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Original Paper

Down-Regulation of Lncrna MALAT1 Attenuates Neuronal Cell Death Through Suppressing Beclin1-Dependent Autophagy by Regulating Mir-30a in Cerebral Ischemic Stroke

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Key Words
Lncrna MALAT1 • Neuronal cell death • Autophagy • MiR-30a • Beclin1 • Cerebral ischemic stroke

Abstract
Background/Aims: LncRNA metastasis associated lung adenocarcinoma transcript 1 (MALAT1) was reported to be highly expressed in an *in vitro* mimic of ischemic stroke conditions. However, the exact biological role of MALAT1 and its underlying mechanism in ischemic stroke remain to be elucidated. ***Methods:*** The roles of MALAT1 and miR-30a on cell death and infarct volume and autophagy were evaluated in experimental ischemic stroke. The relationships between miR-30a and MALAT1, Beclin1 were confirmed by luciferase reporter assay. The autophagy inhibitor 3-methyadenine (3-MA) was used to examine the impact of autophagy on ischemic injury. ***Results:*** We found that MALAT1, along with the levels of conversion from autophagy-related protein microtubule-associated protein light chain 3-I (LC3-I) to LC3-phosphatidylethanolamine conjugate (LC3-II), as well as Beclin1 were up-regulated and miR-30a was down-regulated in cerebral cortex neurons after oxygen-glucose deprivation (OGD) and mouse brain cortex after middle cerebral artery occlusion-reperfusion (MCAO). Down-regulation of MALAT1 suppressed ischemic injury and autophagy *in vitro* and *in vivo*. Furthermore, MALAT1 may serve as a molecular sponge for miR-30a and negatively regulate its expression. In addition, MALAT1 overturned the inhibitory effect of miR-30a on ischemic injury and autophagy *in vitro* and *in vivo*, which might be involved in the derepression of Beclin1, a direct target of miR-30a. Mechanistic analyses further revealed that autophagy inhibitor 3-methyadenine (3-MA) markedly suppressed OGD-induced neuronal cell death and MCAO-induced ischemic brain infarction. ***Conclusion:*** Taken together, our study first revealed

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that down-regulation of MALAT1 attenuated neuronal cell death through suppressing Beclin1-dependent autophagy by regulating miR-30a expression in cerebral ischemic stroke. Besides, our study demonstrated a novel lncRNA-miRNA-mRNA regulatory network that is MALAT1-miR-30a-Beclin1 in ischemic stroke, contributing to a better understanding the pathogenesis and progression of ischemic stroke.

Introduction

Stroke is a leading cause of serious long-term neurological disability in developed countries, along with an incidence of approximately 0.25%-4% and a mortality rate of about 30% [1]. Ischemic stroke occupies approximately 80%-85% of all strokes, usually resulting from atherothrombotic or embolic occlusion of a cerebral artery [2]. Extensive evidence has indicated that ischemia is accompanied by a series of neurological events, such as hypoxia, oxidative stress, and inflammatory response [3], and eventually results in neuronal cell acute necrosis, apoptosis and autophagy in the ischemic brain [4, 5]. Up to date, thrombolysis by intravenous recombinant tissue plasminogen activator (tPA) is the only clinically effective therapeutic strategy for ischemic stroke [6]. Therefore, more efforts are still needed to make in identifying the delicate mechanism of stroke cerebral stroke underlying stroke-induced cell death and neurological dysfunction.

Approximately 90% of human genome is transcribed into RNA, but the majority of them are transcribed into non-coding RNAs (ncRNAs) with no protein-encoding capacity [7]. Generally, ncRNAs, including long non-coding RNAs (lncRNAs) and small non-coding RNAs, are abundantly expressed in mammalian central nervous system (CNS) [8]. Currently, it is well documented that more than 20% of microRNAs (miRNAs) are abnormally expressed in the ischemic brain, suggesting that miRNAs are implicated in the pathogenesis and development of ischemic stroke [9, 10].

LncRNAs are a set of ncRNAs with more than 200 nucleotides that regulate gene expression at transcriptional, epigenetic, or translational levels [11]. RNA-seq analyses have indicated that the altered expression of lncRNAs is involved in the brain development and generally contributes to diverse neurological disorders, such as Alzheimer's disease (AD), Parkinson's disease (PD) [12] and ischemic stroke [13]. LncRNA metastasis associated lung adenocarcinoma transcript 1 (MALAT1), also referred to as non-coding nuclear-enriched abundant transcript 2 (NEAT2) [14], was initially identified as a sign of metastasis in lung cancer [15]. Accumulating evidence reveals that MALAT1 is up-regulated in many solid tumors and associated with cancer metastasis and recurrence [16]. More interestingly, MALAT1 was reported to be highly expressed in an *in vitro* mimic of ischemic stroke conditions [17]. However, the role of MALAT1 in ischemic stroke and its underlying mechanism have not been elucidated.

Autophagy is a cellular self-cannibalization process that breaks down organelles and macromolecules through lysosomal degradation, whereas its failure always leads to cell death [18]. During autophagy, the conversion from a cytosolic form of microtubule-associated protein 1 light chain 3-I (LC3-I) to LC3-phosphatidylethanolamine conjugate (LC3-II) promotes autophagosome formation [19]. Beclin-1, the mammalian homologue of yeast autophagy-associated protein 6 (Atg6), is a key autophagy-associated protein that strongly induces autophagy, which plays pivotal roles in regulating neuronal survival or death in cerebral ischemia [20, 21]. It is well documented that several miRNAs can regulate autophagy by modulating the expression of autophagy-related genes [22, 23]. A previous study reported that down-regulation of miR-30a alleviated ischemic injury by promoting Beclin-1-mediated autophagy [24].

In the present study, we investigated the role of MALAT1 and the interaction among MALAT1 miR-30a and Beclin1 in ischemic stroke, contributing to a better understanding of the pathogenesis of ischemic stroke.

Materials and Methods

Reagents

Adult male C57BL/6 J mice (weighing 22-25 g) were obtained from the Experimental Animal Center of the Chinese Academy of Medical Sciences, People's Republic of China. siRNA against MALAT1 (si-MALAT1), siRNA control (si-control), pcDNA-MALAT1, pcDNA empty vector (vector), miR-30a mimics (miR-30a), miRNA control (miR-control), anti-miR-30a, and anti-miR-control were all purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The Cell Counting Kit-8 was obtained from Dojindo Laboratories (Tokyo, Japan). The primary antibodies against Beclin1, LC3-I, LC3-II and β-actin were purchased from Abcam (Cambridge, MA, USA). All other chemical reagents were purchased from Sigma unless indicated otherwise.

Primary cerebral cortex neuronal culture and oxygen-glucose deprivation (OGD)

Primary cerebral cortex neuronal cultures were obtained from embryonic day 17 C57BL/6 mice as described previously [25]. Primary cortical neurons were isolated and seeded into 24-well plates (1.5×10^5 cells/well) and maintained in a neurobasal medium (Gibco, Grand Island, NY, USA) containing 2% B27 (Gibco), 0.5 mM of glutamine and 50 U/mL of penicillin/streptomycin at 37°C with 5% CO_2. OGD model was established according to previously reported methods with slight modification [26]. In brief, the primary cortical neurons were washed with glucose-free DMEM medium (Gibco) and incubated with glucose-free DMEM medium in an anaerobic chamber containing 5% CO_2 and 95% N_2 at 37°C. After 3 h of OGD treatment, the cell culture medium was changed back to normal neurobasal medium under normoxic conditions for 3 h recovery. In sham group, neurons were treated identically without OGD exposure. In addition, neurons were treated with 5 mM 3-MA for 48 h prior to OGD treatment.

MCAO-induced ischemic stroke mouse model

The animal experimental procedures were approved by the Animal Care Committee of the First Affiliated Hospital of Zhengzhou University and performed in accordance with the guidelines set forth in the First Affiliated Hospital of Zhengzhou University. The C57BL/6 J mice were housed in an environmentally controlled room under a 12h light/dark cycle with free access to food and water ad libitum. Ischemic stroke mouse model was induced by MCAO, as described previously [27]. Briefly, the mice were anesthetized with pentobarbital sodium and the left common carotid artery (CCA) and left external carotid arteries (ECA) were exposed and ligated through a ventral midline neck incision. A 4-0 surgical nylon filament with a blunt tip (0.23 mm in diameter) was inserted into the internal carotid artery to occlude the middle cerebral artery (MCAO). The MCAO-induced ischemic stroke mouse models were prepared through 6 h MCAO without reperfusion followed by 24 h reperfusion. The sham group animals were received the same surgical exposure of the carotid arteries without MCAO. The brains of the mice were removed for further analysis after 24 h reperfusion. In addition, 24 mg/kg of 100 μL autophagy inhibitor 3-methyadenine (3-MA) dissolved in saline was injected intraperitoneally every 5 d for five times. Injection with 100 μL saline was injected as the control.

Quantitative real-time PCR (qRT-PCR)

Total RNA was isolated from cultured cortical neurons after OGD and ischemic tissues after MCAO using TRIzol reagent (Invitrogen). One microgram of extracted RNA was then reverse transcribed into cDNA according to the manufacturer's instructions of a reverse transcriptase kit (Takara, Dalian, China). The RT-PCR was performed to quantify the expressions of MALAT1 and Beclin1 with SYBR Green Real-time PCR Master Mix (Takara, Dalian, China) and miR-30a with the miScript SYBR Green PCR Kit (Qiagen, German) on an ABI 7500 fast real time PCR system (Applied Biosystems, Foster City, CA, USA). The procedures of PCR were performed as follows: 40 cycles of 94°C for 30 s, 60°C for 30 s, and 72°C for 30 s. The relative quantitative of value was determined using the $2^{-\Delta\Delta Ct}$ method and GAPDH expression was used as an internal control.

Western blot analysis

Total protein was extracted from cultured cortical neurons and cerebral cortex using ice-cold RIPA lysis buffer together with a protein inhibitor mixture. For gel electrophoresis, total extracted proteins (30

μg) of each samples were separated by 10% sodium dodecyl sulfate polyacrylamide gel (SDS-PAGE) and then transferred to nitrocellulose membranes (Millipore, Billerica, MA, USA), followed by blocking with 5% nonfat milk for 2 h at room temperature. Subsequently, the membranes were incubated with primary antibodies against Beclin1, LC3-I, LC3-II and β-actin as a control. After three washes, the membranes were further incubated with specific horseradish peroxidase conjugated secondary antibodies (Jackson ImmunoResearch, Inc., West Grove, PA, USA).

Primary cortical neuron transfection

Primary cortical neurons were grown on 24-well plates (1×10^5 cells/well) and maintained for overnight before transfection. Cell transfection with si-MALAT1, miR-30a, anti-miR-30a, pcDNA-MALAT1 or corresponding controls, or cotransfection with miR-30a or miR-control and pcDNA-MALAT1 or vector into cortical neurons was performed by Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA).

Cortical injection of miR-30a, pcDNA-MALAT1 or lentiviral sh-MALAT1

Briefly, mice were anesthetized as above and placed on a stereotactic apparatus. Lentivirus sh-MALAT1 or its control (10^9 TU/mL; GenePharma, Shanghai, China) were mixed with the cationic lipid polybrene (4 μg/μL, GenePharma) and incubated at 37°C for 15 minutes before MCAO. Subsequently, mixture (7 μL) was delivered into the cortex of ischemia region and cerebral cortex of normal mice (n = 6) by microliter syringes (Hamilton CO., Reno, NV, USA) for > 20 mins. miR-30a or miR-control was mixed with pcDNA-MALAT1 or vector, incubated for 20 min, and injected into cortex for 10 min before MCAO exposure.

Cell counting Kit- (CCK-) 8 assay for neuronal death

Cells at a density of 2×10^4 cells/well were seeded in 96-well plates and incubated at 37°C, 5% CO2 overnight. After treatment, CCK-8 solution (Beyotime, Shanghai, China) was added (10 μM/well) to each well, and incubated at 37°C for an additional 4 h. Then, the number of cells per well was determined by measuring the absorbance at 450 nm using Elx800 Reader (Bio-Tek Instruments Inc., Winooski, VT, USA) following OGD.

Neurological score

The foot fault test was performed to evaluate sensorimotor function of affected rat limbs after treated with MCAO according to a published method [28]. Animals were trained for 3 consecutive days before surgery, and the score on the day before injury was used as the baseline. The test was performed at up to 28 d after surgery. Scores of foot fault test were determined as the average of three trials. Neurological scores are defined as the total scores of left forelimb and hindlimb foot fault tests.

Measurement of infarct volume

Cerebral infarction volume was determined using 2, 3,5 triphenyltetrazolium chloride (TTC) staining of brains after MCAO as previously descripted [29]. The dissected brain splices (2 mm thick) were incubated with a 2% TTC solution at 37°C for 20 min. TTC-stained splices were later photographed using a Nikon E950 digital camera attached to a dissecting microscope and the percentage of the infarct volume in the total brain volume was determined with digitized images using the Quantity One software package (Bio-Rad CA).

Luciferase reporter assay

The sequences of wild-type 3'-UTR of Beclin1 gene (WT) and a mutant (MUT) containing the putative binding sites of miR-30a predicted by the TargetScan (http://www.targetscan.org) and the putative miR-30a target binding sequences in MALAT1 wild-type (WT) and a mutant (MUT) predicted by Starbase v.2.0 and miRcode were synthesized and cloned into the downstream of luciferase gene in the pmirGLO dual luciferase reporter vectors (Promega, Madison, WI, USA). The constructed luciferase vectors were named as pmirGLO-Beclin1-3'-UTR-WT, pmirGLO-Beclin1-3'-UTR-MUT, pmirGLO-MALAT1-WT, and pmirGLO-MALAT1-MUT, respectively. Cortical neurons were cotransfected either pmirGLO-MALAT1-WT or pmirGLO-MALAT1-MUT with miR-30a or miR-control, or either pmirGLO-Beclin1-3'-UTR-WT or pmirGLO-Beclin1-3'-UTR-MUT with miR-30a, miR-control, miR-30a + pcDNA-MALAT1 or miR-30a + vector using Lipofectamine2000 (Invitrogen). Firefly and Renilla luciferase activities were measured consecutively by Dual Luciferase Assay (Promega) at 48 h after transfection. Renilla luciferase activity was used as the normalization.

Statistical analysis

All quantitative data were presented as the mean ± SD from at least 3 independent experiments. Statistical significances were evaluated by multivariate analysis of variance (ANOVA) in GraphPad Prism version 5.0 (GraphPad Software, La Jolla, CA, USA). In all cases, a level of $P < 0.05$ was considered statistically significant.

Results

MALAT1, Beclin1 and LC3 conversion were up-regulated and miR-30a was down-regulated in in vitro and in vivo models of ischemic stroke

Cerebral cortex neurons exposed to OGD and MCAO mice were used as *in vitro* and *in vivo* models of ischemic stroke to examine the effects of MALAT1, miR-30a and Beclin1 on regulating ischemic brain damage. The expression status of MALAT1, miR-30a and Beclin1 in *in vitro* and *in vivo* models of ischemic stroke was firstly evaluated by qRT-PCR. The qRT-PCR analyses showed that the expressions of MALAT1 (Fig. 1A and 1F) and Beclin1 (Fig. 1C and 1H) were elevated and miR-30a (Fig. 1B and 1G) was reduced in both cerebral cortex neurons after OGD and mouse brain after MCAO compared with sham groups. Furthermore, to confirm

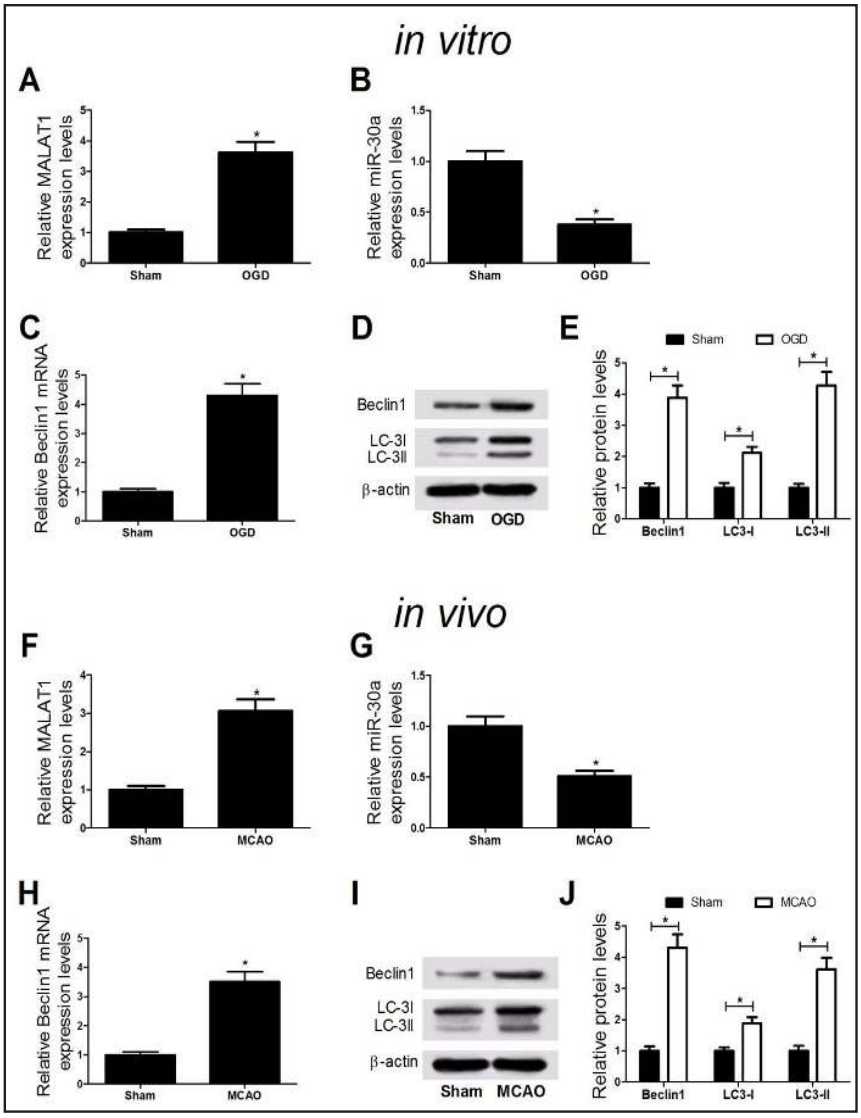

Fig. 1. Expression status of MALAT1, miR-30a, Beclin1 and LC3 conversion in in vitro and in vivo models of ischemic stroke. The expressions of MALAT1 (A and F), miR-30a (B and G), and Beclin1 (C and H) were examined by qRT-PCR in cerebral cortex neurons after OGD and mouse brain after MCAO. The levels of Beclin1, LC3-I and LC3-II in cerebral cortex neurons after OGD (D and E) and mouse brain after MCAO (I and J) were determined by western blot. *$P < 0.05$.

the existence of autophagy in ischemic stroke, the levels of Beclin 1 and LC3 conversion were detected by western blot and the results indicated that the levels of conversion from LC3-I to LC3-II and Beclin1 were significantly improved in cerebral cortex neurons after OGD (Fig. 1D and 1E) and mouse brain after MCAO (Fig. 1I and 1J), suggesting a crucial role of autophagy in ischemic stroke.

Down-regulation of MALAT1 suppressed ischemic injury and autophagy in vitro and in vivo

To explore the biological roles of MALAT1 in ischemic stroke, cortical neurons were transfected with si-MALAT1 or si-control prior to OGD and cerebral cortex were injected with lentivirus sh-MALAT1 or sh-control before MCAO. As shown in Fig. 2A, neuronal cell death was significantly increased after OGD in comparison with sham group while MALAT1 down-regulation dramatically reversed this effect compared with si-control group. In addition, western blot analyses of autophagy-related proteins indicated that OGD treatment markedly improved the conversion of LC3-Ito LC3-II and Beclin1 level in cortical neurons (Fig. 2B and 2C) and cerebral cortex (Fig. 2G and 2H) while MALAT1 down-regulation conspicuously inhibited the increase of conversion of LC3-Ito LC3-II and Beclin1 level by OGD, suggesting that MALAT1 down-regulation strikingly suppressed autophagy of ischemic stroke *in vitro* and *in vivo*. Furthermore, the brain sections were analyzed for infarct formation by TTC staining to investigate the effect of MALAT1 down-regulation on ischemic injury *in vivo*. The TTC staining results exhibited that cerebral ischemia induced a more severe infarct than sham group while MALAT1 down-regulation by sh-MALAT1 led to a significant decrease of infarct volume compared with sh-control group (Fig. 2E and 2F). Neurological outcomes data showed that knockdown of MALAT1 significantly improved long-term neurological outcomes after stroke compared to sh-control treated rats up to 28 d after stroke (Fig. 2D). Taken together, down-regulation of MALAT1 results in an obvious suppression on ischemic injury and autophagy *in vitro* and *in vivo.*

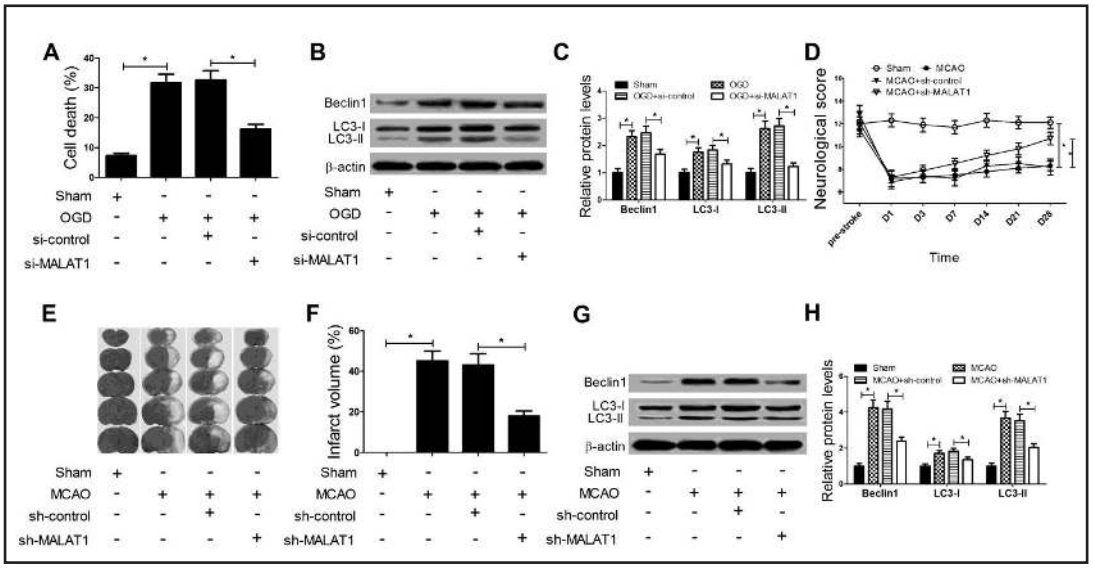

Fig. 2. Effect of MALAT1 down-regulation on ischemic injury and autophagy in vitro and in vivo. Cortical neurons were transfected with si-MALAT1 or si-control prior to OGD and cerebral cortex were injected with sh-MALAT1 or sh-control before MCAO. (A) CCK-8 assay was performed to detect cell survival in transfected cortical neurons after OGD. Western blot analyses were employed to determine the levels of LC3-I, LC3-II and Beclin1 in transfected cortical neurons after OGD (B and C) and injected cerebral cortex after MCAO (G and H). (D) The foot fault test was performed to assess long-term neurological outcomes after MCAO. (E and F) TTC staining of brain sections and quantitative analysis of brain infarct volume. *$P < 0.05$.

MALAT1 may serve as a molecular sponge for miR-30a and negatively regulate its expression

Accumulating evidence has indicated that lncRNAs contain complementary binding sites to miRNAs and competitively inhibit miRNA expression and function [30, 31]. To examine whether MALAT1 had a similar mechanism in cortical neurons, we employed online software including RNAhybird and Starbase v.2.0 to explore the potential miRNAs for MALAT1. The prediction results showed that miR-30a could bind to MALAT1 and the binding sites between them were presented in Fig. 3A. In order to confirm the interaction between MALAT1 and miR-30a, luciferase reporter assay was carried out and the results demonstrated that miR-30a overexpression dramatically inhibited the luciferase activity of pmirGLO-MALAT1-WT (Fig. 3B) and anti-miR-30a markedly improved the luciferase activity of pmirGLO-MALAT1-WT (Fig. 3C), but both had no obvious inhibitory effects on pmirGLO-MALAT1-MUT. The expression of miR-30a in cortical neurons transfected with si-MALAT1, pcDNA-MALAT1 or respective controls was evaluated by qRT-PCR and we noticed that the expression of miR-30a was substantially improved by si-MALAT1 (Fig. 3D) and significantly reduced by pcDNA-MALAT1 (Fig. 3E). These data demonstrated that MALAT1 may serve as a molecular sponge for miR-30a and negatively regulate its expression.

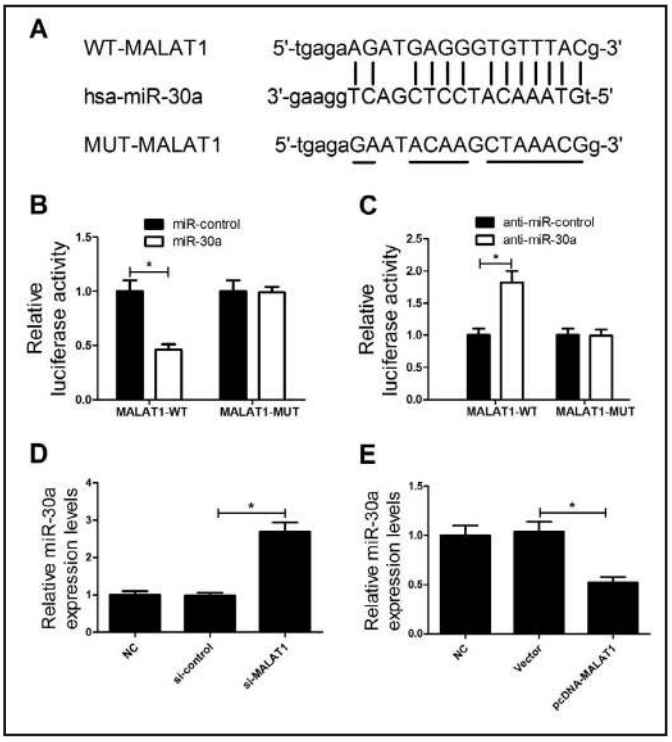

Fig. 3. The interaction between MALAT1 and miR-30a. (A) The predictive binding sites between MALAT1 and miR-30a. (B and C) The luciferase reporter vectors (pmirGLO-MALAT1-WT and pmirGLO-MALAT1-MUT) were cotransfected with miR-30a, anti-miR-30a or matched controls into cortical neurons and luciferase activity was detected by luciferase reporter assay. (D and E) The expression of miR-30a in cortical neurons transfected with miR-30a, anti-miR-30a or corresponding controls was examined by RT-PCR. **P* < 0.05.

MALAT1 overturned the inhibitory effect of miR-30a on ischemic injury and autophagy in vitro and in vivo

To further understand the impact of MALAT1 on the function of miR-30a, cortical neurons were cotransfected with miR-30a or miR-control and pcDNA-MALAT1 or vector prior to OGD. miR-30a or miR-control were mixed with pcDNA-MALAT1 or vector and injected into cerebral cortex after MCAO. As demonstrated by CCK-8 assay, miR-30a overexpression significantly repressed neuronal cell death after OGD in comparison with miR-control-transfected cells (Fig. 4A). In contrast, MALAT1 overexpression conspicuously relieved miR-30a-induced neuronal cell death inhibition. Additionally, the conversion of LC3-I to LC3-II and Beclin1 level were obviously decreased in both miR-30a-transfectedcortical neurons after OGD (Fig. 4B and 4C) and miR-30a-injected cerebral cortex after MCAO (Fig. 4G and 4H). However, MALAT1 overexpression strikingly reversed miR-30a-mediated the decrease of conversion of LC3-I to LC3-II and Beclin1 level. As shown in the figure 4E and 4F, miR-30a overexpression led to a dramatic reduction of MCAO-induced infarct volume of ischemic

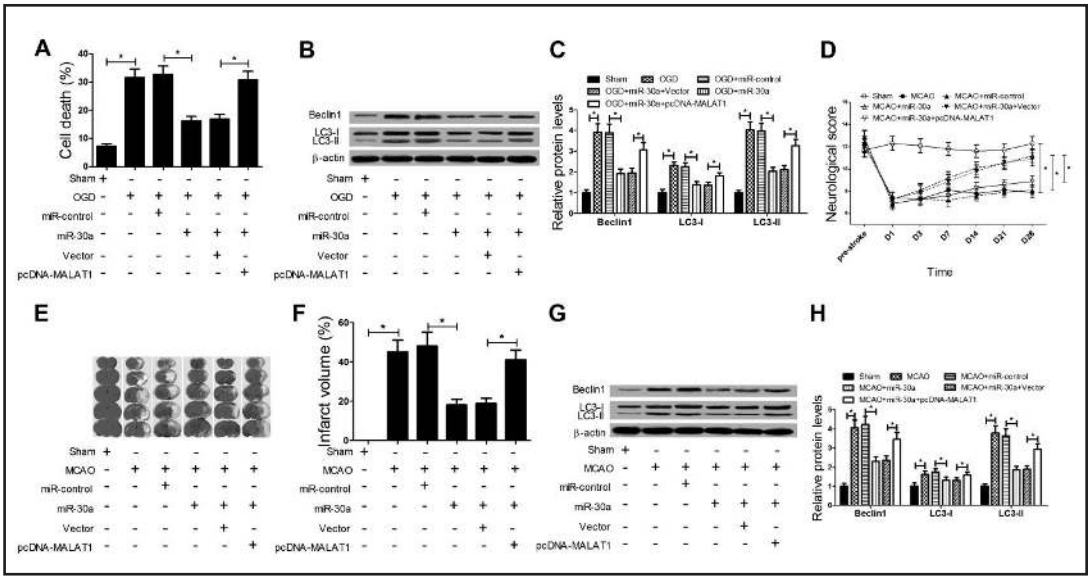

Fig. 4. Effect of miR-30a overexpression or combined with MALAT1 overexpression on ischemic injury and autophagy in vitro and in vivo. Cortical neurons were cotransfected with miR-30a or miR-control and pcDNA-MALAT1 or vector prior to OGD. miR-30a or miR-control were mixed with pcDNA-MALAT1 or vector and injected into cerebral cortex before MCAO. (A) Neuron cell survival in treated cortical neurons was determined by CCK-8 assay. The levels of LC3-I, LC3-II and Beclin1 were detected by western blot in treated cortical neurons (B and C) and cerebral cortex (G and H). (D) The foot fault test was performed to assess long-term neurological outcomes after MCAO or other treatment at the same time. (E and F) The infarct volume of ischemic brain induced by MCAO was evaluated by TTC staining. *$P < 0.05$.

brain while MALAT1 overexpression significantly alleviated this effect. Furthermore, miR-30a overexpression led to an improvement of MCAO-induced neurological deficit of rats while MALAT1 overexpression significantly alleviated this effect (Fig. 4D). Taken together, MALAT1 conspicuously reversed the inhibitory effect of miR-30a on ischemic injury and autophagy *in vitro* and *in vivo*.

MALAT1 positively regulated the derepression of Beclin1 by sponging miR-30a.

A previous study has shown that miR-30a could directly target Beclin1 in cortical neurons [22]. In view of the inhibitory effect of MALAT1 on miR-30a expression and function, we further examine the relationship between MALAT1, miR-30a and Beclin1. Bioinformatics analyses by TargetScan predicted the binding sites between miR-30a and Beclin1 (Fig. 5A). In agreement with the previous study, miR-30a overexpression significantly inhibited luciferase activity of pmirGLO-Beclin1-3'-UTR-WT in cortical neurons, but the luciferase activity of pmirGLO-Beclin1-3'-UTR-MUT was unaffected, confirming that Beclin1 was a target of miR-30a (Fig. 5B). More interestingly, the luciferase activity of pmirGLO-Beclin1-3'-UTR-WT was partly restored in miR-30a + pcDNA-MALAT1-transfected cortical neurons, as compared with miR-30a and miR-30a + vector treatment groups. The effects of si-MALAT1 and pcDNA-MALAT1 on Beclin1 expression were analyzed by western blot. As displayed in Fig. 5C and 5D, si-MALAT1 remarkably restrained the level of Beclin1 and pcDNA-MALAT1 conspicuously promoted Beclin1 level. Importantly, a significant inhibition of Beclin1 level was observed in miR-30a-transfected cortical neurons while pcDNA-MALAT1 dramatically overturned this effect (Fig. 5E). Taken together, these finding strongly demonstrated that MALAT1 positively regulated the derepression of Beclin1 by sponging miR-30a.

MALAT1 positively regulated the derepression of Beclin1 by sponging miR-30a in vivo.

To further confirm the mutual regulatory relationship between MALAT1, miR-30a and Beclin1 *in vivo*, lentivirus sh-MALAT1 or sh-control was injected into cerebral cortex of six

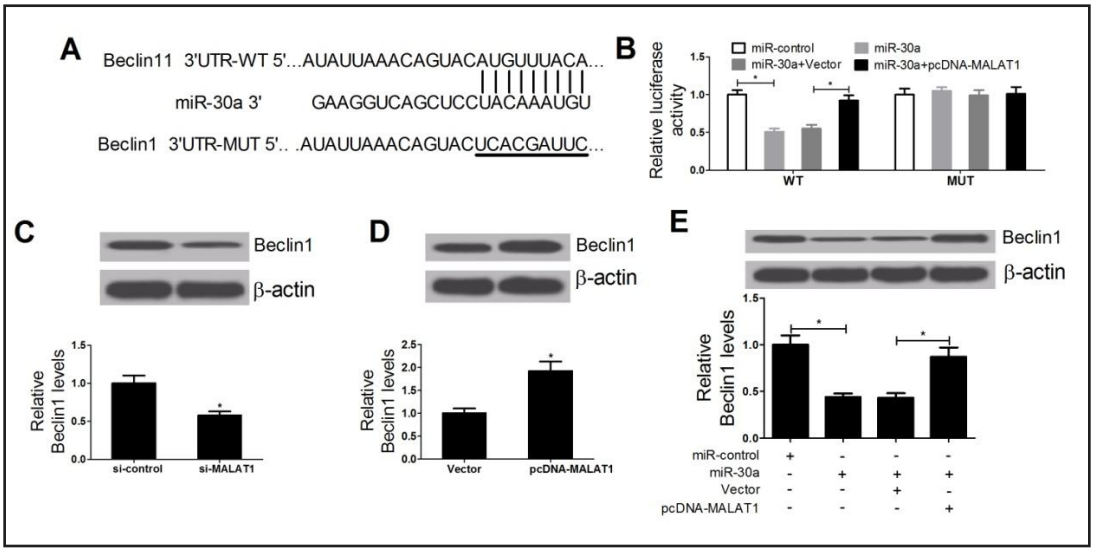

Fig. 5. The mutual regulatory relationship between MALAT1, miR-30a and Beclin1. (A) The predicted binding targets between miR-30a and Beclin1. (B) Cortical neurons was cotransfected with luciferase reporters (pmirGLO-Beclin1-3'-UTR-WT and pmirGLO-Beclin1-3'-UTR-MUT) and miR-30a or miR-control or combined with pcDNA-MALAT1 or vector and the luciferase activity was measured by luciferase reporter assay. (C and D) Western blot was performed to determine the level of Beclin1 in cortical neurons transfected with si-MALAT1, pcDNA-MALAT1 or respective controls. (E) The level of Beclin1 was detected by western blot in cortical neurons transfected with miR-30a, miR-control or combined with pcDNA-MALAT1 or vector. *$P < 0.05$.

normal mice respectively to analyze the expression level of miR-30a and Beclin1. As compared with sh-control group, an obvious increase of miR-30a (Fig. 6A) and a significant decrease of Beclin1 (Fig. 6B) were observed in sh-MALAT1-injected cerebral cortex. Meanwhile, the levels of Beclin1 in sh-MALAT1-injected cerebral cortex of normal mice (n = 6) was all lower than that in sh-control treatment group (Fig. 6C). These results indicated that MALAT1 positively regulated the derepression of Beclin1 by sponging miR-30a *in vivo.*

3-MA markedly suppressed OGD/MCAO-induced ischemic injury

To evaluate the effect of autophagy on ischemic injury, autophagy inhibitor 3-MA was employed to treat cortical neurons prior to OGD and cerebral cortex before MCAO. The CCK-8 assay results suggested that 3-MA dramatically inhibited OGD-induced neuronal cell death (Fig. 7A). Besides, 3-MA exhibited a significant decrease of infarct volume of MCAO-induced

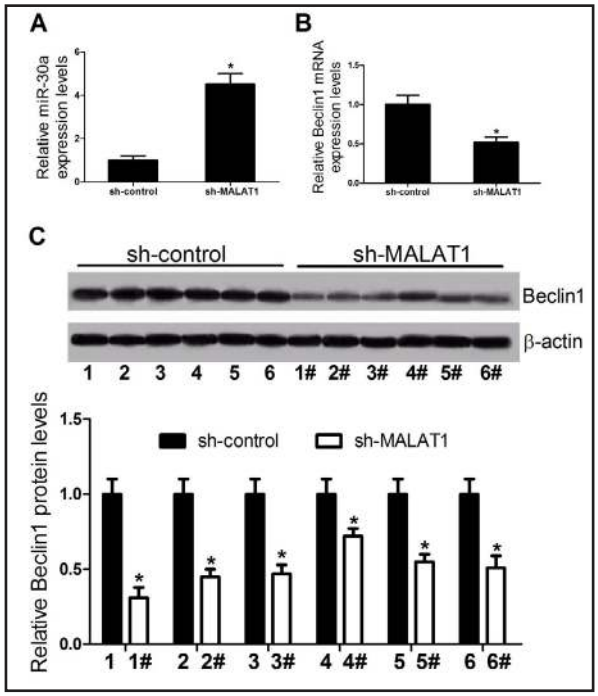

Fig. 6. Effect of sh-MALAT1 on the expression levels of miR-30a and Beclin1 in vivo. qRT-PCR was carried out to assess the expressions of miR-30a (A) and Beclin1 (B) in sh-MALAT1-injected cerebral cortex of six normal mice. (C) Western blot was employed to detect the level of Beclin1 in sh-MALAT1-injected cerebral cortex of six normal mice. *$P < 0.05$.

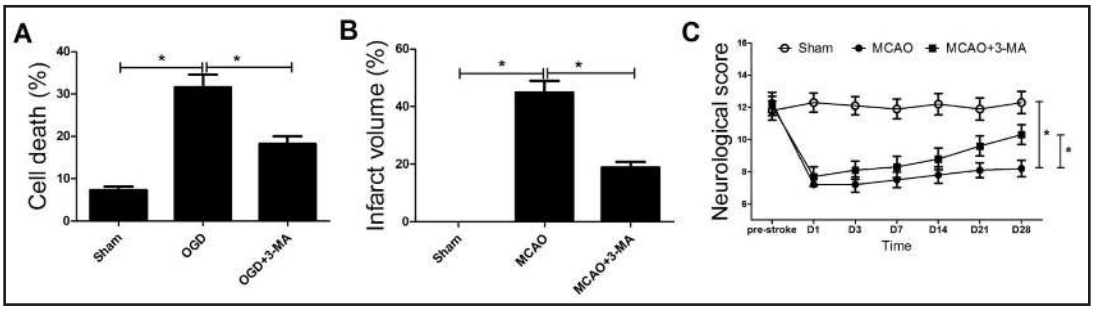

Fig. 7. Effect of 3-MA on OGD/MCAO-induced ischemic injury. (A) CCK-7 assay was used to detect neuron cell survival in cortical neurons treated with 3-MA and OGD. (B) TTC staining was used to detect the brain infarct volume of cerebral cortex treated with 3-MA and MCAO. (C) The total neurological scores were used to assess long-term neurological outcomes after MCAO or combined with 3-MA. *$P < 0.05$.

ischemic brains (Fig. 7B). Furthermore, 3-MA treatment significantly improved long-term neurological outcomes after stroke compared to only MCAO-induced ischemic brains (Fig. 7C). These data suggested that autophagy played an important role in ischemic injury.

Discussion

Recently, advances in epigenomics are focusing on the neuroprotective effects of ncRNAs in ischemic stroke [32]. For example, Yan et al. reported that the up-regulated lncRNA maternally expressed gene 3 (MEG3) functioned as a cell death promoter in ischemic stroke and physically and functionally interacted with p53 to mediate ischemic neuronal death in stroke [33]. Mehta et al. exhibited that lncRNA Fos downstream transcript (FosDT) promoted ischemic brain injury by interacting with REST-associated chromatin-modifying proteinsSin3a and coREST [34]. Wu et al. found that lncRNA-N1LR promoted neuroprotection against ischemic stroke by inhibiting p53 phosphorylation and served as a potential target for therapeutic intervention following ischemic brain injury [35]. In the present study, we found that MALAT1 was significantly up-regulated in MCAO mice and cortical neurons subjected to OGD, consistent with a previous study [17]. More importantly, MALAT1 down-regulation dramatically restrained the conversion of LC3-I to LC3-II and Beclin1 level *in vitro* and *in vivo*, indicating MALAT1 down-regulation suppressed autophagy in ischemic stroke. Meanwhile, MALAT1 down-regulation markedly ameliorated ischemic injury by suppressing OGD-induced neuronal cell death and MCAO-induced ischemic brain infarction. These findings indicated that MALAT1 promises to be a novel therapeutic target for ischemic stroke.

Although the biological role of MALAT1 in ischemic stroke has been explained, the precise regulatory mechanism by which MALAT1 modulated ischemic stroke remains largely unclear. The recent studies demonstrated a novel regulatory mechanism that lncRNAs serve as a miRNA sponge, thus modulating the derepression of miRNA target at a post-transcriptional level [36, 37]. For example, Yu et al. found that MALAT1 functioned as a competing endogenous RNA (ceRNA) to regulate Rac1 expression by sponging miR-101b, thereby influencing the proliferation, cell cycle and activation of primary hepatic stellate cells [38]. Xiao et al. reported that MALAT1 promoted proliferation and metastasis of clear cell kidney carcinoma through sponging miR-200s to regulate ZEB2 expression [39]. Chou et al. uncovered that MALAT1 served as a ceRNA of cdc42 in inducing migration and invasion of breast cancer cells through competitively binding miR-1 [40]. In our study, we demonstrated that MALAT1 could directly interact with miR-30a and negatively regulated its expression. Moreover, function analyses revealed that ectopic overexpression of MALAT1 partly overturned the inhibitory effect of miR-30a overexpression on ischemic injury and autophagy *in vitro* and *in vivo*, suggesting that MALAT1 served as a sponge of miR-30a, inhibiting both miR-30a

expression and function. Additionally, autophagy biomarker Beclin1 was demonstrated to be a target of miR-30a, which was consistent with the previous studies [24]. More importantly, luciferase reporter system further revealed that MALAT1 overexpression partly abolished miR-30a-induced repressing activity on the Beclin1 3'-UTR, as well as protein expression of Beclin1. Besides, an obvious increase of miR-30a and a significant decrease of Beclin1 were observed in sh-MALAT1-injected cerebral cortex. Collectively, these data suggested that MALAT1 regulated the derepression of Beclin1 by sponging miR-30a. Of considerable interest, mechanistic analyses further showed that autophagy inhibition by 3-MA repressed OGD-induced neuronal cell death and MCAO-induced ischemic brain infarction, indicating that autophagy played a regulatory role in neuronal survival and death in ischemic stroke [20]. Overall, these results uncovered that down-regulation of MALAT1 exerted its biological roles through inhibiting Beclin1-dependent autophagy by regulating miR-30a expression in ischemic stroke.

Conclusion

Our study first revealed that down-regulation of MALAT1 attenuated neuronal cell death *in vitro* and *in vivo* through suppressing Beclin1-dependent autophagy by regulating miR-30a in cerebral ischemic stroke, providing a new regulatory mechanism in ischemic stroke. Therefore, MALAT1 may serve as a novel promising therapeutic target for the treatment of ischemic stroke.

Disclosure Statement

The authors have declared that no competing interests exist.

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