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SETD5-AS1 stimulates neuron death in stroke via promoting PTEN expression

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Abstract. – OBJECTIVE: To investigate the specific role of long non-coding RNA (lncRNA) SETD5-AS1 in regulating stroke development, and its underlying mechanism.

MATERIALS AND METHODS: Middle cerebral artery occlusion (MCAO) model and OGD/R (oxygen-glucose deprivation/reoxygenation) model were constructed for exploring the mechanism of ischemia-reperfusion injury induced by ischemic stroke. SETD5-AS1 expression in brain tissues of ischemic stroke mice and control mice was detected by quantitative Real-time-polymerase chain reaction (qRT-PCR). Proliferation and apoptosis of N2a cells were detected after transfection of overexpression plasmid or siRNA SETD5-AS1. The downstream gene of SETP5-AS1 was predicted by Starbase and PTEN was screened out. Both mRNA and protein expressions of PTEN in MCAO model and OGD/R model were detected. Furthermore, the binding condition of SETD5-AS1 and PTEN was verified by dual-luciferase reporter gene assay, RNA pull-down assay and RNA binding protein immunoprecipitation (RIP). The regulatory effect of SETD5-AS1 on PI3K/AKT pathway was detected by Western blot.

RESULTS: SETD5-AS1 was highly expressed in the ischemia-reperfusion injury model. Overexpression of SETD5-AS1 in N2a cells resulted in increased apoptosis and decreased proliferation. PTEN expression was upregulated in MCAO model and OGD/R model. Dual-luciferase reporter gene assay indicated that SETD5-AS1 can promote PTEN transcription. The binding condition of SETD5-AS1 and PTEN was further verified by RNA pull-down assay and RIP. Overexpression of SETD5-AS1 in N2a cells inhibited PI3K/AKT pathway.

CONCLUSIONS: SETD5-AS1 is highly expressed in the ischemia-reperfusion injury model. SETD5-AS1 participates in the development of ischemic stroke by activating PTEN and inhibiting PI3K/AKT pathway.

Key Words:
Stroke, SETD5-AS1, PTEN, PI3K/AKT pathway.

Introduction

Stroke is a type of cerebrovascular disease resulted from cerebral blood flow supply disorder. It has extremely high morbidity and mortality, and brings a heavy burden on affected patients and their families. Stroke is pathologically divided into ischemic stroke and hemorrhagic stroke. Ischemic stroke accounts for about 87% of all stroke cases, which has been well studied in recent years[1]. A series of nerve injuries triggered by cerebral ischemia may lead to neuron death, including oxygen supply deficiency, oxidative stress, excitatory amino acid neurotoxicity, inflammatory reactions, and brain tissue edema[2]. The pathophysiological process of ischemic stroke is complicated. Current treatment options for ischemic stroke include thrombolytic therapy, anticoagulant therapy, control of blood pressure, defibrase therapy, and catheter intervention[3]. Recombinant tissue plasminogen activators (rt-PAs) are the only thrombolytic drugs currently approved by the FDA for treatment of acute stroke. However, due to the narrow therapeutic window and intracranial hemorrhage risk of rt-PAs, only about 5% of patients with ischemic stroke have indications for rt-PAs treatment[4,5]. It is of great significance to explore the underlying mechanism of ischemic stroke, so as to better improve the clinical outcomes of affected patients.

Long non-coding RNA (lncRNA) is a type of RNA with over 200 nucleotides in length and could not encode proteins. LncRNA was previously considered as junk DNA during the transcription of the genome. With the advance of microarray technology and high-throughput sequencing technologies, accumulating evidences have proved that lncRNAs are involved in multiple biological processes[6]. LncRNAs also participate in many diseases including tumors[7,8], cardio-

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vascular diseases[9], nervous system diseases[10], and immune system diseases[11]. Therefore, the identification and analysis of lncRNA sequences, as well as its functions in disease development have been well studied in recent years.

LncRNAs are enriched in the transcriptome of mammalian brain tissue. Differentially expressed lncRNAs are closely related to the occurrence and development of various neurological diseases, such as Alzheimer's disease, Parkinson's disease, and Huntington's disease. The relationship between lncRNA and ischemic stroke, however, is rarely reported. In 2012, the potential role of lncRNA in ischemic stroke was first studied using microarrays. It pointed out that abundant lncRNAs were differentially expressed in cerebral cortex of ischemic stroke rats[12]. Our study aims to investigate the specific role of lncRNA SETD5-AS1 in regulating stroke development, and its underlying mechanism.

Materials and Methods

Middle Cerebral Artery Occlusion (MCAO) Model in Mice

Male C57BL/6J mice (7-8-week-old, weighing 18-21 g) were maintained in a standard environment. Animal experiments were approved by Experimental Animal Center of Shandong University. Mice were anesthetized by isoflurane, followed by exposure and ligation of the right common carotid artery, external carotid artery and internal carotid artery. The distal and proximal ends of the external carotid artery were ligated using 5-0 and 2-0 sutures, respectively. 0-0 suture was used to ligate in the middle of the external carotid artery. Subsequently, a transient occlusion of artery flow was established by inserting a 4-0 nylon monofilament (0.23–0.25 mm in diameter) into the right external carotid artery and advancing it to occlude the middle cerebral artery for 60 min before the filament was withdrawn.

Cell Culture

Mouse neuroblastoma N2a cells were obtained from ATCC (American Type Culture Collection) (Manassas, VA, USA) and cultured in DMEM (Dulbecco's Modified Eagle Medium) (Gibco, Rockville, MD, USA) containing 10% FBS (fetal bovine serum) (Gibco, Rockville, MD, USA). N2a cells were maintained in an environment with 5% CO_2 at 37°C. Cell passage was performed every 2-3 days.

OGD/R (Oxygen-Glucose Deprivation/Reoxygenation) Model Construction

After confluence of N2a cells was up to 80-85%, cells were washed with PBS for three times. Sugar-free DMEM medium was replaced, and N2a cells were placed in a three-gas incubator for 3 h. Next, cells were incubated in sugar-containing DMEM with 10% FBS for 24, 48 and 72 h, respectively.

Sample Collection

Mice were sacrificed and brain tissue was collected. After washing in pre-cooled saline, brain tissue at 2 mm and 6 mm in front of prefrontal lobe was sliced in coronal section. The middle part of brain tissue was harvested and sliced along the sagittal plane with a degree of 30°. The lateral cortex was the ischemic core and the medial cortex was the ischemic penumbra. The cortex of the ischemic penumbra was collected for preservation in liquid nitrogen.

RNA Extraction and Quantitative Real-Time-Polymerase Chain Reaction (Qrt-PCR)

Total RNA in treated cells was extracted using TRIzol method (Invitrogen, Carlsbad, CA, USA) for reverse transcription according to the instructions of PrimeScript RT reagent Kit (TaKaRa, Otsu, Shiga, Japan). RNA concentration was detected using spectrometer. QRT-PCR was then performed based on the instructions of SYBR Premix Ex Taq TM (TaKaRa, Otsu, Shiga, Japan). The relative gene expression was calculated using $2^{-\Delta Ct}$ method. Primers used in the study were as follows: glyceraldehyde 3-phosphate dehydrogenase (GAPDH): F: 5′-CACCCACTCCTCCACCTTTG-3′, R: 5′-CCACCACCCTGTTGCTGTAG-3′; SETD5-AS1: F: 5′-GCTTTTCTCGCTATGCTGCC-3′, R: 5′-GTTTGCCATTTGGGTGGTCC-3′; PTEN: F: 5′-TGGATTCGACTTAGACTTGACCT-3′, R: 5′-GGTGGGTTATGGTCTTCAAAAGG-3′.

Cell Transfection

One day prior to cell transfection, cells in good growth condition were seeded into 6-well plates with serum-free medium. Transfection was performed when the confluence was up to 60% following the instructions of Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). In brief, Lipofectamine 2000 and plasmid were diluted in serum-free medium and mixed together at room temperature for 20 min. The mixture was then added in each well for incubation. Culture

medium was replaced 4-6 h later. Plasmids and siRNA used in the study were constructed by GenePharma Co., Ltd. (Shanghai, China). The transfected siRNAs were: si-SETD5-AS1-1: 5′-ACCCGCTGCTGCCAGAGGCCCGAGTC-CGGC-3′, si-SETD5-AS1-2: 5′-TCTTCCGCGG-GGCTCTAGGTTCCACC-3′, si-SETD5-AS1-3: 5′-CATCTATCTCGCCAGCATCCAGG-3′.

Cell Counting Kit-8 (CCK-8) Assay

Cell viability was determined by cell counting kit-8 (CCK-8) assay (Dojindo, Kumamoto, Japan). N2a cells were cultured in 96-well plates, and 10 μL of CCK8 were added in each well. After incubation for 2 h, the absorbance of each well was measured at 450 nm.

Cell Apoptosis Detection

N2a cells were incubated with 10 μL of Annexin V FITC and 5 μL of propidium iodide (PI) in dark. Finally, cells were incubated with 350 μL of binding buffer for 20 min in dark, followed by flow cytometry detection.

Dual-Luciferase Reporter Gene Assay

N2a cells were co-transfected with wild-type PTEN or mutant-type PTEN and pcDNA-SETD5-AS1 or control vector, respectively. 48 h after co-transfection, dual-luciferase reporter gene assay kit (Promega, Madison, WI, USA) was used to detect the luciferase activity.

RNA Pull-Down Assay

T7 RNA polymerase (Promega, Madison, WI, USA) and biotin RNA tagged mixtures (Roche, Basel, Switzerland) were used to label the transcriptional SETD5-AS1 *in vitro*. Biotinylation SETD5-AS1 was denaturalized at 90°C for 2 min, placed on ice for another 2 min and incubated with RNA binding buffer for 25 min. The biotin-coupled RNA complex was pulled down by incubating the cell lysates with streptavidin-coated beads. The expression of SETD5-AS1 in the bound fraction was detected by Western blot.

RNA Binding Protein Immunoprecipitation (RIP)

Cells were washed and cross-linked with 0.01% formaldehyde for 15 min. After centrifugation and cell lysis, cells extracted were incubated with RIP buffer containing protein A/G magnetic beads coated with anti-Ago2 or negative control anti-IgG antibody. After overnight incubation at 4°C, cells were incubated with Protein G-Sepharose for 1 h at 4°C, followed by the isolation of RNA. SETD5-AS1 level was then detected by qRT-PCR.

Western Blot

Cells were lysed with RIPA (radioimmunoprecipitation assay) lysis buffer in the presence of a protease inhibitor (Sigma-Aldrich, St. Louis, MO, USA) to harvest total cellular protein. The protein concentration of each cell lysate was quantified using the BCA (bicinchoninic acid) protein assay kit (Pierce, Rockford, IL, USA). Protein sample was separated by gel electrophoresis and transferred to a PVDF (polyvinylidene difluoride) membrane (Millipore, Billerica, MA, USA). After incubation with primary and secondary antibodies, images of protein bands were captured by the Tanon detection system using ECL (electrochemiluminescence) reagent (Thermo-Fisher, Waltham, MA, USA).

Statistical Analysis

Statistical Product and Service Solutions (SPSS) 19.0 statistical software (IBM, Armonk, NY, USA) were used for data analysis. Data were expressed as mean ± standard deviation ($\bar{x}\pm s$). Measurement data and classification data were compared using the *t*-test and x^2-*t*est, respectively. $p<0.05$ considered the difference was statistically significant.

Results

SETD5-AS1 was Highly Expressed in MCAO Model and OGD/R Model

After construction of MCAO model, mouse ischemic brain tissue was collected. SETD5-AS1 was highly expressed in ischemic brain tissue of MCAO mice than that of controls (Figure 1A). Similarly, SETD5-AS1 was also upregulated in OGD/R cell model (Figure 1B). To further explore the role of SETD5-AS1 in ischemic stroke, overexpression plasmid and siRNA SETD5-AS1 were constructed (Figure 1C). SETD5-AS1 overexpression promoted apoptosis (Figure 1D), but inhibited proliferation of N2a cells (Figure 1E). Subsequently, three siRNA sequences of SETD5-AS1 were tested for their transfection efficacies and the inhibitory effect of si-SETD5-AS1-3 was the most pronounced (Figure 1F). SETD5-AS1 knockdown remarkably inhibited apoptosis (Figure 1G), but promoted proliferation of N2a cells (Figure 1H).

SETD5-AS1 Activated PTEN Expression

The potential binding target of SETD5-AS1 was predicted by bioinformatics and PTEN was

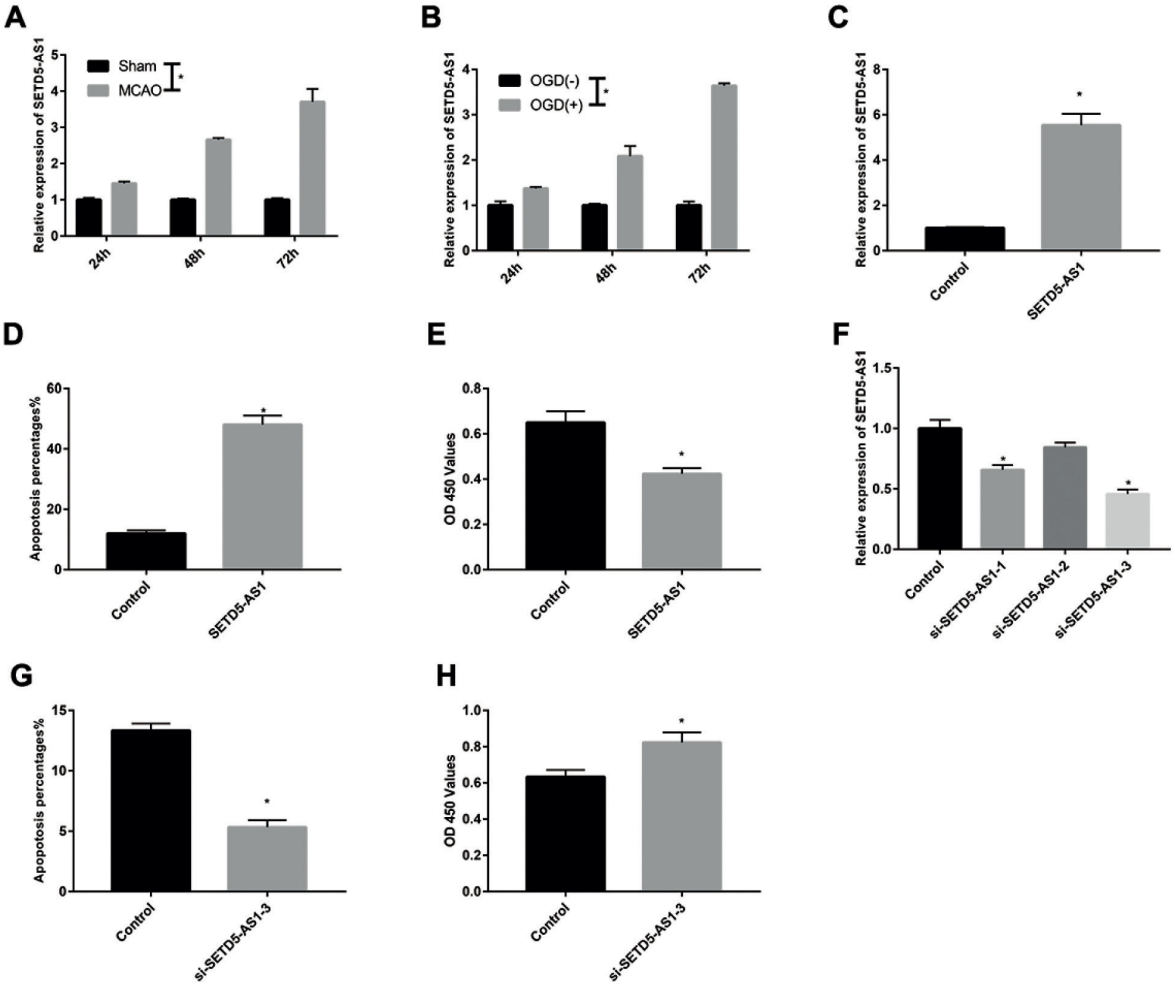

Figure 1. SETD5-AS1 was highly expressed in MCAO model and OGD/R model. ***A,*** SETD5-AS1 was highly expressed in MCAO model. ***B,*** SETD5-AS1 was highly expressed in OGD/R model. **C,** Transfection efficacy of overexpression plasmid of SETD5-AS1 in N2a cells. ***D,*** Overexpression of SETD5-AS1 promoted apoptosis of N2a cells. ***E,*** Overexpression of SETD5-AS1 inhibited proliferation of N2a cells. ***F,*** Transfection efficacy of siRNA SETD5-AS1 in N2a cells. ***G,*** SETD5-AS1 knockdown inhibited apoptosis of N2a cells. ***H,*** SETD5-AS1 knockdown promoted proliferation of N2a cells.

screened out. PTEN was highly expressed in MCAO model (Figure 2A). Overexpression of SETD5-AS1 remarkably elevated mRNA and protein expressions of PTEN in N2a cells (Figure 2B and 2C). Dual-luciferase reporter gene assay indicated that SETD5-AS1 can promote PTEN transcription (Figure 2D). RNA pull-down assay and RIP were performed to further verify the correlation between SETD5-AS1 and PTEN (Figure 2E and 2F). The above data demonstrated that SETD5-AS1 participates in the development of ischemic stroke via activating PTEN.

SETD5-AS1 Inhibited PI3K/AKT Pathway

Since AKT pathway is confirmed to be involved in the development of ischemic stroke, we hypothesized whether SETD5-AS1 could regulate PI3K/AKT pathway in MCAO model. The results indicated that expressions of AKT and PI3K were remarkably upregulated after SETD5-AS1 knockdown in N2a cells (Figure 3A). Overexpression of SETD5-AS1 obtained the opposite results (Figure 3B). The above results indicated that SETD5-AS1 participates in stroke through PI3K/AKT pathway.

Discussion

Due to the extremely complicated pathogenesis, the disability rate and mortality rate of ischemic stroke are relatively high that poses a great challenge for effective treatment. Although many molecules

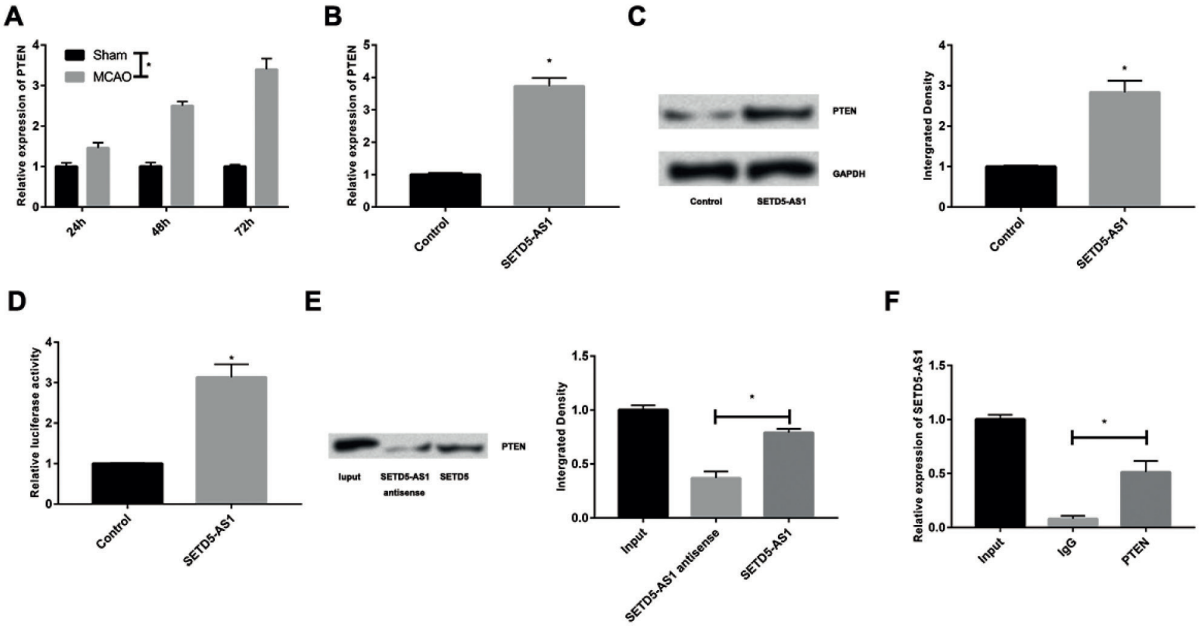

Figure 2. SETD5-AS1 activated PTEN expression. ***A,*** PTEN was highly expressed in MCAO model. ***B,*** Overexpression of SETD5-AS1 increased mRNA level of PTEN. ***C,*** Overexpression of SETD5-AS1 increased protein level of PTEN. ***D,*** SETD5-AS1 activated PTEN transcription. ***E,*** RNA pull-down assay indicated that SETD5-AS1 was bound to PTEN. ***F,*** RIP indicated the binding condition of PTEN and SETD5-AS1.

have been developed for target therapy of ischemic stroke, the treatment efficacy is still unsatisfactory. In recent years, lncRNAs have been found to be involved in the pathological process of ischemic stroke[13-15]. Hence, lncRNAs could be served as novel targets for diagnosing and treating ischemic stroke.

MCAO model and OGD/R model are the frequently applied *in vivo* and *in vitro* models for studying ischemia-reperfusion injury, respectively. MCAO model established by suture-occluded method has advantages of small injury, few complications and low mortality. Additionally, suture-occluded method could flexibly control the ischemia time with definite efficacy. Typically, OGD/R model is constructed using primary neurons[16,17]. In this work, N2a cells were used for constructing OGD/R *in vitro*, which is also the frequently applied cell line in ischemic stroke exploration. Our study found that SETD5-AS1 was highly expressed in MCAO model and OGD/R model.

PTEN is a tumor-suppressor gene with a dual-specificity phosphatase activity, which regulates cell signaling, growth, migration, and apoptosis[18,19]. Scholars[20,21] have shown that PTEN

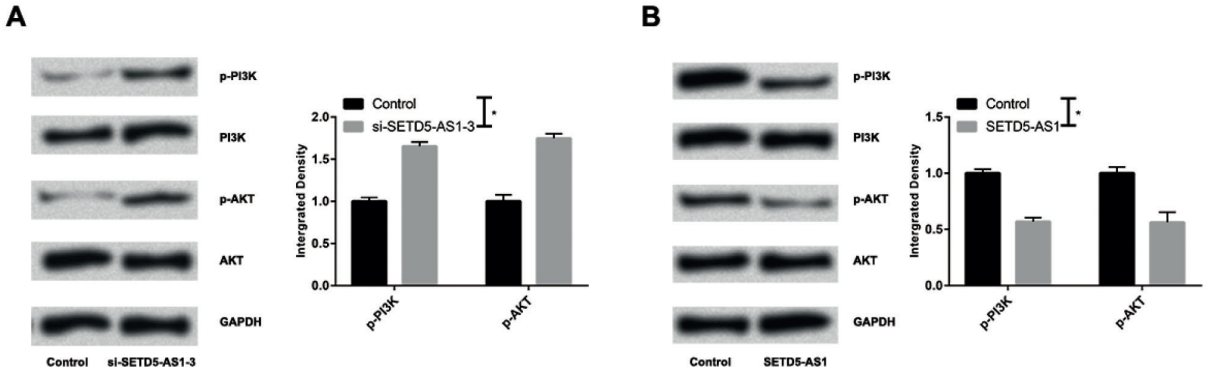

Figure 3. SETD5-AS1 inhibited PI3K/AKT pathway. ***A,*** SETD5-AS1 knockdown activated PI3K/AKT pathway. ***B,*** SETD5-AS1 overexpression inhibited PI3K/AKT pathway.

knockdown exerts a protective role in ischemia-reperfusion injury. In spinal cord injury, PTEN downregulation can promote neuronal axon regeneration[22,23]. These studies have suggested that inhibition of PTEN may protect development of some central nervous system (CNS) diseases. In this study, PTEN was highly expressed in MCAO and OGD/R models, which was activated by SETD5-AS1.

PI3K/AKT pathway participates in the regulation of cell apoptosis, survival and proliferation *via* targeting downstream genes, including Bad, caspase and NF-κB[24-26]. AKT is commonly expressed in normal brain tissue under phosphorylation state. It is found that p-AKT (Ser473) was upregulated in focal and global cerebral ischemia-reperfusion[27]. PTEN, another upstream factor of AKT, negatively regulates AKT expression. The expression level of p-PTEN is decreased in the early stage of ischemia and recovers to normal level within 24 h[28]. Our study confirmed that knockdown of SETD5-AS1 remarkably elevated expression levels of AKT and PI3K in ischemic stroke models.

Conclusions

We showed that SETD5-AS1 was highly expressed in the ischemia-reperfusion injury model. SETD5-AS1 participates in the development of ischemic stroke by activating PTEN and inhibiting PI3K/AKT pathway.

Conflict of Interest

The Authors declare that they have no conflict of interest.

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