MiR-4454 in Combined Allergic Rhinitis and Asthma Syndrome (CARAS): Clinical significance and mechanistic insights into airway inflammation

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Article Info

Abstract

Abnormal expression of non-coding microRNA is associated with the development of combined allergic rhinitis and asthma syndrome (CARAS). However, the function of miR-4454 in CARAS is unknown. Our study aimed to reveal the clinical significance and related mechanism of miR-4454 in CARAS. Blood samples from 38 cases of CARAS and 43 cases of healthy subjects were collected to detect the expression of miR-4454. House dust mite (HDM) sensitization and challenge-induced bronchial epithelial cells to simulate the asthma state model in vitro, miR-4454 mimics and inhibitor transfection to detect the expression level of pro-inflammatory cytokines, cell survival rate and migration ability, flow cytometry and western blot (WB) Detection of cell cycle, apoptosis and inflammation-related protein levels. Compared with healthy controls, the expression of miR-4454 in the blood of CARAS patients was significantly up-regulated, and IL-6 and IL-8 were significantly up-regulated in the HDM treatment group, indicating that the model induction was successful. After overexpression of miR-4454, cell proliferation and migration in the HDM-treated group were significantly inhibited, and the levels of early apoptosis and inflammation-related proteins (IL-17, IL-17RD, TNF-α, GCSF and NF-κB) were increased High; after inhibiting miR-4454, cell proliferation and migration were significantly enhanced, and the levels of apoptosis and inflammation-related proteins were decreased. This study found that inhibiting the expression of miR-4454 can improve HDM-induced cell injury, which may be related to miR-4454 regulating the activation of IL-17/NF-κB inflammatory axis.

Keywords: miR-4454, CARAS, Allergic rhinitis, Airway inflammation, IL-17, NF-κB.

1. Introduction

Combined allergic rhinitis and asthma syndrome (CARAS) is a novel disease associated with lower and upper lung pneumonia. This combined inflammation immediately induces a coordinated response of immune cells and their mediators. Symptoms include airway hyperresponsiveness, mucus hypersecretion, and eosinophil infiltration in the airway, affecting nasal congestion and vascular permeability in patients [1]. Since both allergic rhinitis and asthma are type I allergies and they are very similar in etiology, immunology and pathogenesis, the diagnosis of CARAS is the combined diagnosis of allergic rhinitis and asthma, which can improve the sensitivity of the two diseases at the same time. High diagnostic accuracy can reduce the repeated use of drugs, thereby greatly decreasing the misdiagnosis rate and improving clinical efficacy [2].

The immunological and pathological changes of the upper and lower airways of CARAS are allergic inflammation in the nasal mucosa and bronchial mucosa, respectively. Inflammation of nasal mucosa and bronchial mucosa are very similar in terms of pathogenesis, genetic changes, local pathological changes, abnormal immune function and pathogenesis [3]. Allergic inflammation of nasal mucosa and bronchial inflammation of asthma is usually caused by the same allergen, and the pathology is allergic inflammation characterized by increased airway eosinophils [4]. However, the pathogenesis of CARAS is complicated and has not been fully elucidated.

MicroRNAs (miRNAs) are non-coding RNAs that regulate post-transcriptional modifications and are involved in various physiological and pathological processes [5]. In recent years, more and more studies have shown that abnormal miRNA expression is widely found in patients with rhinitis and asthma and is closely related to the progression of the disease [6-8]. The study shows that miRNA regulation may play a key role in CARAS [9]. The miR-4454 in monocyte-derived dendritic cells [10] and alveolar macrophages [11], is associated with proinflammatory response of macrophages[12]. Studies have also shown that miR-4454 plays an important role in the occurrence and development of various inflammatory diseases and that the abnormally high expression of miR-4454 is related to the induction and activation of NF-κB [13]. In the early stage of this study, the Targetscan online software was used to predict that IL-17RD might be the target gene of miR-4454, and the interaction of IL-17RD regulates the IL-
17 and NF-kB signaling pathways, and the depletion of IL-17RD will impair TNFR2-mediated NF-kB signaling activation [14]. However, the double-luciferase validation gene results showed that miR-4454 could not directly regulate the expression of IL17RD. Therefore, the regulatory mechanism of miR-4454 in cell proliferation, migration and apoptosis and its association with the NF-kB inflammatory pathway need to be further explored. MiR-4454 may be a diagnostic and prognostic tool for invasive aspergillosis in lung transplant recipients [15], and it is highly expressed in asthma [16], but the specific mechanism is not clear. Therefore, our study aimed to explore the molecular mechanism and clinical significance of miR-4454 in regulating CARAS and airway inflammation, in order to provide new ideas and targets for the prevention and treatment of CARAS.

2. Materials and methods

2.1. Material

The ethics committee approved this study of affiliated Changzhou Second People’s Hospital of Nanjing Medical University (2019KY032-01). All study participants gave written informed consent. Fresh bulk blood samples were acquired from 43 healthy subjects and 38 patients with CARAS in the Department of Respiratory and Critical Care Medicine, Changzhou Second People’s Hospital affiliated with Nanjing Medical University.

Human bronchial epithelial cells and human bronchial epithelial cells complete culture medium were bought from Wuhan Punosai (Wuhan, China). Fetal bovine serum (FBS) was bought from Gibco (Rockville, MD, USA). Penicillin/Streptomycin solution, Apoptosis detection kit and Cell cycle detection kit were bought from Meilune (Dalian, China); RPMI-1640 Medium, 0.25% Trypsin and BCA Protein Determination Kit were purchased from Boston biological technology (Wuhan, China). IL-8 and IL-6 ELISA Kit were bought from Fanke Industrial (Shanghai, China). RNA extraction kit and TRizol reagent were purchased from CWbio (Jiangsu, China). A reverse transcription kit and real-time quantitative PCR kit were purchased from Accurate Biology in China. miRNA-related PCR primers were synthesized by Dingguo Biology (Beijing, China); Transwell Kit was purchased from Corning (Corning, NY, USA); MTT and crystal violet staining solution were purchased from Shanghai Biological Engineering (Shanghai, China); mir-4454 mimic, negative control analogue of mir-4454 mimic (Mimic NC), mir-4454 inhibitor analogue and negative control of mir-4454 inhibitor analogue (inhibitor NC) were synthesized by hippoc Biology (Huzhou, China); tumor necrosis factor-α (TNF-α), IL-17, granulocyte colony stimulating factor (GCSF) and NF-kB antibodies were purchased from Bios (Woburn, MA, USA); IL-17RD antibody was purchased from Abcam (Ancaster, ON, Canada); GAPDH antibody was purchased from Proteintech Biology (Rosemont, IL, USA).

2.2. Cell culture, House dust mite (HDM) treatment and grouping

The bronchial epithelial cells from healthy human bronchial tissue were maintained in RPMI-1640 supplement with 10% fetal bovine serum (FBS, Gibco, Carlsbad, CA, USA), 100 U/L penicillin, and 100 mg/L streptomycin. Culture conditions were maintained in a 37°C, 5% CO2, cell incubator. When the cell growth density reaches 80%, digest and pass with trypsin.

The experiment was divided into five groups, including control group; HDM+mimic NC group; HDM+inhibitor NC group; HDM+mir-4454 mimic group and HDM+mir-4454 inhibitor group. Except the control group, normal bronchial epithelial cells were induced with 300 ng/mL HDM for 24 h in the other 4 groups.

2.3. ROC curve analysis

The main function of the receiver operating characteristic curve (receiver operating characteristic curve, ROC) is that it can be used to compare the ability of two or more different diagnostic methods to identify diseases. When comparing two or two diagnostic methods for the same disease, the ROC curves of each diagnostic method can be drawn in the same ROC space (in the same coordinate system), so that various diagnostic methods can be intuitively identified pros and cons. The closer to the lower left corner of the ROC curve, the better the performance of the diagnostic method represented. The ROC curve combines the sensitivity and specificity in a graphical way, which can accurately reflect the relationship between the sensitivity and specificity of an analytical method, and is a comprehensive method representative of the accuracy of a diagnostic method. In this study, ROC curve was used to evaluate the mRNA expression level of miR-4454 to distinguish CARAS from normal population. The pROC package in the R programming language was used for the generation and drawing of ROC curve objects. The confusion matrix corresponding to the optimal threshold point on the ROC curve would be the basis for calculating metrics such as accuracy, specificity, and sensitivity. Typically, we used the Youden index to select the best threshold point. The Jordon index, also known as the correctness index, was the sum of specificity and sensitivity minus 1:

Youden index = Sensitivity + Specificity − 1

The value of the Jordon index range was between 0 and 1, and it was the larger of the Jordon index, the better the performance of the classification model.

2.4. ELISA

After treating bronchial epithelial cells with 300 ng/mL HDM for 24 h, the supernatant was cryopreserved at -80°C. After melting the samples on ice, the absorbance value was tested at 450 nm according to the instructions of ELISA Kit respectively, and the expression level of IL-8 and IL-6 were calculated.

2.5. Cell transfection

The miR-4454 mimics and NC mimics,miR-4454 inhibitor and inhibitor NC were synthesized by Huzhou hippo Biology (Huzhou, China). After HDM treatment, the four groups of bronchial epithelial cells were transfected with mimic NC and miR-4454 mimics, inhibitor NC and miR-4454 inhibitor by Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer’s instructions, respectively. After 24-48 h treatment, total RNA and total protein were extracted.

2.6. RNA extraction and qRT-PCR

According to the manufacturer’s instrument, the total RNA of blood samples from 38 CARAS patients and 43 healthy subjects was extracted with TRizol reagent.
After collecting the cells, pre-cooled D-Hanks (pH=7.2-7.4) was used to wash and resuspend the cell pellet. After adjusting the concentration to 1×10^6 cells/mL, 1mL cell suspension was taken to centrifuge and remove the supernatant, followed by adding 500 μL 75% pre-cooled ethanol to fix the cells at 4°C for 1-4 h. Cells were then centrifuged to remove the supernatant and washed with D-hanks for once or twice. PI staining of cells was performed according to the instructions of the cell cycle staining kit, and sample analysis with the flow cytometry. This experiment was repeated 3 times.

2.10. Western blotting analysis
After treatment with HDM and transfection with miR-4454 mimics, inhibitor, mimics NC and inhibitor NC respectively, the total protein was extracted with pre-cooled RIPA lysis method. BCA kit (Thermo ScientificTM) was used to determine the concentration and adjusted to an equivalent concentration. SDS-PAGE electrophoresis to separate proteins, wet membrane transfer method to transfer proteins to PVDF membrane. 5% BSA solution was used to block the membrane at room temperature for 1 h and immersed the membrane in TBST for wash. Primary antibody incubation: GAPDH (Proteintech Biology, Rosemont, IL, USA), IL-17RD (Affinity Biology, Ancaster, ON, Canada), TNF-a, NF-κB, GCSF and IL-17 (Bioss, Woburn, MA, USA) (1:5000), which were in horseradish peroxidase-conjugated secondary antibodies (1:3000) were added. The electrochemiluminescence of positive bands was visualized using the enhanced chemiluminescence (ECL) reagent on a ChemiDoc MP imaging system (Bio-Rad, Hercules, CA, USA). Image-Pro Plus software quantified the intensity of the bands and calculated the relative expression of each protein by using the gray value of the target protein band/the gray value of the GAPDH protein band as an internal control. This experiment was repeated 3 times.

2.11. Statistical analysis
Statistical analysis was performed using Statistic Package for Social Science (SPSS) 13.0 (SPSS, Inc., Chicago, IL, USA). All data were expressed as the mean ± standard deviation or percentage. Comparison of inter-groups was performed with Student’s unpaired t-test, and one-way analysis of variance was used for multiple-group comparison. Pearson’s correlation analysis was used to evaluate the relationship between each group, and P<0.05 indicated the statistically significant differences.

3. Results
3.1. Expression and Diagnostic Value of miR-4454 in allergic rhinitis and CARAS patients.
In this study, the miRNA-4454 was evaluated in blood samples from 38 CARAS patients and 43 healthy subjects. The level of miR-4454 in CARAS samples was expressly higher than those in healthy patients (P<0.05) in Figure 1.

Next, we studied if miR-4454 expression in serum could be used as a disease diagnosis biomarker of CARAS. To do this, the ROC curve analysis was performed to evaluate the possibility of miR-4454 as a biomarker for disease diagnosis. The ROC curve reveals the relationship between sensitivity and specificity. The results showed that the cut-off value was 1.064, the sensitivity, spec-


specificity and area under the curve (AUC) of miR-4454 as a biomarker for CARAS were 65.8%, 93.1% and 0.896. As shown in Figure 2.

3.2. HDM-induced asthma disease model

To construct the in vitro-induced asthma disease model, we treated bronchial epithelial cells with 300 ng/mL HDM for 24 h, and the expressions of IL-6 and IL-8 were detected by ELISA. On the ELISA detector, at 450 nm (410 nm if ABTS is used for color development), measure the OD value of each well after zeroing the blank control well. If it is greater than 2.1 times the OD value of the specified negative control, it is positive. The results revealed that the expressions of IL-6 and IL-8 were significantly up-regulated after HDM treatment, indicating that the bronchial epithelial cells were in an inflammatory state and could be used as an in vitro disease model for follow-up studies. See Figures 3A and 3B.

3.3. Effect of miR-4454 on proliferation of HDM-treated bronchial epithelial cell

The proliferation of HDM-treated human bronchial epithelial cells was observed with MTT assay. The results showed that compared with NC mimic group, the proliferation of bronchial epithelial cells in miR-4454 mimic group was significantly reduced with the trend over time, while miR-4454 inhibitor treatment could significantly promote the proliferation of human bronchial epithelial cells. The statistically significant difference suggested that miR-4454 could inhibit the proliferation of human bronchial epithelial cells, as shown in Figure 4.

3.4. Effect of miR-4454 on migration of bronchial epithelial cells

The effect of miR-4454 on migratory potential of HDM-induced asthmatic bronchial epithelial cells was evaluated by cell scratch wound test and transwell assay. Cell scratch wound test showed that the migration ability of miR-4454 treated bronchial epithelial cells significantly decreased after 24 h and 48 h compared with mimic NC control group, while the migration rate of miR-4454 inhibitor group was increased, as shown in Figure 5A and 5B. And transwell migration experiment showed that after 24

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**Fig. 1.** The miRNA expression of 4454 in blood samples from 38 CARAS patients and 43 healthy subjects. normal: healthy subjects; CARAS: CARAS patients. *P<0.05; **P<0.01; ***P<0.001.

**Fig. 2.** Diagnostic Value of miR-4454 expression in CARAS patients serum.

**Fig. 3.** The expression of IL-6 and IL-8 in HDM-treated and control groups. Control: bronchial epithelial cells; HDM: bronchial epithelial cells treated with 300 ng/mL HDM for 24 h. (3A) IL-8; (3B) IL-6. *P<0.05; **P<0.01; ***P<0.001.

**Fig. 4.** The value of OD490 at different miRNA treatment groups in bronchial epithelial cells for different time points (24 h-96 h). HDM+mimic NC: bronchial epithelial cells treated with 300 ng/mL HDM for 24 h then transfected with mimic normal control; HDM+miR-4454 mimic: bronchial epithelial cells treated with 300 ng/mL HDM for 24 h then transfected with miR-4454 mimic; HDM+inhibitor NC: bronchial epithelial cells treated with 300 ng/mL HDM for 24 h then transfected with inhibitor normal control; HDM+miR-4454 inhibitor: bronchial epithelial cells treated with 300 ng/mL HDM for 24 h then transfected with miR-4454 inhibitor; *P<0.05; **P<0.01; ***P<0.001.
40% of allergic rhinitis cases are concomitant with pathological changes, immune function and pathogenesis. Asthma and allergic rhinitis are similar in pathogenesis, genetic changes, local pathological changes, immune function and pathogenesis [3, 18].

### Discussion

CARAS is a new medical concept proposed in recent years [17], and two symptoms of allergic rhinitis and asthma are similar in pathogenesis, genetic changes, local pathological changes, immune function and pathogenesis [3, 18]. 40% of allergic rhinitis cases are concomitant with asthma while up to 80% of asthma patients are affected by allergic rhinitis [19-21]. The incidence of CARAS is increasing worldwide year by year and CARAS has become a global health problem [22], especially very common in clinical practice in China [23]. Suffering from CARAS with serious symptoms, patients tend to have recurrent episodes [24], yet there is still a lack of effective treatment [20], and the pathogenesis is also not fully understood.

MiRNAs participate in biological processes such as cell generation, proliferation, differentiation and apoptosis, then become involved in the process of diseases [5, 25]. Overexpression of miR-4454 in drug-resistant tumors is a key precursor of the post-transcriptional repression of GNL3L in the progression of human chemotherapy-resistant tumors. **P<0.01; ***P<0.001.

### 3.5. Effect of miR-4454 on bronchial epithelial cell cycle arrest and apoptosis

In order to study the effect of miR-4454 on bronchial epithelial cell cycle arrest and apoptosis, HDMA-induced asthmatic bronchial epithelial cells were treated with miR-4454 for 24 h. And the Flow cytometry results showed that there was no significant change in cell cycle distribution after miR-4454 treated bronchial epithelial cells 24 h. These results indicated that miR-4454 could not block the cell cycle of HDM-treated bronchial epithelial cells, as shown in Figure 6. But, the Flow cytometry analysis showed that compared with NC mimic group, miR-4454 mimic could promote the early apoptosis of HDM-treated bronchial epithelial cells, while miR-4454 inhibitor could inhibit the apoptosis, as shown in Figure 7.

### 3.6. Effects of miR-4454 on the expression of inflammatory factors

To explore the effect of miR-4454 on the expression of inflammatory factors, western blot assay was used to evaluate the expression of IL-17, IL-17RD, TNF-a, GCSF and NF-κB. And the results showed that miR-4454 mimic could promote the expression of all of the inflammatory factors above in the HDM-treated bronchial epithelial cells, while miR-4454 inhibitor can reverse this phenomenon. It indicated that overexpression of miR-4454 may activate the NF-κB inflammatory pathway and promote the expression of related genes by activating the IL-17/NF-κB inflammatory axis, as shown in Figure 8.

### 4. Discussion

CARAS is a new medical concept proposed in recent years [17], and two symptoms of allergic rhinitis and asthma are similar in pathogenesis, genetic changes, local pathological changes, immune function and pathogenesis [3, 18]. 40% of allergic rhinitis cases are concomitant with...
resistant colorectal cancer, and miR-4454 as a microRNA-based therapeutic to silence GNL3L may significantly reduce the dependence on GNL3L/ Cancer cell survival by NF-κB signaling, making miR-4454 a drug candidate for the treatment of metastatic human colorectal cancer [26]. The regulatory role of microRNA in CARAS has attracted more and more attention [27-29], and several miRNAs have been shown important influence on various biological mechanisms which lead to asthma pathology and symptoms [30-33]. It has been reported that miR-4454 is highly expressed in allergic asthma [16, 34]. QRT-PCR results in this study showed that miR-4454 expression was significantly higher in patients with allergic rhinitis and asthma than in healthy subjects, which was consistent with previous studies. It suggests that miR-4454 may be associated with CARAS disease, but its specific mechanism still remains more puzzling.

It has been reported that bronchial epithelial cells play an important role in asthma [35], and the proliferation of bronchial epithelial cells in patients with asthma is usually inhibited with the occurrence of apoptosis [35, 36]. Therefore, in this study HDM treated bronchial epithelial cells were used to explore the role of miR-4454 in the pathogenesis of CARAS. The results showed that miR-4454 inhibited the proliferation and migration of bronchial epithelial cells, promoted early apoptosis, and had no obvious blocking effect on the cell cycle. Besides, miR-4454 inhibitors can reverse these effects.

NF-κB pathway plays a key role in the regulation of inflammation in various cell types [37, 38]. It has been suggested that the abnormal expression of miR-4454 is related to the activation of NF-κB, which can promote the development of various inflammatory diseases such as arthritis [39]. Other studies have demonstrated that CARAS airway inflammation is closely related to NF-κB pathway [40, 41]. Qu et al found that IL-17 can induce activation of NF-κB pathway [42, 43]. Based on previous studies, we speculated that miR-4454 may affect CARAS by increasing the expression of IL-17RD to promote the function of the IL-17/NF-κB inflammatory axis. Western blot results showed that miR-4454 mimic could increase the expression of IL-17, IL-17RD, TNF-α, GCSF and NF-κB in HDM-treated bronchial epithelial cells, while miR-4454 inhibitor could inhibit these expression. It is suggested that abnormally high expression of miR-4454 may stimulate the function of IL-17/NF-κB inflammatory axis by activating the NF-κB inflammatory pathway and further enhance the expression of inflammation-related genes, thereby exacerbating airway inflammation of CARAS.

At present, it has been proved that miRNA plays an important role in the diagnosis and treatment of asthma [44-47]. ROC analysis showed that in peripheral blood miR-4454 may be a new diagnostic marker for predicting sensitivity of treatment for CARAS. These results suggest that miR-4454 may play a predictive role in CARAS with different characteristics.

5. Conclusions
In summary, we found that miR-4454 was abnormally high expressed in patients with asthma and rhinitis, and may serve as a new diagnostic marker to predict different types of allergic rhinitis and determine the sensitivity of treatment for CARAS. MiR-4454 affected the function of bronchial epithelial cells and may aggravate airway inflammation of CARAS by activating the IL-17/NF-κB inflammatory axis. The results of this study can provide a new research perspective for the prevention and treatment of CARAS, but the specific molecular mechanism needs to be further explored. However, the limitation of this study is that the target gene of miR-4454 and the specific mechanism of regulating cell phenotype could not be identified.

Conflict of interest
All authors declare they have no conflict of interest.

Consent for publications
The author read and approved the final manuscript for publication.

Ethical approval
The ethics institute of affiliated Changzhou No.2 People’s Hospital of Nanjing Medical University (2019KY032-01).

Informed consent
All study participants gave written informed consent.

Data availability
Data of the present research are available upon reasonable request to the Corresponding Author.

Authors’ contributions
Zhengdao Mao and Qian Zhang designed the study and performed the experiments, Zhiguang Liu and Chuang Sun collected the data, Yujia Shi, Zhipeng Wang and Lianzheng Zhou analyzed the data, Zhengdao Mao, Qian Zhang prepared the manuscript. All authors read and approved the final manuscript.
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