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ORIGINAL RESEARCH

Functional BDNF rs7124442 Variant Regulated by miR-922 is Associated with Better Short-Term Recovery of Ischemic Stroke

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Background: Brain-derived neurotrophic factor (BDNF) is the most abundant neurotrophin, which contributes to the neuronal survival and synaptic plasticity. This study investigated the associations of *BDNF* polymorphisms at the 3′-untranslated region with risk and outcome of ischemic stroke in a Chinese Han population.

Methods: 500 patients and 520 controls were enrolled for *BDNF* rs7124442 genotyping. The binding of miR-922 to *BDNF* rs7124442 was examined by luciferase assay; *BDNF* expression was assessed using qRT-PCR.

Results: Alcohol consumption, cigarette smoking, diabetes, hypertension (all $P < 0.001$) and higher serum triglycerides concentration ($P = 0.009$) were associated with an increased risk of developing ischemic stroke. After adjusted for age and sex, logistic regression analysis showed that IS patients harbored with rs7124442 TC genotype had a milder initial stroke (Dominant model: OR = 0.45, 95% CI = 0.25–0.81, P = 0.015), and also showed a better short-term recovery (Dominant model: OR = 0.39, 95% CI =0.24–0.68, P = 0.003). Furthermore, we found that co-transfection of hsa-miR-922 mimics with *BDNF* 3′-UTR containing the mutated allele C changed luciferase activity when compared to co-transfection with *BDNF* 3′-UTR containing the wild-type allele. Besides, patients carrying *BDNF* rs712444 TC or CC genotype had an increased level of *BDNF* compared with patients with the TT genotype.

Conclusion: Our study demonstrates that the SNP rs7124442 in *BDNF* 3′-UTR, through affecting the regulatory role of miR-922 in *BDNF* expression, might act as a protective factor for the outcome of patients with ischemic stroke.

Keywords: ischemic stroke, polymorphism, brain-derived neurotrophic factor, *BDNF*, miR-922, outcome

Introduction

Stroke is a major reason for death and disability of people in the world.[1] Ischemic stroke (IS), which is usually caused by blockage of blood vessels, accounts for about 80% of all strokes. Previous data demonstrated that 6.9 million patients had ischemic stroke in 2013.[2] In China, the estimated mortality rate caused by stroke is 157 per 100,000 every year,[3] and the stroke burden continues to increase. Traditional risk factors of stroke include diabetes mellitus, hypertension, high blood cholesterol, etc., suggesting the important role of genetic factors in stroke.[4]

Brain-derived neurotrophic factor (BDNF) is the most abundant neurotrophin, which contributes to the neuronal survival, synaptic plasticity, angiogenesis and outgrowth of peripheral and central neurons.[5,6] In experimental model studies of stroke,

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both intravenous and intraventricular BDNF injections could reduce infarct size and showed neuroprotective effects.[7] In addition, evidence showed that *BDNF* is associated with some neuropsychiatric disorders.[8] The most well-studied *BDNF* single nucleotide polymorphism (SNP) rs6265 has been reported to be related to brain morphology changes and cortical plasticity.[9] In the context of stroke, Qin et al[6] and Vliet et al[10] reported that *BDNF* rs6265 polymorphism was associated with motor recovery of stroke patients. Thus, this study mainly focused on the contributions of *BDNF* genetic variants to ischemic stroke.

Micro RNAs (miRNA) can degrade mRNA by binding to its 3′-untranslated region (3′-UTR), which is one of the post-transcriptional gene regulation mechanisms.[11] Previous studies have reported that SNPs in 3′-UTR of mRNA were associated with the occurrence of different diseases.[12,13] In this study, we investigated the unreported SNPs in the 3′-UTR of *BDNF*. With the bioinformatics software (http://www.bioguo.org/miRNASNP/), we predicted all candidate SNPs that may regulate *BDNF* expression. Polymorphism rs7124442 was identified as the only potential SNP via regulating the binding of miR-922 and its relationship with ischemic stroke was further investigated.

Materials and Methods

Study Subjects

In this study, we included 500 ischemic stroke patients from Jiangyin People's Hospital (Jiangyin, China) between June 2010 and August 2016. 520 age- and gender-matched healthy controls were also recruited. Ischemic stroke was diagnosed based on the World Health Organization criteria.[14] A questionnaire was filled by all patients and controls. It contains demographical information, medical history such as diabetes mellitus and hypertension, and other vascular risk factors. The definition of vascular risk factors has been detailed described in a previous study.[16] This study was conducted in accordance with the Declaration of Helsinki. An informed consent was signed by all participants and the Ethics Committee of Jiangyin People's Hospital approved this study (Project identification code: 2016–011).

Stroke Severity and Outcome Measures

At the time of presentation and 3 months post-stroke, the National Institutes of Health Stroke Scale (NIHSS) was scored to measure stroke severity and functional changes of patients. The NIHSS score was skewed and not normally distributed, and thus it was dichotomized for logistic regression analysis. The mild and severe IS cutoff score was set at 6, which is consistent with previous studies.[15,16] The score change from presentation to discharge (ΔNIHSS) was used to measure the recovery of ischemic stroke. It was also skewed and dichotomized for logistic regression analysis. Its cutoff score was set at 0 and <0 means clinical improvement.

DNA Extraction and Genotyping

Total DNA of patients and controls was extracted on the basis of a standard protocol.[17] The TaqMan allelic discrimination assay was used to genotype the SNP (Applied Biosystems, San Diego, CA, USA). The PCR conditions were 50°C for 2 mins, 95°C for 10 mins followed by 45 cycles of 95°C for 15 s and 60°C for 1 min. The allelic discrimination mode of the SDS 2.3 software was used to calculate the results.

Quantitative Reverse Transcription-Polymerase Chain Reaction (qRT-PCR)

The *BDNF* mRNA levels were determined by qRT-PCR. Briefly, a Trizol reagent was used to isolate total RNA from 293T cells (Invitrogen, Carlsbad, CA, USA). After that, a TaqMan Reverse Transcription Kit was used to reverse transcribe the RNA into cDNA (ABI, CA, USA). With Realmaster Mix (SYBR Green I) (TAKARA, Dalian, China), the qPCR amplifications were then performed under the following condition: 95°C for 10 mins followed by 40 cycles of 95°C for 30 s, 55°C for 40 s and 72°C for 30 s, and finally 4°C for 30 mins.

Prediction of miRNAs Binding to the SNP

The potential miRNAs that could be affected by SNPs in the 3′-UTR of *BDNF* were predicted by a bioinformatics software (http://bioinfo.life.hust.edu.cn/miRNASNP2/index.php). In brief, the target sites of SNPs in 3′-UTR were predicted using two different methods. Four kinds of results are categorized as SNP targetscan site (ST), SNP miranda site (SM), wild targetscan site (WT), wild miranda site (WM). If a target shows both in ST and SM, but not in WT or WM, we called the gene gained the target site. On the other side, the gene lost the target site.

Construction of Plasmids and 3′-UTR Reporter Assay

The 3′-UTR fragments containing the C or T allele were amplified using PCR and the results were verified by DNA sequencing. Then, we cloned the PCR productions into the

pGL3-promoterless luciferase-based plasmid (Promega, CA, USA) at the cloning site between BamHI and XhoI. For reporter assay, Lipofectamine 2000 (Invitrogen Corp, CA, USA) was used to transfect cells with 100 ng of pGL3-*BDNF* wild, pGL3- *BDNF* mutant and miR-922 mimics, respectively. Renilla luciferase vector pRL-SV40 (5 ng) was co-transfected as a control for transfection efficiency.

ELISA of Serum BDNF Levels

ELISA kit (Green Stone, Bern, Switzerland) was used to measure BDNF levels according to the manufacturer's instructions. Concentrations of serum BDNF were calculated by mean absorbance of each sample at 460 nm.

Statistical Analysis

Demographic data were compared using two-sample *t*-tests and chi-square (χ^2) test. Univariate and multivariate logistic regression analysis were used to analyze the relationships between different genotypes and IS risk, severity and outcome. Luciferase activities and serum BDNF levels between different genotypes were compared using independent-sample *t*-test. All statistical analyses were performed using Stata/SE (V.12.0 for Windows; StataCorp LP, College Station, TX, USA) and two-tailed $P < 0.05$ was considered statistically significant.

Results

Demographic Characteristics of Patients and Control Subjects

Table 1 shows the demographic characteristics of IS patients and controls. Significantly higher proportion of drinking ($P < 0.001$), smoking ($P < 0.001$), suffering from diabetes ($P = 0.003$) and hypertension ($P < 0.001$) were observed in patient group compared with control group. As expected, these observations confirmed that drinking, smoking, hypertension and diabetes were risk factors for IS. As compared with control group, a significant higher serum triglycerides concentration was also observed in the patient group ($P = 0.009$).

Table 1 Demographic Characteristics of IS Patients and Controls

Characteristics	Case (n=500)	Control (n=520)	P-value
Age (year)	65 (51–78)	67 (50–81)	0.188
Sex (male) (%)	295 (59.0)	323 (62.1)	0.309
Smoking (%)	192 (38.4)	120 (23.1)	<0.001
Drinking (%)	238 (47.6)	116 (22.3)	<0.001
Diabetes (%)	116 (23.2)	68 (13.1)	<0.001
Hypertension (%)	317 (63.4)	201 (38.7)	<0.001
BMI ≥25 kg/m^2 (%)	175 (35.0)	174 (33.4)	0.605
Total cholesterol (mmol/L) (mean± SD)	4.28± 1.23	5.27 ± 1.11	<0.001
Triglycerides (mmol/L)(mean+SD)	2.18 ± 1.27	1.98 ± 1.16	0.009
HDL-C (mmol/L) (mean ± SD)	1.29 ± 0.57	1.34 ± 0.32	0.083
LDL-C (mmol/L) (mean ± SD)	2.57 ± 0.78	2.63 ± 0.62	0.173

Abbreviation: SD, standard deviation.

Identification of BDNF SNPs in 3′-UTR

To identify candidate BDNF SNPs in 3′-UTR, NCBI single nucleotide polymorphism database (https://www.ncbi.nlm.nih.gov/snp) was searched. The following searching parameters were used: Organism (Homo sapiens); Function Class (3′-UTR); Global MAF (0.05–0.4); Validation Status (by-1000 Genomes). Finally, three BDNF SNPs were identified (Table 2).

Table 2 SNPs Located in the *BDNF* Gene 3′-UTR

Gene	SNP	Chromosome	HGVS Names
BDNF	rs11030099	11:27656036	CM000673.2: g.27656036C>A
BDNF	rs7124442	11:27655494	CM000673.2: g.27655494C>T
BDNF	rs11030100	11:27656039	CM000673.2: g.27656039G>T

Associations of BDNF rs7124442 with IS Risk, Severity, Outcome

We then assessed genotype frequencies of these three *BDNF* SNPs in 500 ischemic stroke patients and 520 healthy controls and further investigated their associations with IS severity and outcome in patient group. There were no significant differences observed in the genotype distributions of the three SNPs between patients and controls before and after adjusting the risk factors including age, sex, smoking, drinking, diabetes, hypertension, total cholesterol, triglycerides. Moreover, when the rs7124442 was further analyzed under dominant and additive models, still no significant difference was found between patients and controls (Table 3). This suggested that *BDNF* rs11030099, rs7124442 and rs11030100 were not risk factors for ischemic stroke. However, when we next investigated the associations of the three SNPs with IS severity and outcome, there was a significant difference of rs7124442 distribution observed in mild and severe patient groups. After adjusted for age and sex, logistic regression analysis showed that patients harbored with rs7124442 TC genotype

Table 3 Genotype Frequencies of the *BDNF* rs7124442 Polymorphism Among Patient Group and Control Group

Genotype	Control (n=520)	Cases (n=500)	OR (95% CI)	P-value
rs7124442				
TT	449 (86.3%)	417 (83.4%)	1.00	
TC	60 (11.6%)	74 (14.8%)	1.23 (0.91–1.89)	0.134
CC	11 (2.1%)	9 (1.8%)	0.94 (0.60–1.47)	0.785
Dominant			1.19 (0.82–1.73)	0.207
Additive			1.17(0.88–1.56)	0.331

Notes: Logistic regression analyses adjusted for age, sex, smoking, drinking, diabetes, hypertension, total cholesterol, triglycerides.

Table 4 Association of *BDNF* rs7124442 Polymorphism with IS Severity

Genotype	Mild (n=337)	Severe (n=163)	OR (95% CI)	P-value
rs7124442				
TT	270 (80.1%)	147 (90.2%)	1.00	
TC	64 (19.0%)	10 (6.1%)	0.29 (0.14–0.61)	0.006
CC	3 (0.9%)	6 (3.7%)	1.93 (0.97–4.65)	0.117
Dominant			0.45 (0.25–0.81)	0.015
Additive			0.66 (0.41–1.06)	0.092

Notes: Logistic regression analyses adjusted for age, sex.

had a milder initial stroke (Dominant model: OR = 0.45, 95% CI = 0.25–0.81, P = 0.015) (Table 4), and also showed a better short-term recovery (Dominant model: OR = 0.39, 95% CI =0.24–0.68, P = 0.003) (Table 5).

miR-922 Binding Altered by rs7124442

We next performed a bioinformatics analysis to determine whether this *BDNF* rs7124442 SNP could have miRNA binding sites. The results showed that rs7124442 polymorphism may potentially alter miR-922 binding to *BDNF* mRNA (Figure 1A). Then, a dual luciferase assay was conducted to confirm this potential regulation. The results showed that co-transfection of hsa-miR-922 mimics with *BDNF* 3′-UTR containing the mutated allele C changed luciferase activity when compared to co-transfection with *BDNF* 3′-UTR containing the wild-type allele (Figure 1B). *BDNF* mRNA levels in IS patients were then examined. The results showed that patients carrying *BDNF* rs712444 TC or CC genotype had an increased level of *BDNF* mRNA when compared to patients carrying TT genotype (Figure 1C). Next, we tested the patients' serum BDNF concentrations. Mean BDNF serum concentrations of 21.53 ± 6.05 ng/mL in the rs7124442 TT group and 25.06 ± 3.27 ng/mL in the rs7124442 TC/CC group were measured. Student's *t*-test revealed significantly higher BDNF protein concentrations in the TC/CC group compared to TT group ($t = 5.165$, $df = 498$, $P < 0.001$). Considering the *BDNF* rs7124442 polymorphism was associated with IS severity and outcome; we then analyzed the serum BDNF concentrations in patients with different stroke severity and outcome. Mean BDNF serum concentrations of 27.11 ± 7.25 ng/mL in mild stroke group and 20.32 ± 4.67 ng/mL in severe stroke group were measured. Student's *t*-test revealed significantly higher BDNF protein concentrations in mild stroke group compared to severe stroke group ($t = 10.91$, $df = 498$, $P < 0.001$). In addition, mean BDNF serum concentrations of 26.78 ± 6.19 ng/mL in IS improvement group and 19.37 ± 5.81 ng/mL in IS deterioration group were measured. Student's *t*-test revealed significantly higher BDNF protein concentrations in IS improvement group compared to IS deterioration group ($t = 13.68$, $df = 498$, $P < 0.001$). These results showed some consistency with previous rs7124442 association analysis.

Discussion

In this study, the relationships between *BDNF* rs7124442 polymorphism and IS risk and outcome were investigated in a Chinese Han population. We observed that drinking, smoking, hypertension and diabetes were all associated with an increased IS risk. We further found that *BDNF* rs7124442 C-carriers had a milder initial stroke and a better short-term

Table 5 Association of *BDNF* rs7124442 Polymorphism with IS Short-Term Recovery

Genotype	Improvement (n=276)	No Change/Deterioration (n=224)	OR (95% CI)	P-value
rs7124442				
TT	215 (77.9%)	202 (90.2%)	1.00	
TC	58 (21.0%)	16 (7.1%)	0.30 (0.17–0.55)	<0.001
CC	3 (1.1%)	6 (2.7%)	1.48 (0.73–2.98)	0.312
Dominant			0.39 (0.24–0.68)	0.003
Additive			0.55 (0.36–0.86)	0.011

Notes: Logistic regression analyses adjusted for age, sex.

A

SNP in gene 3'UTR	miRNA	SNP location and Target site on UTR	Energy change (kcal/mol)	miRNA/SNP-target duplexes	Effect by SNP on 3'UTR
BDNF; rs7124442 (G/A)	hsa-miR-922	2326 2305-2327	Wild: -22.90 SNP: 0.00	miRNA: 3' cugcAUCAGGAUAAGAGACGACg 5' UTR: 5' ggaaUGCUUGGAAUAUCUGCUAu 3' rs7124442: G --> A	loss

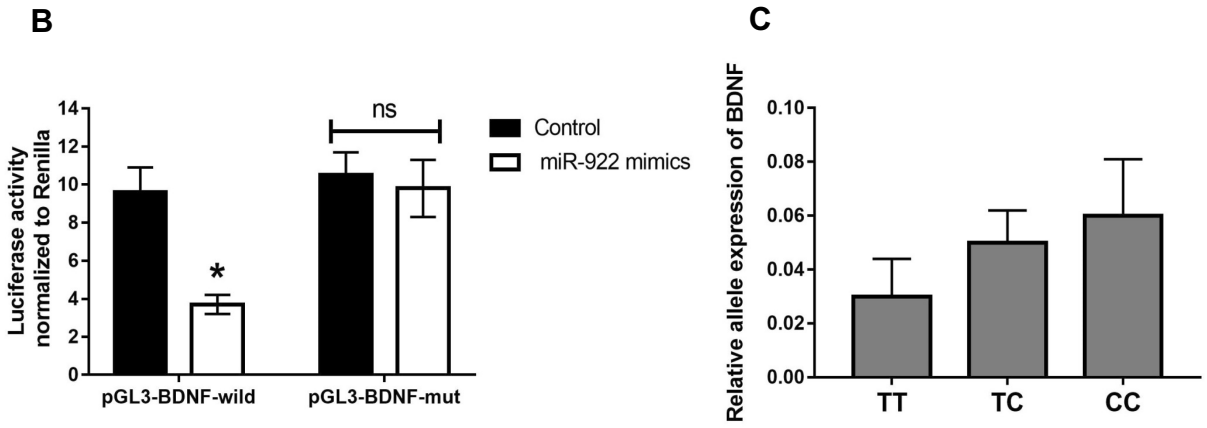

Figure 1 Binding of miR-922 to rs7124442-mutated allele upregulated the expression of *BDNF*. (**A**) Bioinformatics analysis predicted potential binding alteration caused by rs7124442 polymorphism. (**B**) Luciferase readout from wild-type or mutated *BDNF* 3′-UTR reporter co-transfected with miR-922 mimics or control. (**C**) qRT-PCR. The *BDNF* mRNA level was measured by qRT-PCR in IS patients carrying different genotypes. Data were presented as mean ± SE. *P<0.05.

recovery. In addition, our data were first to show that *BDNF* rs7124442 polymorphism could alter the binding of miR-922 to positively regulate *BDNF* expression, thus providing a post-transcriptional mechanism for gene regulation.

Previously, clinical studies have demonstrated that low circulating concentrations of BDNF are associated with poor long-term functional outcome,[18] as well as short-term functional outcome and mortality of ischemic stroke.[19] Genetic studies have revealed that *BDNF* Val66Met (rs6265), the most well-studied *BDNF* polymorphism so far, is associated with several neurological disorders. The *BDNF* rs6265 polymorphism involves a G to A substitution at position 196 (G196A), which results in the conversion of valine to methionine in coding area.[6] Evidence suggests rs6265 is associated with cortical and hippocampal plasticity, as well as brain morphology changes.[9,20–22] Clinical studies have also shown that this polymorphism is associated with motor recovery in stroke patients.[6,10] In addition, the less studied rs7124442 polymorphism is reported to affect acute mortality in patients with traumatic brain injury.[23]

Mounting evidence suggests that SNPs localize in 3′-UTRs may affect the binding of miRNAs to their target genes, causing post-translational expression changes of target mRNAs and alteration of susceptibility to disease.[13] For example, previous studies showed that rs4143815 and rs4819388 SNPs in the 3′-UTR of B7-H1 and B7-H2 genes, respectively, contribute to development of gastric cancer.[24–26] Our study firstly demonstrates the interactions between miR-922 and SNP in *BDNF* 3′-UTR.

There are some limitations needing to be considered. Our study only studied the short-term outcome of patients, and the contributions of the SNPs to the long-term recovery and functional outcomes need to be further studied. Our results should therefore be confirmed in independent samples or samples from another ethnic population. Moreover, our findings also need to be further verified in future studies with larger sample sizes.

Conclusion

In summary, our current study showed that the genetic variant of *BDNF* rs7124442 was associated with milder initial stroke severity, and also with a better short-term recovery after ischemic stroke. Furthermore, miR-922 could regulate SNP rs7124442, resulting in increased *BDNF* expression in C-allele mutants. Thus, *BDNF* rs7124442 might act as a protective factor for the outcome of patients with ischemic stroke.

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Disclosure

The authors report no conflicts of interest in this work.

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