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ORIGINAL RESEARCH

Functional Genetic Variation in the 3′-UTR NTRK2 is Associated with Risk of Ischemic Stroke

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Background: Stroke is a leading cause of death and disability worldwide. It remains difficult to treat brain injury and improve functional rehabilitation after cerebral ischemia. Brain-derived neurotrophic factor (*BDNF*) is involved in ischemic stroke (IS) through interactions in the *CREB1-BDNF-NTRk2* pathway. In this study, we aimed to determine the association of *NTRK2* gene polymorphisms and the effects of intergenetic interactions in the Chinese population.

Materials and Methods: A total of 400 patients diagnosed with IS and 400 healthy controls were enrolled for genotyping. Detailed sequence-based analysis was predicted through bioinformatical investigation. Polymorphisms associated with miRNA were analyzed by a dual-luciferase reporter assay system.

Results: Analysis of clinical characteristics revealed that IS was highly associated with exposure to cigarette smoking, alcohol intake, as well as metabolic diseases, such as diabetes, hypertension, and higher serum triglyceride concentration. Three polymorphisms in *NTRK2* located in the 3′-untranslated region (3′-UTR) were genotyped. Logistic regression analysis showed that IS patients with rs11140793, rs7047042, and rs1221 polymorphisms had a higher risk of stroke and indicated a worse short-term recovery. The mRNA level of *NTRK2* was suppressed in a mutant genotype compared with wild genotype. The suppression of *NTRK2* was induced by the gain-of-binding ability of certain miRNAs through the direct binding of 3′-UTR.

Conclusion: Our research indicated that, by influencing the expression of *NTRK2*, the SNPs rs11140793, rs7047042, and rs1221 in the 3′UTR of *NTRK2* can be used as risk factors for IS patients.

Keywords: bioinformatics, *NTRK2*, ischemic stroke, homocysteine, miRNA

Introduction

Approximately 795,000 Americans suffer a stroke each year, and nearly a quarter (185,000) of the strokes are recurrent.[1,2] In China, about 1.6 million people die from stroke every year. In other words, about 157 people die from strokes per 100,000 people. Therefore, stroke is the leading cause of disability and death among Chinese adults.[3,4] Ischemic stroke (IS) is the most common of all stroke types in China. The most common cause is the de-arterialization of blood in the brain. The detailed pathophysiological causes of IS are not known, but some risk factors such as lifestyle, environment, immunity, inflammation, and genetic factors have been reported.[5] Recent data on the inclusion of genetic markers in prediction models of stroke have been varied.[6] Genome-wide association studies (GWAS) have been used to investigate the relationship between some genetic variants and IS

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risk.[7] Evidence from studies of twins and family history demonstrates that genetics also influences the pathogenesis of IS and it should be responsible for a large part of IS risk.[8] Besides, the data from genome-wide association study (GWAS) have shown that dozens of single nucleotide polymorphism (SNP) are related to IS.[9] The most well-studied *BDNF* single nucleotide polymorphism (SNP) rs6265 has been reported to be related to brain morphology changes and cortical plasticity.[10] In the context of stroke, it was also identified that *BDNF* rs6265 polymorphism was associated with motor recovery of stroke patients.[11]

A more promising approach has been to intervene with the brain-derived neurotrophic factor (*BDNF*), for instance, in pathways regulated by *BDNF*.[12] *BDNF* is the most abundant neurotrophic factor, contributing to nerve cell survival, synaptic plasticity, angiogenesis, and peripheral and central nerve cell growth.[13] *BDNF* is involved in neuro regenerative biological behaviors including synapses in the central nervous system.[14] The *BDNF* genotype is associated with either primary or secondary IS. Reduced serum *BDNF* levels are associated with IS[15] and the *BDNF/TrkB* signal becomes abnormal. Researcher has proved that in experimental model studies of stroke, both intravenous and intraventricular *BDNF* injections could reduce infarct size and showed neuroprotective effects.[16] Besides, A recent study demonstrated that cerebral ischemia in rats made a difference to the regulation of *BDNF* isoforms and their associated proteins in a time-dependent manner, and the balance between *BDNF* and *proBDNF* in the ischemic cerebral cortex may have an impact on the pathogenesis and recovery from ischemia, such as balance, motor coordination and physical condition.[17] Permanent *BDNF* reduction was found in the core area of cerebral infarction, while long-term upregulation was found in the half area of cerebral infarction, which is considered a neuroprotective mechanism.[15,18] The miRNAs can be suppressed by binding to the 3′-untranslated region (3′-UTR), a gene regulation mechanism by transcription. Previous studies have confirmed that functional polymorphisms in 3′-UTR of mRNA played important roles in the occurrence of human diseases.[19]

Here, we mainly have investigated the single nucleotide polymorphisms (SNPs) in the 3′-UTR of *NTRK2*. The TrkB polymorphisms (rs11140793AC, rs7047042CG, rs1221CT, rs2277193TC, and rs2277192AG genotypes) were reported to be significantly associated with post-stroke depression.[20] However, whether these SNPs were associated with the risk of IS lacked thorough investigation. It is not known whether these mutant genotypes will induce the dysregulation of expression. Here we focused on SNPs located in the 3′-UTR of *NTRK2*, aiming to explore whether these SNPs were associated with the risk of IS. We also predicted all candidate miRNAs that may interact with *NTRK2* through bioinformatical analysis.

Materials and Methods

Clinical Information

From July 2010 to October 2016, 400 patients diagnosed with IS were enrolled in the study. The diagnosis of acute IS was based on neurological examination and then validated by CT scan or magnetic resonance imaging (MRI). IS was divided into subtypes according to TOAST criteria. In addition, 400 healthy subjects of the same period were selected as the control group and matched according to age and sex. The control group all went to our hospital for physical examination every year and had no IS. Fasting blood glucose after oral glucose stimulation ≥7.0 mmol/L and/or 11.1 mmol/L at 2 hours after oral glucose or receiving anti-diabetes medication, was defined as diabetes. Smokers were defined as more than ten cigarettes per day with continuous smoking for five years. Drinking exposure was defined as more than 50 mL of alcoholic beverages per day for 5 years. The National Institutes of Health Stroke Scale (NIHSS) score was used to quantify stroke severity at the time of presentation and discharge. This study was approved by the Ethics Committee of Kunshan Rehabilitation Hospital and The Sixth People's Hospital of Nantong. Written informed consent was obtained from all participants. All experiments on human subjects were conducted in accordance with the Declaration of Helsinki.

Genotyping

Fasting venous blood was drawn from patients and controls. The total DNA of leukocytes was extracted by salt fractionation. The ABI PRISM 7900HT sequence detection system (Applied Biosystems, CA, USA) and the TaqMan allele identification method were used for SNP genotyping. Each 384-well format is allocated five replicate samples and two blank controls for quality control. The allelic discrimination model of the SDS 2.3 software package (Applied Biosystems) was used to calculate the genotyping results.

Bioinformatic Analysis

Bioinformatics software (http://bioinfo.life.hust.edu.cn/miRNASNP/#!/) was used to detect the candidate SNPs that could affect *NTRK2* gene regulation via miRNAs.

Cell Line, Culture, and Transfection

Human 293T cells were purchased from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China). The culture medium included 10% FBS (Invitrogen, Carlsbad, CA, USA) with RMPI-1640 (Gibco, Gaithersburg, MD, USA), and cells were cultured in a humidified 5% CO_2 incubator at 37°C. Mimic and control vectors were designed and cloned by Genescript (Nanjing, China). Cells were transfected with Lipofectamine 2000 (Invitrogen, CA, USA) according to the manufacturer's protocol.

Construction of Luciferase-Based Reporter Plasmids and Luciferase Reporter Assay

The 3′-UTR fragment containing wild or mutant allele SNPs was amplified by Genscript (Nanjing, Jiangsu). PCR products were cloned into a miR-reporter luciferase system (Ambion, Thermo Fisher Scientific, Waltham, MA, USA). The amplified fragment was verified by Genscript sequencing. PRL-TK containing Renilla luciferase was used for normalization. The 3′-UTR sequence of *NTRK2* predicted to interact with miRNAs or the mutant sequence of the predicted target were inserted into the PIR-reporter luciferase HindIII and SacI sites (Genscript, Nanjing, China). HEK-293T cells cultured with 24-well plates were co-transfected with pmIR-reporter vectors containing wild-type or mutant *NTRK2*-UTR fragments and control vectors, and PRL-TK containing Renilla luciferase was used for normalization. These miRNA mimics and controls were co-transfected into cells.

Statistical Analysis

The statistical analyses were performed using Stata/SE (StataCorp LP, TX, USA). The Student's *t*-test and chi-square ($\chi 2$) test were used to compare demographic data. Multivariate logistic regression analysis was used to adjust. Odds ratios (OR) and 95% CI were used to estimate the relationship between genotype and IS clinical parameters. $P < 0.05$ was considered significant.

Table 1 Clinical Characteristics of Ischemic Stroke Patients and Healthy Controls

Characteristics	Cases (n=400)	Control (n=400)	*p* value[a]
Age			>0.05
>60	349	351	
<60	51	49	
Gender			>0.05
Male	276	244	
Female	124	156	
Smoking exposure			<0.001
Yes	301	181	
No	99	219	
Drinking exposure			<0.001
Yes	279	199	
No	121	201	
Diabetes			0.007
Yes	241	203	
No	159	197	
Hypertension			<0.001
Yes	311	129	
No	89	271	
Total cholesterol (mmol/L)	4.88 (4.11–5.14)	4.79 (4.12–5.13)	>0.05
HDL-C(mmol/L)	0.87 (0.44–1.71)	1.52 (1.08–1.87)	<0.001
LDL-C(mmol/L)	4.99 (4.11–6.21)	2.83 (1.77–3.31)	<0.001

Note: [a]Student *t*-test for continuous variable, Chi-squared test for categorical variable

Results

Subject Characteristics

As shown in Table 1, there was no significant difference in age and sex distribution between IS patients and healthy controls. Smoking and alcohol exposure, diabetes, hypertension, and other common risk factors were found to have an increased risk of IS compared with the control group ($P<0.05$). In addition, traditional cardiovascular and cerebrovascular disease biomarkers such as total cholesterol, HDL-C, and LDL-C are also related to the occurrence of IS.

Genotype and Allele Analysis

Three polymorphisms including rs11140793, rs7047042, and rs1221 were found to locate in the 3′-UTR region of

Table 2 Genotype Frequencies of the *NTRK2* Polymorphisms in is Patients and Healthy Controls

Genotype	Cases (n =400)		Controls (n = 400)		OR (95% CI) [a]	*P* Value[a]
	N	%	N	%		
rs11140793						
AA	322	80.5	331	82.7	1.00	
AC	53	13.3	51	12.7	1.68 (0.83–1.87)	0.03
CC	25	6.2	18	4.6	1.79 (0.91–1.99)	0.01
rs7047042						
CC	371	92.7	382	95.5	1.00	
CG	23	5.8	16	4	1.12 (1.01–1.65)	0.02
GG	6	1.5	2	0.5	1.31 (1.08–1.78)	0.02
rs1221						
CC	344	86.0	351	87.7	1.00	
CT	38	9.5	37	9.3	1.33 (1.19–2.33)	0.01
TT	18	4.5	12	3	1.41 (1.21–2.45)	0.005

Note: [a]The ORs, 95% CIs and *P* value were calculated after adjusting for smoking, drinking and other characteristics listed in Table 1.

NTRK2. We explored the effects of the three candidate SNPs on IS susceptibility in our case-control participants. The results of chi-square statistical analysis showed that the genotypes of the three SNPs in the healthy control group all conformed to the Hardy-Weinberg equilibrium distribution pattern. As presented in Table 2, logistic regression analysis showed that the sensitivity of the AC genotype and CC genotype to IS was significantly higher than that of the AA genotype (OR=1.68, 95% CI=0.83–1.87; OR=1.79, 95% CI=0.91–1.99). We also found that polymorphisms in rs7047042 indicated a different distribution compared with the control group. Logistic regression analysis showed that *NTRK2* rs7047042 CG and GG genotypes were significantly correlated with the increased risk of IS compared with CC genotypes (OR: 1.12; CG genotype, 95% CI: 1.01–1.65, OR: 1.31; GG genotype, 95% CI: 1.08–1.78). For rs1221 polymorphisms, we also found that patients with the CT or TT genotype have a higher IS risk than patients with the CC genotype (OR: 1.33; CT genotype, 95% CI: 1.19–2.33, OR: 1.41; TT genotype, 95% CI: 1.21–2.45).

The miR-Binding Was Attenuated by SNPs

Because *NTRK2* acted in a beneficial role during the progression of IS, increasing levels of *NTRK2* were associated with a lower risk of IS. Here we found that all the three SNPs were highly associated with the increased risk of IS, indicating that all the SNPs could be associated with a lower level of *NTRK2*. Next, we investigated the mRNA level of *NTRK2* in plasma samples. We found that patients harboring mutant *NTRK2* DNA presented a decreased level of *NTRK2* mRNA (Figure 1A). Whether the suppression of NTRK2 was associated with miRNA-associated interactions are unknown. As predicted by bioinformatics analysis, the miRNAs which have the potential to bind with the 3′-UTR of *NTRK2* are presented in Table 3. A total of six candidate miRNAs that may interact with rs11140793 through binding were listed according to the expression correlation value by prediction software. Three miRNAs were predicted for rs7047042 whereas four miRNAs were predicted for rs1221. We then cloned the 13 candidate miRNA mimics and transfected cells with either wild-type or mutant type *NTRK2*. A luciferase assay confirmed that *miR-181a-3p* could directly bound with the *NTRK2* mutant (CC genotype of rs11140793) causing the suppression of *NTRK2* luciferase (Figure 1B), *miR-127-5p* could interact with the *NTRK2* mutant (GG genotype of rs7047042, Figure 1C), and *miR-483-3p* could interact with the TT genotype of rs1221 (Figure 1D).

Polymorphisms Predicted a Worse Outcome in is Patients

Because the increase of *NTRK2* expression can promote tHcy, it may lead to a better prognosis in IS patients. Next, we evaluated the relationship between polymorphism and also the outcome of short-term IS. The

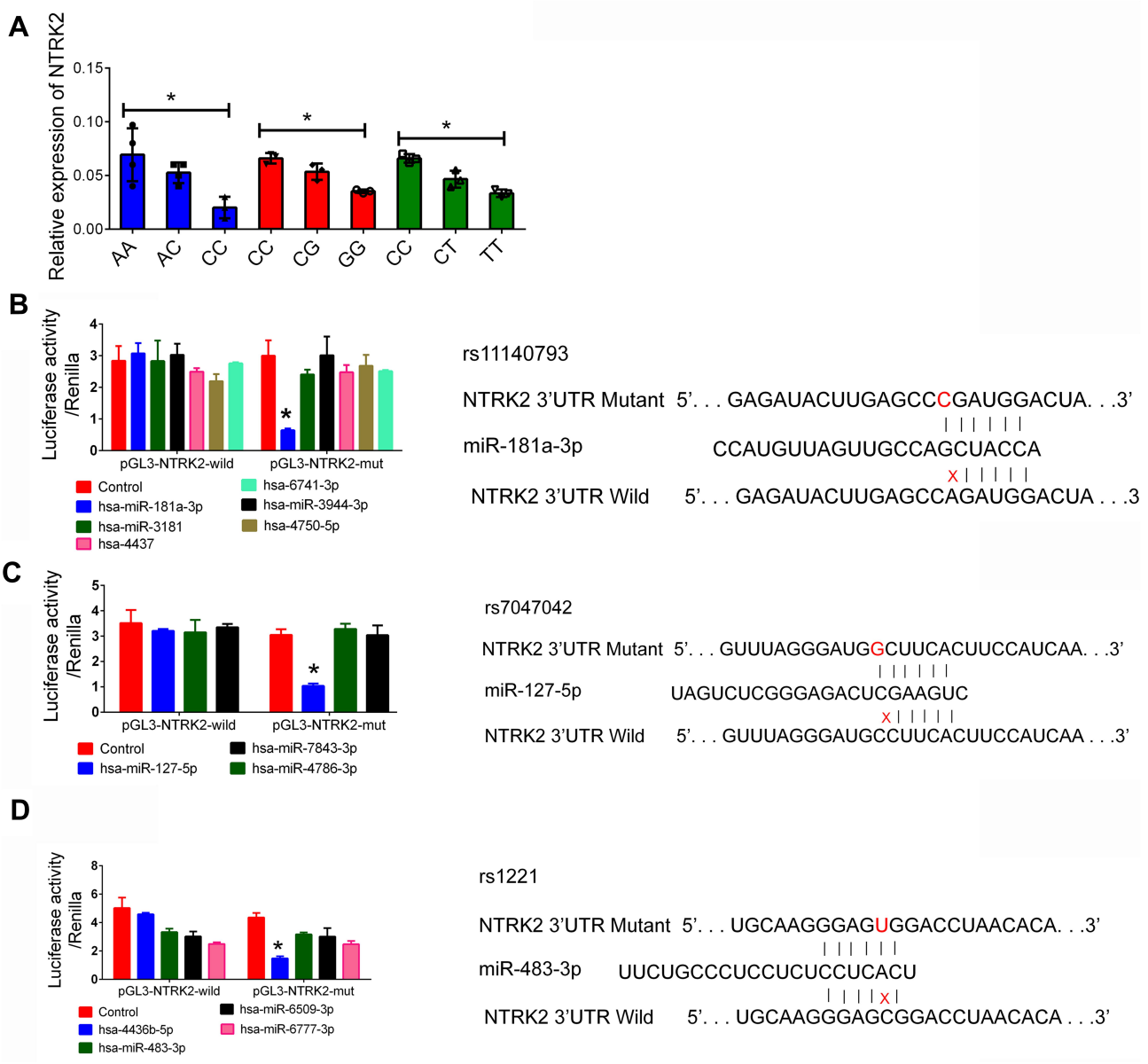

Figure 1 The function miRNAs associated with *NTRK2* polymorphisms. (**A**): Relative expression of *NTRK2* mRNA in patients with different genotypes of IS. (**B**): Luciferase of rs11140793 was detected by miRNA. (**C**): Luciferase of rs7047042 was detected by miRNA. (**D**): Luciferase of rs1221 was detected by miRNA. 293T cells were co-transfected with miRNAs simulator or control Renilla luciferase vector PRL-SV40 for 48 h. Luciferase activity in fireflies and renal cells was determined using the luciferase assay kit. The luciferase firefly signal was normalized to the renal luciferase signal. Data are expressed as mean ± standard deviation. *P < 0.05.

evaluation used the NIHSS. According to the NIHSS measurement, different genotypes had significant differences in the initial severity of stroke in the acute phase. A negative value indicated a clinical improvement in IS outcome. We found that all three polymorphisms predicted a worse prognosis in IS patients. *NTRK2* mRNA expression and circulating tHcy concentration were also examined. Our data showed that *NTRK2* expression was low and *tHcy* level was increased in patients with mutant *NTRK2* (Table 4).

Discussion

It is important to identify the risk of stroke recurrence in patients because about a quarter of strokes recur and have a poor response to therapy.[21] Cerebral or systemic ischemic events may highly increase morbidity and mortality.[22] Therefore, secondary prevention in patients with recent cerebrovascular events is of critical importance.[23] IS endangers the health and quality of life of patients, and at the same time, it also brings a heavy burden to patients, their families, and society. At present, it

Table 3 Candidate miRNAs Associated with NTRK2 Polymorphisms Predicted by Bioinformatics Analysis

Gene	SNP	miRNA-ID	ΔG Binding (kCal/mol)
NTRK2	rs1140793	hsa-miR-181a-3p	−15.32
		hsa-miR-3181	−14.60
		hsa-miR-3944-3p	−17.90
		hsa-4437	−20.35
		hsa-4750-5p	−18.49
		hsa-6741-3p	−14.67
	rs7047042	hsa-miR-127-5p	−9.01
		hsa-miR-4786-3p	−14.83
		hsa-miR-7843-3p	−19.25
	rs1221	hsa-4436b-5p	−18.36
		hsa-miR-483-3p	−15.99
		hsa-miR-6509-3p	−13.95
		hsa-miR-6777-3p	−12.41

Note: ΔG binding (kCal/mol): binding energy based on ensemble free energy.

is worth noting that it may be possible to enhance survival pathways by binding to *TrkB* receptors, such as the pathways found to be regulated by *BDNF*. In addition, studies have shown that *BDNF/TrkB* has a beneficial effect after acute stroke and can stimulate later nerve repair.[24]

NTRK2 consists of 24 exons and is located on chromosome 9 (q22.1), with a length of about 24 KB.[25] It encodes a subtype of the *BDNF* receptor, *TrkB*, which is bound to *mBDNF* with a high affinity.[26] The *BDNF/TrkB* signaling pathway enhances neuroplasticity including neurogenesis, neurite dendrite, and synapse. *BDNF* promotes neuronal cell survival through dimerization, increased tyrosine kinase activity, and full-length *TrkB* phosphorylation of the high-affinity receptor.[27] *NTRK2* can activate and

Table 4 Clinical Outcome Correlation of NTRK2 Polymorphisms with is Patients

Feature	Genotype (rs1140793)			AC vs AA* *P* value	CC vs AA* *P* value
	AA	AC	CC		
NIHSS ac (Mean/SD)	3.1/1.1	4.2/1.5	4.8/1.1	0.02	0.005
NIHSS 3m (Mean/SD)	0.6/0.1	0.8/0.2	1.1/0.2	0.03	**0.002**
tHcy (mmol/L)	12.3/1.8	13.5/1.5	15.5/1.4	0.01	**0.001**
NTRK2 (Mean/SD[b])	0.08/0.022	0.06/0.018	0.04/0.11	0.02	**0.003**
Feature	**Genotype (rs7047042)**			**CG vs CC* *P* value**	**GG vs CC* *P* value**
	CC	**CG**	**GG**		
NIHSS ac (Mean/SD)	3.2/1.1	3.5/1.3	4.1/2.1	0.04	0.02
NIHSS 3m (Mean/SD)	0.7/0.1	0.9/0.2	1.0/0.2	0.03	0.01
tHcy (mmol/L)	11.8/1.9	12.1/1.6	14.2/1.8	0.01	0.04
NTRK2 (Mean/SD[b])	0.09/0.012	0.06/0.021	0.04/0.19	0.02	**0.03**
Feature	**Genotype (rs1221)**			**CT vs CC* *P* value**	**TT vs CC* *P* value**
	CC	**CT**	**TT**		
NIHSS ac (Mean/SD)	3.5/1.1	3.6/1.7	4.2/2.1	0.12	0.03
NIHSS 3m (Mean/SD)	1.1/0.4	1.1/0.6	1.3/0.8	0.23	0.02
tHcy (mmol/L)	10.8/1.9	13.2/0.2	13.1/0.3	0.001	0.002
NTRK2 (Mean/SD[b])	0.06/0.032	0.03/0.021	0.01/0.011	0.01	0.03

Notes: *Student *t*-test for either genotype distributions or allele frequencies in IS patients. [b]Relative expression of *NTRK2* normalized with *GAPDH*. NIHSS ac, initial stroke severity during the acute phase; NIHSS 3m, stroke severity after 3 months; *MTHFR*: Relative expression of MTHGR mRNA in IS patients.

trigger three related cascade signaling pathways, including *MAPK/ERK, PI3K/Akt,* and *PLCγ*[28]

There is also increasing evidence that association analysis based on genes and pathways is more effective than association studies based on SNPs because they increase the ability to identify true associations. However, it should be pointed out that analysis based on gene-gene interactions is rarely applied to IS.[29,30] Functional SNPs located at miRNA binding sites can affect the binding of miRNA to target genes, by reverse inhibition or activating the expression of target genes, and altering susceptibility to human diseases.

There were some limitations to consider in this study. Our study only followed up the short-term prognosis of patients. The influence of SNPs on the long-term rehabilitation and functional prognosis of patients needs further research. Therefore, our results need to be confirmed in independent samples or other populations.

Conclusion

In summary, our current research showed that *NTRK2* SNP gene variants were associated with more severe initial stroke severity and worse short-term recovery after IS. In addition, miRNAs could regulate these three SNPs, resulting in decreased expression of *NTRK2*. This may be a favorable factor in the prognosis of patients with IS.

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Disclosure

The authors declare that they have no conflicts of interest.

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