



# Corin is regulated by circ-0012397/miR-200a-3p and inhibits the oxygen-glucose deprivation-induced apoptosis of SHSY5Y neuroblastoma cells

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**Background:** As a type II transmembrane serine protease, corin plays a role in several important physiological and pathological processes. We conducted a bioinformatics analysis to explore the roles of both corin and circ-0012397/miR-200a-3p in ischemic stroke.

**Methods:** We established an *in vitro* model using oxygen-glucose deprivation (OGD)-induced SHSY5Y cells. The proliferation and apoptosis of SHSY5Y cells was determined using Cell Counting Kit-8 (CCK-8) and flow cytometry/Hoechst 33258 staining, respectively. The RNA and protein level was tested using Real Time Quantitative Polymerase Chain Reaction (RT-qPCR) and western blot, respectively. The regulatory relationship of corin and circ-0012397/miR-200a-3p were detected by dual-luciferase reporter assays.

**Results:** We found that OGD downregulated the expression of corin in a time-dependent manner; this change was inversely proportional to the rate of apoptosis of the SHSY5Y cells. Further, high expression levels of corin enhanced the proliferation of SHSY5Y cells and inhibited the apoptosis of SHSY5Y cells by downregulating the expression of cleaved caspase-3, B-cell lymphoma 2 (BCL-2)-associated death promoter, extracellular-regulated protein kinase (ERK), and protein 38 (p38), and upregulated the expression of Bcl-2. Further, the dual-luciferase reporter assays and RT-qPCR showed that corin expression was regulated by circ-0012397/miR-200a-3p. Corin expression was affected by changes in circ-0012397 and miR-200a-3p expression, which were overexpressed or inhibited. Further, corin exerted different regulatory effects on apoptosis signaling-related proteins, including AD Bcl-2, cleaved caspase-3, ERK, and p38, under different expression levels of circ-0012397 and miR-200a-3p.

**Conclusions:** Corin promotes the cell proliferation and inhibits OGD-induced apoptosis of SHSY5Y cells, and that its expression is regulated by circ-0012397/miR-200a-3p. Thus, corin may be a potential target for ischemic stroke patients.

**Keywords:** Corin; ischemic stroke; circ-0012397; miR-200a-3p; apoptosis

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## Introduction

As a major neurological disease, ischemic stroke is mainly caused by atherosclerosis, cardiac embolism, and cerebral small-vessel occlusion (1). Cerebral artery occlusion can cause cerebral ischemia and hypoxia, leading to neuronal apoptosis and brain parenchyma necrosis (2). These pathological changes may lead to neurological dysfunction, disability, or even death. Ischemic strokes are more prevalent in young people as a result of obesity, inactivity, and excessive drinking (3), making it a major disease in humans.

Neuronal apoptosis after ischemic stroke has been widely studied to salvage dysfunctional neurons (2). Generally, apoptosis caused by stroke is mediated through numerous pathways, such as the mitochondrial pathway and the protein 53 (p53)-mediated apoptosis pathway (4). In the former pathway, B-cell lymphoma 2 (BCL-2)-associated X (Bax) and BCL-2-associated death promoter (Bad) of BCL-2 family proteins are mediated in a receptor-independent manner, which leads to nuclear deoxyribonucleic acid (DNA) degradation and apoptosis (4). In the latter pathway, cell death cascades are triggered by genes involved in the mitogen-activated protein kinase (MAPK) family (4,5). These signals serve as important potential therapeutic targets to improve neuronal apoptosis after stroke.

Corin is mainly expressed in heart tissue, mediates many important physiological, and pathological processes, and is associated with an increased risk of cardiovascular disease (6). Notably, corin overexpression reduces the size of myocardial infarction and regulates the apoptosis of cardiomyocytes and the expression of Bcl-2 family proteins (7). Upon detachment from myocardial cells, corin can enter

the blood stream. Thus, corin may be detected in the sera of patients with heart failure, hypertension, and obesity (8). Stroke, as a co-induced cardiovascular disease, can significantly reduce serum corin expression (9). Additionally, a deficiency in serum-soluble corin predicts severe disability in the 3 months following acute stroke (8), which suggests that corin could affect the progression of stroke. In this way, we speculate corin could influence the progress of stroke.

Circular ribonucleic acids (circRNAs) are important non-coding RNAs that are highly conserved and expressed in human cells. Recent studies have shown that transient cerebral ischemia and oxygen-glucose deprivation (OGD) significantly alter the expression profiles of circRNAs in neurons (10-12), but the specific regulatory mechanism by which this occurs has yet to be identified. A previous study has shown that microRNA could interact with circRNAs and directly affect the stability of messenger RNA and the expression of their encoded downstream proteins (13). There is increasing evidence that miRNAs, such as miR-125a-5p, miR-125b-5p, and miR-143-3p, play important roles in the process of stroke (14). Further, research has shown that miR-200a-3p plays a critical role in ischemic stroke modulated by long non-coding RNAs (15); however, it is unclear whether miR-200a-3 interacts with circRNAs to affect the process of stroke.

As a member of the natriuretic peptide family, corin has been presumed to be epigenetically regulated (16); thus, a better understanding of the epigenetic control mechanism for corin will help clarify its role in the development of common cardiovascular and cerebrovascular diseases and lead to the identification of new therapeutic targets. We sought to investigate the effects of corin on OGD-induced SHSY5Y cells and to determine the underlying mechanisms. We present the following article in accordance with the MDAR reporting checklist (available at <https://atm.amegroups.com/article/view/10.21037/atm-22-4943/rc>).

### Highlight box

#### Key findings

- Circ-0012397/miR-200a-3p/corin axis regulates SHSY5Y cell proliferation and inhibits OGD-induced apoptosis.

#### What is known and what is new?

- CircRNAs and miRNAs have proved to regulate the process of ischemic stroke.
- Circ-0012397/miR-200a-3p/corin axis also plays a key role in regulate OGD-induced SHSY5Y cell apoptosis.

#### What is the implication, and what should change now?

- Circ-0012397/miR-200a-3p/corin axis could be a potential target for ischemic stroke patients.

## Methods

### Cell cultures and OGD

We purchased the SHSY5Y cell line from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China). After resuscitation, the cells were cultured in Eagle's minimum essential medium/F12+ 10% fetal bovine serum medium (Gibco, California, USA) at 37 °C under 5% carbon dioxide (CO<sub>2</sub>) in an incubator. When 90% cell confluence was reached, the cells were passaged at a 1:3 ratio. The cells were

**Table 1** Sequences of miRNA and CircRNA

Name	Sequence
siRNA	5'-GCAGUGUAAUGGCUACAAUTT-3'
si-NC	5'-UAGCACCAUUUGAAAUCAGUGUU-3'
OV-CircRNA	5'-GAACTCGAGATTGGCGTCCAACATGGAC-3'
OV-NC	5'-GTCCATGTTGGACGCCAATCTCGAGTTC-3'
miRNA mimic	5'-UAACACUGUCUGGUAACGAUGU-3'

NC, negative control; OV, over expression.

then added to 96-well plates (density =  $2 \times 10^5$ ) for OGD treatment and cultured in sugar-free medium under hypoxic conditions (94% nitrogen, 5% CO<sub>2</sub>, and 1% oxygen at 37 °C for 2 h, 4 h, and 6 h), after which they were cultured in normal medium under normoxia for 24 h.

### Cell transfection

The small-interfering RNAs (siRNAs) specific for different sequences of corin mRNA, si-negative control (NC), overexpressed (OV)-circRNA, OV-NC, and a miRNA mimic were designed by Shanghai GenePharma Co., Ltd (see *Table 1*). SHSY5Y cells were added to a 96-well plate (density =  $2 \times 10^5$ /well), transfected with constructs in the presence of riboFECTCP transfection reagent (RiBoBio, Guangzhou, China), and cultured at 37 °C with 5% CO<sub>2</sub>. Before OGD treatment, the transfected cells were incubated for 24 h. Subsequently, transfection efficiency was measured using Real time-quantitative polymerase chain reaction (RT-qPCR).

### Cell viability assays

SHSY5Y cell proliferation was detected using Cell Counting Kit-8 (CCK-8; Beyotime, Shanghai, China). In brief, the SHSY5Y cells were seeded in a 96-well plate (density =  $2 \times 10^4$ /well) and transfected for 24 h. Next, 10 µL of the CCK-8 with solution was added to each well, and the cells were then incubated for 2 h incubation at 37 °C. Finally, absorbance was calculated at 450 nm.

### Flow cytometry analysis

The cytoflex flow cytometer (Beckman Coulter, Inc., California, USA) and Annexin V-FITC Apoptosis Detection Kit (Sigma-Aldrich, Missouri, USA) were used for apoptosis

measurement. In brief, the cells (approximately  $2 \times 10^5$ ) were centrifuged at 1,000 ×g for 5 min. After which, the supernatant was removed, and the cells were re-suspended in 500 mL of the conjugation solution. Next, annexin V-FITC and propidium iodide were add by sequential.

### Hoechst 33258 staining

After the medium was removed, the cells were fixed in paraformaldehyde (4%) for 20 mins. Next, after the fixative was removed, the cells were washed with phosphate buffered solution (PBS) 3 times, and incubated with 0.5 mL of Hoechst33258 staining solution for 5 min. After re-washing with PBS, a fluorescence-quenching sealing solution was added. The sample was then covered with a cover slip and observed under a fluorescence microscope.

### RT-qPCR analysis

The cytoplasmic and nuclear RNAs were extracted using an RNA purification kit (Norgen BioTek, Thorold, Canada). Reverse transcription was performed using the EasyScript® One-Step gDNA Removal and cDNA Synthesis SuperMix (TransGen Biotech, Beijing, China), followed by amplification using the TransStart® Top Green qPCR SuperMix (TransGen Biotech) and the CFX96 Touch RT-qPCR Detection System (Bio-Rad, California, USA). The assays were repeated 3 times using the cognate specific primer sequences for hsa\_circ\_0012397, hsa\_circ\_0002995, hsa-miR-3163, hsa-miR-200a-3p, hsa-miR-141-3p, corin, and U6 (see *Table 2*). The relative expression levels of the above-mentioned miRNAs were normalized and calculated using the  $2^{-\Delta\Delta Ct}$  method.

### RNase R assays

After the RNAs of the SHSY5Y cells were isolated using the RNAeasy system (RiboMinus Kit, Invitrogen, California, USA) (17). A denatured sample was first heated to 70 °C and then cooled to 40 °C, and 1.7 µL of RNase R buffer was then added and whisked for 1 h at 40 °C. In 3 wells, RT-qPCR was performed to evaluate the human mRNA expression levels of hsa\_circ\_0012397 and Fas Associated Factor 1 (FAF1).

### Dual-luciferase reporter assays

Website tools, such as miRDB (<http://mirdb.org/>),

**Table 2** Primer sequence

Primer name	Primer sequence
hsa_circ_0012397 F	5'-TCATGGCTGCAATGGAGAT-3'
hsa_circ_0012397 R	5'-GGGTCTGTGCAGGTGAAGAT-3'
hsa_circ_0002995 F	5'-CCATGAACAACCTGGAAA-3'
hsa_circ_0002995 R	5'-GTCCTGGAGTAATGATGG-3'
hsa-miR-3163 F	5'-GCGGCCGCCATTCTCACACGTGCCTAAG-3'
hsa-miR-3163 R	5'-TCTAGATGAT TACCCAACCTACTGCT-3'
hsa-miR-200a-3p R	5'-AACACTGTCTGGTAACGATGTCGT-3'
hsa-miR-200a-3p F	5'-ACGACATCGTTACCAGACAGTGTT-3'
hsa-miR-141-3p R	5'-GGTCTAACACTGTCTGGTAAAGTGG-3'
hsa-miR-141-3p F	5'-CCAGTGCAGGGTCCGAGGT-3'
Corin F	5'-TGCCCAAGCGGAAGTGAG-3'
Corin R	5'-GACGGATGGTCCAGGTTGTTT-3'
U6 F	5'-CTCGCTTCGGCAGCACA-3'
U6 R	5'-AACGCTTCACGAATTTGCGT-3'

TargetScan 7.2 ([https://www.targetscan.org/vert\\_80/](https://www.targetscan.org/vert_80/)), and starBase 3.0 (<https://www.starbase.info/index.html>), were used to predict the potential binding sites of *CORIN* and hsa\_circ\_0012397 in miR-200a-5p. The pGL3 vector (Promega, Wisconsin, USA) was applied to clone the target DNA sequences of wild-type and mutant hsa\_circ\_0012397, and wild-type and mutant *CORIN* 3'-UTR, miRNA mimics, and miRNA NC, respectively. These constructs were used to validate the interactions between hsa\_circ\_0012397 and miR-200a-5p, miR-200a-5p, and *CORIN*. We used a dual-luciferase reporter assay system (Promega) to measure fluorescence intensity after adding the SHSY5Y cells to 96-well plates and co-transfecting them with the above plasmids for 48 h.

### Western blotting analysis

RIPA Lysis Buffer (Applygen Technologies Inc., Beijing, China) was used to extract the total proteins from the SHSY5Y cells. A BCA kit (MultiSciences, Hangzhou, China) was used to measure the protein concentrations. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis

(15% or 8%, MultiSciences) was used for target protein separation, and the polyvinylidene fluoride membranes (Millipore, Darmstadt, Germany) were then transferred and blocked with 5% skimmed milk powder at 25 °C for 90 min. The membranes were incubated overnight at 4 °C with the following primary antibodies: anti-Bad (ab32445, 1:1,000), anti-Bcl-2 (ab182858, 1:2,000), anti-Cleaved caspase-3 (ab2302, 1:400), anti-extracellular regulated protein kinase (ERK) (ab184699, 1:8,000), anti-phospho-ERK (ab201015, 1:1,000) (Abcam, Cambridge, UK), anti-protein 38 (p38) (#9212, 1:1,000, CST), anti-phospho-p38 (#9216, 1:2,000, CST), and glyceraldehyde-3-phosphate dehydrogenase (ab8245, 1:8,000, Abcam). The membranes were then incubated with horse radish peroxidase-conjugated secondary antibodies (1:1,000, MultiSciences) at 25 °C for 90 min. Next, the bands were visualized.

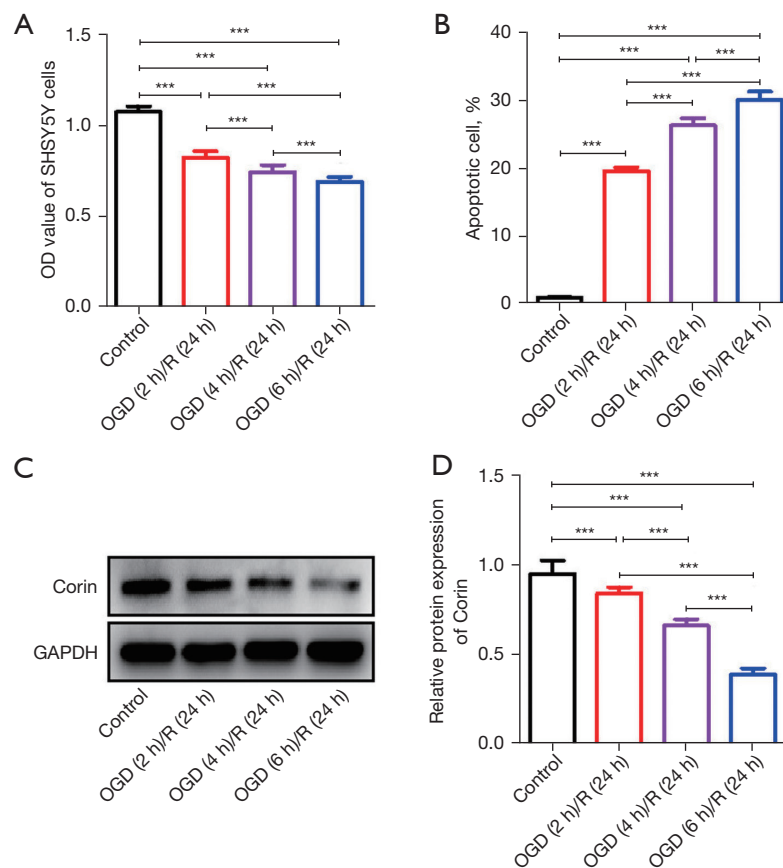
### Statistical analysis

All the experiments were repeated 3 times. The data were presented as the mean  $\pm$  standard deviation (SD). Differences in relative expression levels of cytoplasmic and nuclear hsa\_circ\_0012397 (or FAF1) (or RNase R- and RNase R+) were evaluated using the un-paired 2-sided *t*-test or a 1-way analysis of variance (ANOVA) with Tukey multiple comparisons. A *P* value <0.05 indicated a statistically significant difference.

## Results

### *The protein expression of corin was significantly downregulated in the OGD-induced stroke model*

Recently, corin has been reported to be associated with stroke (6,9); however, the role of corin expression in stroke, particularly ischemic stroke, is unclear. In the present study, SHSY5Y cells deprived of glucose and oxygen were used to establish a stroke model to investigate the role of corin. As *Figure 1A* shows, the longer the duration of OGD, the fewer SHSY5Y cells survived as indicated by the lower optical density (OD) value detected by the Cell Counting Kit-8 (CCK-8) assays. Conversely, the number of apoptotic cells was significantly increased (see *Figure 1B*). Further, western blotting also revealed that the expression of corin protein was significantly downregulated with the prolongation of OGD (see *Figure 1C,1D*). These findings are consistent with the decrease in serum-soluble corin described above.



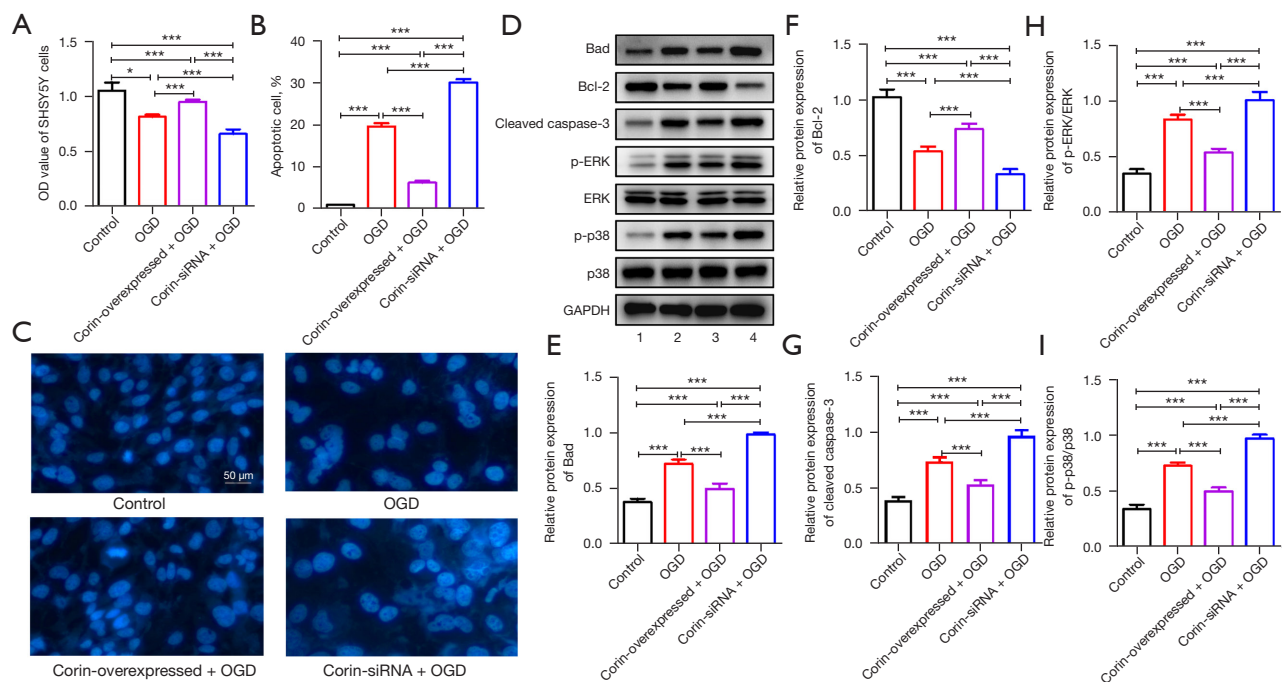
**Figure 1** Corin expression was significantly downregulated in the OGD-induced SHSY5Y cells. A significant decrease in the OD value of CCK-8 was observed in the OGD-induced SHSY5Y cells over time (A), while the number of apoptotic cells significantly increased (B). Mean  $\pm$  SD, the data were analyzed using a 1-way ANOVA *vs.* Control, \*\*\*,  $P < 0.01$ . A western blot analysis of corin expression revealed significant downregulation with the prolongation of OGD in SHSY5Y cells (C,D) ( $n=3$ ). Mean  $\pm$  SD, the data were analyzed using a 1-way ANOVA with Tukey multiple comparisons, \*\*\*,  $P < 0.001$ . OGD/R: oxygen-glucose deprivation/reoxygenation. OD, optical density; ANOVA, analysis of variance; OGD, oxygen-glucose deprivation.

### ***Corin protects cell survival by inhibiting apoptosis-related proteins and ERK/p38 under OGD***

To further examine the role of corin in stroke, we explored the effects of the overexpression and silencing of corin on OGD-induced SHSY5Y cells. We found that the number of surviving cells increased and that of apoptotic cells decreased significantly when corin was overexpressed. Conversely, the number of surviving cells decreased and that of apoptotic cells significantly increased when corin was silenced (see *Figure 2A,2B*). Further, similar results were observed in Hoechst 33258-stained cells, and large numbers of apoptotic cells were observed in the OGD and corin-siRNA + OGD groups, accompanied by pyknosis of the nucleus, deep staining, or fragmented deep staining.

However, apoptotic cells were not detected in the control and corin-overexpressed + OGD groups (see *Figure 2C*).

We also analyzed the expression levels of apoptosis-involved proteins in each group (see *Figure 2D-2I*), and found that the expression levels of Bad, cleaved Caspase-3, p-ERK/ERK, and p-p38/p-38 proteins were significantly higher in the OGD and corin-siRNA + OGD groups than the control group (see *Figures 2E,2G-2I*), and the expression level of Bcl-2 was significantly downregulated (see *Figure 2F*). The protein expression levels of Bad, cleaved Caspase-3, p-ERK/ERK, and p-p38/p-38 were significantly lower in the corin-overexpressed + OGD group than the OGD and corin-siRNA + OGD groups, while the expression level of Bcl-2 was higher compared to that of the



**Figure 2** Corin promotes the survival of SHSY5Y cells subjected to OGD by inhibiting the expression of apoptosis-related proteins and ERK/p38. There was a significant increase in CCK-8 OD in the corin-overexpressed + OGD group, while that in the corin-siRNA + OGD group significantly decreased (A). The number of apoptotic cells significantly decreased in the corin-overexpressed group, but increased in the OGD group (B). Hoechst 33258 staining showed that the number of cells in the control and corin-overexpressed + OGD groups did not differ significantly (C). Protein bands (D). The expression levels of Bad (E), cleaved caspase-3 (G), p-ERK/ERK (H), and p-p38/p38 (I) in the corin-overexpressed + OGD group were significantly upregulated, but that of Bcl-2 (F) was significantly downregulated; however, opposite results were obtained for the corin-siRNA + OGD group. Mean  $\pm$  SD,  $n=3$ , the data were analyzed using a 1-ANOVA with Tukey multiple comparisons *vs.* Control, \*,  $P<0.05$ ; \*\*\*,  $P<0.01$ . OGD, oxygen-glucose deprivation; OD, optical density; ANOVA, analysis of variance.

OGD and corin-siRNA + OGD groups (see *Figure 2D-2I*). Thus, corin appears to protect cells from apoptosis and ERK/p38 activation under OGD.

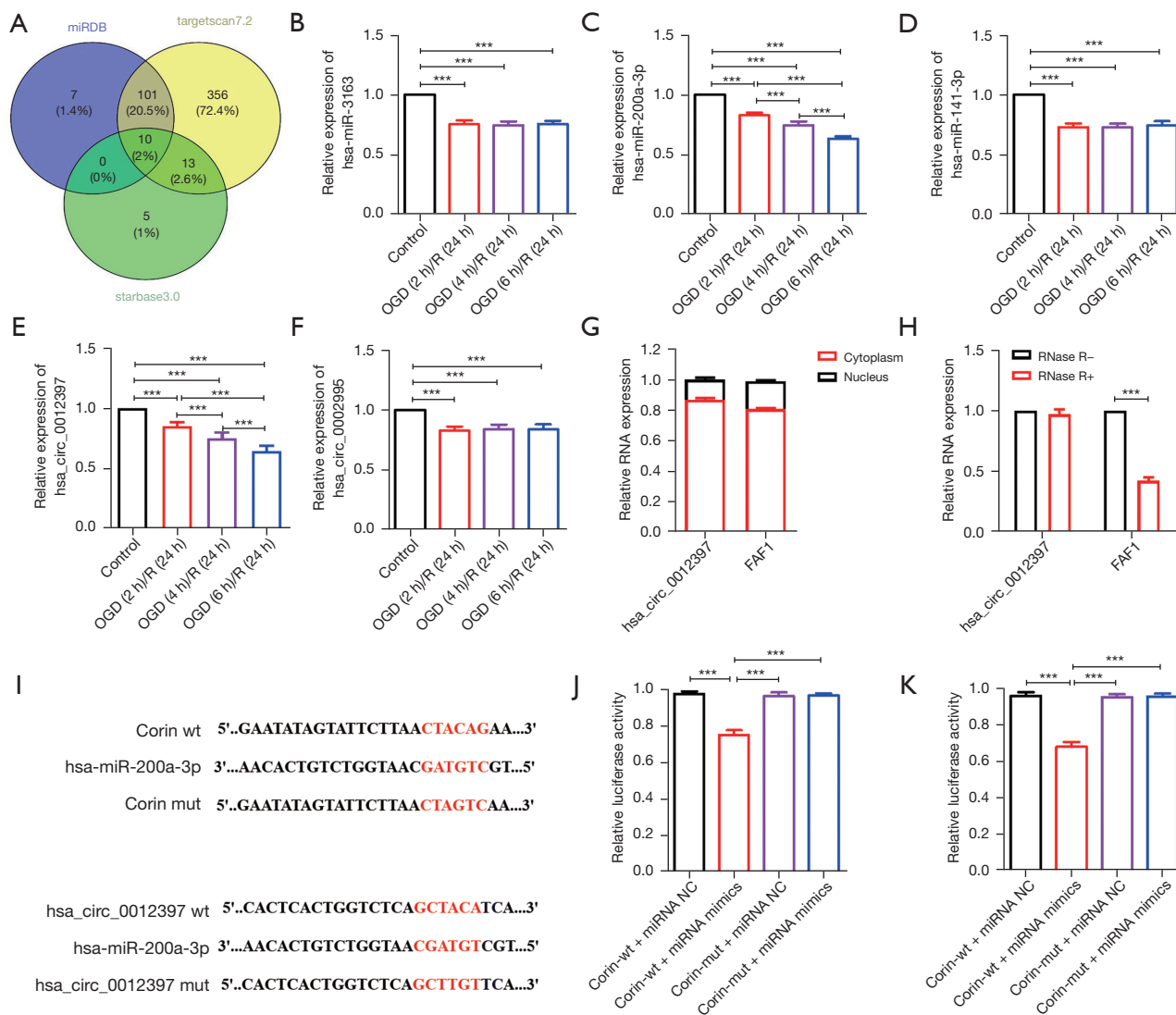
### *Corin regulates hsa\_circ\_0012397/hsa-miR-200a-3p in OGD-induced SHSY5Y cells*

To further clarify the regulatory mechanism of corin, we performed a bioinformatics analysis to predict its upstream-binding miRNAs. In total 10 candidate miRNAs were identified from miRDB, TargetScan 7.2, and starBase 3.0 (see *Figure 3A*). In miRDB, the miRNAs hsa-miR-3163, hsa-miR-200a-3p, and hsa-miR-141-3p had target scores  $\geq 80$ . The RT-qPCR analysis showed that the temporal expression of hsa-miR-200a-3p decreased with the prolongation of OGD, while the expression of hsa-miR-3163 and hsa-miR-141-3p did not change

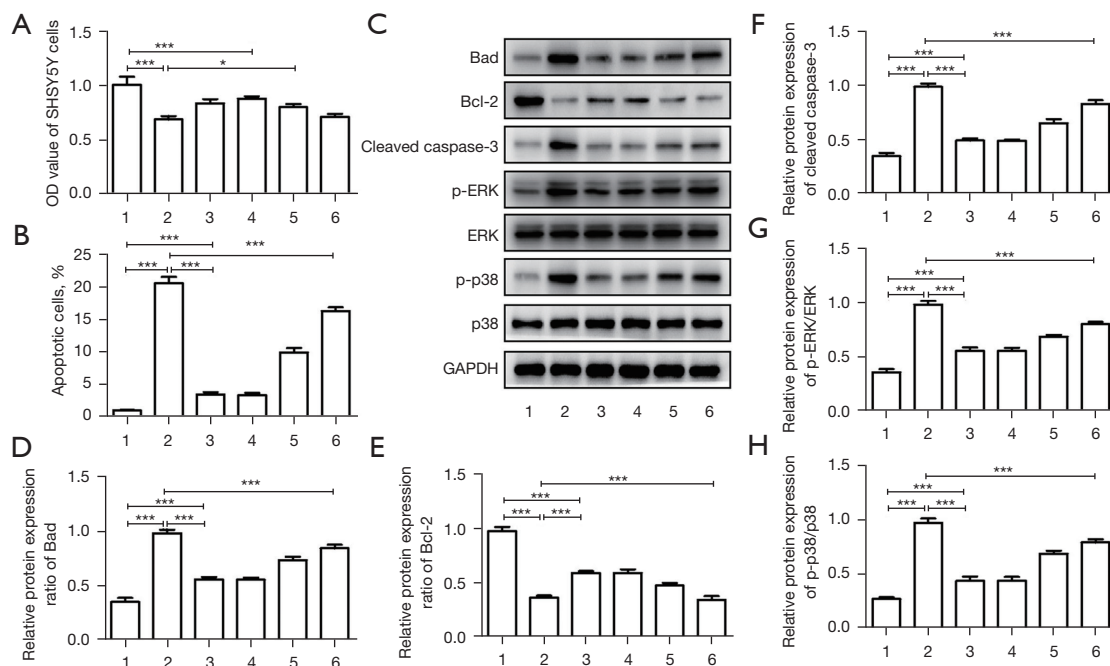
significantly (see *Figure 3B-3D*). Next, we analyzed the potential circRNAs regulating hsa-miR-200a-3p, and identified hsa\_circ\_0012397 and hsa\_circ\_0002995 from the Human circRNA database. However, only the former was considered a regulator of hsa-miR-200a-3p in this model, as the expression of the latter did not change significantly, while the former was significantly downregulated with the prolongation of OGD (see *Figure 3E,3F*).

We also found that hsa\_circ\_0012397 and its target gene FAF1 were mainly expressed in the cytoplasm and nucleus (see *Figure 3G*). Additionally, RNase R assays confirmed the circularity of hsa\_circ\_0012397. As *Figure 3H* shows, the expression of hsa\_circ\_0012397 was unaffected by RNase R<sup>+</sup>, while the expression of FAF1 was significantly downregulated by RNase R<sup>+</sup>, suggesting that hsa\_circ\_0012397 is indeed a circRNA.

We then used dual-luciferase reporter assays to explore



**Figure 3** Corin is regulated by hsa\_circ\_0012397/hsa-miR-200a-3p in OGD-induced SHSY5Y cells. Using bioinformatics technology, we identified 10 upstream miRNA candidates for corin from 3 databases (A). 2 miRNAs and 2 circRNAs were identified, and a RT-qPCR analysis of these RNAs was conducted in the SHSY5Y cells treated with OGD. hsa-miR-3163 (B), hsa-miR-200a-3p (C), hsa-miR-141-3p (D), hsa\_circ\_0012397 (E), and hsa\_circ\_0002995 (F). Mean  $\pm$  SD; the data were analyzed using a 1-way ANOVA, \*\*\*,  $P < 0.001$ . Hsa\_circ\_0012397 and *FAF1* were mainly expressed in the cytoplasm of SHSY5Y cells (G). Hsa\_circ\_0012397 was resistant to RNase R+, but *FAF1* was not (H). Mean  $\pm$  SD; the data were analyzed using an un-paired 2-sided *t*-test, \*\*\*,  $P < 0.001$ . Dual-luciferase reporter assays (I) were used to determine the presence of a direct interaction between corin and hsa-miR-200a-3p (J) and hsa-miR-200a-3p and hsa\_circ\_0012397 (K). Mean  $\pm$  SD; the data were analyzed using a 1-way ANOVA with Tukey multiple comparisons *vs.* Control, \*\*\*,  $P < 0.01$ . OGD, oxygen-glucose deprivation; RT-qPCR, real-time quantitative polymerase chain reaction; ANOVA, analysis of variance.



**Figure 4** Corin expression is regulated by *hsa\_circ\_0012397/hsa-miR-200a-3p* to increase the apoptosis of OGD-induced SHSY5Y cells. The proliferation (OD) of SHSY5Y cells transfected with different recombinant plasmids differed (A), as did the number of apoptotic cells (B). Western blot analysis of the expression of apoptosis-related proteins in SHSY5Y cells (C). Relative expression of Bad (D), Bcl-2 (E), cleaved caspase-3 (F), p-ERK/ERK (G), and p-p38/p38 (H). 1. Control; 2. OGD (oxygen-glucose deprivation); 3. OV-CircRNA (CircRNA over expression) + OGD; 4. miRNA inhibitor + OGD; 5. OV-CircRNA + miRNA mimics + OGD; 6. OV-CircRNA + miRNA mimics + corin-siRNA + OGD. Mean  $\pm$  SD,  $n=3$ , the data were analyzed using a 1-way ANOVA with Tukey multiple comparisons *vs.* Control, \*,  $P<0.05$ , \*\*\*,  $P<0.01$ . OGD, oxygen-glucose deprivation; OD, optical density; ANOVA, analysis of variance; OV, over expression.

whether there was a direct regulatory relationship between corin expression and *hsa-miR-200a-3p*, *hsa-miR-200a-3p*, and *hsa\_circ\_0012397* (see *Figure 3I*). As *Figure 3J* shows, the relative luciferase activity of corin-wt + miRNA mimics was significantly lower compared to that of corin-wt + miRNA NC, but there were no significant difference in the relative luciferase activities between corin-mut + miRNA mimics and corin-mut + miRNA NC. Similar results were observed for circRNA-wt + miRNA mimics and CircRNA-wt + miRNA NC, CircRNA-mut + miRNA mimics, and CircRNA-mut + miRNA NC (see *Figure 3K*). Thus, corin expression in the OGD-induced SHSY5Y cells was regulated by *hsa\_circ\_0012397/hsa-miR-200a-3p*.

#### **Corin is regulated by *hsa\_circ\_0012397/hsa-miR-200a-3p***

To confirm the function of the *hsa\_circ\_0012397/hsa-miR-200a-3p/corin* pathway, we constructed different recombinant plasmids that were used to transfect SHSY5Y

cells to find the effect of OGD. As *Figure 4A* shows, the OD values of the control, OV-CircRNA, miRNA inhibitor, and OV-CircRNA + miRNA mimic groups were significantly higher than those of the model group, suggesting that the numbers of viable cells in these groups were significantly higher than that of the model group. Similarly, the numbers of apoptotic cells in the control, OV-CircRNA, miRNA inhibitor, OV-CircRNA + miRNA mimics, and OV-CircRNA + miRNA mimics + corin-siRNA groups were significantly lower than those of the model group, but those of the OV-CircRNA + miRNA mimics and OV-CircRNA + miRNA mimics + corin-siRNA group were significantly higher than that of the control, OV-CircRNA, or miRNA inhibitor groups (see *Figure 4B*).

We further analyzed the expression levels of relative proteins. As shown in *Figure 4C-4H*, the protein expression level of Bad in the model group was significantly higher than those of the other groups, while those of the OV-CircRNA + miRNA mimics or OV-CircRNA + miRNA



mimics + corin-siRNA groups were significantly higher than those of the control, OV-CircRNA, or miRNA inhibitor groups (Figure 4C,4D). The protein expression levels of cleaved Caspase-3, p-ERK, and p-p38 in each group were similar to those of Bad (see Figure 4F-4H). However, the protein expression level of Bcl-2 in each group differed to that of Bad, cleaved caspase-3, p-ERK, or p-p38. The level of Bcl-2 protein expression of the model group was lower than those of the control, OV-CircRNA, miRNA inhibitor, or OV-CircRNA + miRNA mimics groups, and those of the OV-CircRNA and miRNA inhibitor groups were significantly higher than those of the OV-CircRNA + miRNA mimics or OV-CircRNA + miRNA mimics + corin-siRNA groups (see Figure 4E). Thus, corin is regulated by hsa\_circ\_0012397/hsa-miR-200a-3p, such that OGD-induced SHSY5Y cells undergo apoptosis.

## Discussion

Ischemic stroke is a major disease associated with the highest rates of disability and mortality worldwide (18). In the minutes after stroke, irreversible tissue damage and subsequent cell death occur (18). Notably, the incidence of ischemic stroke in young people has increased since the 1980s (3). Mechanical thrombectomy within 6 or 24 h after the onset of ischemic stroke is beneficial to the recovery of patients (19); however, it is critical to reduce the necrosis or apoptosis of nerve cells within this time to the greatest extent possible. A previous study has shown that numerous miRNAs play important roles in the regulation of ischemic stroke, including certain non-coding RNAs and circRNAs (20). We predicted and verified the regulatory mechanism of corin, whose serum concentration is significantly decreased in stroke patients. We showed that corin was regulated by has\_circ\_0012397/hsa-mir-200a-3p during ischemia and hypoxia, which in turn regulated apoptosis-related proteins, such as Bad and cleaved caspase-3, and MAPK signaling proteins (p-ERK and p-38), thereby inhibiting neuronal apoptosis in OGD-induced SHSY5Y cells. Further, we found that corin was regulated by circ-0012397/miR-200a-3p in the SHSY5Y cells subjected to OGD.

Corin is a serine protease selectively anchored to the heart membrane. Its essential activity is to cleave and activate pro-atrial natriuretic peptide and pro-B-type natriuretic peptide. Thus, corin plays key roles in maintaining saline balance and regulating heart function and blood pressure (21,22). Similar to other transmembrane

proteases, cardiac corin enters the circulation when it is released by apoptotic cells. A significant decrease in the corin levels in the heart and blood has been reported in patients with heart failure in clinical and experimental settings, and the extent of the decrease is related to the severity of the cardiac dysfunction (23). However, numerous studies have shown that the serum levels of corin significantly change in the presence of diseases, such as obesity (24), hypertension (25), hyperlipidemia (26), preeclampsia (27), diabetic nephropathy (28), and stroke (6,9). Because of the special location of corin, it is used as a biomarker for the diagnosis of cardiovascular disease (29). The corin protein is mainly expressed in heart tissue and enters the circulatory system after shedding; however, a previous study has detected the expression of its gene in dopaminergic neural progenitor cells (30). Moreover, striatal dopaminergic neurons are potential targets for the treatment of ischemic stroke associated with Glial cell line-derived neurotrophic factor (GDNF) (31). Thus, the control of protein expression of corin in dopamine nerves and the mechanism require further research.

Corin has been reported to have anti-inflammatory role in natriuretic peptides treating Congestive heart failure (32). IL-1 $\beta$  and TNF- $\alpha$  could increase the level of urinary corin in patients with primary proteinuric kidney diseases (33). Recent studies have reported that corin participates in regulating several physiological signal transduction pathways, such as the MAPK, PI3K/Akt, and NF- $\kappa$ B pathways (4,5,28). These signaling pathways are closely associated with apoptosis; for example, the activation of the ERK and the p38 pathways phosphorylates p53 to promote the cell death cascade. These findings suggest that corin plays a regulatory role in apoptosis, and corin activates the PI3K/Akt and NF- $\kappa$ B signaling pathways to inhibit hydrogen peroxide-induced apoptosis (34). PI3K/Akt and NF- $\kappa$ B have also been reported to regulate neuroinflammation and oxidative stress (35). Thus, the therapeutic effect of corin may be due to its regulation of neuroinflammation and oxidative stress through PI3K/Akt and NF- $\kappa$ B pathway.

Apoptosis is a pathological or physiological process initiated after the occurrence of ischemic stroke; thus, corin may regulate apoptosis after ischemic stroke. We showed that the expression of corin in OGD-induced SHSY5Y cells was significantly decreased, while the number of apoptotic cells was significantly increased. We also found that corin regulated the expression of apoptosis-related proteins, such as Bad, cleaved caspase-3, and Bcl-2, which suggests

that the regulation of corin expression may contribute to the prognosis of ischemic stroke patients. Corin exerts protective role in OGD treated cells. Thus, the low corin level might indicate poor prognosis. Promoting the expression of corin in the brain could be a potential method in treating stroke.

Numerous miRNAs, non-coding RNAs, and circRNAs are involved in the process of ischemic stroke (20). Thus, we conducted a bioinformatics analysis of potential miRNAs regulating the expression of corin and identified 3 candidate miRNAs (i.e., hsa-miR-3163, hsa-miR-200a-3p, and hsa-miR-141-3p). However, further verification showed that only hsa-miR-200a-3p was associated with corin expression. In this model, the expression levels of hsa-miR-3163 and hsa-miR-141-3p were not absolutely associated with corin expression, which may be related to different experimental cells or the fact that they require stimulation *in vivo*. This issue requires further research.

Evidence indicates that circRNAs can be used as competitive endogenous RNAs to adsorb miRNAs and regulate gene expression by binding to proteins (17,36). Thus, we also analyzed potential circRNAs that adsorb hsa-miR-200a-3p to regulate the protein expression of corin, such as hsa\_circ\_0012397 and hsa\_circ\_0002995. Our subsequent experiments revealed that hsa\_circ\_0012397/hsa-miR-200a-3p regulated the differential expression of corin when overexpressed or silenced. Further, the corin-mediated regulation of the expression of apoptosis-related proteins, including Bad, cleaved caspase-3, and Bcl-2, resulted in changes in OGD-induced SHSY5Y cells.

There were some limitations of this study. Although we confirmed corin was regulated by hsa\_circ\_0012397/hsa-miR-200a-3p in SHSY5Y cells, there was no animal models were used in this study. To establish the role of corin in ischemic stroke, further studies of primary neurons in animal models and clinical studies need to be conducted. The expression profiles of hsa\_circ\_0012397 and hsa-miR-200a-3p should also been detected in the *in vivo* experiments.

## Conclusions

We showed that, in SHSY5Y cells, corin was regulated by hsa\_circ\_0012397/hsa-miR-200a-3p, which in turn regulated the expression of apoptosis-related proteins, such as Bax, cleaved caspase-3, and Bcl-2. Additionally, corin reduced apoptosis in OGD-induced SHSY5Y cells. Our findings provide further insights into the role of corin in

ischemic stroke and its potential as a potential therapeutic target.

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## Footnote

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