LncRNA FOXD2-AS1 Protects Against Cerebral Ischemia-Reperfusion Injury Through miR-3144-5p/KCTD15 Axis

Qi Gao

Affiliated Huai'an No1 People's Hospital of Nanjing Medical University: Huaian First People's Hospital

Yanfeng Wang (✉ yfwang_ha@163.com)

Affiliated Huai'an No1 People's Hospital of Nanjing Medical University: Huaian First People's Hospital

<https://orcid.org/0000-0001-6414-7253>

Research Article

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Abstract

Background: Long non-coding RNAs (lnc-RNAs) and microRNAs (miRNAs) play key roles in the development of stroke. LncRNA FOXD2-AS1 has been reported to be important in many cancers. However, the role of LncRNA FOXD2-AS1 in stroke is limited known.

Methods: Real-time polymerase chain reaction (real-time PCR) assays were used to measure expressions of LncRNA FOXD2-AS1, miR-3144-5p and KCTD15. Western blot assays were employed to examine Bax and Bcl2 protein expression. The cell viability was measured by cck8 assay. The cell apoptosis was detected by TUNEL staining assay. The interaction between FOXD2-AS1, miR-3144-5p and KCTD15 was confirmed by site-directed mutagenesis and luciferase assays.

Results: The expression of LncRNA FOXD2-AS1 was down-regulated in blood sample from stroke patients and MAO mice tissues. In addition, LncRNA FOXD2-AS1 was decreased on OGD/R treated cells. LncRNA FOXD2-AS1 overexpression promoted cell viability and reduced apoptosis in OGD/R-Induced PC12 Cells. LncRNA FOXD2-AS1 could interact with miR-3144-5p. Meanwhile, inhibition of miR-3144-5p alleviated OGD/R-induced neuronal injury in PC12 Cells. Dual luciferase reporter assay verified that KCTD15 was a target of miR-3144-5p. And level of KCTD15 was positive with LncRNA FOXD2-AS1.

Conclusion: Taken together, LncRNA FOXD2-AS1 protected against cerebral ischemia-reperfusion injury by acting as a sponge of miR-3144-5p to modulate KCTD15 level.

Introduction

Acute ischemic stroke (AIS) occurs when blood flow loss in the brain happens, being expected to result in neurologic dysfunction. A cerebral vessel was occluded by thrombosis or embolism, which subsequently supplies a specific area in the brain. Due to the fact that this kind of damage is irreversible, blood flow abrupt reduction in the brain bringing about normal function loss(1995; Hacke, Kaste et al. 2008; Goyal, Menon et al. 2016; Albers, Marks et al. 2018; Nogueira, Jadhav et al. 2018). All over the world, the number of people being affected by a stroke per year is higher than 13.7 million and approximately one in six people will have a stroke in their lifetime. Additionally, there are 5.8 million patients died from stroke each year(Phipps and Cronin 2020). Furthermore, it estimated that around 70% of incident strokes are ischemic(Benjamin, Muntner et al. 2019). Nevertheless, improvements in current treatments for cerebral ischemia are restricted by many factors, particularly a narrow therapeutic window and an incomplete understanding of the cellular and molecular changes following acute ischemic stroke (AIS)(Fonarow, Zhao et al. 2014; Saver, Goyal et al. 2016). On account of this, it is urgent to unravel the pathogenesis and underlying mechanisms of cerebral ischemic injury, which might provide us a hint of developing innovative diagnostic and therapeutic targets for patients with AIS.

Many pathophysiologic processes are involved in ischemic stroke. Upon an ischemic offence, a cell starts with the programmed death or the unprogrammed pathway(Bursch, Ellinger et al. 1996). Programmed cell death pathway is made up of apoptosis and autophagy, playing a vital role in the normal cellular

function(Clarke 1990). As for necrosis which belongs to the unprogrammed cell death pathway, it is different fundamentally. On top of that, it has been corroborated that the activation of apoptosis pathway could have an influence on other pathways simultaneously within the same population of cells(Martin, Al-Abdulla et al. 1998). After ischemia, sudden reduction of perfusion of brain tissue leads to a decrease in oxygen, ATP, and glucose, causing cell death. While in the penumbra, this depletion hampers cellular physiological functioning but does not induce an irreversible change. Cells within the penumbra undergo apoptosis and autophagocytosis(Lipton 1999).

Recently, long none-coding RNAs (lncRNAs) have been attached great importance to regulatory functions, particularly gene expression regulation in transcriptional and post-transcriptional level(Iyengar, Choudhary et al. 2014; Yin, Hamblin et al. 2014). In a transcriptional level, lncRNAs make a contribution to chromatin modifications through histones deacetylation or methylation(Bao, Szeto et al. 2018). Besides, lncRNAs could serve as a microRNA (miRNA) sponge, which regulate their functions in the post-transcriptional level in the cells, including splicing, translation, and degradation by interacting with miRNA directly(Cesana, Cacchiarelli et al. 2011; Salmena, Poliseno et al. 2011). Correspondingly, it has been verified that many lncRNAs are involved in ischemia, indicated for their vital roles in the pathogenic mechanisms of ischemic stroke(Zhang, Yuan et al. 2016). According to previous studies, it is reported that CaMK2D-associated lncRNAs, C2dat1 and C2dat2, were identified to have the potential promote neuronal survival in oxygen-glucose deprivation/reoxygenation (OGD/R) status. Mechanically, C2dat1 could interact with C2dat2 to upregulate the production of CaMKII δ and IKK α/β , subsequently activating the NF- κ B signaling pathway(Ye, Das et al. 2019). Apart from that, lncRNA N1LR was supposed to enhance the ability of cell proliferation and promote cell cycle progression, consequently alleviating cell apoptosis in mouse neuronal cells. Comparatively, cells survive by upregulation of H19 under OGD/R circumstances(Wu, Wu et al. 2017).

Here, we first detected FOXD2-AS1 in blood samples diagnosed with stroke. Additionally, we demonstrated that FOXD2-AS1 acted as a ceRNA to regulate KCTD15 expression through sponging miR-3144-5p. It is suggested that FOXD2-AS1 overexpression could protect against cell apoptosis following cerebral I/R injury via miR-3144-5p/KCTD15 axis. Henceforth, it is essential to uncover the mechanism of FOXD2-AS1 in ischemia stroke, which may strike inspiration for us to find a novel target for ischemia stroke diagnose and treatment.

Methods And Materials

Clinical experiments and grouping

A total of 30 paired peripheral blood samples were collected from patients with stroke and healthy volunteers at the Affiliated Huaian No.1 People's Hospital of Nanjing Medical University. Patients were excluded if any other central nervous system disease was identified. Venous blood samples (5 ml) were drawn from all participants. The serum was isolated by centrifuging at 1,000 $\times$ g at 4°C for 5 min and

then stored in liquid nitrogen. The study received approval from the the Affiliated Huaian No.1 People's Hospital of Nanjing Medical University (YX-2021-071-01).

Cell lines and culture

Human PC12 cells cells lines (rat adrenal pheochromocytoma cell line) and 293T cells were purchased from ScienCell company (San Diego, USA). Cells were maintained in Dulbecco's modified Eagle's medium (DMEM; Invitrogen, USA) supplemented with 10% (v/v) fetal bovine serum (FBS; Gibico, USA) in a humidified atmosphere of 5% CO₂ at 37 °C.

OGD/R model

To model OGD/R injury, the medium was discarded and cells were washed with phosphate buffer saline (PBS) three times and incubated in Serum/glucose-free DMEM. The cells were subsequently subjected to a hypoxic chamber at 37°C (95% N₂ and 5% CO₂). Cells in the Control group were cultured under normoxic conditions in the meantime. After OGD exposure, all cells were re-perfused for 12 h under normal culture conditions at 37°C (95% O₂ and 5% CO₂).

RT-qPCR

To assess the levels of miR-3144-5p, FOXD2-AS1 and KCTD15 in ischemic stroke patients, peripheral blood samples were drawn from these stroke patients within 3 hours of stroke onset, as well as the healthy individuals. All blood samples underwent RT-qPCR to measure miR-3144-5p, FOXD2-AS1 and KCTD15 levels in serum by using TaqMan miRNA assays (Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol.

Total RNA was extracted using TRIzol (Invitrogen; Thermo Fisher Scientific, Inc.) and further cDNA synthesis was performed with a reverse transcription kit (Promega Corporation, Madison, WI, USA). The relative expression levels of RNA were calculated using $2^{-\Delta\Delta C_t}$ method.

Transfection and grouping

Before OGD/R, PC12 cells were transfected with miR-3144-5p mimic, miR-3144-5p inhibitor, shR-FOXD2-AS1 or FOXD2-AS1 plasmid by Lipo 3000 Transfection Reagent (Thermo Fisher Scientific, Inc.) as previously described. After 6 hours, the medium was replaced by fresh DMEM.

Dual luciferase reporter assay

Luciferase reporter plasmids including the wild-type and mutant sequences of FOXD2-AS1 and KCTD15 were constructed (Genewiz, China). Cells were each seeded with a proper density in 12-well plates. Then, all plasmids were transfected into the prepared cells for 24 h. After post-transfection, cells were harvested and the firefly luciferase activity was measured using a dual-luciferase reporter assay system (Promega,

USA). The relative luciferase activity was normalized to Renilla luciferase activity. Transfection was repeated in triplicate.

Cell proliferation assay

Cell viability was measured by using the Cell Counting Kit-8 (CCK-8) assay (Beyotime, China). All treated cells were seeded into 96-well plates and were starved in a serum-free medium for another 24 h. Following cells were incubated with CCK-8 reagent, the absorbance at 450 nm was detected via a microplate reader.

Western blot analysis

Briefly, cells were lysed with RIPA buffer (Beyotime, China). After centrifuge, the BCA Protein Assay Reagent (Beyotime, China) was applied to quantify the protein concentration of lysates. Western blot was performed in accordance with the standard protocol anywhere. BAX, BCL2 and tubulin antibodies were obtained from Cell Signal Technology (CST, USA). Then, the membrane was incubated with horseradish peroxidase-conjugated secondary antibodies. Finally, the blots were visualized with a commercially available enhanced chemiluminescence (ECL) analysis kit (Merck Millipore, USA). The bands were analyzed with Image J® software.

TUNEL assay

Neuronal apoptosis was detected by using terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay. Briefly, the cells fixed with ice-cold 4% paraformaldehyde followed by permeabilization with 0.1% (v/v) Triton X-100 (Sigma) for 5 min. Then, cells were incubated with TUNEL Detection Kit (Beyotime, China) in a wet and dark environment. The nuclei were then stained with 4,6-diphenylamine-2'-phenylhydrazine hydrochloride (DAPI). Afterwards, the number of apoptotic cells were determined. Positive TUNEL staining was visualized on the confocal microscope and were quantified using ImageJ. The apoptotic index is the percentage of TUNEL-positive cells (positive cells/100% total cells).

Statistical analysis

All statistical analyses were carried out by using SPSS v20.0. Data are represented as the mean $\pm$ standard deviation (SD). Statistical significance between groups was determined using Student's t-test or the one-way ANOVA method. All experiments were repeated at least three times. Differences with a P-value less than 0.05 were defined as being statistically significant.

FOXD2-AS1 Was Down-Regulated in stroke patients blood samples and OGD/R-Induced PC12 Cells, and It Mitigated OGD/R-Induced Neuronal Injury in PC12 Cells

In order to figure out the function of FOXD2-AS1 in IS, we firstly determined the relative expression of FOXD2-AS1 in stroke patients blood samples and in brain tissues of MCAO mice by RT-qPCR. As shown in Figure 1A, a significant reduction of FOXD2-AS1 was observed in stroke patients blood samples

compared with healthy controls . In addition, similar trend can be obtained when the level of FOXD2-AS1 in the brain tissues of mice with or without MCAO treatment were detected. (Figure 1B). Equivalent to previous results, the expression of FOXD2-AS1 in PC12 cells show a decrease with the increase of OGD/R treatment time, at 4 h, 8 h, 12 h and 24h respectively ly (Figure 1C). Cell viability and apoptosis were detected by CCK-8 assay and tunnel staining, respectively. The results showed that OGD/R treatment inhibited the cell viability and increased the apoptosis in PC12 cells (Figure 1D-E).

FOXD2-AS1 promoted cell viability and reduced apoptosis in OGD/R-Induced PC12 Cells

Next, we investigated the role of FOXD2-AS1 in OGD/R-Induced PC12 Cells by gain or loss functions of FOXD2-AS1. As shown in Figure 2A, FOXD2-AS1 was up-regulated by transfection of pcDNA-FOXD2-AS1 and down-regulated by transfection of sh-FOXD2-AS1 in PC12 cells. CCK-8 analysis revealed that overexpression of FOXD2-AS1 notably promoted the proliferation of PC12 cells no matter they exposed to OGD/R or not. (Figure 2B). Moreover, the cell viability was furthermore reduced by downregulated FOXD2 AS1 under OGD/R treatment (Figure 2C). As for TUNEL Assay, there was a significant reduction of TUNEL-positive cells in the FOXD2 AS1 overexpression group under OGD/R stimulation. (Figure 2D). However, when PC12 cells was transfected with the sh-FOXD2-AS1 , a reverse phenomenon that apoptotic cells markedly increased under OGD/R environment could be observed (Figure 2E). On top of that,we detected the expression of BCL2 and Bax, which were considered as apoptosis marker. The results suggested that FOXD2-AS1 overexpression offered PC12 cells protection against OGD/R stimulation while FOXD2-AS1 knockdown showed opposite results that cell apoptosis significantly elevated. Taken together, it is indicated that the augmented cell viability from FOXD2-AS1 overexpression under OGD/R stress was attributable to the reduced apoptosis.

FOXD2-AS1 Could Serve as a Competing Endogenous RNA (ceRNA) for miR-3144-5p

To further explore the function of FOXD2-AS1 in IS, miRDB and LncBase Predicted v.2. were used to investigate the potential target genes of FOXD2-AS1. There are 74 microRNAs in the two databases in total, and miR-3144-5p gained the highest score in both databases (Figure 3A-C). As shown in the Figure 3D, FOXD2-AS1 contained potential binding sites for miR-3144-5p (Figure 3D). Then the hypothesis about interaction between FOXD2-AS1 and miR-3144-5p was confirmed by DLR assay. Dual-luciferase report assay revealed that miR-3144-5p mimics reduced the luciferase activity of FOXD2-AS1 WT, but not of FOXD2-AS1 mut vector. (Figure 3D).

In addition, in order to verify whether FOXD2-AS1 takes part in regulating expression of miR-3144-5p, we overexpressed or knocked down FOXD2-AS1 in PC12 cells. The RT-qPCR showed that the level of miR-3144-5p was increased significantly after FOXD2-AS1 knockdown, and decreased when overexpressing FOXD2-AS1 in PC12 cells (Figure 3E). Meanwhile, we detected the level of miR-3144-5p in between stroke patients and healthy people blood samples. Consistent with the results mentioned before, blood samples from stroke patients had higher level of miR-3144-5p (Figure 3F). Furthermore, Pearson's correlation analysis was performed to evaluate the relationship between expression of miR-3144-5p and FOXD2-AS1. The analysis showed that the level of miR-3144-5p was negative correlated with FOXD2-AS1 (Figure 3G).

Inhibition of miR-3144-5p Alleviated OGD/ R-Induced Neuronal Injury in PC12 Cells

The cell viability of PC12 cells in the miR-3144-5p overexpression group was decreased in comparison with the NC group under OGD/R treatment, whereas the viability of PC12 cells was significantly increased by FOXD2-AS1 overexpression, although transfected miR-3144-5p (Figure 3H). Importantly, we also detected the expression of BCL2 and Bax, it indicated that the cell apoptosis was increased by miR-3144-5p, while inhibited by FOXD2-AS1 overexpression (Figure 3I). Meanwhile, the TUNEL staining showed in accordance with the previous results (Figure 3J).

KCTD15 Was Targeted by miR-3144-5p

Subsequently, we predicted the target genes of miR-3144-5p with miRDB. It indicated that KCTD15 gained the highest score in the list (Figure 4A). It was observed that KCTD15 had a conserved binding region of miR-3144-5p (Figure 4B). The result of DLR revealed that relative luciferase activity of PC12 cells was reduced by co-transfection of KCTD15 WT vector and miR-3144-5p mimics (Figure 4B), whereas relative luciferase activity of PC12 cells showed no significant change after co-transfection of KCTD15 mut vector and miR-3144-5p mimics (Figure 4B). The result of RT-qPCR showed that KCTD15 was down-regulated when cells were transfected with miR-3144-5p mimics (Figure 4C). Furthermore, KCTD15 was decreased gradually under OGD/R treatment at 4 h, 8 h, 12 h and 24 h in PC12 cells, respectively (Figure 4D). There was a positive correlation between KCTD15 and FOXD2-AS1 in stroke patients and healthy people blood samples (Figure 4E).

Discussion

Ischemic stroke, which occupies about 70% of all strokes, is the result of cerebral artery occlusion giving rise to cerebral blood flow loss(Bulli, Dettori et al. 2021). Due to the reduction of oxygen and glucose supply to cells in the brain, patients suffered rapid loss of brain function like neurologic dysfunction(Mendelson and Prabhakaran 2021).

With regard to the death reason, ischemic stroke ranks third in developing countries and is a leading cause of disability especially in China(Ahsan, Liu et al. 2021). The morbidity of stroke has increased with increased life expectancy. Multiple risk factors contributed to stroke, including genetics and the environment. Ischemic stroke is the result of cerebral artery occlusion giving rise to cerebral blood flow loss. Due to the reduction of oxygen and glucose supply to cells in the brain, patients suffered rapid loss of brain function like neurologic dysfunction(Zimmer, Korotkov et al. 2021). However, the pathogenesis of stroke remains poorly understood. It is therefore important to explore the pathogenic and protective mechanisms associated with stroke.

In the present study, cell apoptosis was assessed in PC12 cells following OGD/R treatment by TUNEL staining. The results demonstrated that FOXD2-AS1 knockdown accelerates cell apoptosis and augment OGD/R-induced injury, whereas FOXD2-AS1 overexpression has protective effect reversing the phenomenon. Collectively, these findings indicate that FOXD2-AS1 upregulation may serve an important

neuroprotective role in OGD/R injury. Furthermore, transfection with miR-3144-5p mimic reduced luciferase activity in cells expressing WT but not MUT vectors, while overexpression or suppression of miR-3144-5p resulted in a decrease or increase in KCTD15 expression during OGD/R injury, respectively. These data suggest that KCTD15 is a potential direct target gene of miR-3144-5p. Apoptosis and autophagy programmed cell death are the main routes by which nerve cell death occurs in cerebral ischemia reperfusion injury and are regulated by apoptosis-related genes and their products.

In summary, these findings suggest that FOXD2-AS1 was declined while miR-3144-5p elevated in the setting of OGD/R, and promotion of FOXD2-AS1 reduced OGD/R induced apoptosis. Further, we also elucidated that FOXD2-AS1-miR-3144-5p-KCTD15 axis played a critical role in OGD/R induced apoptosis. Thus, regulating of FOXD2-AS1-miR-3144-5p-KCTD15 axis to attenuate apoptosis in IS, which sheds new light on the diagnosis and treatment of ischemic stroke.

Declarations

Ethics approval

The study received approval from the the Affiliated Huaian No.1 People's Hospital of Nanjing Medical University (YX-2021-071-01).

Data availability

All data generated or analyzed during this study are included in this published article.

Funding

Sponsors had no such involvement.

Competing interests

The authors declare that they have no competing.

Authors' contributions

Yanfeng Wang designed the research; Zengjin Ren wrote the paper; Qi Gao performed the in vitro experiments; Qi Gao, Yanfeng Wang analyzed data.

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Figures

Figure 1

FOXD2-AS1 in stroke patients and OGD/R treated PC12 cell lines. (A) RT-qPCR was carried out to detect the level of FOXD2-AS1 in blood samples obtained from stroke patients (n=30) and healthy individuals. (B) Relative expression of FOXD2-AS1 was detected by RT-qPCR in MCAO mice brain tissues. (C) Relative expression of FOXD2-AS1 in oxygen and glucose deprivation/reoxygenation (OGD/R)-induced PC12 cells was detected by RT-qPCR. (D) Proliferation of PC12 cells was detected by CCK-8 assay. (E) Apoptosis of PC12 cells was detected by TUNEL staining assay. All values were represented by the mean $\pm$ standard deviation. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Figure 2

FOXD2-AS1 reduced cell apoptosis induced by OGD/R in PC12 cell lines. (A) RT-qPCR was made to measure the expression of FOXD2-AS1 after transfecting pcDNA-lnc and shR-lnc into PC12 cell lines. (B-C) Proliferation of PC12 cells after transfecting pcDNA-lnc and shR-lnc was detected by CCK-8 assay. (D-E) Apoptosis of PC12 cells after transfecting pcDNA-lnc and shR-lnc was detected by TUNEL staining assay. (F) Bcl2 and Bax expression was detected by western blot. All values were represented by the mean $\pm$ standard deviation. * $p < 0.05$, ** $p < 0.01$, *** $P < 0.001$.

Figure 3

FOXD2-AS1 sponges miR-3144-5p. (A-B) The microRNAs lists and scores on predicted by using DIANA and miRDB databases. (C) Venn diagrams showed the same microRNAs between two databases. (D) Predicted miR-3144-5p binding sites in 3'UTR of FOXD2-AS1 and dual luciferase report assay in WT- FOXD2-AS1 or MUT- FOXD2-AS1 co-transfected with miR-NC or miR-3144-5p mimics. (E) Level of miR-3144-5p in cells transfected with shR- FOXD2-AS1 or oe FOXD2-AS1. (F) miR-3144-5p expression in blood from stroke patients (n=30) and healthy individuals. (G) Pearson's correlation analysis was used to check the relationship between FOXD2-AS1 and miR-3144-5p. (H) Proliferation of OGD/R treated PC12 cells after transfecting miR-3144-5p with oe FOXD2-AS1 was detected by CCK-8 assay. (I) Bcl2 and Bax expression in OGD/R treated PC12 cells after transfecting miR-3144-5p with oe FOXD2-AS1 was detected by western blot. (J) Apoptosis of OGD/R treated PC12 cells after transfecting miR-3144-5p with oe FOXD2-AS1 was detected by TUNEL staining assay. All values were represented by the mean $\pm$ standard deviation. * $p < 0.05$, ** $p < 0.01$, *** $P < 0.001$.

Figure 4

miR-3144-5p interacts with KCTD15 and reduce its expression. (A) The microRNAs lists and scores predicted by using miRDB database. (B) Schematic indicating the miR-3144-5p sites in KCTD15 and dual

luciferase assay in WT- miR-3144-5p or MUT- miR-3144-5p cells co-transfected with miR-NC or miR-3144-5p mimics. (C) The KCTD15 level was detected by RT-qPCR. (D) Relative expression of KCTD15 in oxygen and glucose deprivation/reoxygenation (OGD/R)-induced PC12 cells was detected by RT-qPCR. (E) Pearson's correlation analysis was used to check the relationship between KCTD15 and miR-3144-5p. All values were represented by the mean $\pm$ standard deviation. * $p < 0.05$, ** $p < 0.01$, *** $P < 0.001$.