

MiR-202-5p Promotes M2 Polarization in Allergic Rhinitis by Targeting MATN2

Li Wang Xueyan Liu Xicheng Song Lei Dong Dawei Liu

Department of Otorhinolaryngology Head and Neck Surgery, Yuhuangding Hospital, Yantai, China

Keywords

Allergic rhinitis · miR-202-5p · MATN2 · Macrophage · Polarization

Abstract

Background: The aim of this study was to investigate the effect of MiR-202-5p on macrophages involved in allergic rhinitis. **Methods:** Thirty allergic rhinitis patients and 10 healthy controls were recruited. Nasal lavage was utilized to collect mucosal tissues and mucus-derived macrophages. Flow cytometry was used to detect and sort type 2 macrophages, and qRT-PCR and Western blot were adopted to determine the expression of miR-202-5p and MATN2. Luciferase reporter assay and biotin-RNA pulldown were performed to testify the direct binding of miR-202-5p with MATN2. MATN2 was further overexpressed to test its effect on M2 polarization. **Results:** miR-202-5p, which can promote M2 polarization, was highly expressed in allergic rhinitis mucosal tissues and macrophages compared with the healthy controls. MATN2 could be directly targeted by miR-202-5p and can reverse miR-202-5p-mediated M2 phenotype polarization in allergic rhinitis. **Conclusions:** miR-202-5p could target MATN2 to induce M2 polarization involved in allergic rhinitis.

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Introduction

Allergic rhinitis (AR) can be diagnosed with medical history and clinical symptoms, such as pruritus, sneezing, obstruction, and nasal discharge driven by type 2 helper T (Th2) cells and IgE-mediated mast cell degranulation against inhaled allergens, with a particular profile of cytokine secretion and mediator release in the nasal mucus [1–3]. As a chronic airway disorder with a high prevalence and economic burden, AR is also considered as one of the most prominent risk factors for the development of bronchial asthma.

Some ongoing mechanical and clinical investigations in AR are focused on how the Th2 cytokine microenvironment could affect and process AR-related pathology [4, 5]. Recent studies have shown that type 2 macrophages (M2) can influence IL-4 secretion and the IgE class switch process in cedar pollen-sensitized AR mice [6, 7], which suggests that M2 macrophages might have a role in the initiation and development of AR.

Not only might intracellular kinases and transcription factors control the activation and polarization of macrophages [8, 9], but also microRNAs (miRNAs) emerge as

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L.W. and X.L. contributed equally to this work.

critical regulators to rewire functional phenotypes of macrophages [10–12], which could be utilized as biomarkers or therapeutic targets against diverse immunologic and inflammatory diseases. While little miRNA research has been done in AR, the aim of this investigation is to determine the role of miR-202-5p in AR.

Methods

Participants

AR was diagnosed according to symptoms, skin-prick test, and serum IgE assay on the basis of the 2016 revision of Allergic Rhinitis and its Impact on Asthma (ARIA) Guidelines [13]. Thirty AR patients (male 70.0%, mean age 32.3 years, range 18–53) and 10 healthy controls (male 80.0%, mean age 30.9 years, range 19–55) were recruited between February 2017 and February 2018 in Yuhuangding Hospital. The participants with nasal polyps, chronic rhinosinusitis, infectious rhinitis, and age below 4 and over 60 years were excluded. Informed consents were obtained from the participants.

Tissue Sampling

Nasal lavage was performed to collect nasal mucus as previously described [14]. Briefly, 5 mL of prewarmed saline was used to flush the nostrils with the subject in a head-down position. The flush solution was aspirated into a suction collector with the help of nasal endoscopy and further centrifuged (4,000 g, 5 min) to get the supernatants and cell pellets. CD14 microBeads (Miltenyi Biotec) were used for the positive selection of human macrophages from the cell pellets.

Flow Cytometry

Monoclonal anti-human antibodies such as CD45 (HI30, BioLegend, San Diego, CA, USA), CD3 (HIT3a, BD Pharmingen, San Diego, CA, USA), CD14 (HCD14, BioLegend) for macrophages, and CD206 (15–2, Bio-Rad, Hercules, CA, USA) were incubated for 30 min on ice. TruStain FcX (eBioscience, San Diego, CA, USA) was utilized to block nonspecific binding. Fluorescence-activated cell sorting (FACS) was run with a FACS Canto II (BD Biosciences, Franklin Lakes, NJ, USA). CD3–CD14+ macrophages were gated on CD45+ leukocyte cells, and a marker for alternative activation (CD206) was further sorted and analyzed with FlowJo v.10 (Treestar Inc.).

qRT-PCR

The biotinylated miR-202-5p/mRNA complex was pulled down and extracted with DNase I (Sigma-Aldrich, St. Louis, MO, USA) and ISOGEN-LS (Nippon Gene, Tokyo, Japan) to assay MATN2 expression. TRIzol reagent (Invitrogen, Thermo Fisher Scientific, Waltham, MA, USA) was applied to extract total RNA containing miRNA to quantify the expression in mucosal tissues and macrophages. ReverTra Ace-α kit was used to reverse-transcribe mRNA into cDNA, which was further amplified in a 20-μL volume system containing SYBR-Green Master Mix (Toyobo Co. Ltd., Osaka, Japan) in an Applied Biosystems 7500 (40 cycles of 95 °C for 15 s and 58 °C for 5 min). The $2^{-\Delta\Delta C_q}$ method was adopted to calculate the relative expression. U6 and GAPDH were used as the control.

Western Blot

Primary macrophage extracts were separated with 10% Tris-glycine/SDS-polyacrylamide gels and electrotransferred to Hybond® ECL™ nitrocellulose membranes which were further blocked with 5% nonfat milk and incubated with anti-MATN2 (Abcam, Cambridge, MA, USA) and anti-β-actin (Abcam) antibodies in a 1:1,000 dilution. Horseradish peroxidase-conjugated second anti-mouse IgG (Cell Signaling Technology, Danvers, MA, USA) was adopted to develop signal with an ECL system (GE Healthcare Life Sciences, Little Chalfont, UK).

Luciferase Reporter Assay

Luciferase reporter assay was utilized to confirm the direct binding of miR-202-5p with MATN2. The wild or mutant type of the 3'-untranslated region (3'-UTR) of MATN2 was inserted into the pGL3 promoter vector (Invitrogen). Then, the wild or mutant type of 3'-UTR of MATN2 and miR-202-5p mimic or miR-202-5p inhibitor were transfected into HEK293T cells with Lipofectamine 2000. Subsequently, the Dual-Luciferase Reporter Assay System (Promega Corp., Madison, WI, USA) was applied to measure luciferase activities.

Statistical Analysis

Data are presented as the mean ± standard deviation (SD) and analyzed with the Student *t* test or one-way ANOVA analysis followed by a Tukey post hoc test. *p* < 0.05 was considered to be statistically significant.

Results

Highly Expressed MiR-202-5p in AR Patients

The participants were classified into two groups: AR patients (*n* = 30) and healthy controls (*n* = 10). The miR-202-5p level was assayed in mucosal tissues via qRT-PCR. The higher expression of miR-202-5p was identified in AR patients when compared with the normal tissues (Fig. 1a, *p* < 0.01). After sorting with FACS (Fig. 1b), macrophages derived from AR nasal mucus showed an increase of miR-202-5p compared with the healthy controls (Fig. 1c). Moreover, ovalbumin (OVA)-stimulated macrophages (5 mg/mL, 24 h) showed a time-dependent upregulation of miR-202-5p (Fig. 1d). All of these indicated that the aberrant miR-202-5p expression in macrophages might be involved in the recall response of AR.

MiR-202-5p Promotes M2 Polarization

To investigate whether the upregulated miR-202-5p was associated with AR patients with M2 polarization, M2-specific phenotypic and secretory signature was detected. The relative expression of M2-related molecular signature (Arg-1, IL-10, and Fizz-1) is depicted in Figure 2a. The total events (Fig. 2b) and mean fluorescence in-

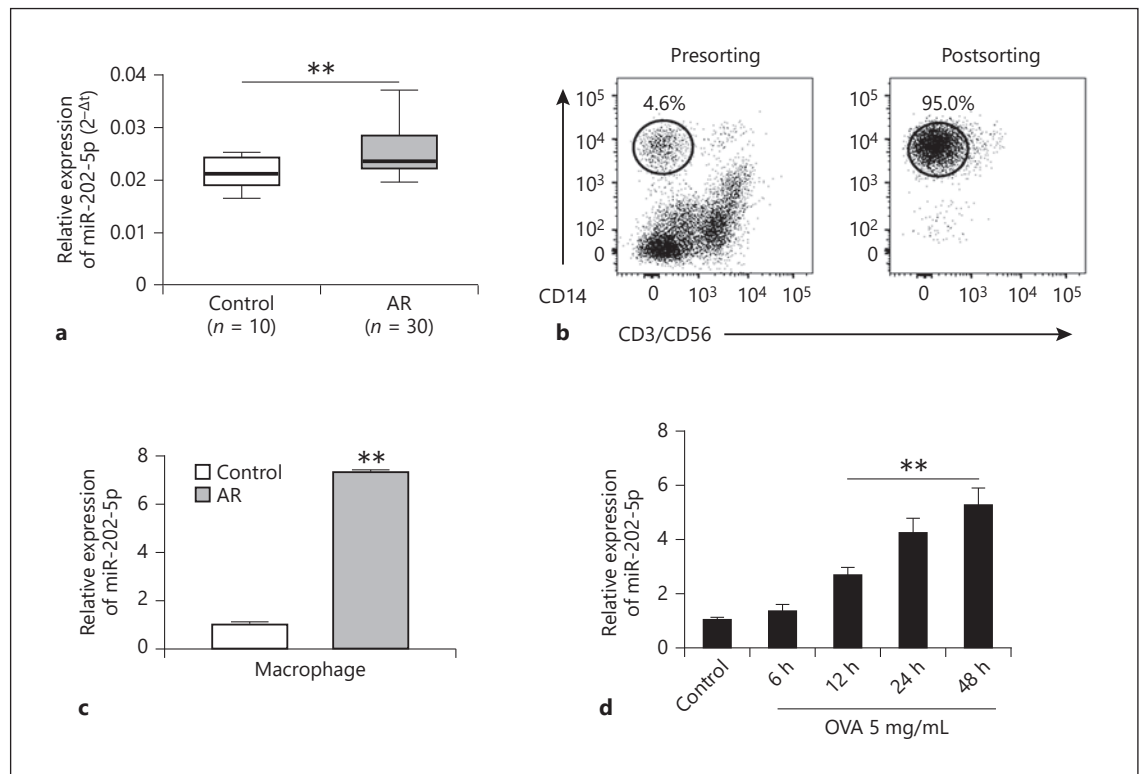


Fig. 1. Highly expressed miR-202-5p in mucosal tissues and macrophages from AR patients. **a** MiR-202-5p expression in mucosal tissues from 30 AR patients and 10 healthy controls were measured by qRT-PCR. Whisker plots (min. to max.). **b** Macrophages of nasal mucus from AR patients or healthy controls were sorted by FACS (gating on CD45+ cells). **c** qRT-PCR analysis of the relative expression of miR-202-5p in macrophages of nasal mucus from

AR patients or healthy controls. **d** The sorted macrophages from healthy controls were treated with OVA (5 mg/mL) for 6, 12, 24, or 48 h. The relative expression of miR-202-5p was determined by qRT-PCR. The data represent the mean $\pm$ SD. Experiments were repeated in triplicate; ** $p < 0.01$. Student t test (**a**, **c**); one-way ANOVA analysis (**d**).

tensity (MFI; Fig. 2c) of CD206 were analyzed by FACS to confirm M2 polarization. All of these data indicated that the macrophages from AR patients showed a typical M2-prone phenotype when compared with healthy controls as all the signatures of the M2 phenotype we detected were upregulated in AR patients. miR-202-5p-overexpressed macrophages exhibited a typical M2 phenotype, characterized by strong induction of Arg-1, IL-10, Fizz-1 (Fig. 2d), and CD206, both in terms of total events of positive cells (Fig. 2e) and MFI (Fig. 2f), compared with the mimics of negative control treated macrophages.

MiR-202-5p Directly Targets MATN2 3'-UTR

miRNA prediction software (TargetScan 7.0) was utilized to search the potential targets of miR-202-5p. The results indicated that Matrilin-2, a protein encoded by the

MATN2 gene, was the potential target of miR-202-5p. The MATN2 3'-UTR region predicted to be complementary to the miR-202-5p seed sequence and mutated MATN2 3'-UTR region were aligned in Figure 3a. By co-transfection with miR-202-5p mimics, miR-202-5p inhibitor or their corresponding negative controls, and MATN2 3'-UTR or the mutant one in HEK293T cells, the luciferase assay showed that upregulation of miR-202-5p significantly decreased the luciferase activity of MATN2. However, miR-202-5p mimics failed to induce the luciferase activity elicited by the reporter carrying the mutant MATN2 3'-UTR. Additionally, miR-202-5p inhibitor significantly increased the luciferase activity of MATN2, but this enhanced effect was abrogated in cells transfected with mutated MATN2 3'-UTR (Fig. 3b). The relative enrichment of MATN2 mRNA in the mRNA/miRNA/AGO2 complex pulled down with biotinylated miR-202-

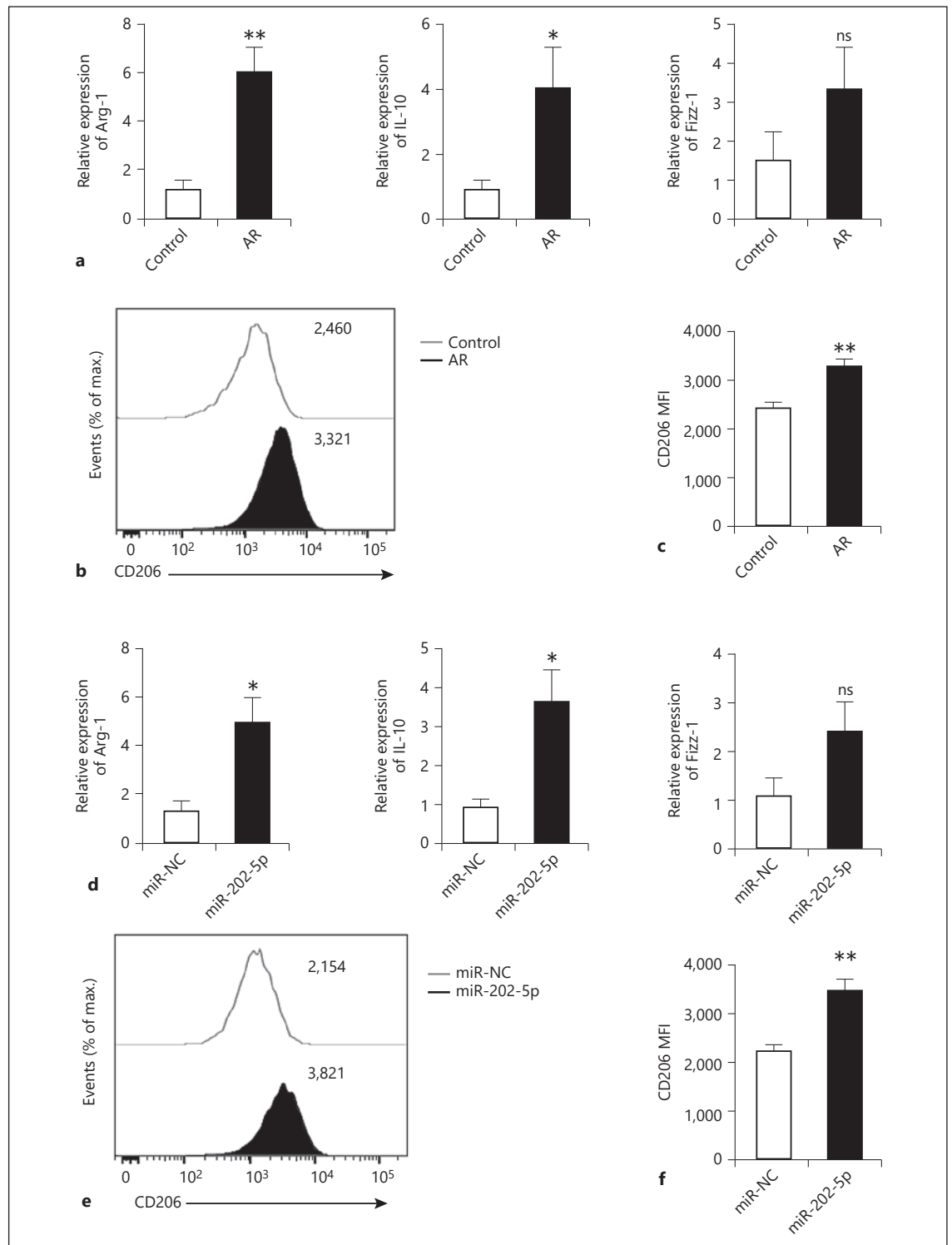


Fig. 2. MiR-202-5p promoted M2 polarization. **a–c** Macrophages of nasal mucus from AR patients or healthy controls were sorted by FACS. qRT-PCR analysis of the relative expressions of IL-10, Arg-1, and Fizz-1 in macrophages (**a**); FACS analysis of CD206 expression on macrophages (**b**), and counted MFI of CD206 (**c**). **d–f** MiR-202-5p was overexpressed in macrophages from healthy

donors and the mRNA was extracted. The relative expressions of IL-10, Arg-1, and Fizz-1 were analyzed by qRT-PCR (**d**); CD206 expression was analyzed by FACS (**e, f**). The data represent the mean $\pm$ SD. Experiments were repeated in triplicate; * $p < 0.05$; ** $p < 0.01$. Student t test.

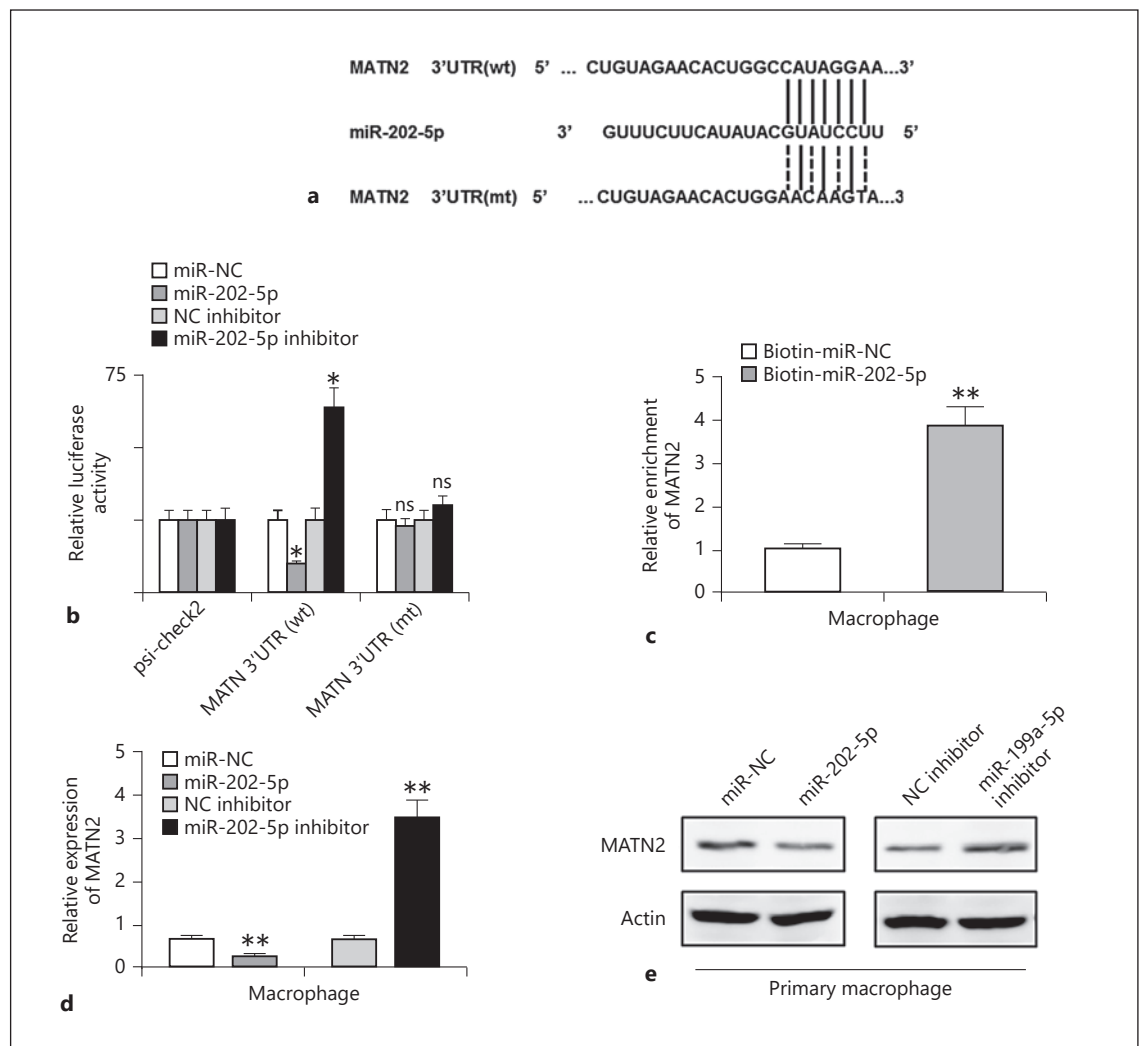


Fig. 3. MiR-202-5p directly targeted MATN2. **a** Schematic of predicted wild-type or mutated miR-202-5p binding sites in the 3'UTR of MATN2. **b** Luciferase reporter assays of HEK293T cells cotransfected with constructed luciferase reporter vectors (MATN2 3'UTR [wt] and MATN2 3'UTR [mt]) and miR-202-5p and miR-202-5p inhibitor or their negative control (miR-NC, NC

inhibitor). **c** MATN2 mRNA detection in biotinylated miRNA/target mRNA complex. **d, e** The relative expression of MATN2 in the sorted macrophages transfected with miR-202-5p mimics or miRNA inhibitors was detected by qRT-PCR and Western blot. The data represent the mean $\pm$ SD. Experiments were repeated in triplicate. One-way ANOVA analysis (**b**); Student *t* test (**c, d**).

5p was significantly higher in AR macrophages than those of the control (Fig. 3c). Furthermore, the effect of miR-202-5p on endogenous MATN2 mRNA and protein expression was determined. Overexpressed miR-202-5p consistently reduced MATN2 mRNA and protein expression, whereas miR-202-5p inhibition increased MATN2 mRNA and protein expression (Fig. 3d, e). All of these data indicated that miR-202-5p negatively regulated MATN2 expression.

MATN2 Downregulates in Mucosal Tissue and Macrophages from AR Patients

qRT-PCR and Western blot were utilized to determine the relative expression of MATN2 in mucosal tissues (Fig. 4a) and sorted macrophages (Fig. 4b, c) from nasal mucus, which showed consistent downregulation of MATN2 in AR patients compared to healthy controls. Moreover, MATN2 expression was also determined in the OVA-stimulated macrophages. As expected, OVA treatment led to a time-dependent decrease in the levels

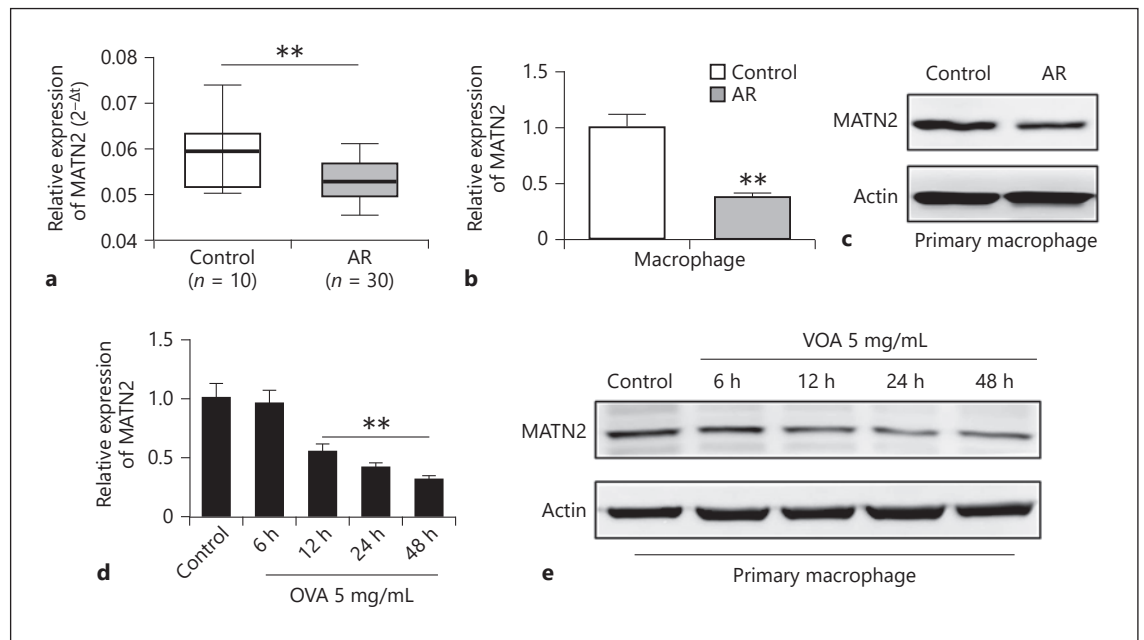


Fig. 4. MATN2 was downregulated in mucosal tissues and macrophages from AR patients. **a** MATN2 expression in mucosal tissues from 30 AR patients and 10 healthy controls were measured by qRT-PCR. Whisker plots (min. to max.). **b, c** Relative expression of MATN2 in sorted macrophages of nasal mucus from AR patients or healthy controls was analyzed with qRT-PCR and West-

ern blot. **d** The sorted macrophages from healthy controls were treated with OVA (5 mg/mL) for 6, 12, 24, or 48 h. The expression of MATN2 was determined by qRT-PCR and Western blot. The data represent the mean $\pm$ SD. Experiments were repeated in triplicate. * $p < 0.05$; ** $p < 0.01$. Student *t* test (**a, b**); one-way ANOVA analysis (**c**).

of expression of MATN2 mRNA (Fig. 4d) and protein (Fig. 4e).

MATN2 Reverses MiR-202-5p-Mediated M2 Polarization

Since miR-202-5p could promote M2 polarization and MATN2 could be regulated by miR-202-5p, whether the upregulated MATN2 could reverse the M2 phenotype induced by miR-202-5p was investigated. Following PcDNA3.1-MATN2-induced overexpression, the relative expression of Arg-1 and IL-10 (Fig. 5a) were inhibited in macrophages from the nasal mucus of AR patients, and CD206 was decreased both in terms of total events (Fig. 5b) and MFI (Fig. 5c) by FACS analysis. The enhanced effects of miR-202-5p on M2-type molecules release (Fig. 5d) and surface CD206 marker expression (Fig. 5e, f) were inhibited by MATN2 overexpression. These data indicated that miR-202-5p promoted an allergic M2 phenotype switch at least in part through inhibition of the MATN2 signaling pathway.

Discussion

This investigation indicates that highly expressed miR-202-5p in mucosal tissues and macrophages from AR patients can promote M2 polarization. Luciferase reporter assay testifies that MATN2 could be targeted by miR-202-5p and is downregulated in macrophages from AR patients, which can reverse miR-202-5p-mediated M2 phenotype in AR.

The alternative pathway induced by Th2-producing cytokines (IL-4 and IL-13) for macrophage activation can induce M2 macrophages, which express low levels of MHC class II and CD86 and high levels of CD206, Ym-1, Fizz-1, and Arg-1. It is worth noting that M2 macrophages can release IL-4 and IL-13 (Th1-antagonizing cytokines) to further promote the differentiation of Th2 and class switch of IgE.

miRNAs are testified to regulate cell activation, proliferation, and differentiation processes [15, 16]. It is worth noting that the miRNA regulation network is complex, since one miRNA can regulate hundreds of genes and one gene can be targeted by multiple miRNAs. At the same

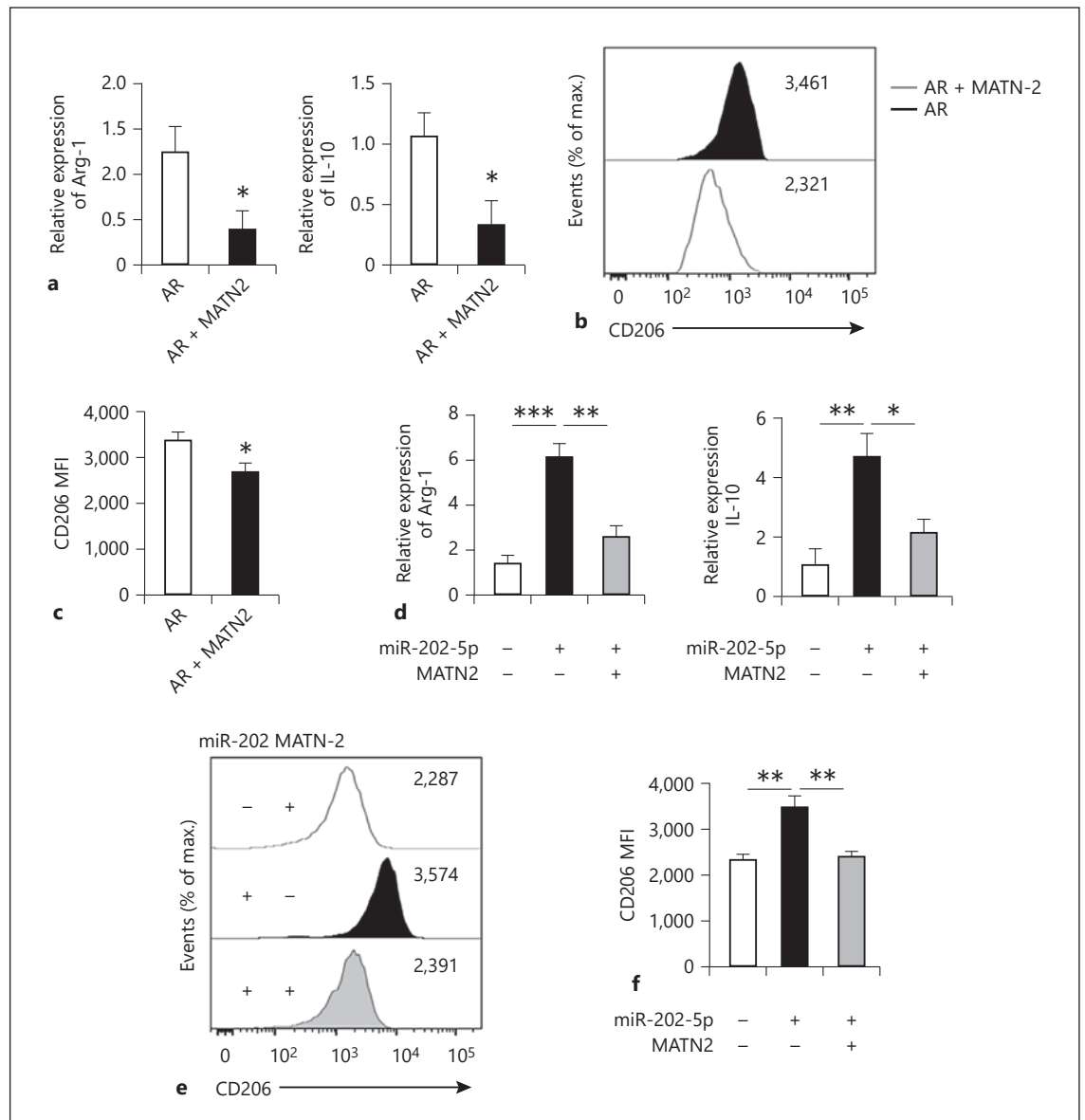


Fig. 5. MATN2 reversed MiR-202-5p-mediated M2 phenotype in AR. **a–c** MATN-2 was overexpressed in macrophages from the nasal mucus of AR patients and the mRNA was extracted. The relative expressions of IL-10 and Arg-1 were analyzed by qRT-PCR (**a**), while CD206 expression was analyzed by FACS (**b, c**). **d–f** Macrophages of nasal mucus from healthy donors were sorted. MiR-202-

5p and MATN-2 were overexpressed alone or together. The mRNAs of IL-10 and Arg-1 in macrophages were analyzed by qRT-PCR (**d**); FACS analysis of CD206 expression in macrophages from each group (**e**), and counted MFI of CD206 (**f**). The data represent the mean $\pm$ SD. Experiments were repeated in triplicate; * $p < 0.05$; ** $p < 0.01$. Student *t* test (**a, c**); one-way ANOVA analysis (**d, f**).

time, miRNA can also affect DNA methylation and histone acetylation processes to regulate gene expression globally [17, 18]. Therefore, well-positioned miRNAs that may regulate AR need further investigation.

In this study, miR-202-5p was testified to promote M2 polarization, which was clarified to be actively involved

in the regulation of AR in children [19]. Comparative genomics analysis of bone marrow-derived macrophages in Balb/c mice shows upregulated mmu-miR-202-3p in polarized M1 compared with M2, although such a result has not been further testified by RT-qPCR [20]. The inconsistency of in vivo and in vitro results may be due to the

transcriptional diversity, or other variables need to be further investigated.

Recent investigations indicate that extracellular matrix molecules, such as versican [21], biglycan [22, 23], and hyaluronan [24], can act as damage-associated molecular pattern molecules, which can be released following tissue stress or damage and stimulate macrophage/microglia activation [25]. In this study, noncollagenous extracellular matrix protein MATN2 was testified to be regulated by miR-202-3p and can reverse miR-202-3p-induced M2 phenotype polarization. Neuron-macrophage coculture results also indicate that exogenous MATN2 could specifically signal through toll-like receptor-4 and directly induce macrophage proinflammatory molecule expression and promote axonal damage [26]. We hypothesize that MATN2 might function as a proinflammatory molecule or damage-associated molecular pattern to induce proinflammatory molecule expression in macrophages, and miR-202-5p could inhibit the expression of MATN2 and promote the differentiation of M2 macrophages involved in AR.

Conclusions

MiR-202-5p could promote M2 polarization in AR by targeting MATN2.

Acknowledgments

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Statement of Ethics

The investigation was approved by the Ethics Committee of the Department of Otorhinolaryngology Head and Neck Surgery, Yuhuangding Hospital. All patients provided signed written consent forms before enrollment and agreed to the anonymous data utilization policy.

Disclosure Statement

The authors have no conflicts of interest to declare.

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