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Expression Analysis of *BDNF* Gene and *BDNF-AS* Long Noncoding RNA in Whole Blood Samples of Multiple Sclerosis Patients: Not Always a Negative Correlation between Them

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ABSTRACT

Multiple sclerosis (MS) is a chronic inflammatory disorder of the central nervous system (CNS), in which axonal damage is a deteriorative factor. Brain-Derived Neurotrophic Factor (BDNF) is described as a neuronal-survival gene, also capable of exerting pleiotropic effects on the immune cells. Here, we aimed to investigate expression levels of *BDNF* and its antisense RNA, *BDNF-AS*, in Iranian MS patients.

Our case-control study was based on collecting 50 whole blood samples of relapsing-remitting MS patients and 50 healthy controls. Then, expression analysis of *BDNF* and *BDNF-AS* was performed by Real-time quantitative PCR.

We found a strong and positive correlation between *BDNF* and *BDNF-AS* in MS patients. This is while no significant difference in *BDNF* and *BDNF-AS* expression levels was seen between MS patients and controls ($p>0.05$). A significant and strong positive correlation was found between the expression levels of *BDNF-AS* and *BDNF* ($r=0.785$, $p<0.0001$). Further, significant positive moderate correlations of *BDNF* and *BDNF-AS* with other lncRNAs (*GSTT1-AS1* and *IFNG-AS1*) and genes (*TNF* and *IFNG*) were revealed ($p<0.0001$). Additionally, there was no correlation between the *BDNF* and *BDNF-AS* expressions and disease duration, age at onset, and Expanded Disability Status Scale of Kurtzke (EDSS) ($p>0.05$).

BDNF and *BDNF-AS* expression levels revealed insignificant discrepancies in patients and controls. We found a strong and positive correlation between *BDNF* and *BDNF-AS* in MS patients, which is, based on previous studies, a quit novel finding and can be further discussed by future works to unravel its possible application in MS. We suggest evaluation of different leukocytes

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subsets separately along with large cohort studies comprising a higher number of individuals from different ages to unravel the effects of other possible aspects.

Keywords: Brain-derived neurotrophic factor; Brain-derived neurotrophic factor antisense RNA; Gene expression; Multiple sclerosis

INTRODUCTION

Multiple sclerosis (MS) is a chronic inflammatory disorder of the central nervous system (CNS) including the brain, spinal cord and optic nerves, which is depicted by inflammatory demyelination and neurodegeneration. Commonly, MS manifests in young adults and develops towards increased disability over time. It is speculated that main cause of MS is attributed to the infiltration of autoreactive T cells that detect and react against autoantigens.[1,2] Several genetic causes are known to contribute to the risk of MS. Meanwhile, environmental aspect might also provoke the onset in genetically predisposed individuals.[3]

The neurobiogenesis pathway appears to be vital in the neurodegeneration process of MS.[4] Recently, new insights into CNS tissue damage and repair in the course of chronic inflammatory disorders have resulted from experimental evidence that some immune responses might play a neuroprotective role.[5,6] Brain-derived neurotrophic factor (*BDNF*) is described as a neuronal-survival and growth-promoting gene also capable of exerting pleiotropic effects on the immune cells within the inflammatory lesions of MS patients.[7,8] Immunohistochemical studies, moreover, showed that *BDNF*, in the context of MS-related inflammatory reactions, is released not only by neurons but also by T cells, microglia, macrophages and reactive astrocytes.[9,10]

Next, we focused on *BDNF antisense RNA* (*BDNF-AS*) which is identified to be a naturally conserved long noncoding RNA (lncRNA). LncRNAs are known as principal regulators of the genome expression and their effects in various physiological and pathological processes have been widely studied.[11,12] It was observed that *BDNF-AS* lncRNA can suppress *BDNF* transcription in numerous cell populations.[13-15] *In vivo* and *in vitro* studies has demonstrated that down-regulation of *BDNF-AS* can inversely up-regulate *BDNF*, suggesting pro-neuronal effects in neuronal differentiation and outgrowth.[14]

Altogether, here we aimed to investigate the expression levels of *BDNF* gene and *BDNF-AS* lncRNA in Iranian MS patients.

MATERIALS AND METHODS

Patient and Control Groups

Here, we recruited 50 Iranian sporadic MS patients (all relapsing-remitting type) and 50 sex- and age-matched control subjects (37 women and 13 men). Patients (38 females and 12 males) were diagnosed by McDonald criteria and MRI (Magnetic Resonance Imaging) via expert neurologists. All patients were treated with Interferon (IFN)-β therapy for at least two years (intramuscular injection of 20 μg of CinnoVex [CinnaGen Co, Tehran, Iran] three-times a week) and were categorized as IFN-β responders.[16,17] Samples were collected from MS Society of Iran and some hospitals from Tehran. Importantly, HLA-DRB1*15, as a critical risk factor for MS, was ruled out in all patients. In the control subject, anyone without a family history of autoimmune disease and cancer was included as control group.

Blood Sampling

The entire protocol and measurements of our study were in line with Ethics Committee of Shahid Beheshti University of Medical Sciences guidelines (IR. SBMU. MSP.REC.1396.876). Blood samples were collected from all participants in this study. Both MS patients and healthy controls gave their informed consent for incorporation in this work. Afterwards, clinical information of patients was obtained.

Quantitative Real-time PCR

Total RNA was isolated by using GeneAll Hybrid-R™ blood RNA extraction kit (cat No. 305-101, South Korea). Then, cDNA synthesis was carried out through kit of Biosystems High-Capacity cDNA Reverse Transcription (PN: 4375575, USA) according to the manufacturer's instruction. Allele ID6 (Premier Biosoft, Palo Alto, USA) was utilized to design the primers

(PCR product lengths and primer sequences have been summarized in Table 1). Beta-2-microglobulin (*B2M*) was considered as a reference gene in order to normalize the expression level for each sample.

SYBR Green-based Real-time quantitative PCR assay was conducted in triplicates in Corbett Rotor Gene 6000 machine (Corbett Life Science, Australia). Routinely, the NTC (No Template Control) sample was included for each primer in each run.

Statistical Analysis

We used SPSS version 18 (Chicago, IL, USA) for statistical analyses. Analysis of differences between two groups was done by independent t-test. Moreover, Pearson coefficient was utilized to study the correlations between variables.

Results were regarded statistically significant if *p* values were <0.05. Spearman correlation test was carried out to assess the possible correlations between *BDNF-AS* and *BDNF* relative expression levels.

Table 1. Sequences of specific primers designed to evaluate the expression levels of *beta-2-microglobulin* (*B2M*), *brain-derived neurotrophic factor* (*BDNF*), and *BDNF antisense RNA* (*BDNF-AS*) in relapsing-remitting multiple sclerosis (RR-MS) patients and healthy controls

Gene name	Primer sequence	Primer length	Product length
B2M	F: AGATGAGTATGCCTGCCGTG	20	104
	R: CGGCATCTTCAAACCTCCA	19	
BDNF	F: GATGCTGCAAACATGTCCATGAG	23	109
	R: TTTTGTCTGCCGCCGTTACC	20	
BDNF-AS	F: GTGGGTCCATTCCGTGTGTG	20	97
	R: AGCTGGTGCAGGTATCAGATTAG	23	

RESUTLS

The clinical information of both MS patients and healthy individuals is provided in Table 2. All the subjects' results were evaluated in the following three categories: results of total subjects (regardless of their sex and age), sex-related results (male or female) as well as age-linked results (<30, 30–40 as well as 40< years). In this way, all of the patients were compared with healthy controls and independently analyzed regarding sex and age.

Relative Expression Level of *BDNF* Gene

The data of all MS patients displayed an elevated, though statistically not significant, level of expression for *BDNF* observed between patients and control groups (Table 3). Of note, the most considerable difference was for male patients >40 years (3.6-fold change) compared with controls.

Relative Expression Level of *BDNF-AS* LncRNA

BDNF-AS relative expression differences in MS and control groups was not significant ($p>0.05$). *BDNF-AS* expression in male patients had a two-fold increase; however, this increment did not reach a statistically significance. Expression ratio of *BDNF-AS* was represented as Table 4.

Correlation Analysis between *BDNF* and *BDNF-AS* with Expanded Disability Status Scale (EDSS)

The correlations between *BDNF* and *BDNF-AS* with EDSS were not statistically significant (r=-0.049, $p=0.739$ and r=-0.087, $p=0.548$, respectively).

Correlation Analysis between *BDNF* and *BDNF-AS* with Disease Duration

The correlation between *BDNF* gene and *BDNF-AS* lncRNA with disease duration did not reach a statistical significance in our analysis (r=0.025, $p=0.861$ and r=-0.056, $p= 0.698$, respectively).

Correlation Analysis of *BDNF* and *BDNF-AS* with Age at Onset

Correlation analysis results showed that there is an insignificant correlation between expression levels of *BDNF* (r=0.121, $p=0.405$) and *BDNF-AS* (r=0.234, $p=0.102$) with age at onset of MS disease.

Correlations between Expression Level of *BDNF* and *BDNF-AS*

After Spearman correlation analysis, a significant and strong positive correlation was found between the expression levels of *BDNF-AS* and *BDNF*, as illustrated in Figure 1 (r=0.785, p<0.0001).

Table 2. Clinical and demographic information of relapsing-remitting multiple sclerosis (RR-MS) patients and healthy controls

Variables	Multiple sclerosis patients	Controls
Female/Male [no. (%)]	38 (76%)/12(24%)	37 (74%)/13(26%)
Age (mean±SD, Y)	36.2±2.7	35.3±2.4
Age range (Y)	17-55	22-60
Age of onset (mean±SD, Y)	31.41±2.8	-
Relapsing-remitting course (no. %)	100 (100%)	-
Duration (mean±SD, Y)	4.58±3.2	-
EDSS* (mean±SD)	3.07±2.5	-

*EDSS: Expanded Disability Status Scale of Kurtzke.

Table 3. The relative expression ratio of *brain-derived neurotrophic factor (BDNF)* in total, sex-based , and age-based categories

BDNF Expression		Control no.	Multiple sclerosis patient no.	Expression ratio	SD	*p*-value	95% Credible interval (CrI)
Total		50	50	1.280869	0.06	0.709	[-0.94, 0.46]
Male		13	12	2.992198	0.84	0.86	[-1.3, 1.7]
Female		37	38	1.039076	-0.17	0.629	[-1.26, 0.47]
<30	Male	0	1	-	-	-	-
	Female	10	6	1.7168	-2.2	0.666	[-5.3, 7.3]
30-40	Male	2	5	1.0378			
	Female	5	15	2.8479	3.7	0.462	[-9.2, 5.8]
>40	Male	11	6	3.6446	0.83	0.705	[-1.83, 2.44]
	Female	22	17	1.1397	-0.11	0.218	[-1.5, 0.55]

Table 4. The relative expression ratio of *brain-derived neurotrophic factor antisense RNA (BDNF-AS)* in total, sex-based, and age-based categories

BDNF- AS1 Expression		Control no.	Multiple sclerosis patient no.	Expression ratio	SD	*p*-value	95% Credible interval (CrI)
Total		50	50	1.0617	0.17	0.513	[-0.94, 0.42]
Male		13	12	2.1055	0.6	0.53	[-0.33, 1.72]
Female		37	38	0.8545	0.06	0.409	[-1.33, 0.24]
<30	Male	0	1	-	-	-	-
	Female	10	6	3.0852	-0.96	0.49	[-5.02, 5.92]
30-40	Male	2	5	0.9852			
	Female	5	15	0.9926	3.1	0.296	[-8.5, 4.1]
>40	Male	11	6	1.7770	.37	0.884	[-2.19, 2.05]
	Female	22	17	0.7734	-0.14	0.056	[-1.79, 0.143]

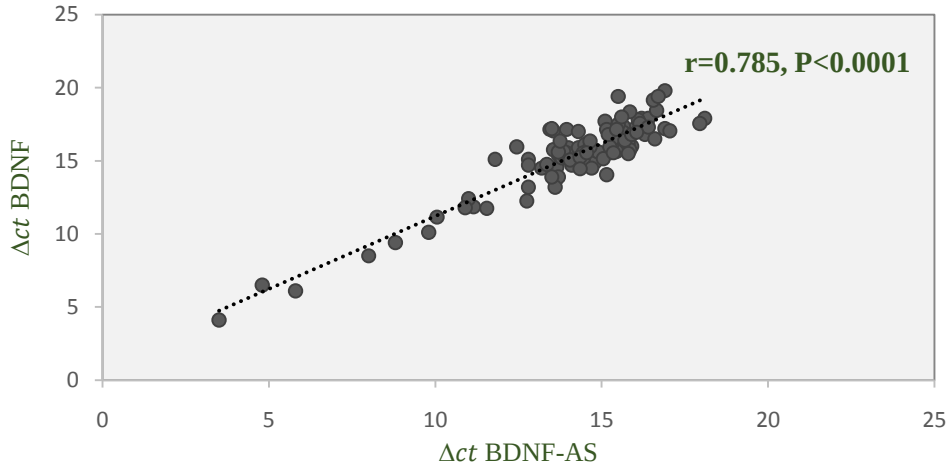

Figure 1. Correlations between the expression levels of *brain-derived neurotrophic factor (BDNF)* and *BDNF antisense RNA (BDNF-AS)* in relapsing-remitting multiple sclerosis (RR-MS) patients.

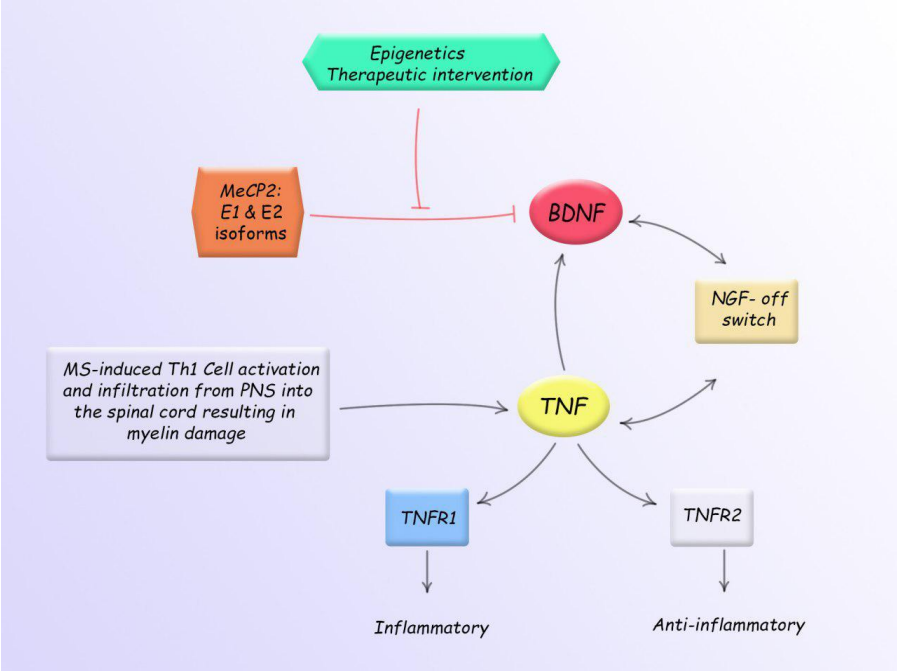

Figure 2. Schematic view of the brain-derived neurotrophic factor (*BDNF*), tumor necrosis factor (*TNF*) and nerve growth factor (*NGF*) signaling triad. Activation of T cells results in *TNF* expression leading to the induction of *BDNF* and *NGF* expressions. *BDNF* acts as a chemo-attractant signal which draws NGF into the regions of increased *BDNF*. *BDNF* moreover induces expressions of *NGF* and *TNF*. Increased *NGF* expression suppresses *TNF* from signaling through tumor necrosis factor receptor 1 (TNFR1). This causes suppressing of the TNF/TNFR1 inflammatory effects- that brings cell damage and apoptosis. On the other hand, increased *NGF* expression promotes the preferential TNF signaling via tumor necrosis factor receptor 2 (TNFR2). The TNF/TNFR2 anti-inflammatory effects promotes remyelination and/or myelin repair. MeCP2 acts as a transcriptional repressor of *BDNF*. Further, therapeutic interventional strategies are recognized to suppress activity of MeCP2 and consequently removing repressive effects of MeCP2 from *BDNF*. This resultant elevation in *BDNF* would restore the homeostatic balance between different cytokines and neurotrophins which is of essential importance in the process of remyelination and/or myelin repair.

DISCUSSION

In this study we compared the expression levels of *BDNF* and *BDNF-AS* in the 50 RR-MS patients versus 50 healthy subjects. We tried to match completely all participants in our sampling. To do so, samples were categorized with regards to sex and age. Also, those patients who were HLA-DRB1*15 positives were excluded due to its major genetic susceptibility for MS.

On the other hand, though expression levels of *BDNF* and *BDNF-AS* have been investigated in different subtypes of blood leukocytes,[6,18-22] to best of our knowledge, no molecular study has been so far performed on whole blood samples comparing their expression levels. Of note, some studies have evaluated BDNF concentrations in serum or plasma, but all of them were restricted to protein assays such as ELISA.[19,20,23,24]

Converging lines of evidence support *BDNF* as a candidate molecular effector in neuroprotective autoimmunity and inflammation, which is produced in peripheral blood and MS lesions by immune cells.[25] It has been recently reported to exert beneficial effects on experimental autoimmune encephalomyelitis (EAE), an animal model that histopathologically and clinically mimics MS. however, *BDNF* has not been demonstrated to have any measurable effect on the clinical progression as well as quantitative magnetic resonance imaging (MRI) parameters.[26]

In the current study, after our analysis, no statistically difference was seen neither in *BDNF* expression level nor that of *BDNF-AS* between groups of patients and healthy subjects. Although there was an increased level for *BDNF* in all subgroups of patients and considerable changes in females between 30-40 years old as well as males>40 years old subgroups (3.6- and 2.8-fold changes, respectively) compared with controls, none of them reached significance. Again, for *BDNF-AS* none of patients' subgroups showed significance even subgroup of females <30 years old (3-fold change). This is well in line with Lindquist and Kalinowska-Łyszczarz works who found no quantitative change in BDNF protein in freshly isolated peripheral blood mononuclear cells (PBMCs) of MS patients,[27,28] and in contrast to those exhibiting significant changes.[19,22] One of the most straightforward explanations for this might be different sample sizes and heterogeneity of MS that, in part, contributes to these results. Meanwhile, there are studies exhibiting that direct effects of *BDNF* on myelination were not found, as suggested by models of spinal cord injury.[29-31] It can be, further, proposed that different mean age of our patients (as collected by random sampling) versus mean age of other studies may come up with different consequences. For instance, Lommatzsch et al observed that BDNF levels in plasma was significantly down-regulated with increasing age.[23] Such conflicting results regarding altered *BDNF* expression in MS patients can be most likely because of inclusion of patients with subclinical disorder activity or different immunotherapy, and also in part due to various biological materials analyzed.[27]

Role of *BDNF-AS1* as a potential biomarker in cancer development in human retinoblastoma has previously been described.[32] Particularly, *BDNF-AS* levels was reported to negatively correlate with the level of *BDNF* Mrna.[14,33] It was found that *BDNF* was normally repressed by *BDNF-AS*.[25] LncRNAs are thought to regulate the expression of target genes through binding to complementary sequences.[34] *BDNF* mRNA contains the *BDNF-AS* complementary sequences. However, caution is required to directly link *BDNF-AS* to *BDNF* in MS. In one study it was noted that stability of *BDNF* sense RNA was not affected by *BDNF-AS*.[14] All these lines of evidence suggest that a more complicated pathway would associate with *BDNF* and *BDNF-AS* regarding their inter-regulation.[13]

Next, our data revealed no correlation with clinical parameters such as disease duration, age at onset and EDSS, which was confirmed by another study at protein level.[18] A strong and positive significant correlation ($r=0.785$, $p<0.0001$) was furthermore observed between *BDNF* and *BDNF-AS*, implying their strong interaction in MS. Interestingly, previous studies suggested a negative correlation between them; nonetheless, our study provided contrary results in whole blood samples of MS remission phase.[14,33] We can speculate due to the fact that our sampling was conducted on patients in remission phase of MS, axonal degeneration might be more emphasized in relapsing phase.[35,36] Indeed, increased levels of BDNF during acute relapses were observed in homogenous groups of either treated or untreated patients.[26,37-39]

It was recently proposed that *BDNF* and *BDNF-AS* can interact with some components of epigenetic pathways and methylation process (Figure 2).[8] Consistent with that, *BDNF* was reported to be regulated at transcriptional level by methyl CpG

binding protein 2 (MeCP2).[8] This can be taken as a new mechanism in re-myelination and/or myelin repair in MS. Partly, activation of T cells can result in *TNF* expression leading to the *BDNF* induction. Then, BDNF acts as a chemo-attractant signal which draws nerve growth factor (NGF) into regions with elevated *BDNF* (Figure 2).[8]

Briefly, *BDNF* and *BDNF-AS* expression levels revealed insignificant discrepancies in patients and controls. We found a strong and positive correlation between *BDNF* and *BDNF-AS* in MS patients, which is, based on previous studies, a quit novel finding and can be further discussed by future works to unravel its possible application in MS. As a limitation of our study it would be better to measure mRNA levels of these genes in different phase of the multiple sclerosis patients. We suggest evaluation of different leukocytes subsets separately along with large cohort studies comprising a higher number of individuals from different ages to unravel the effects of other possible aspects.

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