syndrome, etc. (10). NATs generally originate from the antisense strand of many protein-coding genes and often overlap with the mRNA, promoter, and regulatory regions (11). Studies have shown that BDNF is regulated by an antisense transcript known as BDNF-AS, which is expressed by the opposite strand. BDNF-AS is a long noncoding RNA (lncRNA) and is a natural antisense transcript. The gene is about 191 kb, located on chromosome 11, and contains at least 10 exons transcribed by a promoter (12).

BDNF-AS 5' exons contains about 225 nucleotides that overlap with all BDNF gene variants. Thus, it can form RNA/RNA duplex with BDNF mRNA, and it can be effective in regulating BDNF stability and translation (11), and thus can interfere with the progression of Alzheimer's disease. Both BDNF and BDNF-AS transcripts are coexpressed in many tissues, which indicates a regulatory relationship between them (12). Alzheimer's disease is currently diagnosed when severe and irreversible injuries have occurred in the brain, which makes it impossible to treat and even prevent progression of the disease (13).

Studies have shown that the onset of injuries that lead to Alzheimer's disease dates back to a couple of years ago when changes in the gene expression profiling occurred in the brain. Early diagnosis of these changes may be helpful in preventing disease progression and developing effective therapies (14). However, brain tissue is not available for early diagnosis of gene expression changes. On the other hand, cerebrospinal fluid (CSF) is a good source of research and diagnosis of neurological diseases, including Alzheimer's disease, but its clinical application is limited due to the invasive nature of the procedure, especially in the elderly. In addition, the need for highly skilled people makes it unsuitable for common use.

Therefore, identifying new cost-effective and non-invasive biomarkers that would allow early detection of Alzheimer's disease is necessary. Plasma is a complex body fluid that contains proteins, peptides, lipids, and metabolites that reflects various physiological and pathologic activities of various organs, including the central nervous system (CNS) and can be a good source of biomarkers for various diseases, including neurodegenerative diseases such as Alzheimer's disease (14).

Therefore, the aim of this study is to determine the presence and comparisons of BDNF-AS in plasma of patients with Alzheimer's disease and healthy individuals, and to evaluate its potential as a plasma marker for Alzheimer's disease.

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Methods

After approval by the Ethics Committee of Tabriz University of Medical Sciences with the code TBZMED.REC.1394.1000, this case-control study was conducted among 30 patients referred to Neurology Clinic of Imam Reza Hospital in Tabriz. Diagnosis was performed by an expert in neurological diseases based on the criteria of the National Institute on Aging and the Alzheimer's Association (NIA–AA). The control group included 30 individuals who had no neurological disease and who were matched with the patients in terms of age and were selected by the expert.

In cases of non-satisfaction, lack of matching with Alzheimer's diagnostic criteria, reversible dementia including hypothyroidism, deficiency of vitamins E, B1, B12, alcoholism, use of psychotic drugs, severe depression, liver failure, renal failure, cerebrospinal fluid defects, history of subdural hematoma, history of brain trauma, history of brain infection and encephalitis, NPH, and non-Alzheimer's dementias, such as frontal lobe dementia and lewy body dementia, the patient was excluded. Informed consent was obtained from the participants or their guardian and blood samples were collected.

After collecting blood samples, the plasma of the samples was isolated and transferred to -80 °C freezer. In order to extract RNA, the plasma was first placed out of the -80 °C freezer, and RNA extraction from plasma samples was performed according to the manufacturer's protocols using the TRIzol Reagent (Ambion). After extracting RNA, the concentration and quality of RNA were evaluated using a NanoDrop instruments. Prior synthesizing cDNA to eliminate possible contamination of DNA, treatment with the DNase I (Thermo Scientific) enzyme was performed. After treatment, cDNA was synthesized from the RNA obtained from each sample using the PrimeScript™ RT reagent kit. PCR reaction was used to indicate the presence or absence of BDNF-AS in plasma.

PCR reactions was done in the final volume of 25 microliter under the following conditions; primary denaturation at 94°C for 3 minutes; 35 cycles including denaturation at 94 °C for 30 seconds; primer connection at 60 °C for 30 seconds; expansion at 72 °C for 15 seconds and final expansion at 72°C for 5 minutes.

Proliferation was done by BDNF-AS primers, Forward: 5'-TGTAGAGATGAGCCCAAGGA-3' and Reverse: 5'-TGGTTAGATGAATTTGTGTTGT-3' and RNU6 primers, Forward: 5'-CTCGCTTCGGCAGCACAT-3' and Reverse: 5'-GGAACGCTTCACGAATTTGC-3' as an internal control designed by Gene Runner software. Primers were synthesized by Metabion Company, Germany. To perform the PCR reaction, 5 µl Master mix2x, and 0.3 µl of each forward and reverse primer with a concentration of 10 pmol, 1 µl cDNA and 3.4 µl twice-distilled water were added to each Reaction Vial. The final volume of the reaction is 10 µl. The reaction was performed on the PeQlab machine. In these experiments, the presence or absence of BDNF-AS transcripts in plasma samples was the measured variable, and its measurement criterion was to observe the electrophoresis band created by proliferation in the PCR reaction.

To carry out the real-time PCR reaction, 5 µl Real Q Plus Master 2x (Ampliqon Company), 0.2 µl of each forward and reverse primer with a concentration of 10 pmol, 4 µl of cDNA and 0.8 µl twice-distilled water were added to each Reaction Vial. The final volume of the reaction is 10 µl. The reaction was performed by Step One Plus (ABI Company).

Two repetitions were considered for each sample and the average Ct was calculated for each sample. The RNU6 gene was used as an internal control. Finally, the $2^{-\Delta ct}$ formula was used to estimate the plasma level of BDNF-AS in the samples. Independent t-test with significant level of $p<0.05$ was used for comparison of plasma levels in the two groups.

Results

Investigating the presence of BDNF-AS in plasma: Performing PCR with specific BDNF-AS primers on plasma samples and electrophoresis of PCR products on agarose gel indicated a specific 123 bp band. The experiment was performed on 10 Alzheimer's samples and 5 healthy samples, and proliferation and specific 123 bp band was observed all of them. The results of proliferation and electrophoresis of three samples are shown in Fig 1. Considering the positive result, BDNF-AS transcripts were studied in plasma samples by real-time PCR.

Examining the levels of BDNF-AS in the Plasma of Controls and Patients: A quantitative real-time PCR method was used to determine the levels of BDNF-AS in plasma samples of healthy group and Alzheimer's group. For normalization, the amount of transcripts of RNU6 gene was used as an internal control with almost constant expression in different tissues. The

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results showed that the mean plasma levels in Alzheimer's samples was 0.107 ± 0.021 and in healthy controls was 0.039 ± 0.006. Comparison of these numbers showed that BDNF-AS levels differed significantly between the patients in the patients group and the healthy group, which is higher in patients and this difference is statistically significant ($p < 0.001$) (Fig. 2).

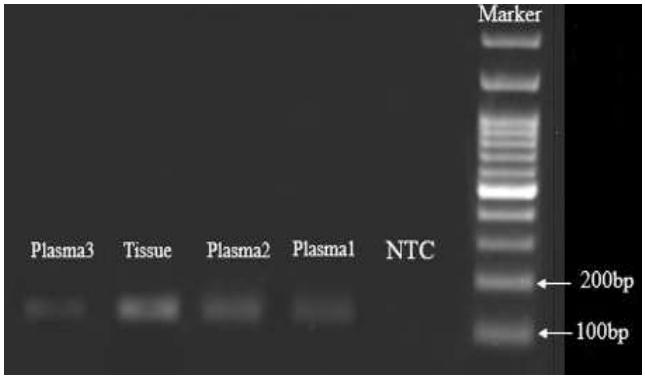

Figure 1. The electrophoresis gel represents the PCR products for BDNF-AS proliferation in various samples. The bands are respectively Marker, NTC (no template control), Plasma 1, 2, and 3 (Plasma samples 1, 2, and 3), and Tissue (Positive PCR Control using control tissue samples).

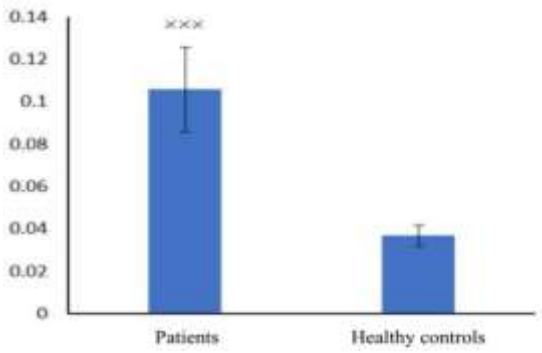

Figure 2. Plasma levels of BDNF-AS in the patient group and control group. (***)indicate a significant level at p<0.001

Investigating BDNF-AS Changes in Men and Women: The mean plasma level of BDNF-AS in men was 0.085±0.021 and in women was 0.063±0.015 (Fig 3). Although the mean plasma levels of BDNF-AS in men is higher than that of women, comparison of these numbers showed that with 95% confidence interval, the observed difference between men and women is not statistically significant. Therefore, plasma levels of BDNF-AS is not associated with the gender of the individuals.

Investigating the relationship between plasma BDNF-AS and age: To examine the relationship between plasma BDNF-AS levels and age, the test samples were divided into two groups of patients over 75 years of age and below 75 years. The mean of relative plasma concentrations in samples over 75 years was 0.085±0.02 and in the group below 75 years was 0.059±0.012. The mean plasma levels of BDNF-AS transcripts in samples over 75 years of age was more than those below 75 years of age. But this difference is not statistically significant (Fig 4).

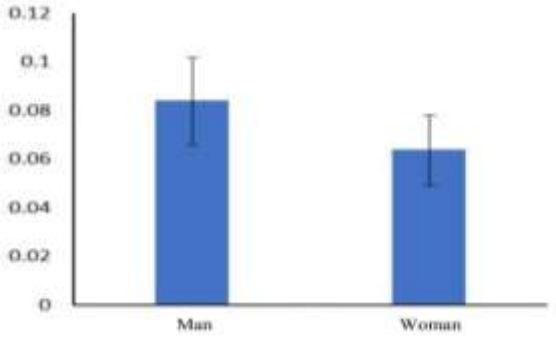

Figure 3. Comparison of BDNF-AS plasma levels between males and females (p > 0.05)

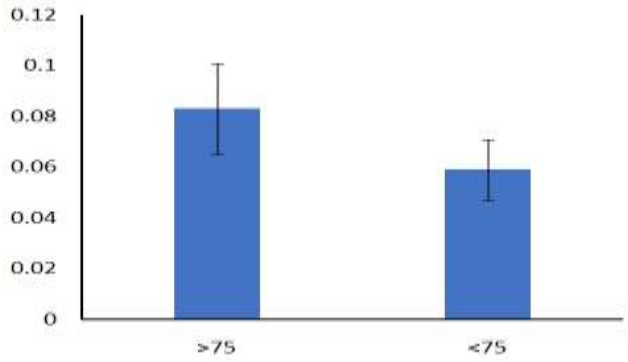

Figure 4. Comparison of plasma levels of BDNF-AS between the two groups of over 75 years and below 75 years (p>0.05)

Discussion

The present study showed that BDNF-AS is present in plasma of healthy individuals and patients with Alzheimer's disease and can be detected by PCR method. In addition, comparing the plasma levels of BDNF-AS transcripts in the patients and healthy individuals showed that the level of this long noncoding RNA in the plasma of patients with Alzheimer's disease is increased compared with healthy subjects. BDNF involvement in memory

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function (16–19) and its expression in Alzheimer's disease (20–22) have been confirmed in previous studies. In addition, the effect of BDNF-AS on BDNF and its role in BDNF function has been proven (11), but until now, there has been no report on the plasma levels of BDNF-AS in both healthy people and in patients with different diseases. However, there are reports on serum levels of BDNF protein and not BDNF-AS in patients with Alzheimer's disease. Borba et al. reported that serum levels of BDNF decreased in patients with Alzheimer's disease (8). Reports about the serum level of BDNF protein are contradictory. However, a meta-analysis conducted in 2016 indicated a reduction in serum levels in patients with Alzheimer's disease (15).

Considering the inhibitory role of BDNF-AS in expressing the BDNF gene (11), we can say that the results of our study, which indicate high levels of BDNF-AS in patients with Alzheimer's disease, are consistent with the results of studies that show low levels of BDNF in the serum of patients with Alzheimer's disease. It has been shown that inhibiting or eliminating BDNF-AS both in vitro and in vivo can increase BDNF levels (11), and naturally, BDNF-AS increase leads to BDNF decline. Considering the higher average life expectancy of women compared to men, the prevalence of Alzheimer's disease among women is higher than males (13). In the present study, comparing plasma levels of BDNF-AS in men and women showed that BDNF-AS plasma levels in patients with Alzheimer's disease were not associated with gender. This finding indicates that the BDNF-AS plasma level is independent of gender and that changes in its levels is due to Alzheimer's disease and thus can be a suitable indicator of Alzheimer's disease. In addition, late-stage Alzheimer's disease is an age-related disease and the chance of developing Alzheimer's disease increases with age (13).

The results of this study regarding the association between BDNF-AS plasma levels and age showed that plasma BDNF-AS level was not significantly correlated with age, and therefore the observed changes in plasma BDNF-AS levels between patients with Alzheimer's disease and healthy controls is not due to age, and is related to Alzheimer's disease. The results of this study showed that BDNF-AS is found in blood and plasma of patients with Alzheimer's disease and healthy people and can be detected by PCR. Moreover, plasma BDNF-AS levels in patients with Alzheimer's disease are significantly higher than healthy subjects, and this increase is not related to the gender and age of individuals. Therefore, BDNF-AS can be considered as a blood / plasma marker for Alzheimer's disease.

Conflict of Interest:

No conflicts of interest.

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