Artery Research

RESEARCH ARTICLE Open Access

The Expression and Regulatory Role of lncRNA CRNDE in Ischemic Stroke

Yuan Cheng[1†], Qiujun Lu[2†], Yuanyuan Su[3], Runxi Xu[3], Shuqi Huang[3], Chong Yang[3], Yunhua Hao[3] and Jun Yang[4*]

Abstract

Background Ischemic stroke (IS) is a disease caused by the occlusion of cerebral arteries with lack of oxygen and blood supply and is characterized by high morbidity and mortality. The role of lncRNA CRNDE in IS remains unclear.

Objective This study focused on investigating the function of lncRNA CRNDE in IS.

Materials and Methods 237 participants were enrolled in this study, including 135 patients with ischemic stroke (Ischemic stroke group) and 102 healthy individuals (Control group). The human brain microvascular endothelial cells (BMECs) treated with oxygen–glucose deprivation (OGD) were used to establish an IS cell model in vitro. The qRT-PCR was used to analyze the expression level of lncRNA CRNDE, miR-451, and MIF. The cell proliferation ability and migration were detected by CCK-8 and Transwell assay, respectively, while cell apoptosis was determined by apoptosis assay kit and flow cytometry. Additionally, the target relationship of CRNDE/miR-451 and miR-451/MIF was verified by dual-luciferase system.

Results LncRNA CRNDE exhibited an up-regulated expression in IS patients and the IS cell model. Down-regulated lncRNA CRNDE not only contributed to cell proliferation ability and migration but also inhibited cell apoptosis. The miR-451 was a potential target of lncRNA CRNDE. The increased MIF was covalently bound to miR-451 and MIF reduction caused by CRNDE silence was significantly rescued by the inhibition of miR-451.

Conclusion Up-regulated lncRNA CRNDE exacerbated IS by regulating proliferation, migration, and apoptosis in IS cell model. MiR-451/MIF was a potential downstream target of lncRNA CRNDE.

Keywords CRNDE, Ischemic stroke, miR-451

1 Introduction

Ischemic stroke (IS) is a disease caused by the occlusion of cerebral arteries with lack of oxygen and blood supply [1] and is characterized by high morbidity and mortality. The IS has been reported to account for 87% of all strokes [2]. Although thrombolysis and mechanical thrombectomy are commonly used in clinical treatment, their application is limited to the narrow time window and secondary bleeding threats [3] and it has been indicated that less than 10% of IS patients receive effective treatment in the appropriate time [4]. In addition, survivors often face the risk of

[†]Yuan Cheng and Qiujun Lu have contributed equally to this work.

*Correspondence:
Jun Yang
yangjun730046@163.com
[1] Geriatric Department 2, Chengdu Dekang Hospital, Chengdu 610091, China
[2] Department of Rehabilitation, Xuzhou Central Hospital, Xuzhou 221000, China
[3] Department of Neurology, Tianyou Hospital, Tongji University, Shanghai 200331, China
[4] Department of Neurosurgery, The No.2 People's Hospital of Lanzhou, No. 388, Jingyuan Road, Chengguan District, Lanzhou 730046, China

disability and even loss of productivity [5]. Therefore, elucidation of the IS pathogenesis is essential, which can provide a reference for early risk assessment and treatment.

Plenty of reports in recent years have indicated the regulation role of long noncoding RNA (lncRNAs) in IS. Previous studies have indicated that the occurrence of IS is influenced by both congenital genetic and acquired factors [3] such as cold exposure [6], dietary habits [7], and lifestyle [8]. Although the detailed pathological mechanism is not clear, the pathophysiological process of IS has been reported to involve apoptosis, oxidative stress, excess calcium ions, as well as inflammation [9]. Analysis on blood samples from IS patients has demonstrated that lncRNAs are significantly altered and differentially expressed in IS [10]. A research from vivo and vitro IS models has confirmed that the lncRNA ZFAS1 inhibits inflammation and mitigates oxidative stress [11]. LncRNA SNHG12 is reported to mediate the angiogenesis after IS by regulating cell viability and migration [12].

Studies have shown blood reperfusion after IS can cause damage to both blood vessels and brain cells, including brain microvascular endothelial cells (BMECs), microglia, and neurons. Moreover, there is also evidence that the damage of brain BMECs is not conducive to the function of cells, the transport of nutrients, as well as the clearance of harmful substances [13]. Thus, the protection of BMECs plays an essential role in IS intervention. Emerging evidence in recent years indicates that lncRNA CRNDE is a widely expressed RNA in a variety of diseases and involves the regulation of various biological processes, including cell proliferation, migration, differentiation, as well as apoptosis [14]. Findings from prostate cancer have manifested that lncRNA CRNDE/miR-146a-5p regulates cell proliferation and apoptosis [15].

Studies related to cardiovascular disease diseases have found that lncRNA CRNDE can regulate the proliferation, migration, and apoptosis of vascular smooth muscle cells [16]. In addition, lncRNA CRNDE has also been reported to regulate the proliferation and apoptosis of vascular endothelial cells [17]. Therefore, we suspected that the abnormally expressed lncRNA CRNDE might be involved in the pathological process of IS.

In this study, we detected the relative expression of lncRNA CRNDE in IS patients and healthy individuals and explored the underlying regulatory role in an IS cell model. We expect this study to provide a theoretical reference for further understanding the pathogenesis of IS and identifying therapeutic targets.

2 Materials and Methods

2.1 Study Population

237 participants were enrolled in this study, including 135 patients with IS (Ischemic stroke group) and 102 healthy individuals (Control group). All participants were more than 18 years old and diagnosed within 24 h after IS onset. IS patients were diagnosed according to the criteria of the fourth National Conference on Cerebrovascular Diseases and were confirmed by CT or magnetic resonance imaging. Exclusion criteria were as follows: (i) with cancer, malignancy, acute infections, autoimmune diseases, or hematological diseases, (ii) traumatic brain injury or hemorrhagic stroke, and (iii) failure or insufficiency of vital organs, such as heart, liver, and kidney. Furthermore, healthy individuals in the control group were from physical examination center. This study was conducted with the approval of the Ethics Committee in our hospital and informed consent from all individuals. Venous blood collection was performed under fasting state. Apart from blood routine examination and the rest was centrifuged to obtain serum at 4000 rpm for 15 min. After centrifugation, these serum were immediately divided into several aliquots and stored at -80℃ refrigerator.

2.2 Cell Culture and IS Model Establishment

The purchased human BMECs were cultured in DMEM with 10% FBS at 37 °C with 5% CO_2. Cells at a confluence of 80–90% were suited for subculture. Cells treated with OGD were used to establish the IS model in vitro. Adherent cells were cultured in the glucose-free DMEM without FBS under anaerobic conditions with 95% N_2 and 5% CO_2 at 37 °C for 2 h. Then, the culture medium was replaced by normal complete medium and cells were placed in normal culture conditions. At different recovery times (6 h, 12 h, 24 h), cells were taken out for expression-level detection of lncRNA CRNDE, miR-451, and MIF.

2.3 Cell Transfection

Adherent human BMECs at a confluence of 80% were suitable for cell transfection. The si-CRNDE was used to inhibit the expression of lncRNA CRNDE with si-NC as the negative control. The miR-451 mimic and miR-451 inhibitor were applied to enhance or suppress the expression of miR-451 with miR-NC as the negative control. Cell transfection was accomplished by lipofectamine 3000 strictly following the specification. Transient transfection efficacy was confirmed by qRT-PCR.

2.4 qRT-PCR

RNA extraction was performed by Trizol reagent for lncRNA CRNDE and MiRcute miRNA Isolation Kit for miR-451. Purity and integrity of RNA were detected by the ratio of A260/A280 in a NanoDrop. Then more stable complementary DNAs were obtained by reverse transcription according to the instructions of the Primescript RT kit. The relative expressions of lncRNA CRNDE and miR-451 were calculated by $2^{-\Delta\Delta Ct}$ methods with U6 as an internal reference with corresponding quantitative PCR kit, while the MIF was measured with the internal reference GAPDH.

2.5 Cell Proliferation Assay

Cell proliferation ability was assessed with the CCK-8 kit. Cell suspension was added to 96-well plates with 1×10^4 cells per well. After 24 h of cell transfection and (or) OGD treatment, CCK-8 regents were dropped to each well with 10 μL for 2 h. The absorbance was measured by the enzyme immunoassay analyzer at 450 nm.

2.6 Transwell Assay

Transfected cells after OGD treatment were seeded in Transwell chambers to test the migration capability. Cells were added to the upper chamber filled with serum-free DMEM medium and the complete medium was added to the lower chamber. After incubation for 24 h, cells were fixed and stained with crystal violet. Then, a microscope was employed for imaging.

2.7 Cell Apoptosis Detection

Transfected cells after OGD treatment were collected and centrifuged for apoptosis detection by the Annexin V-FITC/PI Staining Kit. After centrifugation, the supernatant was discarded and resuspended in binding buffer. Then, the staining solution of Annexin V-FITC and PI were added and incubation in the dark for 15 min was necessary at room temperature. A flow cytometer was applied for cell apoptosis detection within an hour.

2.8 Dual-Luciferase Reporter Assay

After target-gene prediction by the database TargetScan and miRDB, the dual-luciferase system was employed to verify the target relationship between lncRNA CRNDE and miR-451 and the relationship between miR-451 and MIF. The 3'UTR sequence of wild-type lncRNA CRNDE (WT-CRNDE), mutant lncRNA CRNDE (MUT-CRNDE), wild-type MIF (WT-MIF), and mutant MIF (MUT-MIF) was cloned into the vector, respectively. Subsequently, the purified recombined plasmids were co-transfected with miR-NC, miR-451 mimic, or miR-451 inhibitor. After 48 h, the luciferase activity was measured.

2.9 Statistical Analysis

Data were processed by GraphPad Prism 7.0 as well as SPASS 23.0 and results were shown as mean ± SD. One-way ANOVA as well as Student's t tests were utilized for difference analysis between groups. The receiver-operator characteristic (ROC) curve was plotted to assess the identification ability of lncRNA CRNDE. The correlation between lncRNA CRNDE and miR-451 was evaluated by Pearson correlation analysis. $P<0.05$ indicates a statistical difference.

3 Results

3.1 Clinical Characteristics of Study Objects

237 participants were enrolled in this study, including 135 patients with ischemic stroke and 102 healthy individuals. The average age in ischemic stroke group was 60.85 ± 7.78 years and the average BMI was 24.18 ± 1.67 Kg/m^2, exhibiting no difference with the healthy individuals. Besides this, no difference was found in gender, diabetes, smoking, drinking, and HDL-C. However, some characteristics, such as SP ($P=0.007$), DP ($P<0.001$), as well as LDL-C ($P<0.001$), in patients with

Table 1 Clinical characteristics of the study population

Characteristics	Ischemic stroke ($n=135$)	Control ($n=102$)	*P* value
Age (years)	60.85 ± 7.78	62.83 ± 9.56	0.08
BMI (Kg/m^2)	24.18 ± 1.67	24.21 ± 1.45	0.88
Gender, *n* (%)			0.47
Male	65 (48.15%)	54 (52.94%)	
Female	70 (51.85%)	48 (47.06%)	
Diabetes, *n* (%)			0.25
Yes	48 (35.56%)	29 (28.43%)	
No	87 (64.44%)	73 (71.57%)	
Smoking, *n* (%)			0.55
Yes	53 (39.26%)	44 (43.14%)	
No	82 (60.74%)	58 (56.86%)	
Drinking, *n* (%)			0.59
Yes	59 (43.70%)	41 (40.20%)	
No	76 (56.30%)	61 (59.80%)	
SP (mmHg)	130.65 ± 9.32	127.37 ± 8.91	**0.007**
DP (mmHg)	79.58 ± 8.09	72.78 ± 8.45	**< 0.001**
HDL-C (mmol/L)	1.28 ± 0.33	1.32 ± 0.39	0.458
LDL-C (mmol/L)	2.71 ± 0.40	2.41 ± 0.38	**< 0.001**

BMI Body mass index, *SP* systolic pressure; *DP* diastolic pressure, *HDL-C* high-density lipoprotein cholesterol, *LDL-C* low-density lipoprotein cholesterol

The bold represents indicators with significant differences

ischemic stroke presented statistical differences against the control group. More details are recorded in Table 1.

3.2 Aberrant Expression of lncRNA CRNDE in IS Patients

The expression level of lncRNA CRNDE was first detected in IS patients and randomly selected healthy individuals by qRT-PCR to explore its possible correlation to the IS disease. The quantitative data indicated that the relative level of lncRNA CRNDE in IS patients was obviously increased compared to that of the control group (Fig. 1A, $P<0.001$). This abnormally elevated expression suggests that lncRNA CRNDE may be involved in the development of IS. Based on the differential expression between IS patients and healthy individuals, the receiver-operating characteristic (ROC) curve was plotted to estimate the identification ability for IS patients. Further analysis demonstrated that sensitivity was 78.52%, while the specificity was 87.25%. In addition, the area under the curve was 0.8877 (Fig. 1B). It provided further evidence for the association between lncRNA CRNDE and IS. Thus, our subsequent work focused on functional studies of the lncRNA CRNDE in IS disease.

3.3 Effect of Down-Regulated lncRNA CRNDE on Proliferation, Migration, and Apoptosis in Human BMECs

The human BMECs were first treated with OGD to establish an ischemic stroke cell model in vitro. The qRT-PCR results demonstrated with the extension of OGD treatment time, and the relative expression of lncRNA CRNDE showed a gradual increase trend (Fig. 2A). Subsequently, the effect of down-regulated lncRNA CRNDE on the biological behaviors of human BMECs was explored in the ischemic stroke cell model in vitro. In the ischemic stroke cell model, the down-regulated expression level of lncRNA CRNDE was accomplished by interference with si-CRNDE transfection and the efficiency was validated by qRT-PCR (Fig. 2B). The proliferation ability was analyzed by the CCK-8 kit. Results indicated that in ischemic stroke cell model, cell proliferation ability was significantly reduced and down-regulated lncRNA CRNDE contributed a lot to improving the cell proliferation ability (Fig. 2C). A similar effect was observed on cell migration (Fig. 2D). However, an opposite effect appeared on cell apoptosis that down-regulated lncRNA CRNDE diminished the enhanced apoptosis caused by OGD treatment (Fig. 2E).

3.4 LncRNA CRNDE Regulated miR-451 Targetly

The regulatory mechanism of lncRNA CRNDE in human BMECs was explored subsequently. The miR-451 was predicted to be a downstream target by the TargetScan database (Fig. 3A) and a dual-luciferase reporter system was employed to verify the target relationship. Regardless of up-regulated or down-regulated miR-451, luciferase activity was altered only when it was co-transfected with WT-CRNDE instead of MUT- CRNDE (Fig. 3B, C). In addition, in human BMECs treated with OGD, the relative expression of miR-451 was identified to be gradually declined (Fig. 3D) and in the IS cell model down-regulated lncRNA CRNDE was conducive to elevating the reduced expression of miR-451 (Fig. 3E), showing an opposite expression tendency with lncRNA CRNDE compared to the previous results. Moreover,

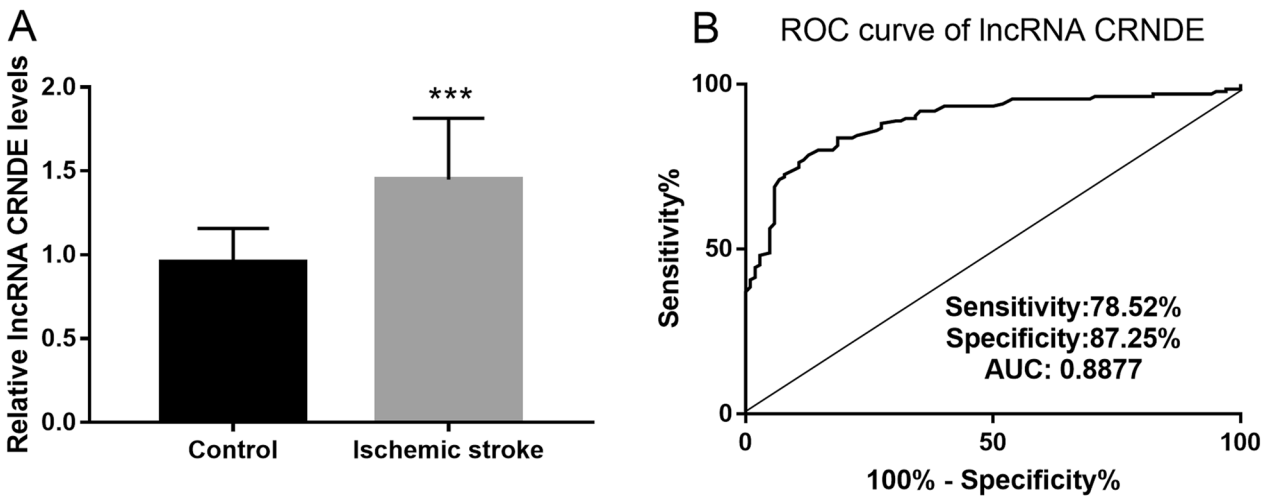

Fig. 1 Expression level and identification ability of lncRNA CRNDE. **A** Expression level of lncRNA CRNDE in ischemic stroke patients (Ischemic stroke, $n=135$) compared to healthy individuals (Control, $n=102$). **B** The receiver-operator characteristic (ROC) curve to assess the identification ability. ***means $P<0.001$

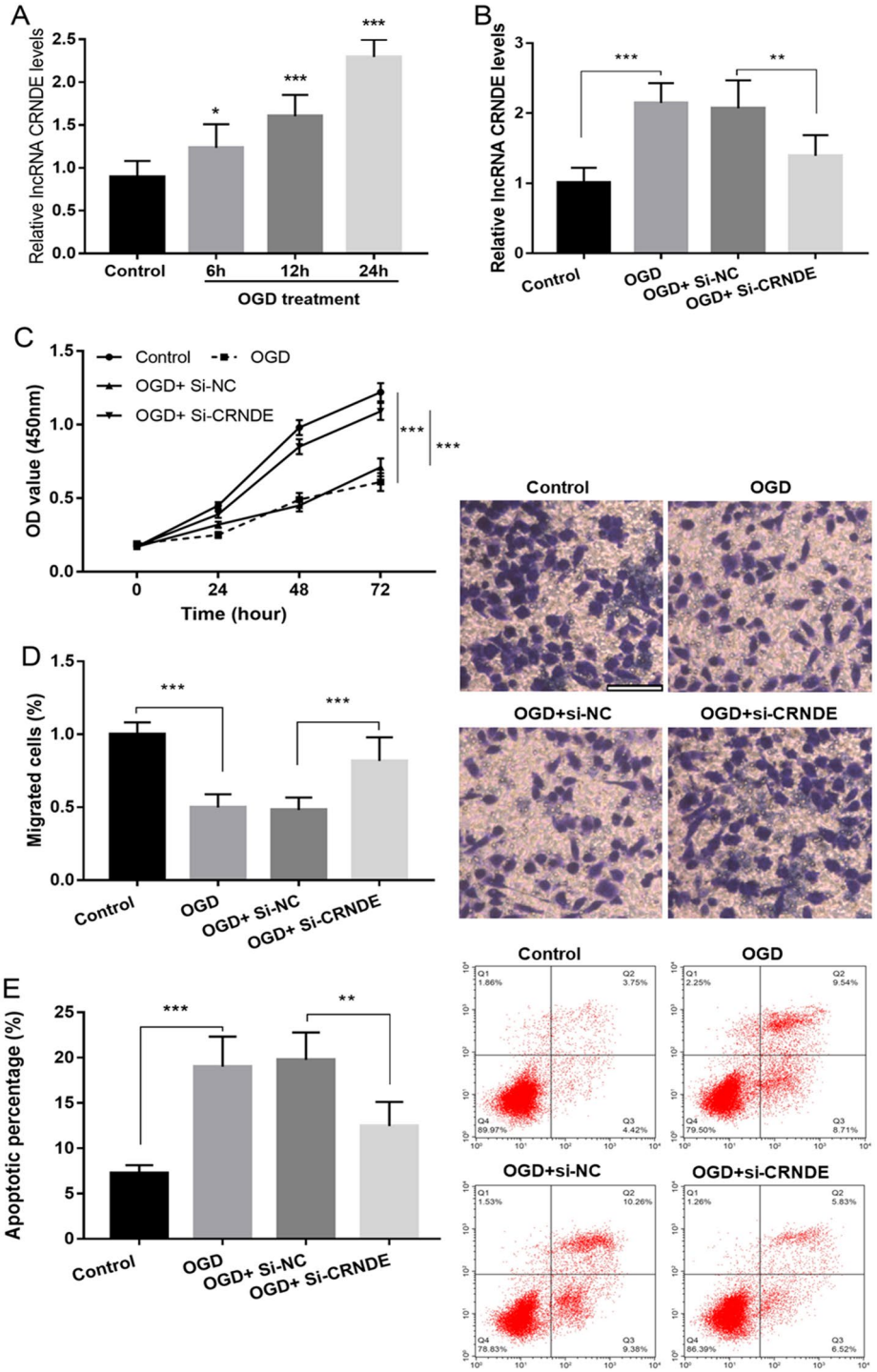

Fig. 2 The effect of down-regulated lncRNA CRNDE on the biological behaviors of human BMECs treated with OGD. **A** Expression level of lncRNA CRNDE at different time points after OGD treatment. **B** The expression level of lncRNA CRNDE after OGD treatment and interference with si-CRNDE. **C** Effect of down-regulated lncRNA CRNDE on cell proliferation ability. **D** Effect of down-regulated lncRNA CRNDE on cell migration. The bar in microscopic images of cell migration was 100 μm. **E** Effect of down-regulated lncRNA CRNDE on cell apoptosis. Flow cytometric analysis on human BMECs. The early apoptosis was presented in the lower right quadrant, while the late apoptosis was in the upper right quadrant. The total cell apoptosis was exhibited by histogram. *means $P < 0.05$, **means $P < 0.01$, ***means $P < 0.001$

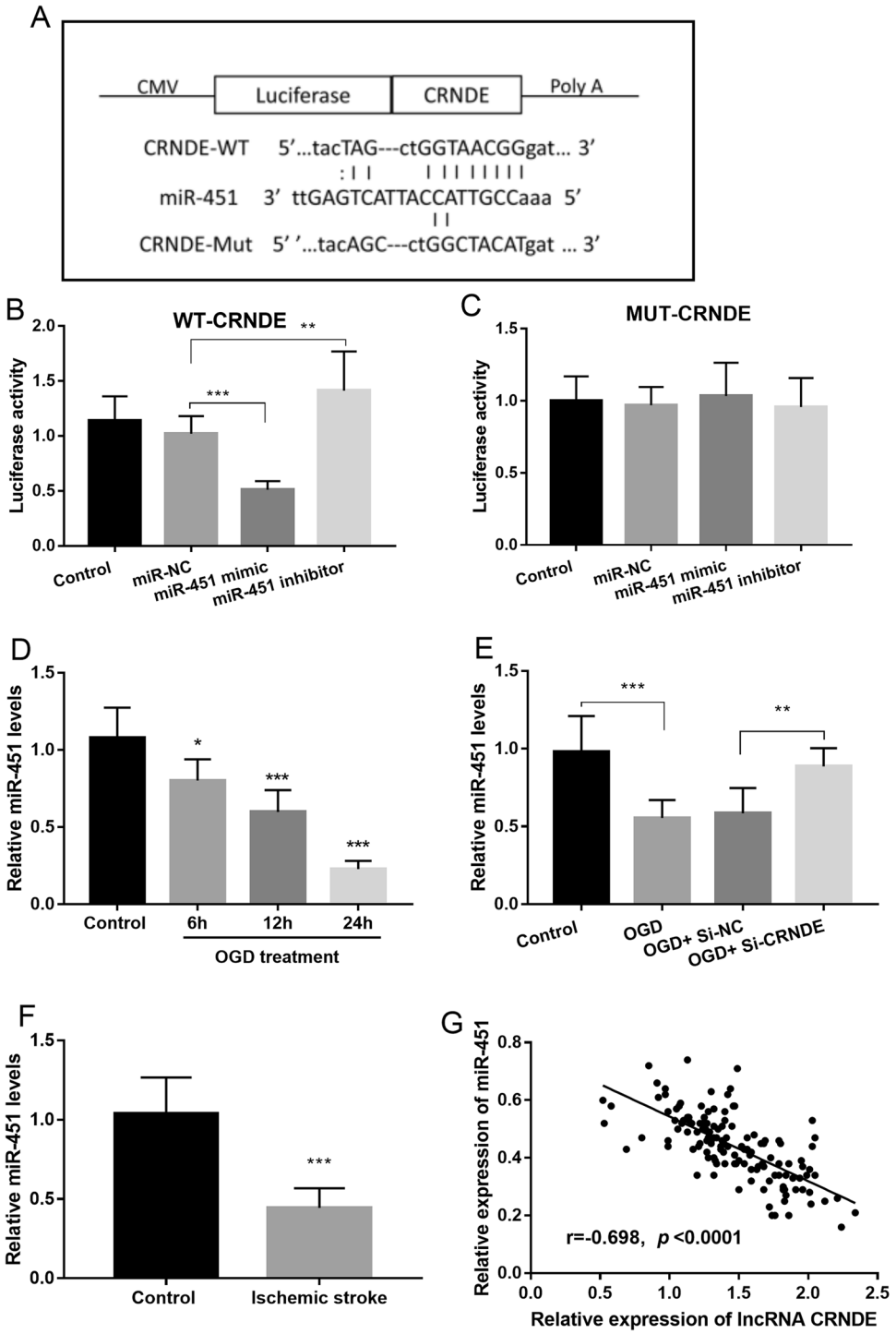

Fig. 3 LncRNA CRNDE interacted with miR-451 in human BMECs. **A** The 3'UTR of lncRNA CRNDE covalently binding miR-451. **B**, **C** Detection of the luciferase activity in human BMECs co-transfected with miR-451 mimic (or inhibitor) and WT-CRNDE/MUT-CRNDE. For cell transfection, si-NC was used as the negative control. **D** Expression level of miR-451 in human BMECs after OGD treatment. **E** Expression level of miR-451 in human BMECs after OGD treatment and si-CRNDE transfection. **F** Relative expression of miR-451 in ischemic stroke patients. **G** Pearson correlation analysis between lncRNA CRNDE and miR-451 in ischemic stroke patients. *means $P < 0.05$, **means $P < 0.01$, ***means $P < 0.001$

the expression of miR-451 in IS patients was significantly decreased against the control group (Fig. 3F), also presenting an inverted expression compared to lncRNA CRNDE (Fig. 1A). Further Pearson analysis proved that miR-451 was negatively related to lncRNA CRNDE on relative expression level in IS patients (Fig. 3G, $r = -0.698$, $P < 0.0001$).

3.5 MiR-451 Regulated the Expression of MIF

To investigate the possible relationship between miR-451 and cell proliferation, migration, and apoptosis, we used an online database to predict the downstream targets. In virtue of the online TargetScan and miRDB databases, 12 common target genes (OSR1, ATF2, MIF, PSMB8, S1PR2, VAPA, CDKN2D, SAMD4B, PMM2, FBXO33, CA39, and TTV) were found. Their targeting relationship with miR-451 were validated by the dual-luciferase system and MIF was covalently bound to miR-451 (Fig. 4B). In human BMECs, regardless of elevation or decline of miR-451, only the luciferase activity of WT-MIF group was affected by miR-451 expression instead of MUT-MIF group (Fig. 4C, D). The expression of MIF was further computed in OGD-treated BMECs. The MIF was enhanced with the extension of OGD treatment (Fig. 4E, $P < 0.001$). Additionally, the relevance among CRNDE, miR-451, and MIF was preliminarily investigated in OGD-treated BMECs. Surprisingly, data indicated that CRNDE silence remarkably decreased the MIF level, which was significantly rescued by miR-451 inhibitor (Fig. 4F, $P < 0.001$).

4 Discussion

The IS is resulted from focal occlusion or stenosis of a single or multiple intracranial or extracranial arteries. Despite the availability of thrombolysis and mechanical thrombectomy, these IS patients usually have poor functional recovery after surgery. This study aims to investigate the function and potential mechanism of lncRNA CRNDE in IS, to provide a theoretical basis for predicting the occurrence and molecular treatment of IS.

As reported, lncRNAs regulate a series of essential cell or molecular events in various diseases. The up-regulated CIRKIL in myocardial infarction aggravates cell injury by promoting cell apoptosis in the cardiac Ischemia/Reperfusion model [18]. Accumulating evidence from recent reports has indicated a close relationship between the aberrant expression of lncRNAs and the pathogenesis of IS. For instance, findings from an IS cell model have

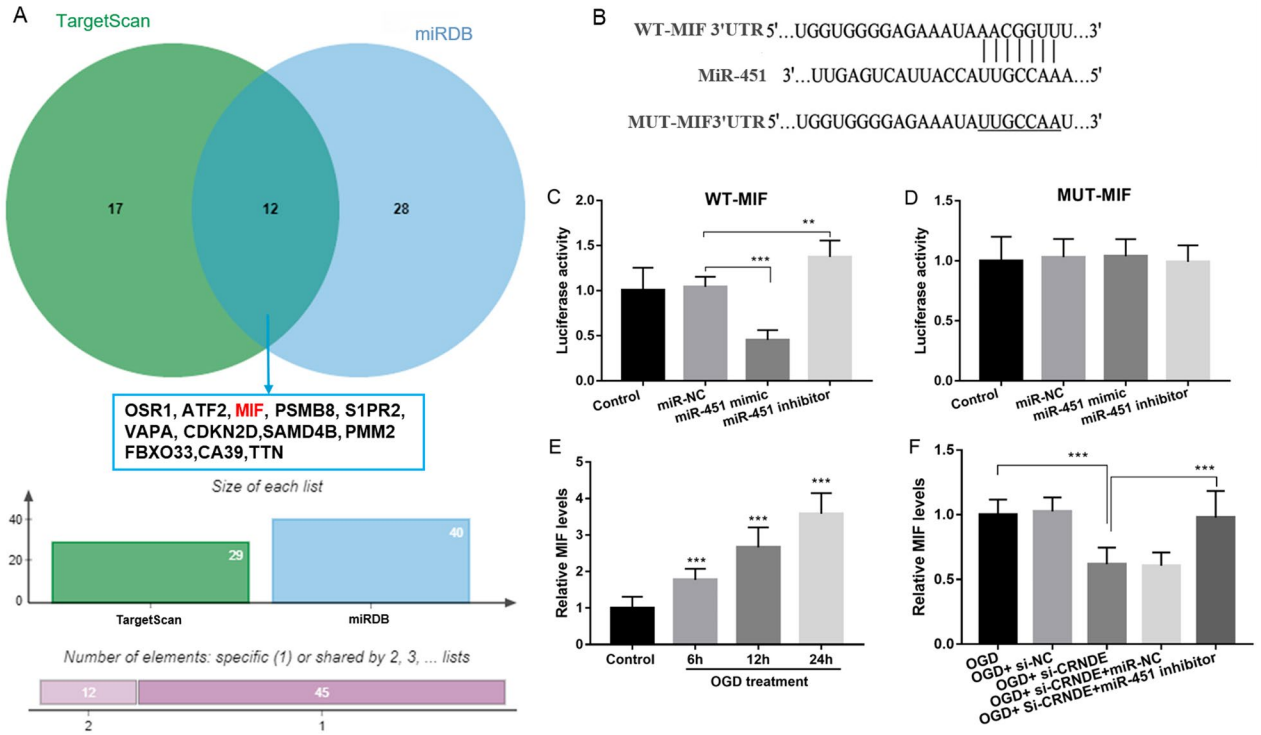

Fig. 4 The miR-451 interacted with MIF in human BMECs. **A** The common target genes of miR-451 were listed in both miRDB and TargetScan databases. **B** The miR-451 covalently binding 3'UTR of MIF. **C**, **D** Detection of the luciferase activity in human BMECs after co-transfection of miR-451 mimic (or inhibitor) with WT-MIF/MUT-MIF. **E** Expression level of MIF in human BMECs after OGD treatment. **F** Expression level of MIF in OGD-treated human BMECs after si-CRNDE transfection and subsequent miR-451 inhibition. ***means $P < 0.001$

suggested that the overexpressed lncRNA SNHG12 contributes to restraining inflammation as well as apoptosis of BMECs and facilitating angiogenesis through interaction with miR-199a [19]. The down-regulated MALAT1 has been reported to stimulate OGD-induced cell apoptosis and suppress cell viability but MALAT1 overexpression leads to opposite results [20]. Data from IS rat model have revealed that lncRNA MEG8 is increased accompanied by enhanced cell apoptosis as well as reduced cell viability, which can be reversed by knockdown of MEG8 [21]. Similar results were observed in our study that lncRNA CRNDE exhibited an enhanced expression in both IS patients and cell model treated with OGD. Moreover, in the IS model, human BMECs showed significantly reduced cell proliferation and migration ability with increased apoptosis. However, lncRNA CRNDE interference by si-CRNDE resulted in opposite biological behavior. Besides, lncRNA CRNDE has proved to be enhanced in various diseases, such as prostate cancer [15] and alcoholic liver disease [22]. Additionally, ROC curve analysis identified its identification ability. Similar role can also be found in the previous reports, such as colorectal cancer [23] and non-small cell lung cancer [24].

Further investigations focused on intermolecular interactions. Here, the online target-gene prediction and validation in the dual-luciferase reporter system indicated that miR-451 was a potential target of lncRNA CRNDE. The target relationship has been declared in the existing reports [25]. In our study, the data from IS cell model and IS patients confirmed that the relative expression of miR-451 was reduced compared to the control. Consistent expression trends can be seen in many previous studies, such as endometriosis [26] and non-small cell lung cancer [27]. Our subsequent Pearson analysis proved that miR-451 was negatively related to lncRNA CRNDE in IS patients, which was another evidence for their target relationship. Moreover, the previous reports have also confirmed the association between miR-451 and IS. A previous study on IS patients reports the possibility of serum miR-451 as a biomarker of IS [28]. Subsequent findings from IS patients and mouse models confirm the neuroprotective function of miR-451 [29]. The recent study demonstrates its role in IS prevention by interacting via Phd3 [30]. The correlation between miR-451 reduction and cell migration and apoptosis has been confirmed in emerging previous reports. For instance, in acute myeloid leukemia, miR-451 reduction regulates cell apoptosis via HMGB1 [31], while it suppresses cell migration in cholangiocarcinoma [32].

MIF is a widely studied cytokine in vascular endothelial cells. Results from the rat IS model confirm that the angiogenesis is regulated by MIF [33]. Recent studies have also reported the role of MIF in regulating cell migration and apoptosis. MIF/RIPK1 regulates cerebrovascular endothelial cell death in mice with perioperative IS [34]. It has also been declared that MIF affects the proliferation, migration, and apoptosis in human osteosarcoma cells by interaction with miR-451 [35]. Here, we found MIF was a downstream target of miR-451. This target relationship can also be discovered in nasopharynx cancer [36]. Additionally, our results proved MIF reduction caused by CRNDE silence was significantly rescued by the inhibition of miR-451, reinforcing the interaction of CRNDE /miR-451/MIF in the regulation of cell proliferation, migration, and apoptosis.

Collectively, we explored the regulatory role of CRNDE in ischemic stroke and miR-451/MIF was a potential interaction target of CRNDE. Our findings shed new light on stroke pathogenesis and offer hope for patient rehabilitation. This study also has its limitations. For example, the role of miR-451 and MIF in cell proliferation, migration, and apoptosis needs to be further investigated. In addition, further animal models need to be established to verify the conclusions from the cell model. We hope to improve them in future work.

Author Contributions

Y.Y. S, R.X. X and Y.H. H conceived and designed the experiments. Y. C, Q.J. L, S.Q. H, C. Y and J. Y performed the experiments. Y. C, Q.J. L, S.Q. H, C. Y and J. Y contributed sample collection and statistical analysis. Y.Y. S, R.X. X and Y.H. H wrote the manuscript. All authors revised it critically for important intellectual content. All authors read and approved the final manuscript.

Funding

No funding was received to assist with the preparation of this work.

Data Availability

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Declarations

Conflicts of interest

The authors declare no competing interests.

Ethics Approval and Consent to Participate

The study protocol was approved by The Ethics Committee of Tianyou Hospital, Tongji University and followed the principles outlined in the Declaration of Helsinki. In addition, informed consent has been obtained from the participants involved.

Received: 9 October 2024 Accepted: 10 April 2025
Published online: 28 April 2025

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