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Overexpression of TUG1 promotes neuronal death after cerebral infarction by regulating microRNA-9

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Abstract. – OBJECTIVE: This study aims at investigating whether TUG1 (Taurine Upregulated Gene 1) can regulate FOXO3 expression through competitive binding to microRNA-9, thus leading to increased neuronal death and promoting the occurrence and development of acute cerebral infarction.

MATERIALS AND METHODS: TUG1 and FOXO3 expressions in cerebral cortical neurons of MCAO mice, control mice and primary neurons were detected by quantitative Real Time-Polymerase Chain Reaction (qRT-PCR). The effects of TUG1 and FOXO3 on neuronal apoptosis were determined by TUNEL after cerebral infarction area was stained with TTC. The binding condition of microRNA-9, TUG1 and FOXO3 was verified by the Luciferase reporter gene assay. Western blot was performed to detect the protein expressions of B-cell lymphoma-2 (BCL-2) and BCL2-Associated X (BAX) after altering the TUG1 or FOXO3 expression in primary neurons.

RESULTS: TUG1 and FOXO3 were overexpressed in cerebral cortical neurons of MCAO mice and primary neurons. The inhibition of TUG1 or FOXO3 resulted in less neuronal apoptosis. Luciferase reporter gene assay demonstrated that TUG1 regulates FOXO3 via TUG1/microRNA-9/FOXO3 regulatory network. Besides, TUG1 inhibited BCL-2 but promoted BAX expression in primary neurons.

CONCLUSIONS: The overexpression of TUG1 can promote neuronal death after cerebral infarction in mice by competitive binding to microRNA-9 and promotion of FOXO3 expression.

Key Words:
TUG1, Cerebral infarction, Neuronal death, ceRNA.

Introduction

Acute cerebral infarction is a condition of ischemic necrosis of brain tissue resulted from intracerebral thrombosis or arteriosclerosis, which is a common clinical cerebrovascular disease[1]. It mainly affects middle-aged and elderly patients, with high morbidity and mortality[2]. Previous studies suggested that necrosis is the major performance of ischemic neurons. However, current reports demonstrated that neuronal apoptosis and delayed neuronal death are observed in I/R (ischemia/reperfusion) animal model[3]. The morphological changes of delayed neuronal death differ from necrosis and apoptosis. Delayed neuronal death indicates the acute stage of ischemia that could not be used to assess the direction of neuronal damage. Hyperplastic endoplasmic reticulum arranges in a lamellar structure in the delayed dead neurons. Besides, swelling endoplasmic reticulum pool is vacuolated with pyknosis. No significant changes in mitochondria are found in delayed neuronal death[4]. B-cell lymphoma-2 (BCL-2) family is currently recognized as being closely related to cell apoptosis. BCL-2 is the major anti-apoptosis gene and BCL2-Associated X (BAX) is a crucial pro-apoptosis gene. BCL-2/BAX reflects apoptosis occurrence[5].

Recent scholars have shown that human genome can be transcribed into non-coding RNA (ncRNA) with limited or no protein-coding function[6]. Long non-coding RNAs (lncRNAs) participate in multiple biological processes at different levels. LncRNA dysfunction is associated with many diseases, including tumors[7,8]. LncRNAs affect epigenetic status through chromatin-modifying complexes and lead to the phenotype alteration required for tumor progression and metastasis[9]. Moreover, lncRNA may serve as a key factor in the regulatory network of oncogenes and tumor suppressors[7]. Therefore, it is necessary to identify tumor-related lncRNAs and their poten-

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tial functions to improve clinical outcomes of tumor patients. Studies have shown that lncRNA regulates target genes at epigenetic, transcriptional, post-transcriptional, and translational levels[10]. In recent years, relative works have found that lncRNA can exert its biological function as competing endogenous RNA (ceRNA). CeRNAs, also known as molecular sponges of miRNAs, exert their biological effects on regulating downstream target miRNAs[11-13].

MiRNAs are a series of small, non-coding, single-stranded RNAs with 18-25 nt in length. They degrade and inhibit transcription of mRNA by complementary binding to target mRNA[14]. Functionally, miRNAs precisely regulate a wide range of biological behaviors such as cell proliferation, apoptosis, migration and immune responses[15]. It has been found that dysregulation of certain miRNAs exerts a key role in the tumor development[16,17].

TUG1 (Taurine Upregulated Gene 1) is located on 22q12. 2 with a total length of 7.1 kb. Previous studies have reported that TUG1 is involved in mouse retinal development[18]. Upstream of TUG1 in tumor cells is directly regulated by p53, which further influences p53-induced cellular functions[19]. Other studies[20,21] showed that TUG1 is differentially expressed in tumors, which affects proliferation and apoptosis of tumor cells. The TUG1 expression is confirmed to be correlated with patient prognosis. Overexpressed TUG1 leads to hypoxia/ischemia-induced brain injury and apoptosis of primary hippocampal neurons. The underlying mechanism of TUG1 in cerebral infarction, however, still remains to be studied. This study aims at investigating whether TUG1 exerts its biological function through upregulating FOXO3 by microRNA-9.

Materials and Methods

Neuron Culture and OGD (Oxygen Glucose Deprivation) Model Construction

Neonatal mice (within 24 h after birth) were placed in Phosphate-Buffered Saline (PBS). Cerebral cortex was exposed for stripping the meninges and blood vessels. Cortex tissues were cut into 1 mm^3, digested with trypsin for 15 min at 37°C and washed with Dulbecco's Modified Eagle Medium (DMEM; Gibco, Rockville, MD, USA) containing 10% Fetal Bovine Serum (FBS) and 10% horse serum (Gibco, Rockville, MD, USA). Tissues were gently blown with DMEM 10 times and filtered with 200-mesh sieve. The mixture was seeded in dishes pre-coated with 0.1 g/L Poly-D-lysine. Primary neurons were maintained in a 5% CO_2 incubator at 37°C. Neurobasal medium (2% B27 and 2 mmol/L L-glutamine) was replaced 30 min later. Half of the medium was replaced every 3 days. After cell culture for 7 days, glucose-free DMEM was added for 30 min-incubation, followed by replacement of neurobasal medium for the following incubation.

MCAO Mouse Model Construction

This study was approved by the Ethics Committee of Wuhan Central Hospital, Tongji Medical College, Huazhong University of Science and Technology. MCAO (Middle Cerebral Artery Occlusion) mouse model was conducted according to the previous work. Briefly, common carotid artery, external carotid artery and internal carotid artery were ligated after mouse anesthesia. The suture (0.23 mm of tip diameter, 0.18 mm of trunk diameter) was inserted in external carotid and flowed upside to arteria cerebri media (12 mm in length). Blood flow from the common carotid artery to the internal carotid artery was restored after obstruction for 1 h. All procedure lasted 80 min. Mice were given free access to water and diet. Enrolled mice were randomly assigned into sham group and MCAO group.

Triphenyltetrazolium Chloride (TTC) Stain

Brain tissues were prepared into 1.5 mm slices and washed with PBS. After TCC stain, slices were fixed with 4% paraformaldehyde for preservation. Ulead Photo Express 2.0 image analysis software was introduced for drawing the outline of cerebral infarction. Infarct volume was calculated by forensic damage measurement software.

Neurological Function Evaluation

Mice were pre-trained before the procedure for consecutive 3 days. Neurological function score calculated on the day before the procedure was considered as the baseline. Based on the modified neurological function evaluation, scores were recorded after I/R for 1 d, 3 d, 7 d, 14 d, 21 d, and 28 d, respectively[22]. 0 point was considered with no neurological deficit symptoms and 15 was mouse death.

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

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). QRT-PCR was then performed based on the instructions of SYBR Premix Ex Taq TM (TaKaRa, Otsu, Shiga, Japan), with 3 replicates in each group. Primers used in the study were as follows: TUG1, F: 5'-TTCCTACCACCTTACTACTGACG-3', R: 5'-GGAGGTAAAGGCCACATC-3'; MicroRNA-9, F: 5'-GTGCAGGGTCCGAGGT-3', R: 5'-GCGCTCTTTGGTTATCTAGC-3'; FOXO3, F: 5'-CGGACAAACGGCTCACTCT-3', R: 5'-GGACCCGCATGAATCGACTAT-3'; GAPDH, F: 5'-AGCCACATCGCTCAGACAC-3', R: 5'-GCCCAATACGACCAAATCC-3'; U6, F: 5'-CTCGCTTCGGCAGCACATATA-3', R: 5' -AAATATGGAACGCTTCACGA-3'. Relative gene expression was calculated by $2^{-\Delta\Delta CT}$ method.

Cell Transfection

Lentiviruses were constructed by Gene Pharma (Shanghai, China). Neurons were centrifuged at 1000 r/min for 5 min and resuspended in TrypLE Express for preparation of cell suspension. 5×10^5 cells were seeded in each well of 24-plate and cell transfection was performed based on the instructions of Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). Fresh medium was replaced 6 h later.

Luciferase Reporter Gene Assay

The binding site of microRNA-9, TUG1 and FOX3 was predicted by Target Scan. Wild-type and mutant-type plasmids were constructed by Ruizhen (Nanjing, China). MicroRNA-9 mimic and negative control were constructed by GenePharma (Shanghai, China). Luciferase activity was detected according to the instructions of relative commercial kit.

Cell Apoptosis Detection

After cell transfection for 48 h, neuronal apoptosis was detected according to the instructions of the TUNEL assay kit. TUNEL-positive neurons were stained with brown particles in the nucleus and apoptotic neurons were calculated.

Western Blot

The total protein was extracted from treated cells by radioimmunoprecipitation assay (RIPA) solution (Beyotime, Shanghai, China). The protein sample was separated by electrophoresis and transferred with Polyvinylidene difluoride (PVDF) membrane (Millipore, Billerica, MA, USA). After membranes were blocked with skimmed milk, the membranes were incubated with primary antibodies (Cell Signaling Technology, Danvers, MA, USA) overnight at 4°C, followed by the incubation of secondary antibody at room temperature for 1 h. The protein blot on the membrane was exposed by enhanced chemiluminescence (ECL).

Statistical Analysis

Statistical Product and Service Solutions (SPSS) 22.0 statistical software (IBM, Armonk, NY, USA) was used for data analysis. All data were expressed as mean ± SEM. Chi-square test and *t*-test were used for classification and measurement data, respectively. $p<0.05$ was considered to be statistically significant.

Results

TUG1 was Overexpressed in Cerebral Infarction

We detected TUG1 expression in cerebral cortical neurons of mice in MCAO group and sham group by qRT-PCR. The results showed that TUG1 was overexpressed in cerebral cortical neurons of MCAO mice than that of controls (Figure 1A). Subsequently, we found that TUG1 was also highly expressed in the OGD model of primary neurons *in vitro* (Figure 1B). Corresponding lentiviruses were constructed and transfection efficacies of LV-shTUG1 and LV-TUG1 were verified (Figure 1C). *In vitro* expression of TUG1 was altered by tail vein injection of LV-shTUG1 or LV-TUG1, respectively. The infarct size was remarkably decreased after LV-shTUG1 injection (Figure 1D). Foot fault test was performed to evaluate mouse neurological function. The results showed that the LV-shTUG1 group had a higher score than that of LV-Vector group, while the LV-TUG1 group and the LV-Vector group had no significant difference (Figure 1E). The results demonstrated that compared with the LV-Vector group, apoptotic cells in the LV-shTUG1 group decreased, while the apoptotic cells in the LV-TUG1 group increased (Figure 1F).

TUG1 Regulated microRNA-9 Expression

Bioinformatics predicted that microRNA-9 was the target gene of TUG1 (Figure 2A). Luciferase reporter gene assay revealed that lower Luciferase activity was found after co-transfection with TUG1-WT and microRNA-9 mimic. However, no significant difference in luci-

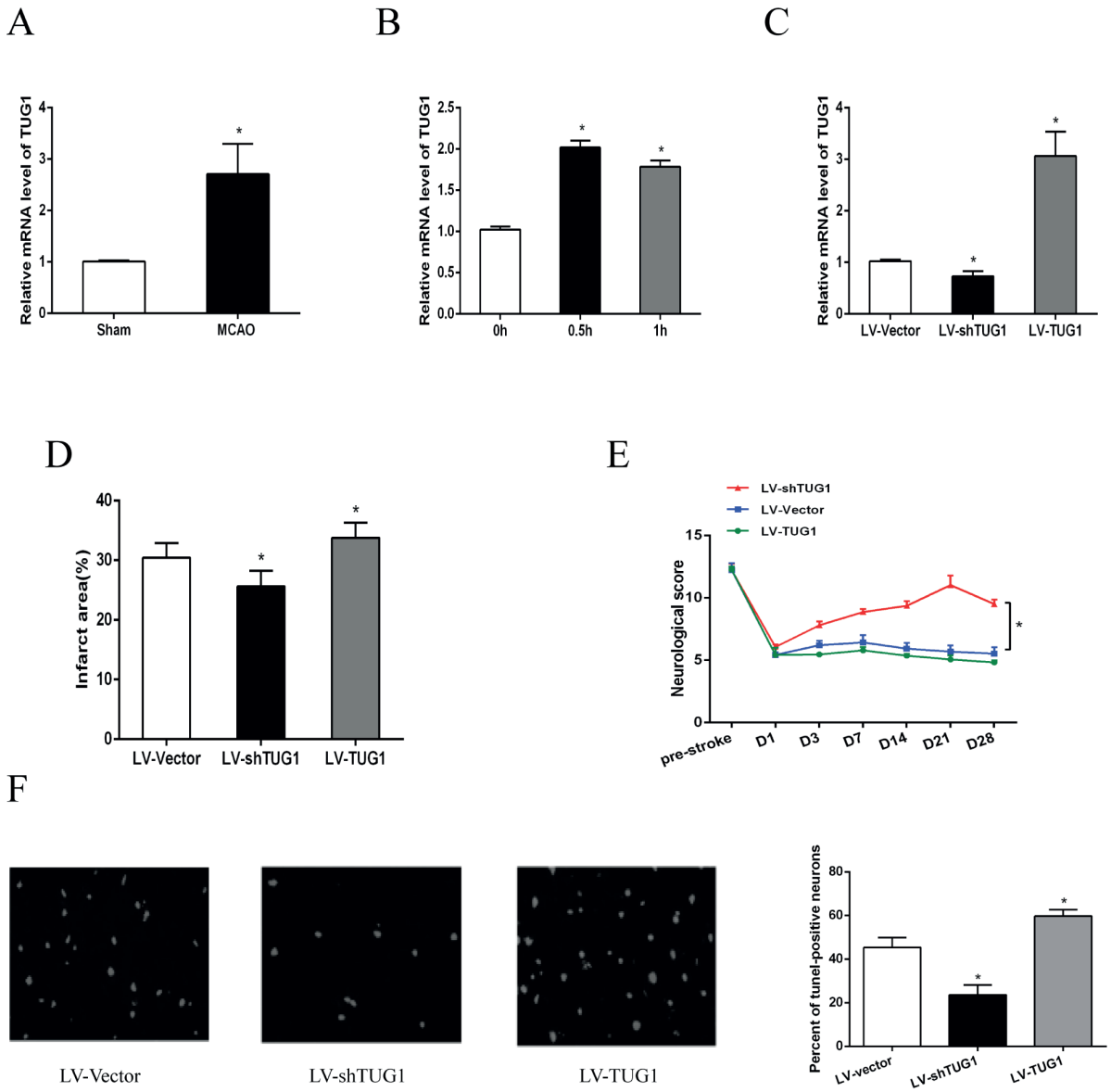

Figure 1. TUG1 was overexpressed in cerebral infarction. ***A,*** TUG1 was overexpressed in cerebral cortical neurons of MCAO mice than that of controls (Figure 1A). ***B,*** TUG1 was also highly expressed in OGD model of primary neurons *in vitro*. ***C,*** Transfection efficacies of LV-shTUG1 and LV-TUG1 were verified. ***D,*** The infarct size decreased after LV-shTUG1 injection. ***E,*** LV-shTUG1 group scored higher than the LV-Vector group, while the LV-TUG1 group and the LV-Vector group had no significant difference. ***F,*** Compared with the LV-Vector group, apoptotic cells in the LV-shTUG1 group decreased, while the apoptotic cells in the LV-TUG1 group increased.

ferase activity was observed in those transfected with TUG1-MUT (Figure 2B), indicating that TUG1 could bind to microRNA-9. Subsequently, we found that TUG1 overexpression resulted in downregulated microRNA-9 in primary neurons, suggesting that TUG1 negatively regulates microRNA-9 expression (Figure 2C). In order to further explore the biological function of TUG1 and microRNA-9 in cerebral infarction, we predicted the target gene of microRNA-9 and explored its underlying mechanism. FOXO3 was screened out to be the target gene of microRNA-9 (Figure 2D). Lower luciferase activity was observed in neurons transfected with FOXO3-WT and microRNA-9 mimic, whereas no significant difference was seen in those transfected with FOXO3-MUT (Figure 2E). The mRNA level of FOXO3 in primary neurons was detected after

transfection with microRNA-9 mimic or inhibitor, respectively. The data indicated that FOXO3 is negatively regulated by microRNA-9 (Figure 2F). Furthermore, the mRNA level of FOXO3 in primary neurons was detected after transfection with LV-shTUG1 or LV-TUG1. The data demonstrated that FOXO3 is positively regulated by TUG1 (Figure 2G). Western blot results obtained the similar results that FOXO3 expression was decreased after microRNA-9 overexpression or TUG1 downregulation (Figure 2H and 2I).

FOXO3 Promoted Neuronal Apoptosis

We first verified transfection efficacies of LV-shFOXO3 and LV-FOXO3 in both mRNA level and protein level (Figure 3A and 3B). Meanwhile, FOXO3 expression in cerebral cortical neurons was detected in MCAO and sham group. QRT-PCR results demonstrated that FOXO3 was overexpressed in cerebral cortical neurons of MCAO mice (Figure 3C). *In vitro* OGD model also elucidated that FOXO3 was overexpressed in primary neurons (Figure 3D). Subsequently, we detected the effect of FOXO3 on

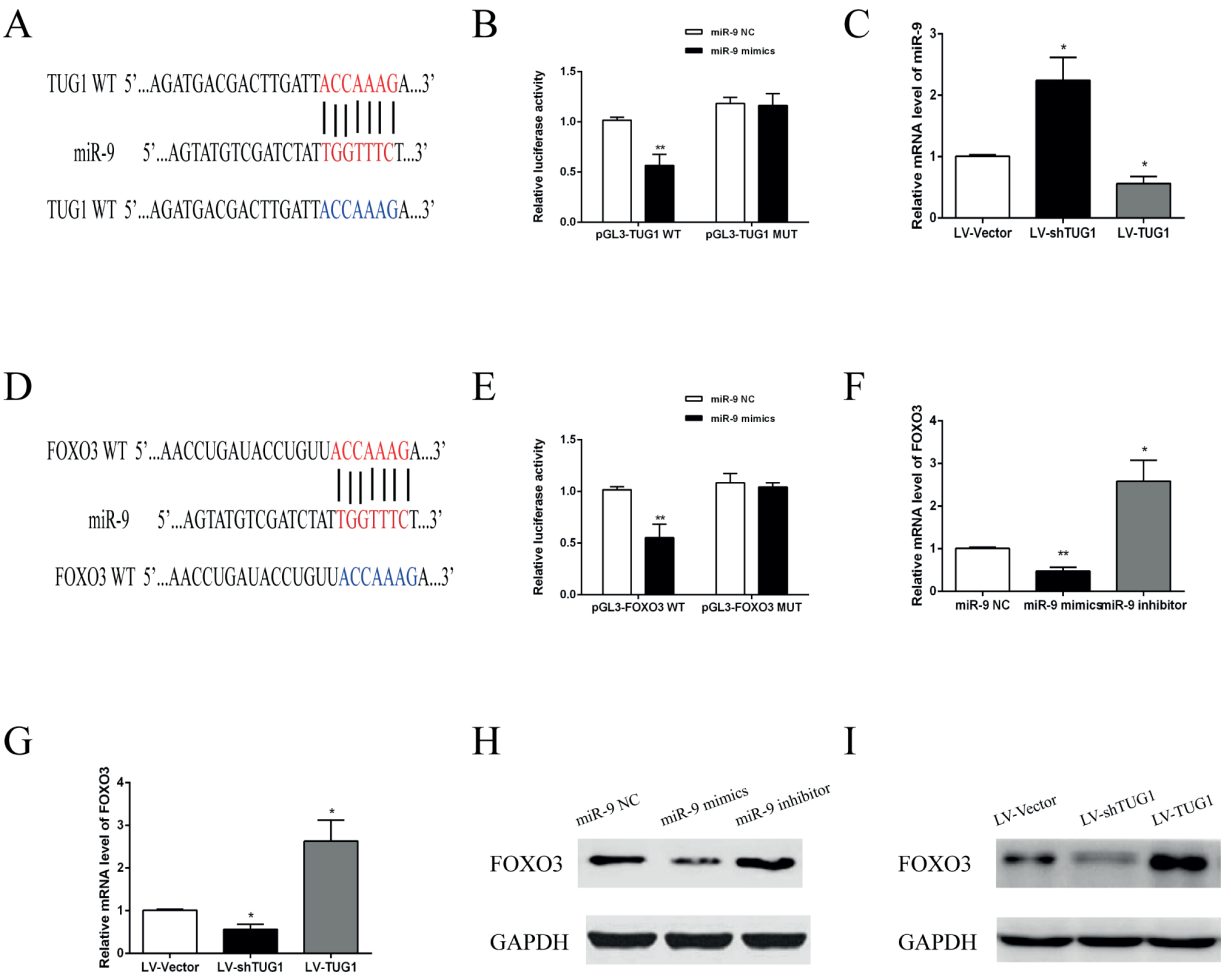

Figure 2. TUG1 regulated microRNA-9 expression. ***A,*** Bioinformatics predicted that microRNA-9 was the target gene of TUG1. ***B,*** Luciferase reporter gene assay revealed that lower luciferase activity was found after co-transfection with TUG1-WT and microRNA-9 mimic. However, no significant difference in luciferase activity was observed in those transfected with TUG1-MUT. ***C,*** TUG1 overexpression resulted in downregulated microRNA-9 in primary neurons. ***D,*** FOXO3 was screened out to be the target gene of microRNA-9. ***E,*** Lower luciferase activity was observed in neurons transfected with FOXO3-WT and microRNA-9 mimic, whereas no significant difference was seen in those transfected with FOXO3-MUT. ***F,*** The data indicated that FOXO3 is negatively regulated by microRNA-9. **G,** FOXO3 is positively regulated by TUG1. ***H, I,*** Western blot results obtained the similar results that FOXO3 expression was decreased after microRNA-9 overexpression or TUG1 downregulation.

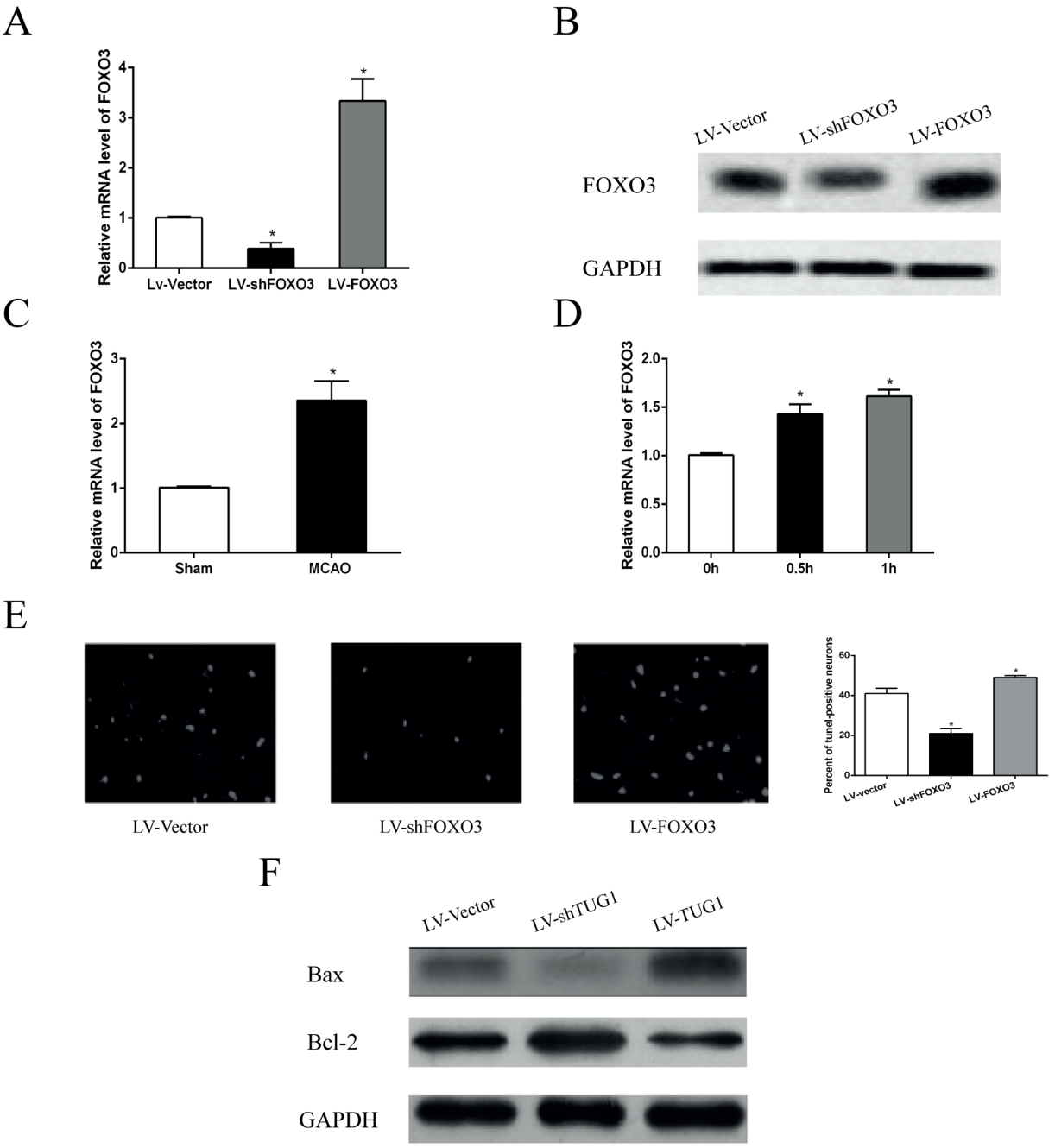

Figure 3. FOXO3 promoted neuronal apoptosis. ***A-B,*** Transfection efficacies of LV-shFOXO3 and LV-FOXO3 in both mRNA level and protein level. ***C,*** QRT-PCR results demonstrated that FOXO3 was overexpressed in cerebral cortical neurons of MCAO mice. ***D,*** *In vitro* OGD model also elucidated that FOXO3 was overexpressed in primary neurons. ***E,*** TUNEL assay indicated that neuronal apoptosis was alleviated after LV-shFOXO3 transfection. ***F,*** Western blot results also indicated that LV-shFOXO3 transfection resulted in downregulated BAX and upregulated BCL-2.

neuronal apoptosis by TUNEL and Western blot. TUNEL assay indicated that neuronal apoptosis was alleviated after LV-shFOXO3 transfection (Figure 3E). Western blot results also indicated that LV-shFOXO3 transfection resulted in downregulated BAX and upregulated BCL-2 (Figure 3F).

Discussion

The deterioration of neurological function in patients with acute cerebral infarction refers to progressive aggravation even after positive and timely treatment. Neurological impairment is evaluated

based on the NIH Stroke Scale (NIHSS)[23]. A recent study showed that advancing stroke, brains welling, cerebral ischemia recurrence and cerebral parenchyma hemorrhage are the main reasons for neurological function deterioration. Acute cerebral infarction has higher morbidity and mortality than other types of stroke. Survivors often have long-lasting and severe neurological impairment. Neurological function is considered as an independent prognostic factor for cerebral infarction patients[24]. At present, the etiology and pathogenesis of acute cerebral infarction are still unclear. Some studies have speculated that infarct volume, brain edema and other relative risk factors are responsible for neurological function deterioration[3].

TUG1 is believed to be involved in the occurrence and development of tumors mainly through competitive binding to miRNAs, regulation of cyclin-dependent kinase inhibitors, and effects on tumor proliferation and apoptosis. For example, TUG1 can directly regulate the blood-tumor barrier *via* altering microRNA-144 expression. The downstream factor heat shock protein transcription factor 2 is subsequently activated to upregulate ZO-1, occludin, and claudin-5, thereafter enhancing blood-tumor barrier and reducing chemotherapy efficacy[25]. TUG1 inhibits miR-144/c-Met axis to promote the metastasis and invasion of gastric cancer cell lines, which eventually promotes tumorigenesis[26]. TUG1 inhibits glioblastoma viability through activating apoptosis induced by caspase-3 and caspase-9[27]. In the present work, we found that TUG1 is overexpressed in cortical neurons isolated from MCAO mice and primary neurons in the OGD model. Overexpressed TUG1 enlarged cerebral infarct size and reduced neurological function in MCAO mice. *In vitro* experiments further confirmed that TUG1 overexpression promotes neuronal apoptosis.

MicroRNAs are a type of endogenous, non-encoded, single-stranded, RNA molecules. They are highly conserved in evolution and widely present in animals and plants. MicroRNAs regulate target genes at post-transcriptional level by complementary base pairing. Differentially expressed microRNAs are closely related to the occurrence and development of cerebral infarction, as well as secondary neuronal death after cerebral infarction. MicroRNA-9 is widely expressed in mammalian embryos and brain, gallbladder, nerves, and pancreas. It is closely related to proliferation and differentiation of neural cells.

BCL-2 and BAX are vital genes that regulate apoptosis of brain cells[28,29]. FOXO3 is located on 6p22. 2, which was previously considered to be related to dyslexia. The expression product of FOXO3 is a bicorticoid cytoplasmic protein that can promote neuronal migration in the interaction with cell membrane microtubules. FOXO3 stimulates neuronal migration to ventricle and cerebral cortex during the embryonic stage and maturation stage, respectively.

Our study found that TUG1 regulates microRNA-9 expression and FOXO3 degradation induced by microRNA-9. FOXO3 was overexpressed in cortical neurons isolated from MCAO mice and primary neurons in the OGD model which promoted neuronal apoptosis. Further researches demonstrated that FOXO3 is regulated by TUG1, so as to regulate the occurrence and progression of cerebral infarction.

Conclusions

We found that overexpression of TUG1 can promote neuronal death after cerebral infarction in mice by competitive binding to microRNA-9 and promotion of FOXO3 expression.

Conflict of Interest

The Authors declare that they have no conflict of interest.

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