Knockdown of NEAT1 induced microglial M2 polarization via miR‑374a‑5p/NFAT5 axis to inhibit inflammatory response caused by OGD/R

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INTRODUCTION

Stroke is caused by sudden blockage of the blood vessels, due to various inducements such as hypertension, insulin resistance, diabetes, etc, resulting in acute cerebral vascular disorders. The pathological phenotype of stroke includes ischemic stroke and hemorrhagic stroke (Hankey, 2017). The incidence and prevalence of stroke in China have been on the rise, with an annual incidence of 185–219/100,000 people over the past 30 years. Microglia cells have long been considered to be involved in the pathological process of neuropathic diseases (Hu et al., 2014; Prinz and Priller, 2014; Ma et al., 2017). In the normal brain, microglia are at rest, and subsequently defined artificially as M1-like microglia that release pro-inflammatory cytokines, and M2-like microglia that release anti-inflammatory cytokines (Kettenmann et al., 2011; Varnum and Ikezu, 2012; Ponomarev et al., 2013; Jonathan et al., 2014). After ischemic brain damage, microglia rapidly migrated to the site of injury, producing cytotoxic substances and inflammatory cytokines, and aggravating tissue injury. Additionally, microglia also promoted tissue repair and re-construction by clearing up debris and producing growth factors and anti-inflammatory cytokines (Shi and Pamer, 2011). However, the precise mechanism underlying microglia in stroke has not been fully elucidated.
The long non-coding RNAs (lncRNAs) are more than 200 nucleotides in length, which simultaneously affect and regulate the occurrence and development of various tumors. It had been confirmed that the lncRNA nuclear enriched abundant transcript 1 (NEAT1) is in many disease processes. Recent research found that NEAT1 up-regulation induced by the transcription factor Yin Yang 1 (YY1) resulted in the oxygen and glucose deprivation/reoxygenation (OGD/R) damage and caused neuro-inflammation injury in microglia (Han and Zhou, 2019). Knockdown of NEAT1 effectively mitigated inflammatory response and death of dopaminergic SK-N-SH neuroblastoma cells (Zhou et al., 2021). In patients with acute ischemic stroke, NEAT1 expression positively correlated with inflammatory factor levels (Li et al., 2020). However, the exact biological role of NEAT1 in stroke and their mechanisms remain unclear.

MicroRNAs (miRNAs) are a class of non-coding single-stranded RNA molecules composed of approximately 22 nucleotides encoded by endogenous genes, which can recognize target genes downstream of the 3’-untranslated region (3’-UTR), thereby leading to mRNA degradation and translation inhibition. MiRNA plays a key role in cerebrovascular ischemic stroke (Giordano et al., 2020). In the hypoxia/reoxygenation (H/R) cardiomyocyte and ischemia/reperfusion (I/R) mouse models, the expression level of miR-374a-5p was reduced. Meanwhile, overexpression of miR-374a-5p could improve cardiomyocyte injury (Huang et al., 2019). Furthermore, miR-374a-5p suppressed the activation of inflammatory signals and then released of inflammatory related cytokines in microglia (Chen et al., 2020). Bioinformatics showed that binding sites existed among miR-374a-5p and NFAT5, and the findings showed that all of them together regulate the inflammatory response of microglia, but their specific regulatory mechanism is still unclear.

This study intends to take an in vitro acute stroke model as the research objective and molecular techniques were used to analyze the mechanism of NEAT1, miR-374a-5p and NFAT5 in OGD/R treated CHME5 microglia cells, in order to provide new molecular targets for the prediction and treatment of acute stroke.

METHODS

Cell culture

CHME5 cells, HEK293T cells were acquired from ATCC (Rockville, MD, USA) and cultured at 37°C in high glucose-dulbecco’s minimal essential medium (DMEM) containing 10% heat-inactivated fetal bovine serum (FBS) and 1% penicillin/streptomycin.

Oxygen and glucose deprivation/reoxygenation

CHME5 cells were treated with oxygen and glucose deprivation/reoxygenation (OGD/R) to simulate in vitro injury. In brief, cells were cultured in serum/glucose-free DMEM medium at 37°C under anoxic conditions containing 95% N2 and 5% CO2. OGD/R processing lasted 4 h. To terminate the effect of OGD/R on cells, the medium was replaced and the cells were further cultured in high-glucose DMEM medium containing 10% FBS and 1% penicillin/streptomycin for 12 h.

Plasmid construction and cell transfection

The short hairpin (sh)-NEAT1 and the negative control (NC) shRNAs were used in gene silencing experiments. The miR-374a-5p mimic or inhibitor and their NCs were purchased from GenePharma. To overexpress NFAT5, the full length of NFAT5 cDNA sequence was inserted into the pcDNA3.1 vector (Life Technologies, US). OE-NFAT5 was used to overexpress NFAT5. 24 h after transfection, related cells were instantaneously transfected with sh-NEAT1 plasmids, OE-NFAT5, miR-374a-5p mimics and inhibitor with their negative control using the Lipofectamine 3000 transfection reagent (Invitrogen, USA). 48 h after transfection, cells were harvested for subsequent assays.

Enzyme-linked immunosorbent assay

The supernatant of CHME5 cells was collected to detect the concentrations of related inflammatory cytokines. Meanwhile, the concentrations of IL-1β, IL-6 and tumor necrosis factor-α (TNF-α) were detected with enzyme-linked immunosorbent assay (ELISA) kit. The color range was measured at the wavelength of
450 nm with a microplate reader. The concentration of inflammatory factors was determined by comparing the optical density (OD) value of the sample with the standard curve.

3-(4, 5-Dimethyl-2-Thiazolyl)-2, 5-Diphenyl-2-H-Tetrazolium Bromide assay

CHME5 cells were seeded in a 96-well plate and cultured in an incubator, after treated with Lipofectamine 3000 for 48 h, the plates were removed and the medium containing 5 g/L 10% MTT (Sigma-Aldrich) was replaced, and the culture was continued for 4 h discard the supernatant. Dimethyl sulfoxide was added to each well to avoid light, and then shaken for 10 min to dissolve formazan crystals. The OD value of each well was measured at a wavelength of 490 nm with a microplate reader.

Bioinformatics analysis and dual luciferase reporter gene assay

We used a common tool starBase online (http://starbase.sysu.edu.cn/) predict target genes, the predicted binding sequence of miR-374a-5p in NEAT1 (NEAT1-WT) or NFAT5 3’-UTR (NFAT-WT) and their mutated sequence (NEAT1-MUT and NFAT5-MUT) were separately cloned into pmir-GLO vectors (Promega, USA). The luciferase reporter vector constructed was co-transfected with miR-374a-5p mimics, miR-374a-5p inhibitor or their NC into HEK293T cells using Lipofectamine 3000. At 48 h after transfection, the cells were collected using a dual luciferase assay kit (KGAF040, Nanjing, China) according to the manufacturer’s instructions and luciferase activity was assessed.

Quantitative real-time polymerase chain reaction

As suggested by the supplier, total RNA was extracted from CHME5 cells using the Pure-Link RNA Mini Kit (Thermo Fisher Scientific, MA). The total RNA was converted to cDNA using the applied Bio-Systems high-capacity cDNA reverse transcription kit, as recommended by the supplier’s specifications. Bio-systems real-time PCR system and the SYBR-Green PCR kit were used to detect the relative RNA levels by $2^{-\Delta\Delta Ct}$. MiR-374a-5p expression level was normalized to U6, and the remaining levels were normalized to GAPDH. See supplementary Table 1 for the sequence of related primers.

Western blot

The total protein samples were isolated from CHME5 cells by cell lysate containing PMSF (ST506; Beyotime). Then a nuclein extraction kit was used to extract related nuclear and cytoplasmic proteins. After quantification with a BCA protein assay kit (P0011; Beyotime), sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gel was used to separate proteins and then transfer them to the PVDF membranes. The membranes were incubated with primary antibodies against NFAT5 (AF7663, Changzhou, China, 1:1000), CD11b (1:1000, Abcam, UK), iNOS (1:1000, Abcam, UK), CD206 (1:1000, Abcam, UK), Arg1 (1:1000, Abcam, UK) and GAPDH (1:3000, Cell signaling, USA, 1:3000) over-

Table 1. The primer forward and primer reverse for qRT-PCR.

| Gene   | Primer forward (5'-3') | Primer reverse (5'-3') |
|--------|------------------------|------------------------|
| TNF-α  | CCCCAGGGACCTCTCTCTAA  | TGAGGTACAGGCCCTCTGAT  |
| IL-6   | ACAGGGAGAGGGAGCGATAA  | GAGAAGGCAACTGGACCGAA  |
| IL-1β  | CGATGCACTGTACGATCAC  | TCTTTCAACCGCAGAGACAG  |
| lncRNA NEAT1 | GAGTTAAGGGGCGCATCCTCA   | AGCACTGCGCCCTGAAAAT   |
| miR-374a-5p | GCCGGGCTTATAATACAACCTGGA | GTCGTATCCAGTGCAAGGTCCAGGT |
| NFAT5  | TGCAGTATGTGGATGGAGGA  | CCGTGTGTAAGCTGAAAGAC  |
| U6     | CTCGCTTCCGGCAGCACA    | AACGCTTACGAAATTTGCGT  |
| GAPDH  | CACGGTTGCTCTCTCTGA    | GCTGTAGCACAATCGTGT   |

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night at 4°C. The membrane was then washed in TBST buffer solution and HRP-conjugated goat anti-rabbit or anti-mouse antibody were incubated at 37°C for 60 min. The ECL solution was used to display western blots results and relative intensity of proteins was analyzed by the ImageJ software.

**Statistical analysis**

Each experiment was repeated independently for 3 times. Experimental data were represented as mean ± standard deviation (SD). The SPSS 20.0 software was used for data analysis. Student T test was used to compare the differences between two independent groups. ANOVA was used for comparison between groups. *P<0.05 was considered statistically significant.

**RESULTS**

**Differential expression of NEAT1, miR-374a-5p and NFAT5 in OGD/R treated microglia**

CHME5 cells were deprived of oxygen glucose for 12 h, and restored to normal oxygen culture for 12 h. Then, we analyzed the relationship among NEAT1, miR-374a-5p and NFAT5. And we found that NEAT1 was enhanced and miR-374a-5p was decreased in CHME5 cells with OGD/R treatment (Fig. 1A and B). The data suggested that both mRNA and protein level of NFAT5 were increased in CHME5 cells with OGD/R treatment (Fig. 1C-E). All the results demonstrated that NEAT1, miR-374a-5p and NFAT5 were involved in the regulation of microglia response to OGD/R.

**Knockdown of NEAT1 contributed to the shifting of OGD/R treated microglia from M1 to M2 and inhibited the expression of inflammatory factors**

To further investigate whether NEAT1 was involved in the shifting of OGD/R model microglia from M1 to M2, we first performed knockdown of NEAT1 using the short hairpin (sh-NEAT1) in CHME5 cells. CHME5 cells were treated with Lipofectamine 3000 for 48 h, MTT assay was used to measure the influence of the Lipofectamine 3000 on cell viability. The results showed that the Lipofectamine 3000 transfection reagent had no effect on cell activity (Fig. S1). Then, these cells undergo OGD/R treatment. The qRT-PCR analysis revealed that NEAT1 was significantly down-regulated after knockdown of NEAT1 (Fig. 2A). For quantitative analysis the effect of NEAT1 on the transformation of microglia from M1-like to M2-like, relative proteins level of M1 microglia markers iNOS and CD11b and M2 microglia markers Arg1 and CD206 were detected by western blot. We found that CD11b and iNOS were increased while CD206 and Arg1 were decreased in OGD group as compared with control group, but knock-
down of NEAT1 inverted these effects (Fig. 2B-C). Inflammatory response is closely related to ischemic stroke. Here, we tested IL-1β, IL-6 and TNF-α related inflammation factors and found that relative mRNA levels of these genes were significantly enhanced in CHME5 cells with OGD/R treatment and knockdown of NEAT1 in cells reversed these effects (Fig. 2D-F). Moreover, ELISA assay demonstrated that the concentrations of IL-1β, IL-6 and TNF-α were dramatically elevated in CHME5 cells with OGD/R treatment, and knockdown of NEAT1 in OGD/R treated CHME5 cells also reversed these effects (Fig. 2G-I). Transfection of CHME5 cells using vector and sh-NC for 48 h did not induce an inflammatory response (Fig. S2A-B). Taken together, our data indicated that knockdown of NEAT1 led to the transformation of OGD/R model microglia from M1 to M2 and suppressed inflammatory response in CHME5 microglia cells.

![Fig. 2](image-url)
NEAT1 targeted miR-374a-5p and suppressed its expression

Since the bioinformatics analysis predicted that NEAT1 might have binding sites with miR-374a-5p 3'-UTR. In this study, a miR-374a-5p mimic and the miR-374a-5p inhibitor were used to overexpress and suppress its expression, respectively. Firstly, we transfected miR-374a-5p mimics or inhibitor and their negative control into CHME5 cells for 48 h, and the data illustrated that the expression level of miR-374a-5p was increased after the miR-374a-5p mimic transfection and decreased after the miR-374a-5p inhibitor transfection compared with the related NC (Fig. 3A). Secondly, we investigated whether miR-374a-5p was a target of NEAT1. To investigate this hypothesis, we did dual luciferase reporter assay and the experiments showed that miR-374a-5p was the target of NEAT1. The results revealed that miR-374a-5p mimics reduced the luciferase activity of NEAT1-WT, whereas miR-374a-5p inhibitor displayed the opposite effect. Both miR-374a-5p mimics and inhibitors showed no effect on the NEAT1-MUT (Fig. 3B-D). Furthermore, knockdown of NEAT1 in CHME5 cells dramatically enhanced the expression level of miR-374a-5p (Fig. 3E). Altogether, the data demonstrated that NEAT1 directly targeted and inhibited miR-374a-5p in microglia.

Suppression of miR-374a-5p inverted the role of NEAT1 downregulation in microglia

To further determine whether miR-374a-5p affected the role of NEAT1, CHME5 cells were transfected with miR-374a-5p inhibitor alone or with sh-NEAT1, and an OGD model was constructed. Western blot experiments showed that inhibition of miR-374a-5p enhanced the protein levels of CD11b and iNOS, and suppressed the inhibitory effect of NEAT1 downregulation on the M1-like microglia. Meanwhile, inhibition of miR-374a-5p decreased the protein levels of CD206 and Arg1, and suppressed the effect of NEAT1 downregulation on the M2-like microglia (Fig. 4A-C). In addition, qRT-PCR results showed that the miR-374a-5p inhibitor promoted the mRNA levels of IL-1β, IL-6 and TNF-α, and decreased the inhibitory effect of NEAT1 downregulation on inflammatory responses (Fig. 4D-F). Similarly, suppression of miR-374a-5p also
Fig. 4. Inhibition of miR-374a-5p reversed the role of NEAT1 knockdown in OGD/R microglia. (A-C) Protein levels of CD11b, iNOS, CD206 and Arg1 were detected by western blot. (D-I) qRT-PCR and ELISA assays were used to detected the mRNA levels and concentrations of IL-1β, IL-6 and TNF-α, respectively. N=3.
enhanced the concentrations of inflammatory factors and effectively reversed the inhibitory effect of NEAT1 downregulation (Fig. 4G-I). Thus, these results strongly suggested that suppression of miR-374a-5p inverted the effect of NEAT1 downregulation on microglia.

**MiR-374a-5p directly targeted and inhibited NFAT5**

In order to study the role of miR-374a-5p and the underlying mechanism, we used qRT-PCR and western blot to detect the expression levels of NFAT5 in CHME5 cells after transfection with miR-374a-5p mimic or inhibitor. We found that the level of NFAT5 was obviously decreased in CHME5 cells after the miR-374a-5p mimic transfection and the NFAT5 level was dramatically elevated after the miR-374a-5p inhibitor transfection (Fig. 5A-C). Next, the dual luciferase reporter assay showed that NFAT5 was the target of miR-374a-5p. miR-374a-5p mimic dramatically reduced the luciferase activity of cells in the NFAT5-WT group, and miR-374a-5p inhibitor obviously enhanced the luciferase activity of cells in the NFAT5-WT group, while there was no difference in NFAT5-MUT group (Fig. 5D-F). Collectively, these findings fully demonstrated the target relationship between NFAT5 and miR-374a-5p.

**miR-374a-5p induced the transformation of OGD/R treated microglia from M1 to M2 and reduced the expression of inflammatory factors by inhibiting NFAT5**

To identify the role of miR-374a-5p and NFAT5 in OGD/R treated CHME5 microglia, CHME5 cells were
Fig. 6. MiR-374a-5p contributed to the shifting of microglia with OGD/R treatment from M1 to M2 and suppressed the expression of inflammatory factors by inhibiting NFAT5. (A-C) The mRNA and protein levels of NFAT5 in microglia after NFAT5 overexpression were detected by qRT-PCR and western blot, respectively. (D-F) Protein levels of CD11b, iNOS, CD206 and Arg1 were detected by western blot. (G-L) qRT-PCR and ELISA assays were used to detect the mRNA levels and concentrations of IL-1β, IL-6 and TNF-α, respectively. N=3.
transfected with miR-374a-5p mimic and OE-NFAT5, then an OGD model was constructed. The overexpression by transfecting with OE-NFAT5 was tested by qRT-PCR and western blot experiments in CHME5 cells, and the data indicated that the mRNA and protein level of NFAT5 was remarkably increased in OE-NFAT5 group as compared with control group (Fig. 6A–C). We then evaluated the effect of NFAT5 on OGD/R treated microglia. Western blot results illustrated that compared with OGD + vector + mimics NC group, miR-374a-5p overexpression reduced the expression of CD11b and iNOS and increased the expression of CD206 and Arg1, while CD11b and iNOS expression were increased, CD206 and Arg1 were decreased after overexpression of NFAT5. However, overexpression of NFAT5 and miR-374a-5p at the same time reversed the role of miR-374a-5p overexpression alone in OGD/R treated CHME5 cells (Fig. 6D–F). Furthermore, qRT-PCR analysis showed that expression levels of IL-1β, IL-6 and TNF-α were reduced after miR-374a-5p overexpression, while expression levels of these factors were increased and inverted the effect of miR-374a-5p overexpression after NFAT5 overexpression compared with the OGD + vector + mimics NC group (Fig. 6G–I). Additionally, the ELISA assay showed that concentrations of the inflammatory factors were increased, and the effects of miR-374a-5p overexpression could also be inverted after NFAT5 overexpression (Fig. 6J–L). Overall, these results significantly demonstrated that miR-374a-5p induced the shifting of OGD/R treated microglia from M1 to M2 and weakened the expression of inflammatory factors by suppressing NFAT5.

**DISCUSSION**

The stroke is one of the leading causes of death and morbidity worldwide and can be classified into ischemic and hemorrhagic types. Ischemic stroke was the main stroke (70-80%), while intracerebral hemorrhage accounted for only 10-20% of all strokes. We urgently need to have a deeper understanding of the molecular mechanism of stroke. Microglia cells are resident macrophages originated from primitive progenitor cells of yolk sac in brain (Ma et al., 2017). Microglia not only can sense very small imbalance of homeostasis in the environment, but also can be activated rapidly by dynamic morphology and polarization characteristics (Prinz and Priller, 2014). In this study, we demonstrated that NEAT1 regulated an in vitro acute stroke model via miR-374a-5p / NFAT5 axis to induce the transformation of microglia from M1 to M2 and inhibit inflammatory responses. This may be a potential treatment target for stroke.

LncRNAs introduce chromatin modifiers, transcription factors, and hetero-nucleotide proteins into DNA to control epigenetic and/or transcriptional processes. So far, some lncRNAs have been identified as epigenetic mediators of inflammation-related diseases. Such as, the lncRNA antisense noncoding RNA in the Ink4 locus (ANRIL) was NF-κB inducible in endothelial cells and upregulated the expression of the inflammatory genes IL-6 and IL-8 through interaction and recruitment of the YY1 transcription factor (Zhou et al., 2016). Additionally, more lncRNAs are involved in stroke, particularly in ischemic stroke. For example, OGD induced PC-12 cell injury and decreased IncRNA ANRIL expression. Overexpression of ANRIL could obviously reduce OGD-induced PC-12 cell injury. In the ischemic stroke, the lncRNA MALAT1 played an important role in promoting angiogenesis, inhibiting apoptosis and inflammation, and regulating autophagy (Wang et al., 2020b). Knockdown of NEAT1 had been recently showed to inhibit microglial polarization towards the M1 phenotype, but does not promote M2 microglial polarization in OGD/R treated BV-2 microglial cells (Ni et al., 2020). This study report is inconsistent with our results. Our data showed that knockdown of NEAT1 induced microglial M2 polarization and obviously inhibited the expression of inflammatory factors in OGD/R-treated CHME5 microglia cells. The above reasons may be due to the different cells used and the different time of OGD/R treatment of cells. They used BV-2 cells and treated them with OGD/R for 2 h, whereas we used CHME5 cells and treated them with OGD/R for 4 h. In addition, NEAT1 was up-regulated under hypoxic conditions, mediating the effect of hypoxia on microglia-mediated inflammation and in the mouse models of peritonitis and pneumonia, NEAT1 deficiency dramatically decreased inflammatory responses (Zhang et al., 2019). The reported is consistent with our findings. And we also found that knockdown of NEAT1 obviously inhibited the expression of inflammatory factors in OGD/R-treated CHME5 microglia cells.

MiRNAs are one of many molecules that lead to functional changes before, during or after ischemic stroke. MiRNAs are very important regulator of post-transcriptional gene silencing in brain developmental physiology and ischemic stroke pathology (Li et al., 2018). Furthermore, miR-203 could suppress the production of inflammatory factors, such as IL-8 and TNF-α, thereby reducing post-ischemic inflammatory damage and neuronal death after OGD (Yang et al., 2015). In experimental allergic encephalomyelitis models, up-regulation of miR-124 might reduce TNF-α and inhibit microglia activation (Ponomarev et al.,
Additionally, IncRNA ZFAS1 had an obvious protective effect on neuronal injury and inhibited oxidative stress, inflammation and apoptosis via regulating miR-582-3p in cerebral ischemia/reperfusion injury (Zhang and Zhang, 2020). In our study, we validated that miR-374a-5p was the target of NEAT1 in CHME5 microglia cells and miR-374a-5p led to the shifting of microglia from M1 to M2 and suppress the expression of inflammatory cytokines in CHME5 cells treated with OGD/R.

As an important transcription factor, NFAT5 is involved in maintaining homeostasis of cells against hypotonic or hypertonic stress (Jeong et al., 2016). NFAT5 might be unaffected by strong acids in inflammation, development, and liver detoxification and tumor metastasis, etc. (López-Rodríguez et al., 2004; Go et al., 2010; Berga et al., 2010; Levy et al., 2010; Halterman et al., 2012). In this study, we found that NFAT5 was elevated in the CHME5 cells with OGD/R treatment and up-regulation of miR-374a-5p mediated the shifting of M1 to M2 and finally inhibited inflammatory responses in OGD/R treated microglia by inhibiting NFAT5.

CONCLUSION

In summary, our work showed that OGD/R led to the upregulation of NEAT1 and NFAT5 while downregulation of miR-374a-5p in treated microglia. Collectively, knockdown of NEAT1 contributed to the shifting of microglia with OGD/R treatment from M1 to M2 and finally inhibited inflammatory responses in an in vitro stroke model, which provided a novel insight for understanding stroke more completely.

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All data generated or analyzed during this study are included in this article. The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

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Fig. S1. After treated with Lipofectamine 3000 for 48 h, the cells were collected, and MTT assay was used to measure the influence of the Lipofectamine 3000 on cell viability. N=3.

Fig. S2. (A) Vector and sh-NC transfected 48 h later, cells were harvested. The mRNA levels of pro-inflammatory factors IL-1β, IL-6 and TNF-α were tested by qRT-PCR. (B) 48 h after transfection with vector and sh-NC, cells were harvested. The concentrations of pro-inflammatory factors IL-1β, IL-6 and TNF-α were detected by ELISA experiments. N=3.