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RESEARCH PAPER

Long non-coding RNA (lncRNA) nuclear enriched abundant transcript 1 (NEAT1) promotes the inflammation and apoptosis of otitis media with effusion through targeting microRNA (miR)-495 and activation of p38 MAPK signaling pathway

Yajuan Hu, Hongjun Dong, Jingjing Huang, Jie Huang, Dandan Tao, Chao Huang, Lei Hu, Hao Xu, and Yan Sun

Department of Otorhinolaryngology, Zhangjiagang TCM Hospital Affiliated to Nanjing University of Chinese Medicine, Suzhou, China

ABSTRACT

Long non-coding RNA (lncRNA) plays a vital role in human inflammatory diseases. Our study aimed to investigate the function of lncRNA nuclear-enriched abundant transcript 1 (NEAT1) in otitis media with effusion (OME). The mRNA levels of NEAT1 and miR-495 were measured by RT-qPCR. The protein levels of p38 MAPK were detected by western blot. The levels of inflammatory cytokines were examined by ELISA. CCK-8 and flow cytometry assays were used to evaluate the cell viability and apoptosis, respectively. The interaction between NEAT1 and miR-495 was determined by luciferase reporter and RIP assays. NEAT1 was highly expressed in OME, and silencing of NEAT1 facilitated the cell proliferation and suppressed levels of inflammatory cytokines and cell apoptosis in LPS-induced HMEECs. Moreover, miR-495 was confirmed as a downstream target of NEAT1. Functional assays revealed that NEAT1 promoted the OME by targeting miR-495. It was further demonstrated that NEAT1 could activate the p38 MAPK signaling pathway by regulating miR-495, and the p38 MAPK inhibitor restored the effects of NEAT1 overexpression on the inflammation levels, cell proliferation, and apoptosis. Our study revealed that lncRNA NEAT1 served as a ceRNA to activate p38 MAPK signaling by targeting miR-495 in OME, which may offer a new target for OME treatment.

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Introduction

Otitis media with effusion (OME) is a common childhood disease characterized by the presence of fluid in the middle ear [1,2]. Persistent OME may lead to long-term changes in the tympanic membrane and middle ear, resulting in some degree of hearing loss. Previous studies have indicated that several factors, such as eustachian tube dysfunction, allergies, immunologic factors, and bacterial infection, are major causes of OME [3–5]. Nevertheless, the exact molecular mechanism of OME is still unclear. Hence, it is important to investigate the underlying molecular mechanism of the pathogenesis of OME.

Long non-coding RNA (LncRNA) is a type of non-coding RNAs (ncRNAs) >200 nt in length without protein-coding ability [6]. Extensive studies have proposed that lncRNAs have essential functions in multiple biological processes, including inflammatory response. For instance, FTX inhibited the inflammatory response of microglia via regulating the miR-382-5p/Nrg1 axis in spinal cord injury [7]. HOXA-AS2 suppressed endothelium inflammation by activating NF-κB signaling pathway [8]. NEAT1 is a well-known lncRNA with various functions in different physiological and pathological processes. For instance, NEAT1 facilitated inflammatory response and cell apoptosis in acute lung injury via the miR-944/TRIM37 axis [9]. Besides, silencing of NEAT1 suppressed inflammation in LPS-treated sepsis by regulating miR-590 [10]. However, the biological function of NEAT1 in the pathogenesis of OME is still unclear.

p38 MAPK is implicated as a critical regulator of the release of inflammatory cytokines and modulates the gene expression involved in the acute phase response, such as IL-1β, IL-6, and TNF-α [11,12]. In addition, multiple extracellular stimuli can activate the p38 MAPK signaling pathway, which further

CONTACT Hao Xu xu_hao098@163.com; Yan Sun sunyan_0@126.com Department of Otorhinolaryngology, Zhangjiagang TCM Hospital Affiliated to Nanjing University of Chinese Medicine, 77 Changan South Road, Zhangjiagang, Suzhou, China

activates a cascade of physiological outcomes, such as apoptosis, proliferative ability, and the transcription of certain genes [13,14]. A recent study implied that upregulated NEAT1 increased the production of numerous cytokines in human lupus through activating p38 MAPK signaling [15], which led us to speculate that NEAT1 exerted its biological role via MAPK signaling in OME.

This work aimed to explore the biological function and molecular mechanism of NEAT1 in OME development. We hypothesized that NEAT1 might play a critical role in the OME progression, and the results indicated that NEAT1 promoted the inflammation response and cell apoptosis through miR-495 and activation of the p38 MAPK signaling pathway in OME. Our data might offer a novel insight into the pathogenesis of OME.

Materials and methods

Clinical sample

Sixty OME patients and 60 sex- and age-matched healthy controls were recruited at Zhangjiagang TCM Hospital Affiliated to Nanjing University of Chinese Medicine from February 2018 to June 2019. The following were the inclusion criteria: (i) Patients provided informed consent; (ii) Patients were diagnosed with OME; (iii) Patients without specific causes of sensorineural hearing loss, such as noise exposure, head injury, and systemic ototoxic drugs. Written informed consent was obtained from the participant. Our work was approved by the Ethics Committee of Zhangjiagang TCM Hospital Affiliated to Nanjing University of Chinese Medicine.

Cell culture and treatment

HMEECs were purchased from Cell Bank of Chinese Academy of Sciences (Shanghai, China) and cultured in 50/50 mixture of DMEM and BEBM (both from Gibco) at 37°C with 5% CO_2. To construct an in vitro OME model, 100 ng/ml lipopolysaccharide (LPS) was used to treat HMEEC cells as previously described [16].

Cell transfection

Short hairpin RNA (shRNA) targeting NEAT1 (shNEAT1) with its control (shNC), miR-495 mimics with its control (NC mimics), miR-495 inhibitor with its control (NC inhibitor), and pcDNA3.1/NEAT1 with pcDNA3.1 were obtained from GenePharma (Shanghai). The treated HMEECs (5×10^6 cells per well) were seeded in 6-well plates and transfected with 50 nM shNEAT1, 50 nM shNC, 50 nM miR-495 mimics, 50 nM NC mimics, 50 nM miR-495 inhibitor, 50 nM NC inhibitor, 50 nM pcDNA3.1/NEAT1, or 50 nM pcDNA3.1 using Lipofectamine 2000 (Thermo Fisher Scientific, Inc.) following the manufacturer's instructions [17].

Reverse transcription-quantitative PCR (RT-qPCR)

Total RNA was isolated using TRIzol reagent (Invitrogen) and reverse-transcribed to cDNA using a PrimeScript RT Reagent Kit (TaKaRa). RT-qPCR was conducted on ABI 7500 real-time PCR system (Thermo Fisher Scientific) with SYBR Green kit. GAPDH or U6 was used as an internal control [18].

Cell counting kit-8 (CCK-8) assay

Cells were seeded into 96-well plates at a density of 2×10^4 cells/well. Subsequently, 10 μL CCK-8 reagent was added to each well and incubated for 4 h at 37°C. The optical density (OD) was determined using a microplate reader at 450 nm [19].

Dual-luciferase reporter assay

The wild-type (WT) and mutant (Mut) sequences of NEAT1 were sub-cloned into pmirGLO vectors (Promega). Then, NC mimics or miR-495 mimics were co-transfected with these vectors into HMEEC cells, and the luciferase activity was detected by Dual-luciferase Reporter System (Promega).

Enzyme-linked immunosorbent assay (ELISA)

The levels of inflammatory cytokines were detected using ELISA kits (R&D Systems, USA) according to the manufacturer's instructions.

Flow cytometry

To detect apoptosis of HMEEC cells, Annexin V-FITC Apoptosis Detection Kit (Yeasen, China) was utilized for flow cytometry assay. Treated HMEEC cells were washed in PBS and resuspended in binding buffer. Thereafter, the cell suspension was supplemented with Annexin V-FITC solution (5 μl) and PI solution (5 μl) followed by 15 minutes incubation in darkness. The cell apoptotic rate was analyzed and was detected by FACSCalibur Flow Cytometer (Becton Dickinson) [20].

Western blotting

Total protein was extracted from cells using RIPA buffer, resolved on SDS-PAGE, and transferred onto a PVDF membrane. The membrane was cultured with primary antibodies against anti-p-p38 and anti-p-38 and anti-β-actin at 4°C. Then, the membrane was incubated with the secondary antibody for another 2 h. Finally, the protein blots were visualized by ECL kit (Pierce Chemical, USA) [21].

Statistical analysis

All experiments were conducted with three replicates. SPSS 17.0 (SPSS, USA) was employed for statistical analysis, and the data are presented as mean ± standard deviation. Statistical differences were analyzed using Student's t-test or one-way ANOVA. Statistical significance was set at $P < 0.05$.

Results

In the current study, we explored the role and molecular mechanism of NEAT1 in OME. Functional assays revealed that NEAT1 promoted the inflammation and apoptosis of OME through targeting miR-495 and activation of p38 MAPK signaling pathway, suggesting that NEAT1 might be a novel target for the diagnosis and treatment of OME.

NEAT1 and inflammation levels are elevated in OME

To examine the expression pattern of NEAT1 in OME, RT-qPCR was employed, and the results indicated that NEAT1 expression was upregulated in serum and middle ear effusion of OME. (Figure 1a). Moreover, the levels of IL-1β, IL-6, IL-8, and TNF-a were increased in OME compared with that in healthy controls (Figure 1b).

NEAT1 regulates inflammation, cell proliferation, and apoptosis in LPS-treated HMEECs

To investigate the effect of NEAT1 on OME, an in vitro OME model was established by HMEECs treated with LPS. NEAT1 expression level was markedly upregulated in LPS-treated HMEEC compared to that in the control group (Figure 2a). Furthermore, the levels of inflammatory cytokines were enhanced in LPS-treated HMEECs (Figure 2b). Subsequently, LPS-treated

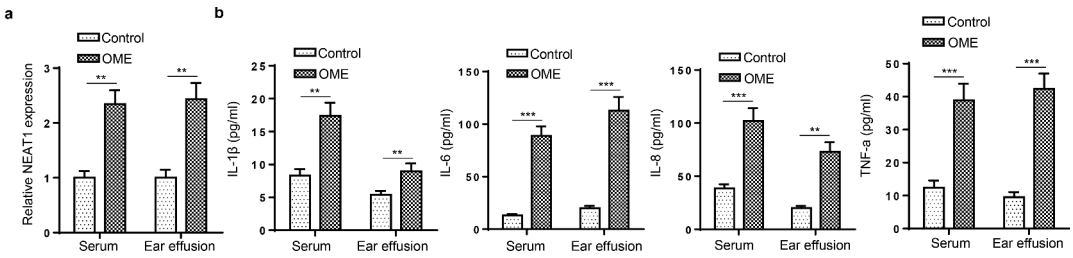

Figure 1. NEAT1 and inflammation levels are elevated in OME.

(a) RT-qPCR showed NEAT1 expression in serum and middle ear effusion of OME. (b) ELISA showed the levels of IL-1β, IL-6, IL-8, and TNF-a in serum and middle ear effusion. $^{**}P < 0.01$, $^{***}P < 0.001$.

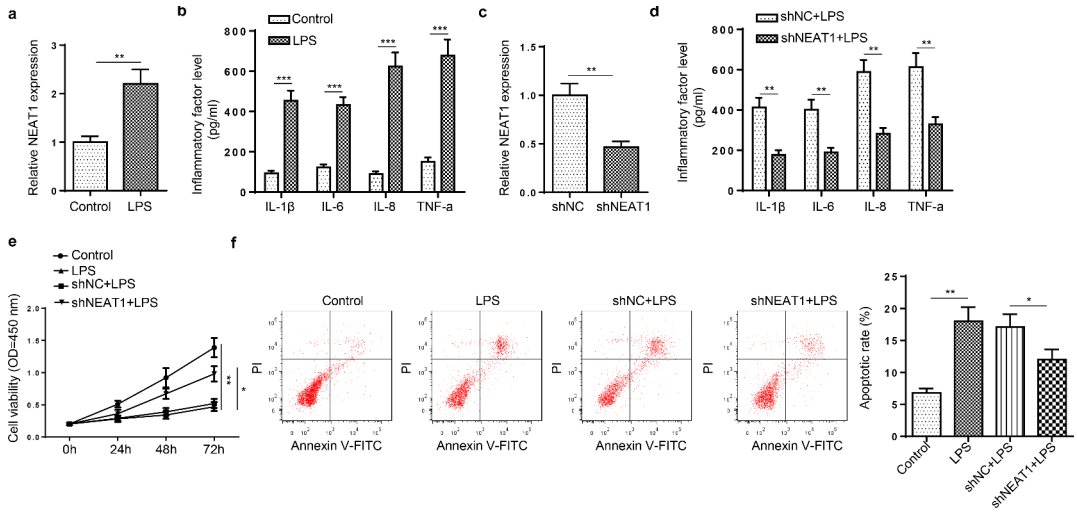

Figure 2. NEAT1 regulates inflammation, cell proliferation, and apoptosis in LPS-treated HMEECs.

(a) RT-qPCR showed NEAT1 expression in LPS-treated HMEEC compared to that in the control group. (b) ELISA showed the levels of IL-1β, IL-6, IL-8, and TNF-a in LPS-treated HMEEC. (c) RT-qPCR showed NEAT1 expression in LPS-treated HMEECs transfected with shNC or shNEAT1. (d) ELISA showed the levels of IL-1β, IL-6, IL-8, and TNF-a in LPS-treated HMEECs transfected with shNC or shNEAT1. (e and f) CCK-8 and flow cytometry assays showed cell proliferation and apoptosis in LPS-stimulated HMEECs transfected with shNC or shNEAT1. *P< 0.05, **P< 0.01, ***P< 0.001.

HMEECs were transfected with shNC or shNEAT1. RT-qPCR indicated that NEAT1 was downregulated by NEAT1 knockdown in LPS-treated HMEECs (Figure 2c). Then, ELISA revealed that inflammation levels were decreased in LPS-treated HMEECs transfected with shNEAT1 (Figure 2d). Moreover, NEAT1 silencing promoted cell proliferation and inhibited apoptosis in LPS-stimulated HMEECs (Figure 2e and f). The above data demonstrated that NEAT1 knockdown inhibited the inflammatory response and apoptosis and promoted cell proliferation in OME.

miR-495 is a target of NEAT1

By using StarBase, the downstream genes of NEAT1 were screened, and the potential binding site between NEAT1 and miR-495 is presented in Figure 3a. Increasing evidence indicated that miR-495 acted as a vital regulator in the inflammation response of various diseases, such as pneumonia [22], inflammatory bowel disease [23], and ankylosing spondylitis [24]. Moreover, RT-qPCR analysis indicated that miR-495 expression was decreased in serum and middle ear effusion of OME (Figure 3b). Besides, we found that miR-495 was decreased in LPS-induced HMEECs (Figure 3c). In addition, miR-495 overexpression suppressed luciferase activity of NEAT1-wt but had no effect on the activity of NEAT1-mut (Figure 3d). RIP assay indicated that miR-495 and NEAT1 were markedly enriched in the Ago2 group (Figure 3e). Furthermore, RT-qPCR uncovered that miR-495 expression was enhanced by NEAT1 depletion in HMEECs (Figure 3f). These data indicated that NEAT1 could negatively regulate miR-495 expression.

NEAT1 promotes OME progression by targeting miR-495 in HMEECs

To further investigate whether miR-495 was a downstream regulator of NEAT1 in OME, LPS-treated HMEECs were transfected with shNC, shNEAT1, shNEAT1+ NC inhibitor, and shNEAT1+ miR-495 inhibitor. ELISA indicated that NEAT1 knockdown significantly decreased levels of inflammatory cytokines, while the inhibition of miR-495 restored these effects (Figure 4a). Moreover, the downregulation of miR-495 partially abrogated the effects of NEAT1 silencing on cell proliferation and

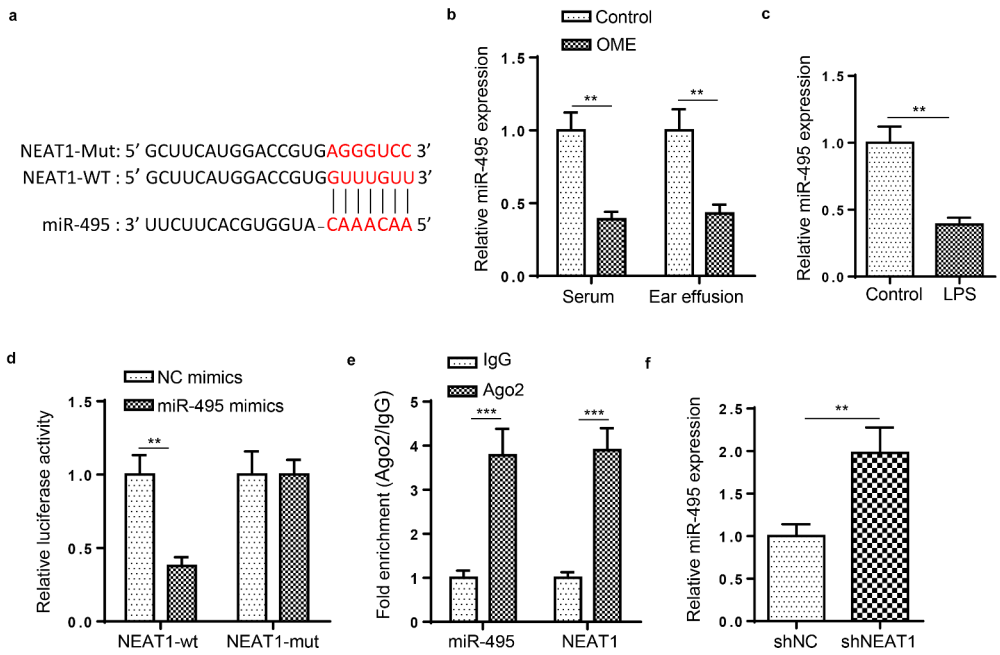

Figure 3. miR-495 is a target of NEAT1.

(a) The binding site between NEAT1 and miR-495 was predicted by starBase website. (b) RT-qPCR analysis showed miR-495 expression in serum and middle ear effusion of OME. (c) RT-qPCR showed miR-495 expression in LPS-induced HMEECs. (d) Luciferase reporter assay was performed to testify the interaction between miR-495 and NEAT1 in HMEECs. (e) RIP assay was performed to determine the enrichment of miR-495 and NEAT1 in anti-IgG and anti-Ago2. (f) RT-qPCR analysis showed miR-495 expression in LPS-treated HMEECs transfected with shNC or shNEAT1. **P< 0.01, ***P< 0.001.

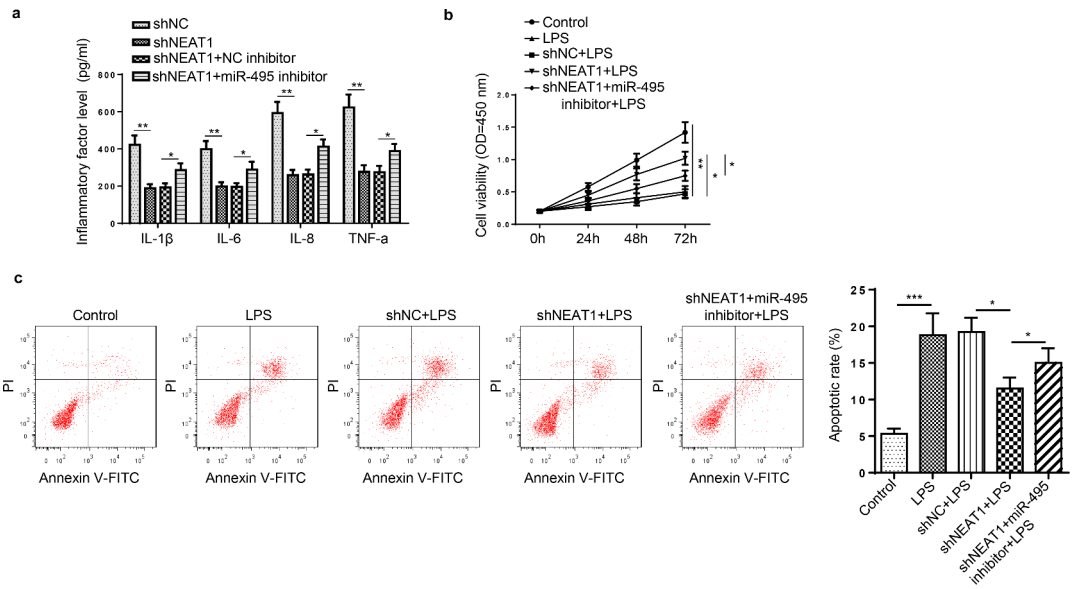

Figure 4. NEAT1 promotes OME progression by targeting miR-495 in HMEECs.

(a) ELISA showed the levels of IL-1β, IL-6, IL-8, and TNF-a in LPS-treated HMEECs transfected with shNC, shNEAT1, shNEAT1+ NC inhibitor, and shNEAT1+ miR-495 inhibitor. (b and c) CCK-8 and flow cytometry assays showed cell proliferation and apoptosis in LPS-treated HMEECs transfected with shNC, shNEAT1, shNEAT1+ NC inhibitor, and shNEAT1+ miR-495 inhibitor. *P< 0.05, **P< 0.01, ***P< 0.001.

apoptosis in LPS-treated HMEECs (Figure 4b and c). These results manifested that NEAT1 modulated OME progression via sponging miR-495.

NEAT1 activates p38 MAPK signaling pathway by targeting miR-495 in LPS-treated HMEECs

Previous studies indicated that miRNAs could inhibit the inflammatory response by inhibiting the activation of p38 MAPK signaling [20]. Western blot showed that the upregulation of NEAT1 alone increased the expression of phosphorylated p38 MAPK, while NEAT1 overexpression combined with miR-495 mimics significantly decreased their expressions (Figure 5a). These data revealed that p38 MAPK might be a downstream modulator of NEAT1. To further determine the effects of p38 MAPK on the pathogenesis of OME, p38 MAPK inhibitor, SB203580 (10 μM), was used to treat LPS-treated HMEECs. The results indicated that SB203580 reversed the effects of NEAT1 overexpression on inflammatory cytokine levels, cell proliferation, and apoptosis (Figure 5b-d).

Discussion

Dysregulated lncRNAs are involved in the pathogenesis and development of various human diseases [25]. Previous researches have unveiled that NEAT1 participated in the inflammatory response through various regulatory mechanisms. For instance, NEAT1 promoted the inflammatory reaction via modulating the intestinal epithelial barrier in inflammatory bowel disease [26]. NEAT1 aggravated inflammatory response through HMGB1/RAGE signaling in acute lung injury [27]. Hence, we speculated that NEAT1 might promote the inflammatory response in OME. Herein, it was found that NEAT1 was highly expressed in OME and inhibited cytokine level and cell proliferation but promoted apoptosis in LPS-treated HMEECs.

LncRNAs are widely reported to serve as ceRNAs by specifically adsorbing miRNAs to participate in the pathogenesis of various diseases [28,29]. For example, lncRNA XIST acted as a ceRNA of miR-599 to increase TLR4 expression in atherosclerosis [30]. LncRNA LOC100912373 promoted the occurrence and development of rheumatoid arthritis by sponging

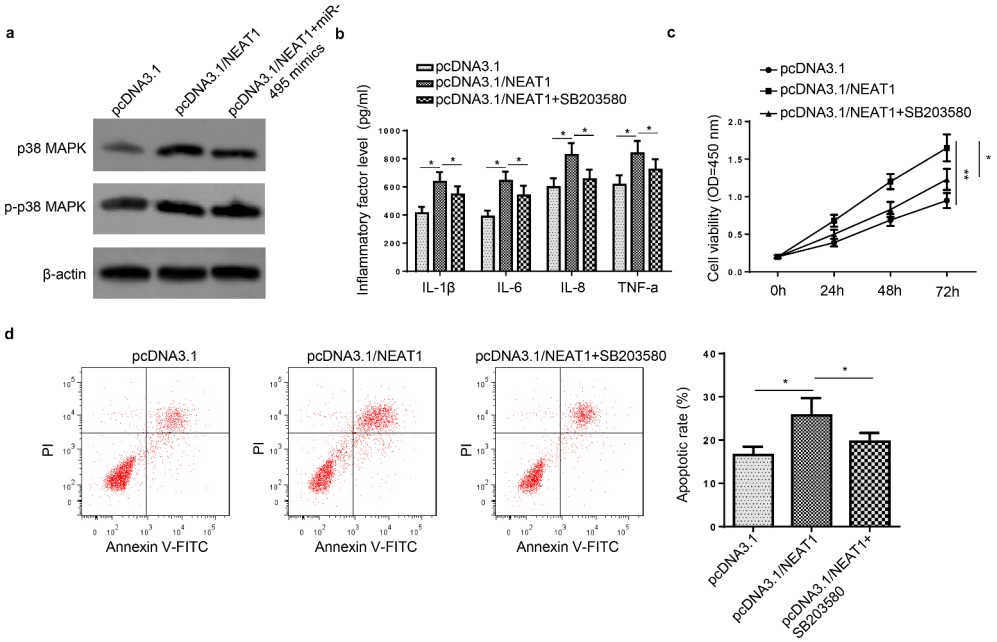

Figure 5. NEAT1 activates p38 MAPK signaling pathway by targeting miR-495 in LPS-treated HMEECs.

(a) Western blot showed the expression of phosphorylated p38 MAPK in LPS-treated HMEECs transfected with pcDNA3.1, pcDNA3.1/NEAT1, pcDNA3.1/NEAT1+ miR-495 mimics. (b–d) ELISA, CCK-8, and flow cytometry assays showed inflammatory cytokine levels, cell proliferation, and apoptosis in LPS-treated HMEECs treated with pcDNA3.1, pcDNA3.1/NEAT1, pcDNA3.1/NEAT1+ SB203580. *P< 0.05, **P< 0.01.

miR-17-5p to upregulate PDK1 [31]. In addition, NEAT1 has been reported to target different miRNAs in various diseases, including miR-23a in rheumatoid arthritis [32], miR-34 c in diabetic nephropathy [33], and miR-27a-3p in acute kidney injury [34]. In this study, our results elaborated that miR-495 was a target gene of NEAT1. Zhou et al. indicated that miR-495 inhibited proliferation and inflammation by regulating β-catenin expression in rheumatoid arthritis fibroblast-like synoviocytes [35]. Du et al. reported that miR-495 relieved cardiac microvascular endothelial cell injury and inflammation by repressing NLRP3 [36]. miR-495 attenuated inflammatory reaction and accelerated bone differentiation of fibroblast-like synovial cells by targeting DVL-2 [24]. Herein, we found that NEAT1 knockdown facilitated cell proliferation and suppressed apoptosis and inflammation by targeting miR-495.

As a subgroup of MAPK pathway, p38 is a vital regulator in diverse cellular processes, including cell apoptosis, proliferation, and inflammation [37]. LPS can induce the activation of MAPK pathway through binding to Toll-like receptors, resulting in inflammatory reaction [38,39]. Studies demonstrated that some miRNA could act as an inflammation suppressor gene through targeting p38 MAPK signaling pathways [40,41]. Herein, we demonstrated that NEAT1 activated p38 MAPK in OME by targeting miR-495. In addition, p38 MAPK inhibitor, SB203580 reversed the effects of NEAT1 overexpression on inflammation levels, cell proliferation, and apoptosis. These results indicated that NEAT1 activated p38 MAPK signaling pathway by targeting miR-495 in LPS-treated HMEECs.

Conclusion

Our study verified that NEAT1 promoted inflammation and apoptosis by targeting miR-495 and activation of p38 MAPK signaling. These findings provided a theoretical basis for the possibility of NEAT1 as a novel target for the diagnosis and treatment of OME. However, the limitation of this work is its lack of mechanistic experiments in animal models to confirm the modulatory effects of NEAT1 on OME. Further study should validate the function of NEAT1 in OME in vivo.

Data availability statement

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

Disclosure statement

No potential conflict of interest was reported by the author(s).

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ORCID

Yan Sun http://orcid.org/0000-0001-5525-6482

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