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## RESEARCH ARTICLE

### THE RELATIONSHIP BETWEEN SERUM RHOA AND MIR-133B IN ACUTE ISCHEMIC STROKE

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#### Abstract

**Objective:** The present study investigates the expressions of miR-133b and RhoA in acute ischemic stroke (AIS) patients.

**Methods:** In the present study, serum samples from AIS patients and healthy controls were collected to determine the regulatory miRNAs and RhoA involved in AIS. The expression levels of miR-133b in serum were detected by real-time quantitative PCR, and the expression levels of RhoA in blood were detected by ELISA. The correlation between the levels of miR-133b and RhoA was analyzed.

**Results:** The results indicate that the RhoA level in the AIS patients is very high compared with that in controls. The level of miR-133b showed the opposite change with that of RhoA. The association between the National Institutes of Health stroke scale (NIHSS) scores with RhoA and miR-133b-related RhoA serum levels was calculated using Pearson's/Spearman's correlation coefficient. The findings revealed a positive correlation between NIHSS scores and RhoA level, whereas a negative correlation was observed between NIHSS scores and miR-133b. In addition, the relationship between serum RhoA and miR-133b was negative in AIS patients.

**Conclusion:** Serum miR-133b and the miR-133b-regulatory RhoA may serve as molecular markers for AIS.

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#### Introduction:-

Acute ischemic stroke (AIS) is the leading cause of adult disability in the world [1]. At present, the most commonly used diagnostic methods for ischemic stroke are clinical physical examination and neuroimaging techniques, but there are no reliable circulating indicators to predict its risk, diagnostic screening and prognosis. In order to find suitable markers for the diagnosis of AIS, many protein markers such as RhoA and albumin had been found to be involved in the pathophysiology of ischemic stroke, [2,3]. However, due to the heterogeneity of ischemic stroke, the role of these protein markers as diagnostic parameters is still unsatisfactory.

Recent studies have suggested that RNA biomarkers may be useful for the diagnosis and evaluation of ischemic stroke [4,5]. As a newly discovered RNA widely distributed in various tissues and body fluids in recent years, miRNA is a type of endogenous non-coding single-stranded RNA consisting of 18-24 nucleotides [6]. MiRNAs acting on target genes can reduce or degrade their translation, thereby regulating the expression of target genes [7]. MicroRNA has been identified by more and more studies as a potential molecular marker for various diseases. There is growing evidence that microRNAs play an vital role in many pathophysiological processes, including ischemic

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stroke and coronary artery disease[8]. Recently, abnormal expression of miR-133b has been identified in renal cancer [9], colorectal cancer [10], breast cancer[11] and non-small cell lung cancer[12]. However, it has not been reported whether miR-133b can be used as a potential biomarker for ischemic stroke.

The purpose of this study was to investigate the relationship between serum miR-133b and RhoA in patients with ischemic stroke, and to evaluate the potential of miR-133b in the diagnosis and prognosis of ischemic stroke.

## **Materials and Method:-**

### **Study population:**

A total of 60 patients with AIS were selected from April 2017 to October 2018. All patients admitted to the hospital were diagnosed by CT or MRI within 72 hours after the onset of illness. Exclusion criteria were acute infection, acute coronary syndrome, tumor or autoimmune disease, arteritis and peripheral vascular disease, valvular disease and atrial fibrillation, liver and kidney dysfunction and severe heart dysfunction, blood disease, and recent surgery or trauma. All patients were scored based on the clinical neurological deficit score of stroke patients, including 24 mild neurological deficits (score <15), 23 moderate neurological deficits (score 16-30), and 13 cases of severe neurological deficits. Neurological deficit (score > 30). In addition, 60 healthy people who underwent a physical examination at the Weihai Medical Examination Center were selected as the control group.

The proportion of history of hypertension, diabetes, hyperlipidemia, and stroke was significantly higher in the patient group than in the control group ( $p < 0.05$ ) (Table 1). There were no significant differences in age, sex, and smoking rates between the two groups ( $p > 0.05$ ).

### **ELISA:**

RhoA was detected by ELISA performed as previously described [13]. Elbow venous blood was collected in the morning and stored in a procoagulant tube. After 2 hours, cubital venous blood was centrifuged at 3,000g for 15 min at 4°C at room temperature and ELISA assay (Nanjing dongji biology co. LTD) follows the manufacturer's agreement. Samples were read at 450 nm using a microplate reader.

### **RNA extraction:**

Total RNA was isolated from whole blood samples (5ml, collected in test tubes containing EDTA) according to the manufacturer's protocol. The concentration of the RNA samples were determined by measuring optical density (OD) 260/OD280.

### **Real-time quantitative PCR:**

A total of 1µg of RNA was reverse transcribed using Moloney murine leukemia virus (MMLV) reverse transcriptase (Nanjing dongji biology co. LTD) and specific primers. PCR amplification was performed in a 10µl reaction system, which contained 5µl SYBR Green Supermix, 0.4µL forward primer, 0.4µl reverse primer, 2.2µl double-distilled H<sub>2</sub>O, and 2µl template cDNA. The temperature protocols used for RT are as follows: 72°C for 10min; 42°C for 60min, 72°C for 5min and 95°C for 2min thermocycling conditions were set as follows: 95°C for 10min followed by 40 cycles of 95°C for 15 seconds and 60°C for one min. DNA fragments were collected to be tested by PCR with primer located in miR-133b promoter: forward 5'-TGCAA-CACTTGCAGCTGAG-3' and reverse 5'-TCTACTCTGCCTGCTTGT TG-3' (198bp).

### **Statistical Analysis:**

Statistical analysis was performed using SPSS 17.0 software. The measurement data are expressed as mean  $\pm$  standard deviation. A one-way ANOVA multiple comparison test followed by a Turkish post hoc test was used to compare two or more groups. Student's test, Pearson's correlation coefficient, and Spearman's rank test were used to evaluate the normality of the distributions of RhoA levels, NIHSS scores, and miR-133b values. The difference was statistically significant  $p < 0.05$ .

## **Result:-**

### **Clinical analysis of AIS patients and control samples:**

One hundred and twenty individuals were enrolled into the present study including 60 patients with AIS and 60 healthy controls. In total, 53% were male, and 47% were female. Age of patients ranged from 45 to 78 years. The

NIHSS assigns scores to stroke patients based on seriousness: 0 indicates stroke symptoms, 1–4 indicates minor stroke, 5–15 indicates moderate stroke, 16–30 indicates severe stroke. The clinical parameters were tabulated and are presented in Table 1.

Table1. Clinical characteristics of the acute ischemic stroke and healthy samples

#### **Serum RhoA level and miR-133b in AIS patients and controls:**

The relationship between RhoA and miR-133b has been clarified in the previous study. It indicated that miR-133b+MSC treatment further significantly decreased RhoA at day 14 after MCAO compared with that after naive MSC treatment, and the RhoA expression after miR-133b-MSC treatment was sustained at a similar elevated level at day 14 after MCAO compared with naive MSC treatment [14]. These findings suggest that miR-133b plays a crucial role in regulating RhoA expression.

In the present study, the serum miR-133b level was lower in AIS patients than that in healthy controls (Fig. 1). While the miR-133b-related RhoA level was higher in AIS patients than that in healthy controls (Fig. 2). The correlation between RhoA and miR-133b was analyzed by Spearman's coefficient. The data revealed a negative correlation between serum RhoA level and miR-133b (Spearman's  $r = -0.632$ ;  $p = 0.009$ ) in AIS patients and healthy controls. These results confirm that miR-133b is the putative target that regulates the expression of RhoA, which may be causative of AIS.

#### **Levels of miR-133b and RhoA related with NIHSS scores:**

The expression levels of RhoA in the patients with severe neurological deficits were significantly higher than those of patients with moderate and mild neurological deficits ( $p < 0.05$ ). At the same time, the levels of RhoA in the patients with moderate neurological impairment were significantly higher than those of patients with mild neurological impairment. ( $p < 0.05$ ) (Fig.3) The level of miR-133b in the above patients with different degrees of neurological impairment showed the opposite change with RhoA (Fig.4). This finding suggests that the serum miR-133b level is associated with the NIHSS scores, which may contribute to AIS diagnosis.

#### **Discussion:-**

RhoA, a kind of bird nucleoside triphosphate binding protein, is a member of the Ras superfamily, whose downstream effector Rho kinase (ROCK) plays a vital role in various physiological and pathological processes, such as contraction of vascular smooth muscle cell, actin cytoskeleton organization, cell adhesion, et al. Increasing evidences show that RhoA/ROCK has played a key role in increasing the permeability of blood-brain barrier (BBB) [15]. Under the activation of cerebral ischemia reperfusion injury, ROCK increased phosphorylation of myosin regulatory light chain 2 (p-MLC2) expression and enhanced the contraction force of actin and myosin, by increasing BBB leakage at the same time, the increase of p-MLC2 may also contribute to the destruction of ZO-1. Consequently the expression of MMP-9 increasing causes hydrolysis of the extracellular matrix, which results in BBB damage and brain edema. Inhibition of RhoA/ROCK pathway can downregulate expression of MMP-2/9, which can reduce the degradation of tight connections and BBB damage [16].

In the present study, the serum RhoA level was significantly increased in patients with AIS. MiRNAs are a class of endogenous non-coding small RNAs consisting of 18-24 nucleotides. They bind to the 3'-UTR of the target gene messenger RNA to regulate degradation of the messenger RNA or inhibit protein translation to regulate the target gene expression [17]. Previous studies have shown that microRNAs are widely distributed in various body fluids, such as serum, plasma, saliva, tears, and amniotic fluid, and that the types and amounts of microRNAs in body fluids vary with physiological and disease conditions [18]. It is involved in growth and development of many diseases [19]. Compared with healthy controls, the levels of serum miR-133b in patient with AIS reduced. Exosomes from MSCs mediate the miR-133b transfer to astrocytes and neurons, which regulate gene expression, subsequently benefit neurite remodeling and functional recovery after stroke [14]. Exosome-mediated transfer of miR-133b overexpression contributes to neurite outgrowth [20]. At the same time, MSCs were used to deliver miR-133b to increase the expression level of miR-133b in ischemic lesion and further improve therapeutic effects [21]. Here, we evaluated for the potential of miR-133b as a biomarker for AIS in clinical practice.

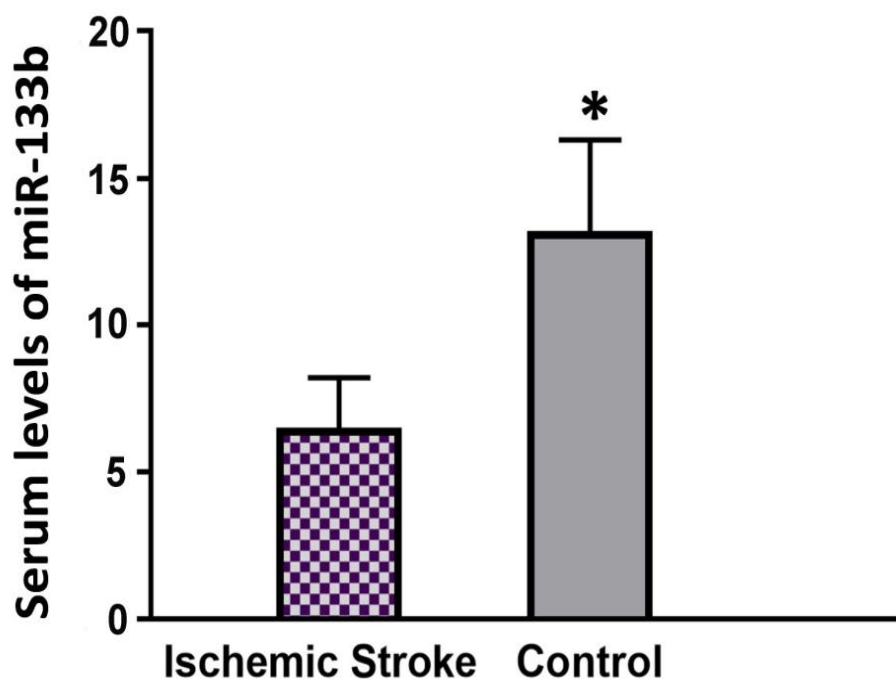
In addition to confirming the downregulation of serum miR-133b expression in the patients with AIS, we also found that there was a significant negative correlation between the expression of miR-133b and RhoA, suggesting that the expression of miR-133b and RhoA can be used as a biomarker for AIS. There is a good correlation between miR-

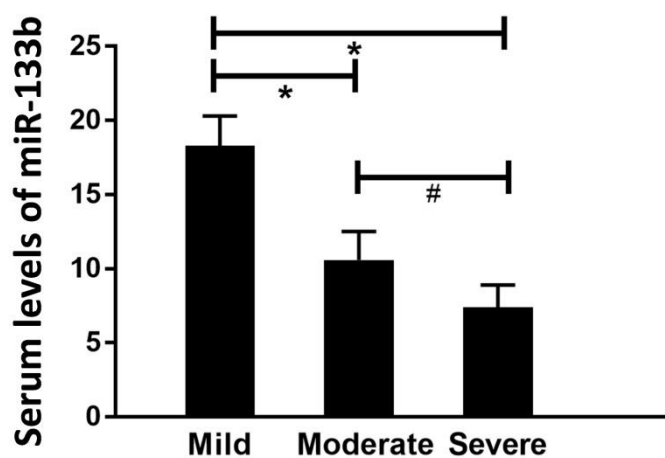
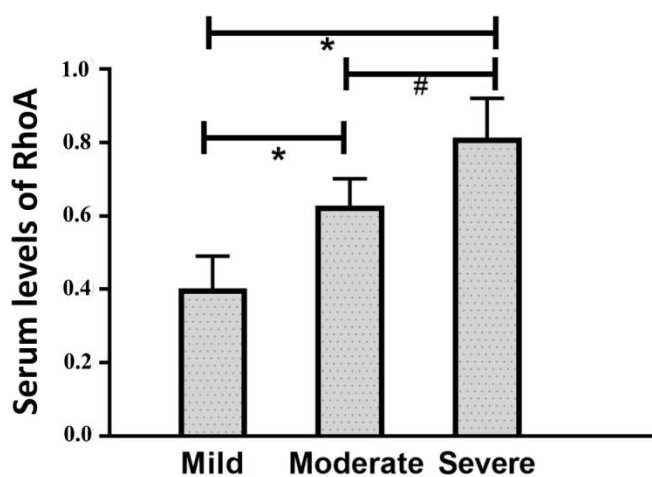
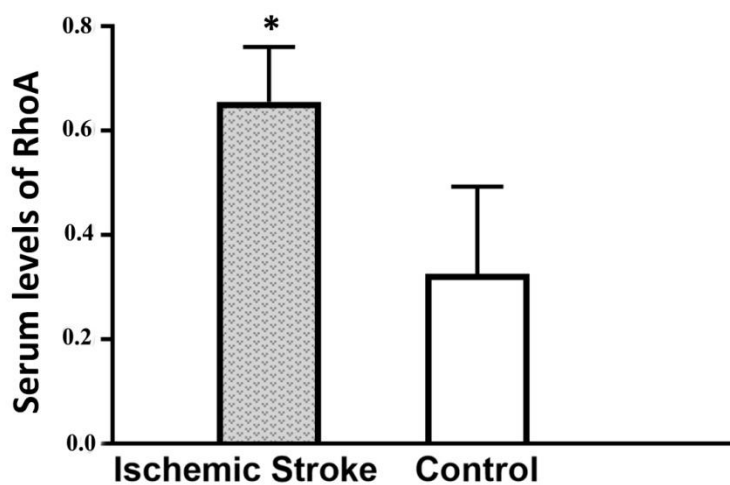
133b and RhoA in patients with moderate neurological deficits, which indicates that miR-133b and RhoA have a high degree of value in estimating to moderate neurological dysfunction in AIS. Therefore, in patients with severe neurological deficits, further exploration of protein markers that are better related to miR-133b will become the focus of future research.

This study suggests that serum miR-133b may play a protective role in the pathogenesis, development, and prognosis of ischemic stroke. Serum miR-133b is a useful biomarker for AIS as well as its degrees of neurological impairment. However, the specific mechanism still remains elusive, further research is needed to determine whether there is a relationship between miR-133b and BBB damage in AIS. Large scale studies are still needed to confirm these findings.

**Table 1:-** Clinical characteristics of the acute ischemic stroke and normal samples.

Group	AIS	HC
N	60	60
Age (years)	64.3 ± 10.3	62.8 ± 9.5
Gender (male/female)	33/27	31/29
Smoke n (%)	16 (26.7%)	14 (23.3%)
Hyperlipidemia n (%)	11 (18.3%)	7 (11.6%)
History of stroke n (%)	8 (13.3%)	6 (10.0%)
Diabetes n (%)	13 (23.3%)	10 (16.7%)
Hypertension n (%)	18 (30.0%)	15 (25.0%)
miR-133b (10 <sup>3</sup> copies/ml)	6.5 ± 2.3	12.2 ± 3.9
RhoA (ng/ml)	0.66 ± 0.10	0.32 ± 0.17



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**Declaration of Interest:**

All authors declare no conflicts of interest.

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