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Downregulation of miR-96-5p Inhibits mTOR/NF-κb Signaling Pathway via DEPTOR in Allergic Rhinitis

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Keywords
MicroRNA-96-5p · DEP domain-containing mammalian target of rapamycin-interacting protein · Inflammatory cytokines · NF-κB · Mammalian target of rapamycin

Abstract
Background: This study aims to investigate the regulatory effect of microRNA-96-5p (miR-96-5p) in the pathophysiological process of allergic rhinitis (AR). ***Methods:*** Nasal mucosal tissue samples were collected from AR patients and healthy controls. An in vitro AR model was established by stimulating human nasal epithelial cells (HNECs) with interleukin (IL)-13. The expressions of target genes and proteins were measured by qPCR, Western blot, or ELISA. Dual-luciferase reporter assay and pull-down assay were performed to confirm the interaction between miR-96-5p and DEP domain-containing mammalian target of rapamycin-interacting protein (DEPTOR). ***Results:*** The level of miR-96-5p was increased while the expression of DEPTOR was decreased in AR patients. The expressions of proinflammatory cytokines were markedly increased and the mammalian target of rapamycin (mTOR)/NF-κB pathway was activated in HNECs following IL-13 stimulation. miR-96-5p downregulation alleviated the stimulated function by IL-13. DEPTOR was the target of miR-96-5p. Knockdown of DEPTOR reversed the function of miR-96-5p inhibitor on IL-13-stimulated HNECs. ***Conclusions:*** The current study showed that miR-96-5p and DEPTOR were aberrantly expressed in AR nasal mucosa. miR-96-5p knockdown inhibited the production of inflammatory cytokines and the activation of mTOR/NF-κB pathway via targeting DEPTOR. These findings suggested that miR-96-5p might be used as a diagnostic marker and therapeutic target for the treatment of AR.

Introduction

Rhinitis is defined as nasal mucosa inflammation and is considered as one of the most common disorder worldwide, affecting 10–20% of the population [1]. Allergic rhi-

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nitis (AR) occurs when the immune system overreacts with allergens in the air, usually environmental allergens, such as pollen, pet hair, dust, or mold [2]. The clinical symptoms of AR include a runny or stuffy nose, sneezing, red, itchy, watery eyes, and swelling around the eyes [3]. These clinical symptoms are known to be induced by inflammatory mediators, such as histamine and leukotrienes, and inflammatory cytokines produced by mast cell, eosinophils, and basophils [4]. The incidence of AR is increasing and this disease has a negative impact on patients' quality of life, work performance, and quality of sleep [5]. Despite the rapid development of various medical treatments for AR, including immunotherapy, intranasal steroids, and anti-histamines, there are still around 20% of the patients with no significant improvement in symptoms [6]. Thus, it is of significant importance to identify new therapeutic targets and improve treatment strategies for AR patients.

The mammalian target of rapamycin (mTOR) is a conserved serine/threonine protein kinase that plays important roles in multiple cellular processes including cell metabolism, growth, proliferation, survival, and angiogenesis [7–9]. The activation of mTOR was negatively regulated by distinct proteins, such as DEP domain-containing mTOR-interacting protein (DEPTOR). DEPTOR was reported to be associated with both mTORC1 and mTORC2 through direct binding [10]. It was reported that mTORC1 inhibited autophagy via phosphorylation of ULK1/RB1CC1/ATG13/ATG101 complex, and thus upregulation of DEPTOR decreased the mTOR activity, induces the autophagy, and inhibits the inflammation [11]. It has been reported that human nasal epithelial cells (HNECs) released interleukin (IL)-33, an endogenous signal of tissue damage, to regulate the excretion of inflammatory factors from mast cells via activating the phosphoinositide 3-kinase (PI3K)/protein kinase B (Akt)/mTOR signaling pathway in AR [12]. The transcription factor NF-κB is an essential mediator of immune responses and inflammation that is often regulated by Akt/mTOR-dependent signaling [13]. A previous study showed that the NF-κB pathway was activated in a mouse model of ovalbumin-induced AR [14]. From these findings, we speculated that the suppression of mTOR/NF-κB signaling by DEPTOR may protect against the progression of AR.

MicroRNAs (miRNAs) are small, single-stranded, noncoding RNAs with 22–24 nucleotides in length, which play critical roles in RNA silencing and post-transcriptional regulation of gene expression in multiple organisms [15]. miRNAs regulate the expression of its target gene by binding to the 3′-UTR of this gene and promote its cleavage [16, 17]. There are approximately 2,200 miRNAs in mammalian genomes, and one-third of human genomes are regulated by miRNAs [18, 19]. Emerging evidence has shown that miRNAs play critical roles in the regulation of the key pathogenic mechanisms of human diseases, including cancer, cardiac failure, and allergic inflammation [20, 21]. MiR-96-5p is identified as an oncogene and biomarker in multiple cancers, such as hepatocellular carcinoma, breast cancer, and prostate cancer [22–24]. It has been reported to activate the PI3K/Akt/mTOR signaling via directly binding to phosphatase and tensin homolog, the main negative regulator of the PI3K/Akt pathway, in head and neck squamous cell carcinoma cells [25]. Interestingly, TargetScanHuman database (http://www.targetscan.org/vert_72/) predicted that DEPTOR was the target of miR-96-5p. However, whether the regulation of inflammation in AR by miR-96-5p is associated with DEPTOR remained unknown.

In the current study, we aimed to explore the regulatory effects of miR-96-5p in the progression of AR and the potential mechanisms involved in this process. Our results showed that the downregulation of miR-96-5p in a cell model of AR inhibited the expression of proinflammatory cytokines and the activation of mTOR/NF-κB signaling pathway via targeting DEPTOR.

Material and Method

Antibodies and Reagents

Anti-p-IκBα (#2859), anti-IκBα (#4812), anti-p-p65 (#3033T), anti-p65 (#3034), anti-p-mTOR (#2974T), anti-mTOR (#2983T), anti-p-4EBP1 (#2855), and anti-4EBP1 (#9452) were obtained from Cell Signaling Technology (Danvers, MA, USA). Anti-β-actin (sc-47778) and anti-DEPTOR (sc-398169) were obtained from Santa Cruz Biotechnology (Dallas, TX, USA). Rapamycin (Rapa) and IL-13 were purchased from Sigma-Aldrich (St. Louis, MO, USA). DEPTOR siRNA and siRNA negative control were purchased from Invitrogen (Shanghai, China). miR-96-5p inhibitor and inhibitor negative control were obtained from Gene Pharma (Shanghai, China). The sequences of siRNA and miRNA inhibitors were si-DEPTOR, 5'-GCCATGACAATCGGAAATCTA-3'; siRNA negative control, 5'-ACGUGACACGUUCGGAGAA-3'; miR-96-5p inhibitor, 5'-UUUGGCACUAGCACAUUUUUGCU-3'; and inhibitor negative control, 5'-CAGUACUUUUGUGUAGUACAA-3'.

Human Nasal Mucosal Samples

Nasal mucosal specimens were obtained from the inferior turbinate tissues of AR patients (n = 24) and healthy controls (n = 24) as previously described [26]. The diagnosis of AR was based on allergic rhinitis and its impact on asthma criteria and a demonstration of sensitization by a positive skin-prick test. No pa-

Table 1. Prime sequences for qPCR

Genes	Prime sequences (5′–3′)
miR-96-5p	
Forward	GCCTTTGGCACTAGCACATT
Reverse	GTGCAGGGTCCGAGGT
IL-1β	
Forward	CTGAGCTCGCCAGTGAAATG
Reverse	TGTCCATGGCCACAACAACT
IL-6	
Forward	ACAGGGAGAGGGAGCGATAA
Reverse	GAGAAGGCAACTGGACCGAA
Eotaxin	
Forward	CCCCTTCAGCGACTAGAGAG
Reverse	TCTTGGGGTCGGCACAGAT
GM-CSF	
Forward	ATGTGGCTGCAGAGCCTGCTGC
Reverse	CTCCCAGCAGTCAAAGGG
GAPDH	
Forward	CCAGGTGGTCTCCTCTGA
Reverse	GCTGTAGCCAAATCGTTGT
U6	
Forward	CTCGCTTCGGCAGCACA
Reverse	AACGCTTCACGAATTTGCGT
DEPTOR	
Forward	GCTCCGTATGCAAGGAAGAC
Reverse	CCGTTGACAGAGACGACAAA

IL, interleukin; DEPTOR, DEP domain-containing mammalian target of rapamycin-interacting protein.

tient received corticosteroid therapy 1 month before recruitment. This study was approved by the Ethics Committee of Hainan General Hospital. All patients provided written informed consent.

Cell Culture, Transfection, and Treatments

Primary HNECs were isolated from the inferior turbinate tissues of AR patients and cultured in Bronchial Epithelial Growth Medium (Lonza, Basel, Switzerland) in a 5% CO_2 humidified incubator at 37°C. When 70–80% confluency was reached, HNECs were passaged. The second-passage cells were used for subsequent experiments.

HNECs were transfected with designated sequences (miR-96-5p inhibitor or inhibitor negative control; si-DEPTOR or si-control) diluted in OptiMEM medium with Lipofectamin 3000 (Invitrogen, USA) following the manufacturer's instructions. After 48 h, all groups of HNECs were treated with or without IL-13 (10 ng/mL) for 48 h in culture medium as previously described [27]. Cells treated with Rapa at 1 μM were served as a positive control.

Quantitative PCR

The total RNAs were isolated from human nasal mucosal samples and primary HNECs using Trizol reagent (Thermo Fisher Scientific, Japan) according to the manufacturer's protocols. Then 2 μg of the RNAs were reverse transcribed to cDNAs using Reverse Transcription Kit (Thermo Fisher Scientific). qPCR was performed using TaqMan primers, and the primers used in this study were listed in Table 1. The reaction condition for qPCR was as follows: denature at 95°C for 10 min, followed by 40 amplification cycles of 95°C for 15 s and 60°C for 1 min. The expression of target genes was normalized to GAPDH, and the data were analyzed using the $2^{-\Delta\Delta CT}$ relative quantification method.

ELISA Assay

The supernatants of HNECs were collected at designated time points after the treatment with IL-13. The expressions of cytokines, including IL-1β, IL-6, eotaxin, and GM-CSF were determined using ELISA kits (Abcam, MA, USA) following the manufacture's protocols.

Dual-Luciferase Reporter Assay

HNECs were co-transfected with pmirGLO-DEPTOR-WT-3′UTR or pmirGLO-DEPTOR-MUT-3′UTR (Promega, Madison, WI, USA) and miR-96-5p mimic or miR-96-5p mimic negative control (GenePharma, Shanghai, China) with Lipofectamin 3000. Forty-eight hours after transfection, the relative luciferase activity was determined using a dual-luciferase reporter assay kit (Promega, Madison, WI, USA).

Western Blot

Proteins from human nasal mucosal samples and HNECs were prepared with the digestion of cell lysis buffer (50 mM Tris-HCl [pH 7.4], 1% Nonidet P-40, 150 mM NaCl, 1 mM EDTA, and 1 mM PMSF). The protein concentration of each sample was measured using Bradford protein dye reagent (Bio-Rad, USA). Protein samples (30 μg) were separated on SDS-PAGE and transferred to polyvinylidene difluoride membranes. Then the membranes were blocked with 5% nonfat dry milk in TBS for 1 h and incubated with primary antibodies overnight at 4°C. After 2 washes, the membranes were incubated with an HRP-conjugated secondary antibody for 1 h at room temperature. The protein expression was visualized using enhanced chemiluminescence procedure following the manufacturer's instructions.

Argonaute Immunoprecipitation Assay

The pull-down assay was performed in HNECs using a Dynabeads Protein G Immunoprecipitation Kit (Thermo Fisher Scientific) according to the manufacturer's instructions. Briefly, a volume of 10 μL of anti-Argonaute2 antibody (#SAB4200085, Sigma-Aldrich) was coupled with 1.5 mg Dynabeads. The protein lysates (100 μg and 200 μL) isolated from HNECs were incubated with the antibody-treated beads at room temperature for 20 min. After 3 washes, target antigens were eluted with the elution buffer. The enrichment of DEPTOR was analyzed by qPCR.

Statistical Analysis

Data were analyzed in GraphPad Prism and presented as means ± SD (standard deviation). All experiments were performed in triplicate and repeated at least 3 times. The statistical significance between 2 groups was evaluated using unpaired 2-tailed Student *t* tests. One-way AVONA with Tukey's post-test was used for the comparison among 3 or more groups. The linear correlation coefficient was used to estimate the correlation in DEPTOR expression versus miR-34b-5p level. A p value ≤0.05 was considered to be statistically significant.

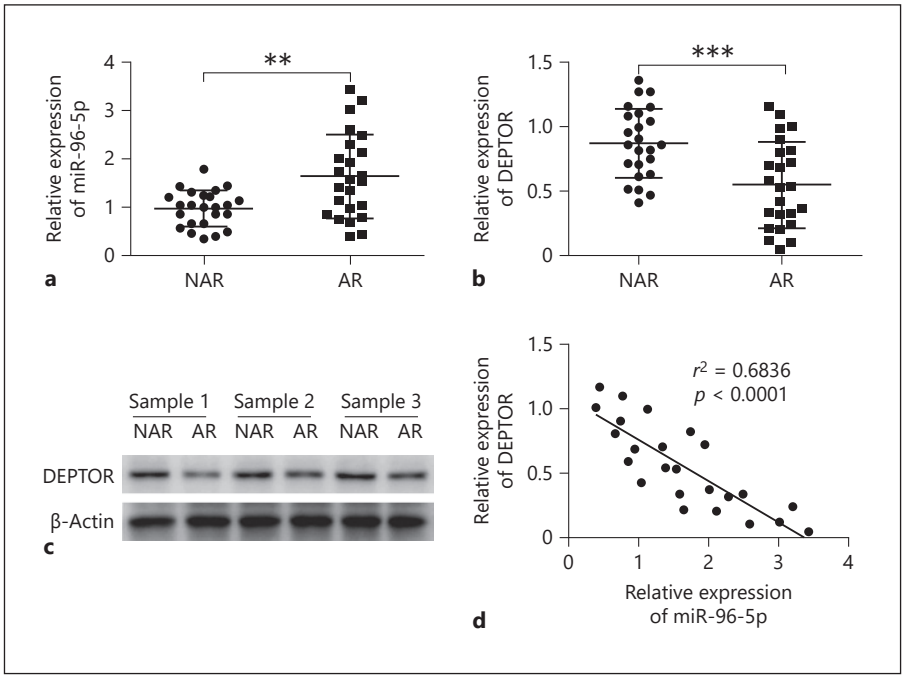

Fig. 1. Expressions of miR-96-5p and DEPTOR in human nasal mucosal tissues. Nasal mucosal samples were obtained from AR patients (n = 24) and healthy controls (n = 24). **a** The expression of miR-96-5p was measured by qPCR. The mRNA (**b**) and protein levels (**c**) of DEPTOR were determined by qPCR and Western blot, respectively. **d** The correlation between DEPTOR expression and miR-96-5p level in the nasal mucosal tissues from AR patients was estimated by the linear correlation coefficient. $^{**}p < 0.01$, $^{***}p < 0.001$. DEPTOR, DEP domain-containing mammalian target of rapamycin-interacting protein; AR, allergic rhinitis; NAR, nonallergic rhinitis.

Results

The Expressions of miR-96-5p and DEPTOR Were Negatively Correlated in the Nasal Mucosal Tissues from AR Patients

To investigate the role of miR-96-5p and DEPTOR in the pathogenesis of AR, the nasal mucosal samples were obtained from AR patients and patients with nonallergic rhinitis (NAR), and the expression of miR-96-5p and DEPTOR were measured. AR patients showed significantly upregulated miR-96-5p as compared to the NAR group (Fig. 1a). However, DEPTOR, the negative regulator of mTOR activation, was significantly lower in AR patients than that in patients with NAR (Fig. 1b). The result from Western blot also showed a downregulated protein level of DEPTOR in AR patients (Fig. 1c). The correlation analysis revealed that DEPTOR expression was negatively correlated with the level of miR-96-5p in the nasal mucosal tissues from AR patients (Fig. 1d). These results indicated that miR-96-5p and DEPTOR may be related to the pathophysiological process of AR.

The Downregulation of miR-96-5p Inhibited the Upregulation of Proinflammatory Cytokines in IL-13-Stimulated HNECs

To further explore the involvement of miR-96-5p in the development of AR, an in vitro cell model was established by stimulating HNECs with IL-13 for 0, 6, 12, 24, and 48 h. The level of miR-96-5p gradually increased with the increase of IL-13 treatment time (Fig. 2a). Then we transfected HNECs with miR-96-5p inhibitor or inhibitor negative control (inhibitor NC) and then treated cells with IL-13 for 48 h. The delivery of miR-96-5p inhibitor significantly inhibited the upregulation of miR-96-5p in HNECs following IL-13 treatment (Fig. 2b). Then we analyzed the expressions of proinflammatory cytokines, including IL-1β, IL-6, eotaxin, and GM-CSF in cells transfected with miR-96-5p inhibitor or inhibitor NC during IL-13 stimulation. Rapa, an mTOR inhibitor, has been shown to alleviate inflammatory responses in cells, and thus NECs treated with Rapa were used as a positive control [28]. Results showed that IL-13 increased the expressions of IL-1β, IL-6, eotaxin, and GM-CSF in HNECs, whereas the downregulation of miR-96-5p by its inhibitor sequence and Rapa significantly suppressed IL-13-induced upregulation of these cytokines (Fig. 2c–f). Consistently, ELISA revealed that insufficient miR-96-5p expression led to decreased protein levels of proinflammatory cytokines in HNECs following IL-13 treatment (Fig. 2g–j). These results suggested the regulatory effect of miR-96-5p on the production of proinflammatory cytokines in the cell model of AR.

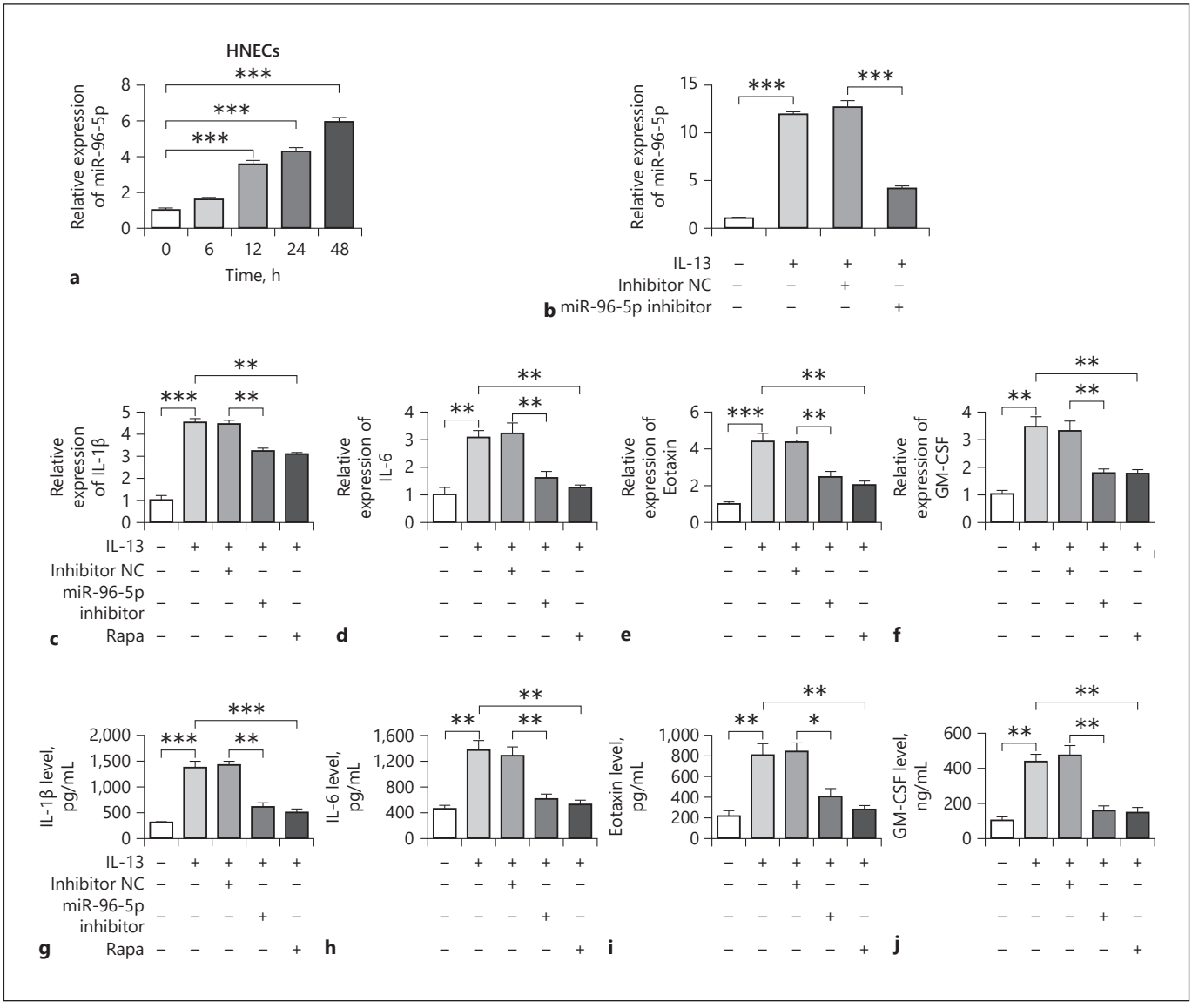

Fig. 2. miR-96-5p inhibitor suppresses IL-13-induced upregulation of proinflammatory cytokines in HNECs. **a** HNECs were stimulated with IL-13 (10 ng/mL) for indicated times (0, 6, 12, 24, and 48 h) and then the expression of miR-96-5p was measured by qPCR. The data were normalized to time 0. **b** HNECs transfected with miR-96-5p inhibitor (or inhibitor negative control) were treated with IL-13 (10 ng/mL) for 48 h. The level of miR-96-5p was determined by qPCR. **c–j** HNECs were transfected with or without miR-96-5p inhibitor (or inhibitor negative control) followed by the stimulation with IL-13 (10 ng/mL) for 48 h. Cells treated with 1 μM Rapa were served as a positive control. The mRNA levels of IL-1β (**c**), IL-6 (**d**), eotaxin (**e**), and GM-CSF (**f**) were assessed using qPCR. The data were normalized to control. The protein expressions of IL-1β (**g**), IL-6 (**h**), eotaxin (**i**), and GM-CSF (**j**) were measured by ELISA kits. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$. IL, interleukin; HNEC, human nasal epithelial cell; Rapa, rapamycin.

The Downregulation of miR-96-5p Inhibits mTOR/NF-Kb Signaling Pathway in HNECs

NF-κB signaling pathway has been widely reported to regulate the production of proinflammatory cytokines [29]. To explore whether miR-96-5p is a mediator of the NF-κB pathway in IL-13-stimulated HNECs, we analyzed the phosphorylation levels of p65 and IκBα in cells with insufficient miR-96-5p expression. Our data showed that IL-13 treatment significantly increased the phosphorylation of p65 and IκBα in HNECs, while miR-96-5p inhibitor decreased the levels of phosphorylated p65 and phosphorylated IκBα without affecting the expressions of p65

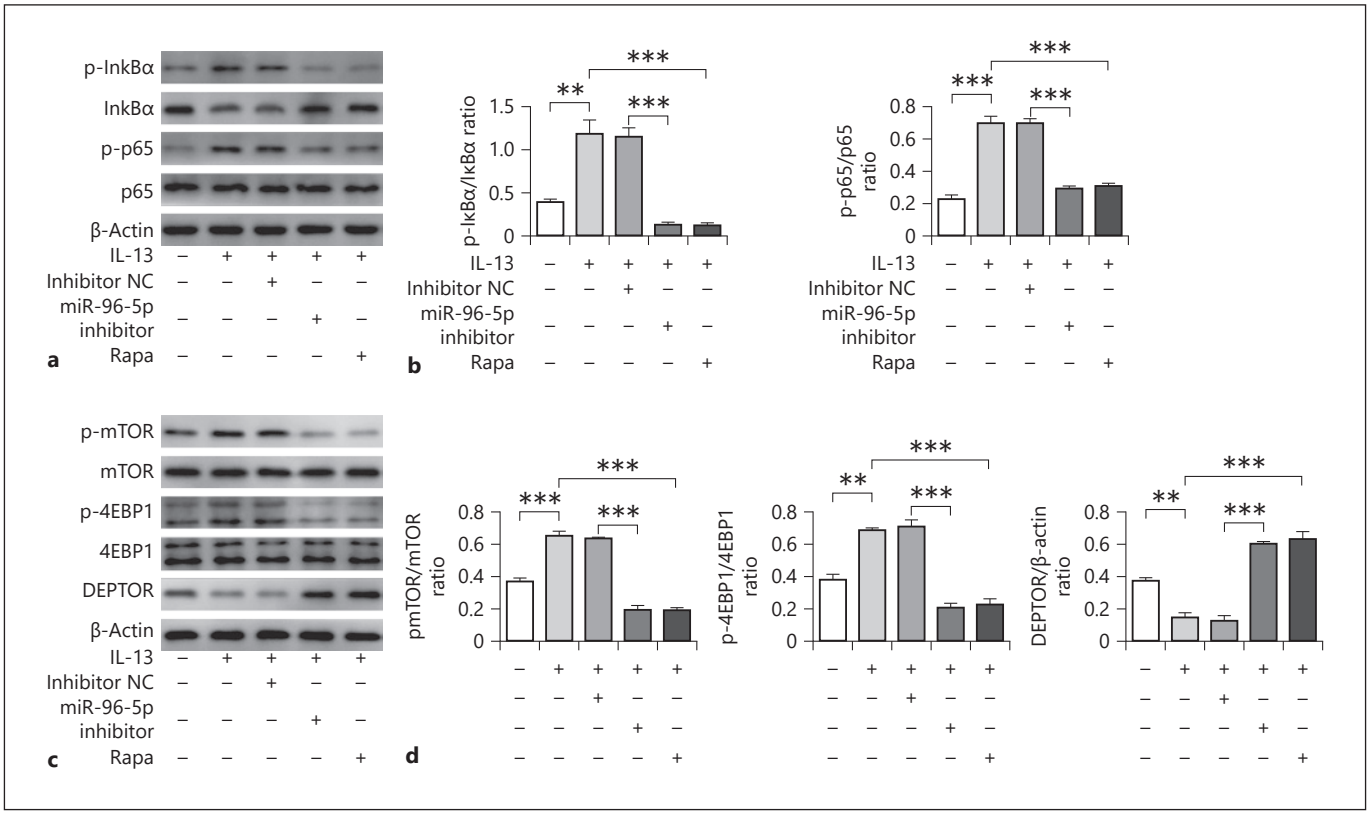

Fig. 3. Downregulation of miR-96-5p inhibits mTOR/NF-κB signaling pathway in HNECs. HNECs were transfected with or without miR-96-5p inhibitor (or inhibitor negative control) followed by the stimulation with IL-13 (10 ng/mL) for 48 h. Cells treated with 1 μM Rapa were served as a positive control. **a** The expressions of p-IκBα, IκBα, p–p65, and p65 were measured by Western blot. **b** The ratios of p-IκBα/IκBα and p-p65/p65 were shown. **c** The expression levels of p-mTOR, mTOR, p-4EBP1, 4EBP1, and DEPTOR were assessed by Western blot. **d** The ratios of p-mTOR/mTOR, p-4EBP1/4EBP1, and the relative expression of DEPTOR were shown. $^{**}p < 0.01$, $^{***}p < 0.001$. mTOR, mammalian target of rapamycin; HNEC, human nasal epithelial cell; IL, interleukin; Rapa, rapamycin; DEPTOR, DEP domain-containing mTOR-interacting protein.

and IκBα (Fig. 3a, b). Moreover, we investigated the regulatory effects of miR-96-5p on mTOR-related proteins. The treatment of HNECs with IL-13 resulted in significantly increased phosphorylation levels of mTOR and 4EBP1 as compared to untreated cells. DEPTOR, an endogenous regulator involved in mTOR signaling pathway, was significantly decreased in IL-13-treated cells (Fig. 3c, d). However, the transfection of HNECs with miR-96-5P inhibitor effectively impeded the activation of the mTOR pathway in HNECs as shown by decreased phosphorylation levels of mTOR and 4EBP1, as well as increased expression of DEPTOR, as compared to the inhibitor NC-transfected cells. Taken together, these data implied that miR-96-5p downregulation inhibited the activation of mTOR/NF-κB signaling pathway in IL-13-stimulated HNECs.

DEPTOR Is a Direct Target of miR-96-5p

The TargetScan and Starbase databases predicted that DEPTOR might be a potential target of miR-96-5p with a putative binding site (Fig. 4a). The transfection of HNECs with miR-96-5p mimic significantly increased the expression level of miR-96-5p compared to cells delivered with mimic NC sequence (Fig. 4b). Then we co-transfected HNECs with WT-DEPTOR reporter (or MUT-DEPTOR) and miR-96-5p mimic (or mimic NC). The dual-luciferase assay showed that the luciferase activity of WT-DEPTOR was significantly lower in cells transfected with miR-96-5p mimic in comparison to mimic NC group, whereas cells transfected with MUT-DEPTOR showed no difference in the luciferase activity between miR-96-5p mimic and control mimic groups (Fig. 4c). The overexpression of miR-96-5p significantly decreased the mRNA level of DEPTOR in HNECs (Fig. 4d). Fur-

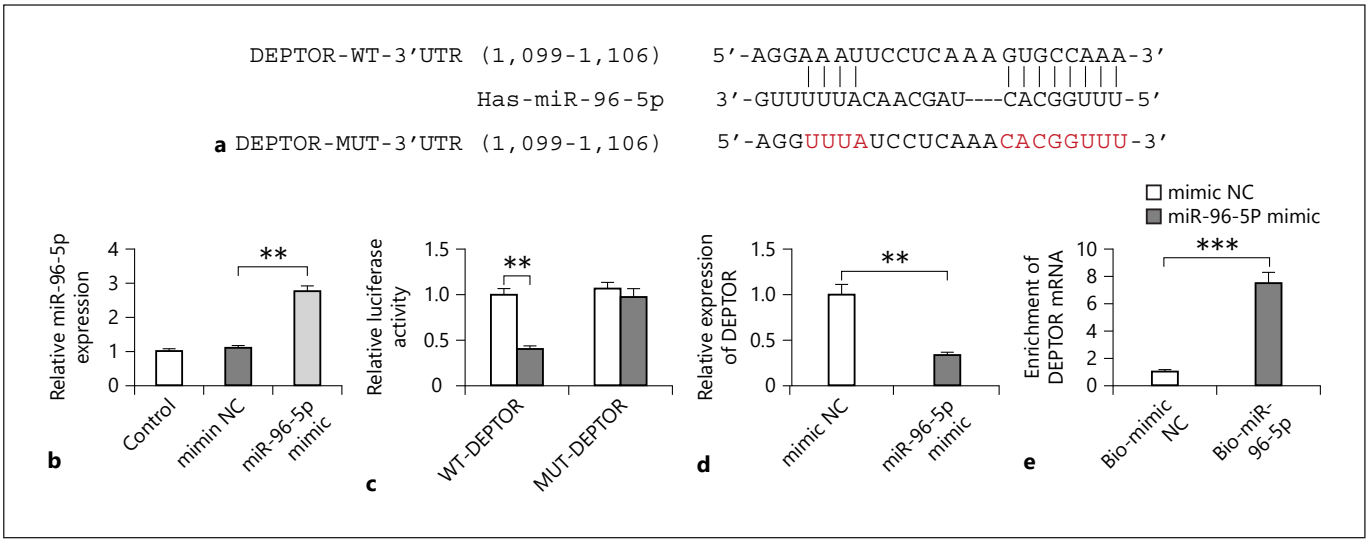

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Fig. 4. DEPTOR is a direct target of miR-96-5p. The putative binding site of miR-96-5p for DEPTOR was predicted and the WT (**a**) and MUT binding sequences were shown (**b**) HNECs were transfected with or without miR-96-5p mimic (or mimic NC), and then the expression of miR-96-5p was detected by qPCR. **c** HNECs were co-transfected with WT-DEPTOR (or MUT constructs) vector and miR-96-5p mimic (or mimic NC). The luciferase activity was determined by luciferase assay. **d** HNECs were transfected with miR-96-5p mimic or mimic NC. The mRNA expression of DEPTOR was measured by qPCR. **e** The enrichment of DEPTOR in HNECs transfected with miR-96-5p mimic or mimic NC was analyzed by pull-down assay. $^{**}p < 0.01$, $^{***}p < 0.001$. DEPTOR, DEP domain-containing mammalian target of rapamycin-interacting protein; HNEC, human nasal epithelial cell.

thermore, the pull-down assay demonstrated that the enrichment of DEPTOR in miR-96-5p mimic-transfected cells was significantly higher than that in the mimic NC group, suggesting the interaction between miR-96-5p and DEPTOR (Fig. 4e). The above findings suggested that DEPTOR was a downstream target of miR-96-5p.

The Knockdown of DEPTOR Reversed the Protective Effect of miR-96-5p Inhibitor on IL-13-Induced Inflammation

To ascertain that miR-96-5p regulated IL-13-induced inflammation in HNECs via targeting DEPTOR, we co-transfected HNECs with miR-96-5p inhibitor and si-DEPTOR. MiR-96-5p inhibitor decreased IL-13-induced elevation in the expressions of IL-1β, IL-6, eotaxin, and GM-CSF, while the downregulation of DEPTOR significantly increased the expression of these cytokines (Fig. 5a). DEPTOR knockdown also attenuated the inhibitory impact of miR-96-5p inhibitor on the activation of NF-κB pathway by increasing the phosphorylation of p65 and IκBα (Fig. 5b, c). In addition, the phosphorylation levels of mTOR and 4EBP1 were also higher in the cells co-transfected with si-DEPTOR and miR-96-5p inhibitor following IL-13 treatment when compared with the group delivered with control siRNA (si-NC) and miR-96-5p inhibitor (Fig. 5d, e). These results indicated that the downregulation of DEPTOR diminished the inhibitory effect of miR-96-5p inhibitor on the activation of mTOR/NF-κB signaling pathway in IL-13-induced HNECs.

Discussion

AR is an inflammatory disorder of mucosa tissues with varied prevalence rates according to the regions and age ranges [30]. The pathogenesis of AR is complex, involving the intense activation of inflammatory cytokines and the infiltration of immune-related cells [1]. In this study, we showed that miR-96-5p was significantly upregulated while the level of DEPTOR was decreased in the nasal mucosal tissues from AR patients in comparison to the group with NAR. The expressions of proinflammatory cytokines, including IL-1β, IL-6, eotaxin, and GM-CSF, and the phosphorylation levels of IκBα, p65, mTOR, and 4EBP1 were markedly increased in primary HNECs following IL-13 stimulation. However, the downregulation of miR-96-5p suppressed the secretion of proinflammatory cytokines and the activation of the mTOR/NF-κB pathway via targeting DEPTOR. The current study, for

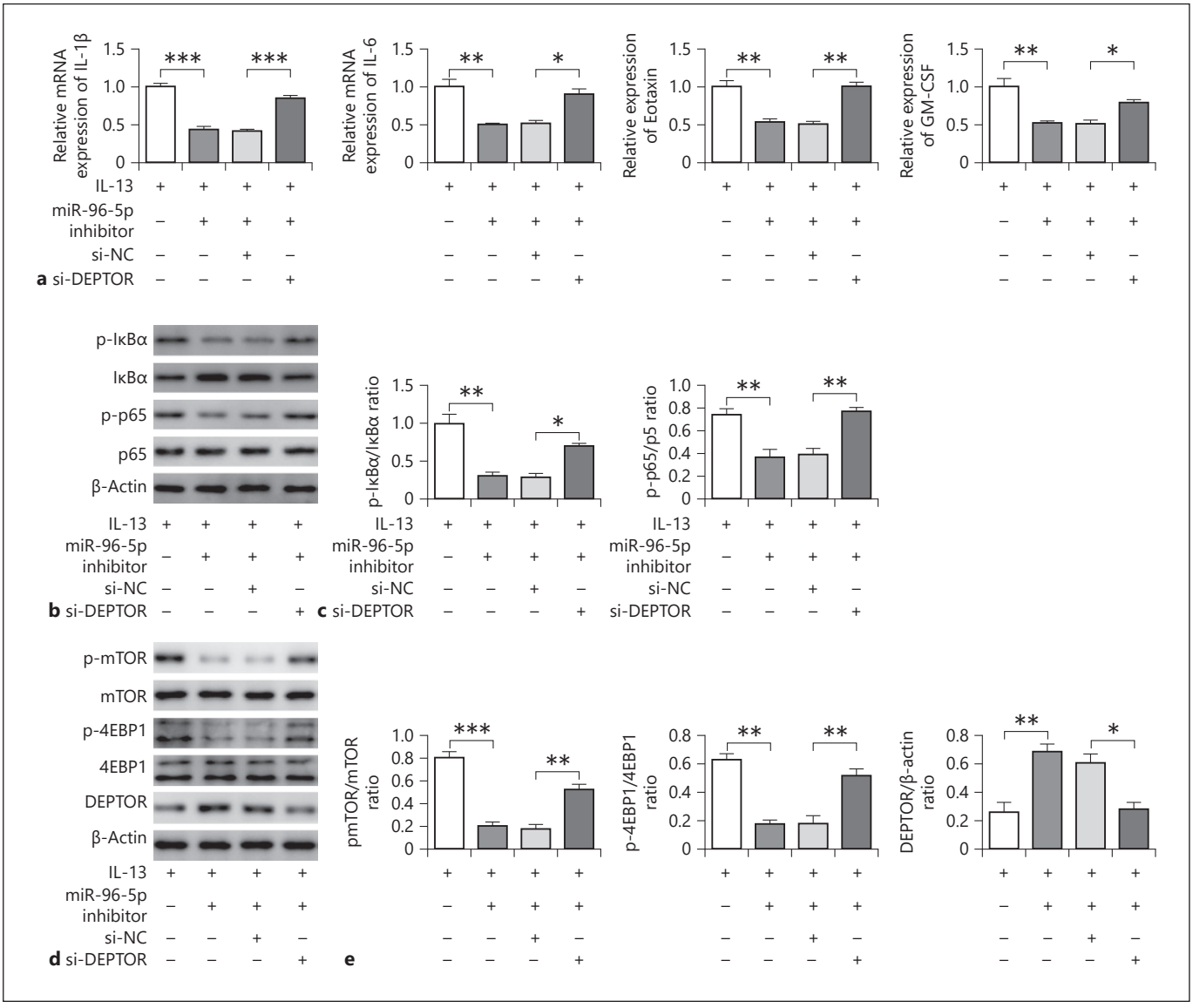

Fig. 5. The knockdown of DEPTOR diminished the suppressive effect of miR-96-5p inhibitor on IL-13-induced inflammation. HNECs were transfected with miR-96-5p inhibitor alone, co-transfected with miR-96-5p inhibitor and si-DEPTOR, or co-transfected with miR-96-5p inhibitor and si-NC. Transfected cells and the control group were treated with IL-13 at 10 ng/mL for 48 h. **a** The mRNA expressions of IL-1β, IL-6, eotaxin, and GM-CSF were determined by qPCR. **b** Western blot was performed to assess the phosphorylation of IκBα and p65. **c** The ratios of p-IκBα/IκBα and p–p65/p65 were shown. **d** The expressions of p-mTOR, mTOR, p-4EBP1, 4EBP1, and DEPTOR were measured by Western blot. **e** The ratios of p-mTOR/mTOR, p-4EBP1/4EBP1, and the relative expression of DEPTOR were shown. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$. mTOR, mammalian target of rapamycin; DEPTOR, DEP domain-containing mTOR-interacting protein; IL, interleukin; HNEC, human nasal epithelial cell.

the first time, showed the aberrant expression and regulatory function of miR-96-5p in AR, suggesting that miR-96-5p might be a potential target for the therapeutic intervention in AR.

The nasal mucosa serves as the first line of defense against environmental allergens [31]. The in vitro models using HNECs exhibit similar responses to exogenous stimuli, and thus are essential in exploring the therapeutic target for AR patients [32]. IL-13 is a Th2 cytokine that has been demonstrated to mediate a series of pathological events in allergic diseases, such as mucus cell metaplasia, subepithelial fibrosis, smooth muscle hypertrophy, and

eosinophil recruitment [33–35]. Teng et al. [27] reported that the IL-13 signaling pathway played a critical role in the pathogenesis of AR by increasing the secretion of inflammatory cytokines, such as eotaxin, GM-CSF, and IL-1β, and inducing mucus production in primary HNECs from AR patients. Our data showed that the level of miR-96-5p was significantly increased in the nasal mucosal tissues from AR patients as compared to the group with NAR. Consistent with previous results, we found elevated expression of IL-1β, IL-6, eotaxin, and GM-CSF in HNECs following IL-13 stimulation. The downregulation of miR-96-5p, however, inhibited the expressions of proinflammatory cytokines in HNECs at both transcriptional and translational levels.

The NF-κB pathway is a classic inflammatory pathway associated with the progression of AR. Gao and Yu [36] showed that miR-16 inhibited IL-13-induced cytokine secretion and mucus overproduction via inhibiting the activation of IκB and p65 in HNECs. Pan et al. [37] found that catechin, an anti-inflammatory component in green tea, inhibited the phosphorylation of IκB and p65 in a murine model of ovalbumin-induced AR and in poly (I:C)-stimulated HNECs. Consistently, our data presented that IL-13 stimulation induced the activation of NF-κB pathway in HNECs, whereas the transfection with miR-96-5p inhibitor effectively impeded the phosphorylation of IκB and p65. And previous evidence suggested that the Akt-dependent regulation of NF-κB pathway is mediated by mTOR [13]. Here, the analysis of the phosphorylation levels of mTOR and its substrate, 4EBP1, demonstrated that the downregulation of miR-96-5p inhibited IL-13-induced activation of both mTOR and 4EBP1 in HNECs.

There are 2 multi-component complexes of mTOR: mTOR complex 1 (mTORC1) and mTOR complex 2 (mTORC2) [38]. mTORC1 plays a pivotal role in the regulation of protein synthesis via mediating the phosphorylation of S6 kinase and EIF4EBP1, while mTORC2 is involved in the regulation of cell survival via regulating AKT phosphorylation [39]. DEPTOR is a key protein that belongs to mTORC1 and mTORC2 complexes and has been shown to negatively regulate the activation of mTOR [10]. In the current study, DEPTOR was confirmed as a downstream target of miR-96-5p and their expressions in the nasal mucosa from AR patients were inversely correlated. By transfecting siRNA against DEPTOR in HNECs with insufficient miR-96-5p, we found that the knockdown of DEPTOR reversed the protective effect of miR-96-5p inhibitor on IL-13-induced AR as evidenced by increased cytokine production and elevated phosphorylation levels of IκB, p65, mTOR, and 4EBP1. These results indicated that miR-96-5p ameliorated the development of AR in HNECs via directly targeting DEPTOR. The regulatory effects of miR-96-5p on DEPTOR expression and the mTOR/NF-κB signaling pathway need to be confirmed in animal AR models.

In conclusion, our study revealed that miR-96-5p is upregulated in the nasal mucosa of AR patients. The downregulation of miR-96-5p inhibited the expression of inflammatory cytokines and the activation of the mTOR/NF-κB signaling pathway via direct interaction with DEPTOR. These findings suggested the potential use of miR-96-5p as a diagnostic marker and therapeutic target for the treatment of AR.

Statement of Ethics

This study was approved by the Ethics Committee of Hainan General Hospital. All patients provided written informed consent.

Conflict of Interest Statement

All authors agree with the presented findings, have contributed to the work, and declare no conflict of interest.

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Author Contributions

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