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Regular paper

Upregulation of miR-22 alleviates oxygen–glucose deprivation/reperfusion-induced injury by targeting Tiam1 in SH-SY5Y cells

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MicroRNA-22 (miR-22) has been reported to exert a neuroprotective effect. However, the specific role and mechanism of miR-22 in ischemia/reperfusion (I/R)-induced brain injury are still not known well. In this study, we evaluated whether miR-22 participates in I/R-induced neuronal injury and the potential mechanism by using an oxygen-glucose deprivation/reperfusion (OGD/R) model *in vitro*. Our results showed that miR-22 was significantly down-regulated in SH-SY5Y cells suffering from OGD/R. Up-regulation of miR-22 by its specific mimic could protect SH-SY5Y cells against OGD/R-induced injury. The luciferase reporter assay demonstrated that T-cell lymphoma invasion and metastasis 1 (Tiam1) was a direct target of miR-22. MiR-22 mimic obviously inhibited Tiam1 expression in OGD/R-exposed SH-SY5Y cells. Tiam1 siRNA could attenuate OGD/R-induced SH-SY5Y cell injury. In addition, Tiam1 siRNA reduced the activation of Ras-related C3 botulinum toxin substrate 1 (Rac1) in OGD/R-exposed SH-SY5Y cells, and up-regulation of Rac1 activity could attenuate the neuroprotective effect of miR-22 up-regulation. Furthermore, OGD/R exposure led to increased methylation of miR-22, and the demethylating agent 5-Aza-dC significantly up-regulated miR-22 expression and inhibited Tiam1 expression and Rac1 activation. Taken together, our results demonstrated that DNA methylation-mediated miR-22 down-regulation aggravated I/R-induced neuron injury by promoting the activation of Tiam1/Rac1 signals. Our findings provide a deeper understanding of I/R-induced brain injury and suggest that miR-22 may be a promising therapeutic target for this disease.

Keywords: miR-22, Tiam1, Rac1, neuronal injury

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Abbreviations: OGD/R, oxygen–glucose deprivation/reperfusion; I/R, ischemia/Reperfusion; HIBD, hypoxic-ischemic brain damage; MSC-exos, Mesenchymal stem cells-derived exosomes; PUMA, P53 upregulated modulator of apoptosis; GEF, Guanine nucleotide-exchange factor; Rac1, Ras-related C3 botulinum toxin substrate 1; Tiam1, T-cell lymphoma invasion and metastasis 1; NMDAR, N-methyl-D-aspartate (NMDA) receptor

INTRODUCTION

Neuron dysfunction and/or death serves a pivotal function in the development and progression of brain-related diseases, such as Parkinson's disease, ischemic stroke and neonatal ischemic hypoxic brain damage (HIBD), which is caused by oxygen deprivation in the infant's brain (Khoshnam *et al*., 2017; Koehn *et al*., 2020; Liu *et al*., 2020a; Salamon *et al*., 2020). The neuronal cell was susceptible to injury or death by various pathophysiological factors (Khoshnam *et al*., 2017; Koehn *et al*., 2020; Liu *et al*., 2020a; Salamon *et al*., 2020). Ischemia is characterized by an insufficient oxygen supply and serves as the main cause of the aggravation of cerebral injury. The treatment for cerebral ischemia usually involves the restoration of blood flow as quickly as possible. However, this can entail secondary injury to the ischemic area, referred to as 'ischemia/reperfusion' (I/R) injury (Ryou and Mallet, 2018). However, the molecular mechanisms underlying I/R-induced neuronal injury are not entirely clear.

It is demonstrated that miRNAs played an important role in I/R-induced injury in various tissues, including brain tissue (Cai *et al*., 2021; Duan *et al*., 2019; Kuai *et al*., 2021; Liu *et al*., 2020b). Previous studies have reported that miR-22 played a protective role in myocardial injury (Du *et al*., 2016; Zhang *et al*., 2019). Up-regulation of miR-22 was shown to protect the cerebra against I/R injury (Wang *et al*., 2020). A recent study discovered that mesenchymal stem cells-derived exosomes (MSC-exos) alleviated I/R-induced brain injury by transferring miR-22 to neurons (Zhang *et al*., 2021b). Jiao *et al* reported that enhanced miR-22 expression reversed I/R-induced apoptosis in PC12 cells (Jiao *et al*., 2020). These findings indicated that miR-22 might provide a potential neuroprotective effect. However, the neuroprotective mechanism of miR-22 in I/R-induced injury is far from fully elucidated.

Ras-related C3 botulinum toxin substrate 1 (Rac1), a Rho-related small GTPase, is ubiquitously expressed throughout the brain (Stankiewicz and Linseman, 2014). Rac1 has been implicated in oxygen-glucose deprivation (OGD)/reoxygenation (OGD/R)-induced pathways responsible for neuronal injury, neuronal degeneration, and cognitive dysfunction (Chen *et al*., 2020; Li *et al*., 2021). The precise spatial and temporal regulation of Rac1 activation depends on its upstream regulators, the guanine nucleotide exchange factors (GEFs) (Marei and Malliri, 2017). T-cell lymphoma invasion and metastasis 1 (Tiam1) is a Rac1-specific GEF, which is stimulated by N-methyl-D-aspartate (NMDA) receptor (NMDAR) activation in a Ca^{2+}-dependent manner (Tolias *et al*., 2005). Previous studies demonstrated that Tiam1-mediated Rac1 activation in hippocampal and cortical neurons mediates differential spine shrinkage in response to OGD (Blanco-Suarez *et al*., 2014). However, whether Tiam1-mediated Rac1 activation is involved in OGD/R-induced neuronal injury remains unknown. Tiam1 was demonstrated to be a target gene of miR-22 in various cells (Li *et al*., 2013; Li *et al*., 2012) and endometrial Tiam1/Rac1 signal was shown to be negatively regulated by miR-22

(Ma *et al*., 2015). Therefore, it is worthwhile to explore whether Tiam1/Rac1 signal is involved in protective effect of miR-22 against I/R-induced neuronal injury.

In vitro OGD/R is a widely accepted experimental model of *in vivo* I/R-induced neuronal injury (Zhang *et al*., 2021a). In this study, we investigated the effect of miR-22 on I/R-induced neuronal injury and its mechanism by using an OGD/R model in SH-SY5Y cells. Our results showed that miR-22 was down-regulated in SH-SY5Y cells. Enforced miR-22 expression could inhibit Tiam1 expression and suppress Rac1 activation, thus contributing to SH-SY5Y cell protection against I/R-induced injury. Furthermore, we also found that methylation of the miR-22 gene promoter suppressed its expression in OGD/R-exposed SH-SY5Y cells.

MATERIALS AND METHODS

Cell culture and OGD/R induction

The SH-SY5Y human neuroblastoma cell line was purchased from Zhong Qiao Xin Zhou Biotechnology Co., Ltd (Shanghai, China). Cells were grown in MEM/F12 (1:1 mixture) supplemented with supplemented with 10% heat inactivated fetal bovine serum, 1% sodium pyruvate, 1% L-alanyl-L-glutamine, and 1% penicillin/streptomycin at 37°C in a humidified atmosphere with 5% CO_2. For neuronal differentiation, SH-SY5Y cells were cultured for 3 days in MEM/F12 medium containing 10 μM retinoic acid and 1% FBS, followed by culturing for a further 3 days with 50 ng/mL brain-derived neurotrophic factor and 2 mmol/L glutamine in MEM/F12 medium containing 1% FBS. For OGD/R induction, SH-SY5Y cells were cultured in glucose-free MEM/F12 under the conditions of 95% N_2, 5% CO_2 and 37°C for 4 h. Thereafter, the cells were then cultured in normal DMEM for 24 h of reoxygenation under normoxic condition. SH-SY5Y cells cultured in DMEM containing glucose under normoxic condition served as a control.

Cell transfection

The miR-22 mimic and its negative control, Tiam1 siRNA and control siRNA were purchased from Genepharma (Shanghai, China) and transfected into SH-SY5Y cells using lipofectamine 2000 (Invitrogen) according to the manufacturer's manual. The expression of target genes was determined after 24 h of transfection and cells were used in further experiments. Sequences of the miR-22 mimic, and its negative control (NC) were as follows: miR-22 sense, 5'-AAGCUGCCAGUUGAAGAACUGU-3' and antisense, 5'-AGUUCUUCAACUGGCAGCUUUU-3'; and NC sense, 5'-UUCUCCGAACGUGUCACGUTT-3' and antisense, 5'-ACGUGACACGUUCGGAGAATT-3'.Sequences of Tiam1 siRNA were as follows: GGCGAGCUUUAAGAAGAAATT (sense) and UUUCUUCUUAAAGCUCGCCGT (antisense). Scrambled siRNA was used as a negative control in all experiments.

CCK-8 assay

The cell viability of SH-SY5Y cells was measured by CCK-8 assay. SH-SY5Y cells were seeded into 96-well plates (5×10^3/well) and treated as described in the text. At the end of treatment, CCK-8 solution (10 μL/well, Chemicon, Temecula, CA, USA) was added to the cultured cells for 2 h incubation at 37°C. The absorbance (OD) at 450 nm was measured using an ELx-800 microplate reader (Bio-TekInc., Winooski, VT, USA).

Hoechst staining

SH-SY5Y cells were seeded onto coverslips in 6-well plates (5×10^3/well) and treated as described in the text. At the end of treatment, cells were fixed with 4% paraformaldehyde for 30 min, then stained with Hoechst 33258 (5 μg/mL) for 20 min at room temperature. After washing with PBS, the cells were observed and photographed under a fluorescence microscope.

Luciferase reporter assay

The wild-type Tiam1 3'-UTR and mutant-type Tiam1 3'-UTR were cloned into the pGL3 basic luciferase reporter vector (Promega, Madison, WI, USA). SH-SY5Y cells were transfected in 24-well plates with miR-22 agomir and WT Tiam1 3'-UTR or miR-22 agomir and Mut Tiam1 3'-UTR respectively using Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol. After 48 h of transfection, the cells were harvested, and the luciferase activity was measured using a Dual-Luciferase Reporter Gene Assay kit (Promega). Renilla luciferase activity was used to normalize the firefly luciferase intensity.

Western blot

At the end of treatment, total proteins were extracted from SH-SY5Y cells using RIPA lysis buffer (Beyotime Biotechnology, Shanghai, China). Proteins were separated by SDS-PAGE and transferred onto polyvinylidene fluoride (PVDF) membranes (Millipore, USA). After blocking with 5% milk for 1 h at room temperature, the membranes were incubated overnight at 4°C with primary antibodies specific to Tiam1, Rac1, Bcl-2, Bax, Bad and GAPDH (all 1:1000; Cell Signaling Technology, Danvers, MA, USA). The membranes were then incubated with secondary antibodies (1:2000, anti-rabbit; 1:5000, anti-mouse) at room temperature for 1 h. The blots were visualized with an enhanced chemiluminescence kit (Pierce; Thermo Fisher Scientific, Inc.). Image J software was used to analyze the band density.

Pull-Down Assay

Pull-down assay was used to detect Rac1 activity and performed as described previously (Stahle *et al*., 2003). Briefly, after treatments, equal volumes of total cellular protein were incubated with GST-PBD beads captured on MagneGST glutathione particles (Promega, Madison, WI) for 1h at 4°C. The particles were then resuspended in SDS and subjected to immunoblotting analysis by using an anti-Rac1 antibody.

Lactate dehydrogenase (LDH) activity detection

At the end of treatment, the supernatants of SH-SY5Y cells were collected and the contents of LDH were measured by an LDH detection kit (Nanjing Jiancheng Bioengineering Institute, Jiangsu, China) according to the manufacturer's manual. Absorbance values were read at 450 nm using an ELx-800 microplate reader (Bio-TekInc., Winooski, VT, USA).

RT-qPCR

Total RNA was extracted from SH-SY5Y cells using RNAeasy™ Small RNA Isolation Kit (Beyotime,

Shanghai, China) according to the manufacturer's manual. cDNAs were synthesized using the OneStep PrimeScript miRNA cDNA Synthesis Kit (Takara Biotechnology, Dalian, China). Quantitative real-time PCR was performed with an ABI 7300 system (Applied Biosystems, USA) using BeyoFast™ SYBR Green qPCR Mix (Beyotime). The relative gene expression level of miR-22 was normalized to U6 and calculated using the $2^{-\Delta\Delta Ct}$ method. Primers used for RT-qPCR were listed as follows: U6 small nuclear RNA was used as internal reference, with upstream: 5'-GGAACAGAGAAGATTA GC-3', and downstream: 5'-TTGGAATCACGAATTCCG-3'.miR-22 upstream: 5'-TGACAACCGTTTTTGACTG-3' and downstream: 5'-TACTGTTTTGAAAATCGTT-3'.

Methylation specific PCR (MSP)

The genomic DNA was extracted and purified by the Genomic DNA Extraction Kit (TaKaRa, Dalian, China). Bisulfite treatment and conversion of DNA for methylation analysis were performed using the EZ-96 DNA Methylation Kit (Zymo Research, Irvine, CA, USA). The PCR reaction conditions were as follows: 98°C for 4 min, 40 cycles of 98°C for 30 s, 56°C for 30 s, and 72°C for 30 s, and then 72°C for 10 min. The PCR products were electrophoresed in 3% agarose gel.

Statistical analysis

SPSS 16.0 statistics software (SPSS, Chicago, IL) was used for statistical analysis. The data were presented as the mean ± standard deviation (S.D.). Significant differences were determined using One-way ANOVA. $P<0.05$ was considered statistically significant.

RESULTS

OGD/R down-regulated miR-22 expression, up-regulated Tiam1 expression and Rac1 activation in SH-SY5Y cells

As shown in Fig. 1A, the expression level of miR-22 was decreased time-dependently in SH-SY5Y cells after OGD/R treatment. The results of the western blot assay revealed that the expression level of Tiam1 was significantly increased after treatment for 12 h or 24 h with OGD/R (Fig. 1B). The activity of Rac1 was obviously increased, while the total Rac1 expression remained unchanged during OGD/R treatment (Fig. 1B).

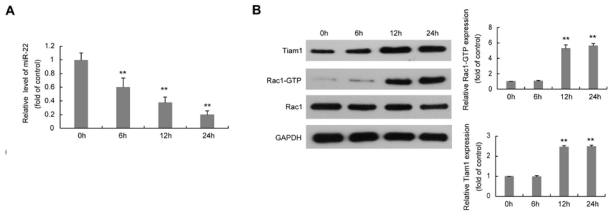

Figure 1. The effects of OGD/R exposure on the activation of Rac1 and the expression levels of miR-22 and Tiam1 in SH-SY5Y cells.
SH-SY5Y cells were exposed to OGD/R for the indicated times, and then the expression level of miR-22 was detected by qRT-PCR (**A**), the expression levels of Tiam1 and Rac1 were measured by western blot, Rac1 activation (Rac1-GTP) was determined by pull-down assay and analyzed by western blot (**B**). $^{**}P<0.01$ compared with 0 h group.

Up-regulation of miR-22 protected SH-SY5Y cells against OGD/R-induced injury

To elucidate the effect of miR-22 down-regulation on OGD/R-induced neuronal injury, SH-SY5Y cells were transfected with miR-22 mimic, which obviously up-regulated the expression level of miR-22 in OGD/R-exposed SH-SY5Y cells (Fig. 2A). CCK-8 assay showed that miR-22 mimic notably elevated the viability of SH-SY5Y cells under OGD/R condition (Fig. 2B). Hoechst staining showed that miR-22 mimic significantly reduced the number of apoptotic cells in OGD/R-exposed SH-SY5Y cells (Fig. 2C). In addition, OGD/R treatment significantly increased the expression levels of Bax and cleaved caspase-3, and reduced the expression level of Bcl-2 in SH-SY5Y cells (Fig. 2D). However, the expression levels of these proteins were notably reversed after treatment with miR-22 mimic in OGD/R-exposed SH-SY5Y cells (Fig. 2D). Furthermore, miR-22 mimic markedly reduced the content of LDH from OGD/R-exposed SH-SY5Y cells (Fig. 2E). These data indicated that up-regulation of miR-22 protected SH-SY5Y cells against OGD/R-induced injury.

MiR-22 alleviated OGD/R-induced injury by directly targeting Tiam1 in SH-SY5Y cells

To investigate the protective mechanism of miR-22, we analyzed its potential targets using TargetScan and

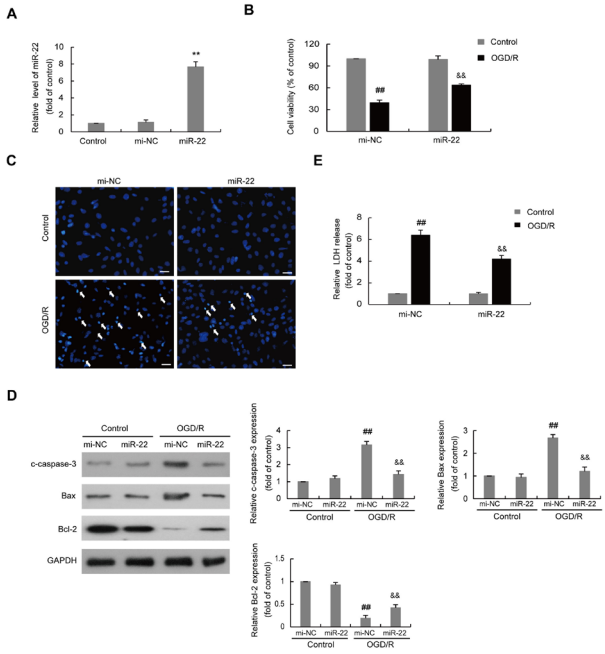

Figure 2. The effect of miR-22 mimics on OGD/R-induced SH-SY5Y cell injury.
(**A**) The expression level of miR-22 was measured by qRT-PCR in OGD/R-exposed SH-SY5Y cells with negative control mimic (mi-NC) or miR-22 mimic (miR-22) transfection, or without transfection (control). (**B**) Cell viability was measured by CCK-8 assay in SH-SY5Y cells with mi-NC or miR-22 transfection under normal (control) or OGD/R conditions. (**C**) Apoptosis was measured by Hoechst staining in SH-SY5Y cells with mi-NC or miR-22 transfection under normal or OGD/R conditions. Scale bar 100 μm. (**D**) The levels of apoptosis-related proteins were measured by western blot in SH-SY5Y cells with mi-NC or miR-22 transfection under normal (control) or OGD/R conditions. (**E**) LDH release was determined by ELISA assay in SH-SY5Y cells with mi-NC or miR-22 transfection under normal (control) or OGD/R conditions. $^{**}P<0.01$ compared with mi-NC group; $^{\#\#}P<0.01$ compared with mi-NC + control group; $^{\&\&}P<0.01$ compared with mi-NC + OGD/R group.

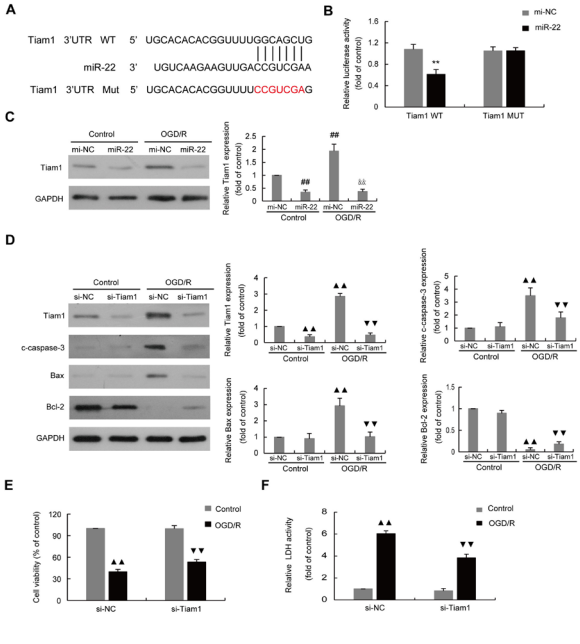

Figure 3. MiR-22 protected SH-SY5Y cells against OGD/R-induced injury by directly targeting Tiam1.
(**A**) 3′-UTR of Tiam1 and its putative binding sites in miR-22. (**B**) The luciferase activity for binding of miR-22 to 3′-UTR of Tiam1 was measured by luciferase reporter assay in SH-SY5Y cells. (**C**) The protein level of Tiam1 was measured by western blot in SH-SY5Y cells with negative control mimic (mi-NC) or miR-22 mimic (miR-22) transfection under normal (control) or OGD/R conditions. (**D**) The protein levels of Tiam1 and apoptosis-related proteins were measured by western blot in SH-SY5Y cells with negative control siRNA (si-NC) or Tiam1 siRNA (si-Tiam1) transfection under normal (control) or OGD/R conditions. (**E**) Cell viability was measured by CCK-8 assay in SH-SY5Y cells with si-NC or si-Tiam1 transfection under normal (control) or OGD/R conditions. (**F**) LDH release was determined by ELISA assay in SH-SY5Y cells with si-NC or si-Tiam1 transfection under normal (control) or OGD/R conditions. $^{**}P$<0.01 compared with mi-NC + Tiam1 WT group; $^{\#\#}P$<0.01 compared with mi-NC + control group; $^{\&\&}$P<0.01 compared with mi-NC + OGD/R group; $^{\blacktriangle\blacktriangle}P$<0.01 compared with control + si-NC group; $^{\blacktriangledown\blacktriangledown}P$<0.01 compared with OGD/R + si-NC group.

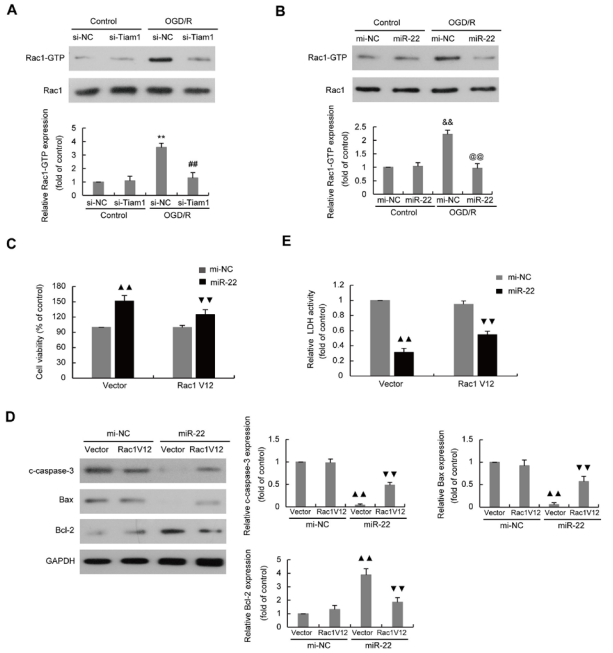

Figure 4. The effect of Rac1 V12 over-expression on the protective effect of miR-22 over-expression during OGD/R-induced injury.
(**A**) Rac1 activation was measured by pull-down assay in SH-SY5Y cells with negative control siRNA (si-NC) or Tiam1 siRNA (si-Tiam1) transfection under normal (control) or OGD/R conditions. (**B**) Rac1 activation was measured by pull down assay in SH-SY5Y cells with negative control mimic (mi-NC) or miR-22 mimic (miR-22) transfection under normal (control) or OGD/R conditions. (**C–E**) After transfection of empty plasmid (vector) or the plasmid containing constitutively active form of Rac1 (Rac1-V12) in mi-NC or miR-22 overexpressed SH-SY5Y cells, cell viability, the levels of apoptosis-related proteins and LDH release were determined by CCK-8 assay (C), western blot (D) and ELISA assay (E), respectively. $^{**}P$<0.01 compared with control + si-NC group; $^{\#\#}P$<0.01 compared with OGD/R + si-NC group; $^{\&\&}P$<0.01 compared with control + mi-NC group; $^{@@}P$<0.01 compared with OGD/R + mi-NC group; $^{\blacktriangle\blacktriangle}P$<0.01 compared with vector + mi-NC group; $^{\blacktriangledown\blacktriangledown}P$<0.01 compared with vector + mi-22 group.

miRDB. Tiam1 was selected for further study as it was predicted to be a potential target of miR-22 by using the two software and was demonstrated to be involved in the regulation of hippocampal neuronal vulnerability to OGD/R (Blanco-Suarez *et al.*, 2014). The possible binding sites between miR-22 and Tiam1 were presented in Fig. 3A. Luciferase reporter assay illustrated that miR-22 mimic notably inhibited the luciferase activity of Tiam1 harboring wild type (WT) 3'UTR, but it had no effect on the luciferase activity of Tiam1 with a mutant type (Mut) 3'UTR (Fig. 3B). Moreover, OGD/R treatment led to a significant increased expression level of Tiam1, which was reversed by the miR-22 mimic (Fig. 3C). These data suggest that Tiam1 is a direct target of miR-22 under the OGD/R condition.

To examine whether Tiam1 is involved in the protective effect of miR-22, SH-SY5Y cells were transfected with Tiam1 siRNA, which markedly inhibited Tiam1 expression in OGD/R-exposed SH-SY5Y cells (Fig. 3D). OGD/R-induced up-regulation of Bax and cleaved caspase-3, and down-regulation of Bcl-2 were reversed by Tiam1 siRNA in SH-SY5Y cells (Fig. 3D). Tiam1 siRNA obviously increased the viability of SH-SY5Y cells under OGD/R condition (Fig. 3E). Tiam1 siRNA also significantly inhibited LDH release in OGD/R-exposed SH-SY5Y cells (Fig. 3F). The above data indicated that miR-22 alleviated OGD/R-induced injury by directly targeting Tiam1 in SH-SY5Y cells.

Rac1 activation reversed the protective effect of miR-22 up-regulation on OGD/R-induced injury in SH-SY5Y cells

As Tiam1 is a specific guanine nucleotide-exchange factor (GEF) of Rac1, the involvement of Rac1 in the protective mechanism of miR-22 was then investigated. As shown in Fig. 4A, Tiam1 siRNA significantly inhibited OGD/R-induced Rac1 activation in SH-SY5Y cells. OGD/R-induced Rac1 activation was also inhibited by miR-22 mimic (Fig. 4B). MiR-22 overexpression significantly elevated cell viability, inhibited LDH release, Bax and cleaved caspase-3 expression, and up-regulates Bcl-2 expression in OGD/R-exposed SH-SY5Y cells (Fig. 4C-4E). However, these parameters were reversed by transfection of Rac1-V12, an active mutant of Rac1 (Fig. 4C-4E). These results suggested that Rac1 activation reversed the protective effect of miR-22 up-regulation on OGD/R-induced injury in SH-SY5Y cells.

Methylation of the miR-22 gene promoter suppressed its expression in SH-SY5Y cells under OGD/R condition

To investigate the underlying mechanism of miR-22 down-regulation in SH-SY5Y cells after OGD/R expo-

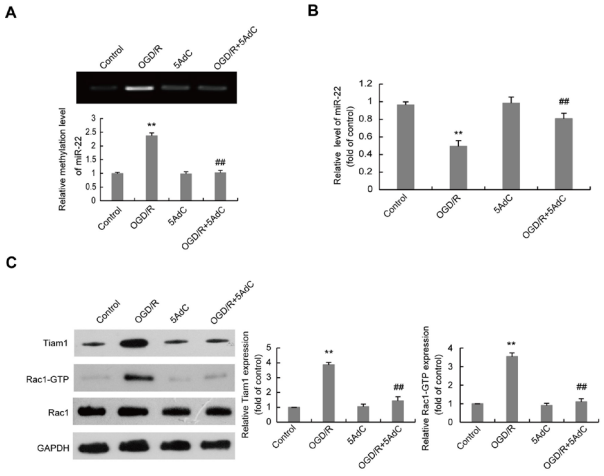

Figure 5. Methylation of the miR-22 gene promoter inhibited miR-22 expression under OGD/R condition.
(**A**) The methylation level of miR-22 in OGD/R-exposed SH-SY5Y cells with or without 5-Aza-dC (5AdC) treatment was analyzed by MSP analysis. (**B**) The expression level of miR-22 in OGD/R-exposed SH-SY5Y cells with or without 5-Aza-dC (5AdC) treatment was examined by RT-qPCR. (**C**) The level of Tiam1 expression and Rac1 activation in OGD/R-exposed SH-SY5Y cells with or without 5-Aza-dC (5AdC) treatment were determined by western blot and pull down assay, respectively. $^{**}P<0.01$ compared with control group; $^{\#\#}P<0.01$ compared with OGD/R group.

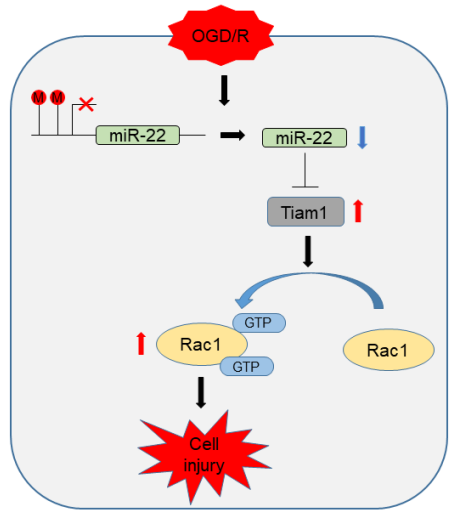

Figure 6. Schematic illustration of OGD/R-induced SH-SY5Y cell injury *via* the miR-22/Tiam1/Rac1 signaling pathway.

sure, the methylation level of the miR-22 gene promoter was measured. As shown in Fig. 5A, OGD/R exposure led to increased methylation of miR-22, which was reversed by treatment with 5-Aza-dC, a demethylating agent. 5-Aza-dC treatment also obviously up-regulated the level of miR-22 in OGD/R-exposed SH-SY5Y cells (Fig. 5B). Moreover, the increased Tiam1 expression and Rac1 activation in OGD/R-exposed SH-SY5Y cells were significantly inhibited by 5-Aza-dC treatment (Fig. 5C). These results suggested that methylation of the miR-22 gene promoter suppressed its expression in SH-SY5Y cells under OGD/R condition.

DISCUSSION

MicroRNAs have been demonstrated to work as critical factors in various brain injuries (Song *et al.*, 2019; Suofu *et al.*, 2020; Zhao *et al.*, 2020). Previous studies have indicated that up-regulation of miR-22 could protect neuron against I/R-induced injury (Wang *et al.*, 2020). Recently, MSC-exos-derived miR-22 was shown to attenuate I/R-induced brain (Zhang *et al.*, 2021b). However, the specific mechanism of miR-22 in I/R-induced neuronal injury is far from clear nowadays. In this study, we constructed an OGD/R model of SH-SY5Y cells and found that the level of miR-22 was decreased. Up-regulation of miR-22 could protect SH-SY5Y cells against OGD/R-induced injury. Our results suggest that miR-22 plays a protective role in OGD/R-induced neuronal injury.

The potential neuroprotective mechanism of miR-22 was then explored by searching for its target gene with targetscan and miRDB. Our results showed that the expression of Tiam1 was negatively correlated with miR-22 and was a potential target of miR-22. Tiam1 was demonstrated to be the target of miR-22 in several types of cells, such as NK/T cells, colon cancer cells and ovarian cancer cells (Huang *et al.*, 2016; Li *et al.*, 2013; Li *et al.*, 2012). In the present study, luciferase report assay and western blot assay indicated that Tiam1 is a direct target of miR-22 in OGD/R-exposed SH-SY5Y cells. Furthermore, Tiam1 siRNA could obviously attenuate OGD/R-evoked injury in SH-SY5Y cells. These results suggested that miR-22 overexpression alleviated OGD/R-induced injury by directly targeting Tiam1 in SH-SY5Y cells. MiR-22 was shown to exert its neuroprotective function by regulating PI3K/AKT signaling pathway in cerebral I/R rats (Wang *et al.*, 2020). Jiao *et al.* (Jiao *et al.*, 2020) reported that miR-22 protects PC12 cells from OGD/R-evoked injury by targeting the p53 upregulated modulator of apoptosis (PUMA). Recently, it is demonstrated that MSC-exos-derived miR-22 attenuated OGD/R-evoked injury by inhibiting KDM6B expression in rat primary cortical neurons (Zhang *et al.*, 2021b). These findings, together with our results, indicated that miR-22 exerts neuroprotective effects by different downstream targets, which depend on cell type. It will be interesting to determine whether the reported targets of miR-22 are involved in our system and their crosstalk with Tiam1.

Tiam1 is known best as a specific GEF for Rac1 activation (Chapelle *et al.*, 2020; Kurdi *et al.*, 2016; Yue *et al.*, 2021). Previous studies showed that Tiam1 mediates an OGD-induced increase in Rac1 activity in hippocampal neurons (Blanco-Suarez *et al.*, 2014; Smith *et al.*, 2017). Furthermore, Rac1 activation was demonstrated to play an important role in I/R-induced injury in different tissues, including brain tissue (Chen *et al.*, 2020; Li *et al.*, 2017; Liang *et al.*, 2018; Liu *et al.*, 2019; Su *et al.*, 2019). Therefore, we speculated that miR-22 exerts its neuroprotective function by inhibiting Tiam1-mdiated Rac1 activation. Our results showed that Rac1 activity was obviously increased in OGD/R-exposed SH-SY5Y cells. Tiam1 siRNA and miR-22 angomir could markedly inhibit OGD/R-induced Rac1 activation. Moreover, Rac1-V12, an active mutant of Rac1, could significantly attenuate the inhibitory effect of miR-22 up-regulation on OGD/R-evoked injury. These data indicated that miR-22 exerts its neuroprotective function on OGD/R-evoked injury by inhibiting Tiam1-mdiated Rac1 activation in SH-SY5Y cells.

The key factors that reduced miR-22 expression in our system were then investigated. It is demonstrated that the CpG island methylation in promoter regions is key to miRNAs expression (Chhabra, 2015; Glaich *et al.*,

2019). Moreover, DNA-methylation was shown to play an important role in I/R-induced brain jury (Deng *et al.*, 2019; Jin *et al.*, 2021; Tang and Zhuang, 2019; Zeng *et al.*, 2020). Thus, we investigated whether the decreased miR-22 expression in OGD/R-exposed SH-SY5Y cells is due to DNA-methylation in its promoter. Our results showed that the methylation level of the CpG island in the miR-22 promoter was significantly increased after OGD/R exposure. 5-Aza-dC treatment could obviously up-regulated miR-22 expression in OGD/R-exposed SH-SY5Y cells. Furthermore, Tiam1 expression and Rac1 activity were significantly inhibited by 5-Aza-dC treatment in OGD/R-exposed SH-SY5Y cells. These results suggested that DNA-methylation in OGD/R-exposed SH-SY5Y cells led to a decreased miR-22 expression and increased Tiam1 expression and Rac1 activation, thereby promoting neuronal injury.

In conclusion, our study demonstrated that DNA-methylation of miR-22 causes miR-22 down-regulation, which results in Tiam1 up-regulation and Rac1 activation in OGD/R-exposed SH-SY5Y cells, therefore leading to cell injury (Fig. 6). MiR-22 up-regulation plays a neuroprotective role in OGD/R-exposed SH-SY5Y cells, which is partly via directly targeting Tiam1 to inhibit Rac1 activation. Although additional *in vivo* studies are needed to verify our findings, our study provides a new insight into the protective effects of miR-22 against I/R-induced brain injury and warrants further study of DNA methylation-mediated silencing of miR-22 may serve as a potential therapeutic strategy for I/R-induced brain injury.

Declarations

Competing interests. The authors declare that they have no competing interests.

Ethical approval. Not applicable.

Statement of Informed Consent. Not applicable.

Availability of data and materials. The datasets used during the present study are available from the corresponding author upon reasonable request.

Authors' contributions. Jiansong Yin wrote the manuscript, performed the experiments and analyzed the experimental data, Yu Wan performed the experiments and analyzed the experimental data, Jing Wang analyzed the experimental data, Mei Xue edited the manuscript and supervised the study. All authors read and approved the final manuscript.

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