



Circ_0067835 regulates allergic inflammatory response in type-2 innate lymphoid cells in allergic rhinitis (AR) via miR-155/GATA3

Xunshuo Jiang¹ · Taojian Huang¹ · Hongbing Liu¹ · Xubo Chen¹ · Hao Zhang¹ · Ke Liu¹ · Jianjian Deng¹ · Chunping Yang¹

Received: 16 December 2020 / Accepted: 9 April 2021
© Japan Human Cell Society 2021

Abstract

Allergic rhinitis (AR) is a familiar respiratory allergic inflammatory disease with higher incidence. The pathogenesis of AR is particularly complex. Therefore, a lot of work is acquired to excavate deep mechanisms, thereby providing effective strategies for AR diagnose and treatment. AR mice model was induced by recombinant murine IL-33 (0.05 µg/µl) on days 1, 3, and 5. The lentiviral vectors carrying si-circ_0067835, miR-155 mimic, si-NC or miR-NC were injected into AR mice. Thus, mice were divided into control, AR, AR + si-NC, AR + si-circ_0067835, AR + si-circ_0067835 + miR-NC, and AR + si-circ_0067835 + miR-155 mimic groups. qRT-PCR experiment was used to measure the expression of circ_0067835 and miR-155. Behavioral test result was quantified to assess AR mice model. Hematoxylin and eosin (HE) staining was performed to analyze histopathological changes. Helper T cell 2 (Th2) cytokines (IL-4, IL-5, IL-9 and IL-13) and percentage of type-2 innate lymphoid cells (ILC2s) in nasal mucosa tissues in AR mice model were evaluated needing western blot, ELISA, and flow cytometry. Besides, the targeting relationship between circ_0067835 and miR-155, or between miR-155 and GATA3, was investigated via luciferase report assay. Circ_0067835 expression levels were raised in the nasal mucosa tissues of AR mice. Inhibiting circ_0067835 could reduce Type2 cytokines and ILC2s levels in AR mice model. Furthermore, circ_0067835 targeted and positively regulated miR-155 expression, and GATA3 was a downstream target of miR-155 and adjusted by circ_0067835/miR-155 axis. In addition, silencing circ_0067835 inhibited cytokines and ILC2s levels by down-regulating miR-155. Circ_0067835 effectively inhibited AR response in ILC2s through participation of miR-155/GATA3 axis.

Keywords Allergic rhinitis (AR) · Type-2 innate lymphoid cells · Circ_0067835 · MiR-155 · GATA3 axis · Inflammatory factors

Introduction

Allergic rhinitis (AR) is a common respiratory allergic inflammatory disease with higher incidence in adults and children [1, 2]. It affects approximately 40% of the world's population and is increasing annually [3]. The main clinical symptoms of AR include nasal itching, sneezing, runny watery nose, and nasal congestion [4]. Although AR cannot directly harm the health of patients, the complications

caused by AR, including asthma, rhinosinusitis, secretory otitis media, and other upper and lower airway diseases, bring great inconvenience to patients and lower their quality of life [5]. AR is an allergen-activated inflammatory disease of the nose mediated by immunoglobulin (Ig) E [6]. Currently, the treatment methods for AR include drug therapy with slow research progress, the most effective immunotherapy in clinical practice, and active and voluntary avoidance of allergens [7]. Therefore, it is significantly important to find out fast and effective treatment strategies of AR.

The activation of Helper T cell 2 (Th2) cells are considered as the most important mechanism involved in both innate and adaptive AR [8]. Innate immune cells are a kind of important body homeostasis regulation factors. As “mirror” cells of innate immune, Group 2 innate lymphoid cells (ILC2s) secreted a variety of Type2 factors involved in multiple of allergic inflammatory pathologic processes.

Xunshuo Jiang and Taojian Huang are co-first authors.

✉ Chunping Yang
yep406@sohu.com

¹ Department of Otorhinolaryngology Head and Neck Surgery, The Second Affiliated Hospital of Nanchang University, No.1 Minde Road, Nanchang 330006, Jiangxi, China

Non-coding RNAs (ncRNAs) are a category of rich RNAs, including circular RNA (circRNA), long ncRNA (lncRNA), and microRNA (miRNA) [9]. ncRNAs are important regulatory factors of gene expression, which participate in nosogenesis of human diseases [10]. For the last few years, it has been found that different types of ncRNAs have different function, but are correlated with each other. ncRNAs may play a key role in the occurrence and development of allergic diseases. Although researches regarding ncRNAs in AR have been reported, these studies principally focused on lncRNA and miRNA [11–13]. However, a body of recent evidence has indicated that circRNAs are involved in AR [14]. In this study, circ_0067835 was found to be abnormally expressed in AR. CircRNAs function as a sponge of miRNA and bind with miRNAs to inhibit effects of miRNAs. MiR-155 was observed to be a downstream target of circ_0067835. Furthermore, our preliminary work has verified that miR-155 expression and its function in AR [15].

In this study, IL-33 treatment was utilized to establish an AR mice model. We explored the functional effects and mechanisms of circ_0067835 in ILC2s cells in AR. The results illustrated that circ_0067835 expression was up-regulated, and circ_0067835 sharpened inflammatory response through positively regulating miR-155.

Materials and methods

Mice and groups

Specific Pathogen-Free (SPF) female BALB/c mice purchased from Hunan SJA Laboratory Animal Co. Ltd (Hunan, China) were maintained in horizontal laminar flow cabinets of the Second Affiliated Hospital of Nanchang University (Nanchang, China) and supplemented with abundant and sterile food and water. After mice were balanced, they were randomly assigned into six groups and there was 6 mice in each group, including control (normal mice treated with saline), AR (AR mice treated with saline), AR + si-NC (AR mice transfected with si-NC), AR + si-circ_0067835 (AR mice transfected with si-circ_0067835), AR + si-circ_0067835 + miR-NC (AR mice transfected with si-circ_0067835 and miR-NC), and AR + si-circ_0067835 + miR-155 mimic (AR mice transfected with si-circ_0067835 and miR-155 mimic) groups. The animal usage protocols were approved in advance by the Animal Care and Use Committees of the Second Affiliated Hospital of Nanchang University.

AR mice model

The mice were simulated by intranasal injection of saline (20 μ L) containing IL-33 (1 μ g) on days 1, 3, and 5 to establish AR model [15].

Cell culture and treatment

The control group of mice was subjected to ether anesthesia. The mice nasal epithelial cell (NEpC) was isolated from the mice, digested by collagenase I at 37 °C for 10 h, and cultured in Dulbecco's modified Eagle medium (DMEM, Gibco, Grand Island, NY) containing 10% fetal bovine serum (FBS, Gibco). NEpC cell was cultured in a humidified incubator at 37 °C with a 5% CO₂ atmosphere.

Cell fractionation assay

Cytoplasmic and Nuclear RNA Purification Kit (Invitrogen, Carlsbad, CA, USA) was used for the collection of cytoplasmic and nuclear RNA. Briefly, NEpC cells were harvested and incubated with lysis solution for 10 min. The supernatant was collected for cytoplasmic RNA and the nuclear pellet was used for nuclear RNA extraction. GAPDH was used as the cytoplasmic endogenous control. The U6 small nuclear RNA was used as the nuclear endogenous control.

Preparation and administration of lentiviral vectors

Small interfering RNA (siRNA) targeted circ_0067835 (si-circ_0067835) and miR-155 mimic were constructed by GenePharma (Shanghai, China). The si-circ_0067835, miR-155 mimic or their respective control (si-NC or miR-NC) was intranasally injected into mice on days 6–12. The samples carrying with lentiviral vectors were collected after 24 h. The sequences of siRNAs and miRNAs were listed as follows. si-circ_0067835-1: 5'-GTG ATA AAA CTG GGA ATT AGT-3', si-circ_0067835-2: 5'-TGT GAT AAA ACT GGG AAT TAG-3', miR-155 mimic: sense: 5'-UUA AUG CUA AUU GUG AUA GGG GU-3', anti-sense: 5'-CCC UAU CAC AAU UAG CAU UAA UU-3', miR-NC: 5'-UCU ACU CUU UCU AGG UUG UGG-3'.

Symptom score

30 min after the final challenge on day 5, a number of behavioral experiments were performed on AR mice according to previous research [16]. According to number of frequency of rubbing and sneezing, scores were calculated as follows: 1—slightly wipes for several times or sneezes less than three times; 2—repeatedly wipes or sneezes more than three times and less than ten times; 3—constantly wipes from nose to face or sneezes more than ten times. AR model was recognized to be successful when

scores were more than 5, and the rest were excluded from further study. The behavioral experiments were performed within 30 min on day 12.

Quantitative reverse transcription polymerase chain reaction (qRT-PCR)

The RNAs from normal control group and various experimental groups were gained through TRIzol reagent (Invitrogen) after mice were euthanized. The collected RNAs could be reserved in the -80°C environment for following detection. The concentration of RNA was confirmed under an ABI RISM7500 system (Applied Biosystems, Carlsbad, CA, USA). The qRT-PCR process was implemented through SYBR ExScript RT-qPCR Kit (Applied Biosystems, Foster City, CA, USA) to determine gene expression status. U6 and GAPDH were, respectively, defined as internal reference of circRNA and miRNA. Endly, and data were analyzed adopting $2^{-\Delta\Delta\text{Ct}}$ method.

Enzyme-linked immunosorbent assay (ELISA)

The blood from the orbital venous plexus of anesthetized mice was gathered and placed at room temperature for 30 min, as well as centrifuged at 2000 rpm/min for 10 min at 4°C to obtain upper layer of the serum. Type2 cytokines-associated IL-4, IL-5, IL-9, and IL-13 concentration was quantified through their respective ELISA kits (from R&D Systems, Minneapolis, MN, USA), according to instruction of the product.

Western blot

Protein samples were collected from nasal mucosal tissues through RIPA lysis buffer, and every 60 μg protein extraction was separated on 12% sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE). After separation of proteins, they were transferred onto nitrocellulose membranes. The membranes were blocked with 5% non-fat milk diluted in Tris-Buffered Saline and Tween-20 (TBST, Beyotime Biotech, Shanghai, China) at room temperature for 2 h and incubated with primary antibodies at 4°C overnight, including IL-4 (MM450C, Invitrogen, Thermo Fisher Scientific, Waltham, MA, USA), IL-5 (MM550D), IL-9 (MA5-23,789), IL-13 (MA5-23,735), GATA3 (PA5-20,892), and β -actin (MA5-15,452). Subsequently, redundant unbound primary antibody was eluted using TBST. After eluting three times, the membranes were incubated with matched secondary antibody (goat anti-rabbit, A16099, Invitrogen), and then, they were again washed three times with TBST. The positive protein signals on membranes were captured utilizing an enhanced chemiluminescence detection kit (Thermo Fisher Scientific Inc.) and data analysis was conducted applying Image J software (NIH, Bethesda, MD, USA).

Histological analyses

Mice were stimulated one last time and killed under conditions of ease. The heads of mice were taken down and immediately fixed in 4% paraformaldehyde (Sigma-Aldrich, St. Louis, MO, USA) at 4°C for 24 h. Then, acquired tissue samples were dehydrated in a series of increasing concentrations of ethanol and finally embedded in paraffin. Nasal mucosa tissues were cut into 4 μm thick sections and stained

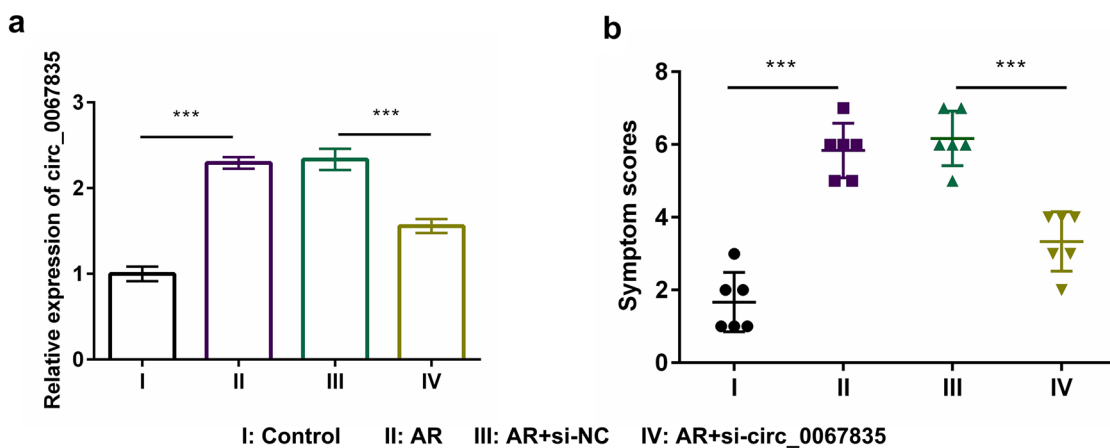


Fig. 1 circ_0067835 expression was detected in AR mice nasal mucosa tissues. AR mice model was induced by recombinant murine IL-33 (0.05 $\mu\text{g}/\text{ul}$) on days 1, 3, and 5. The lentiviral vectors carrying si-circ_0067835 or si-NC were injected into AR mice. Thus, mice were divided into Control, AR, AR+si-NC and AR+si-

circ_0067835 groups. **a** Relative expression of circ_0067835 and **(b)** symptom scores were monitored exploiting qRT-PCR and behavioral tests. Data shown represent mean $\pm$ SD from three independent experiments, $n=6$. ANOVA followed by Tukey's test. *** $P<0.001$

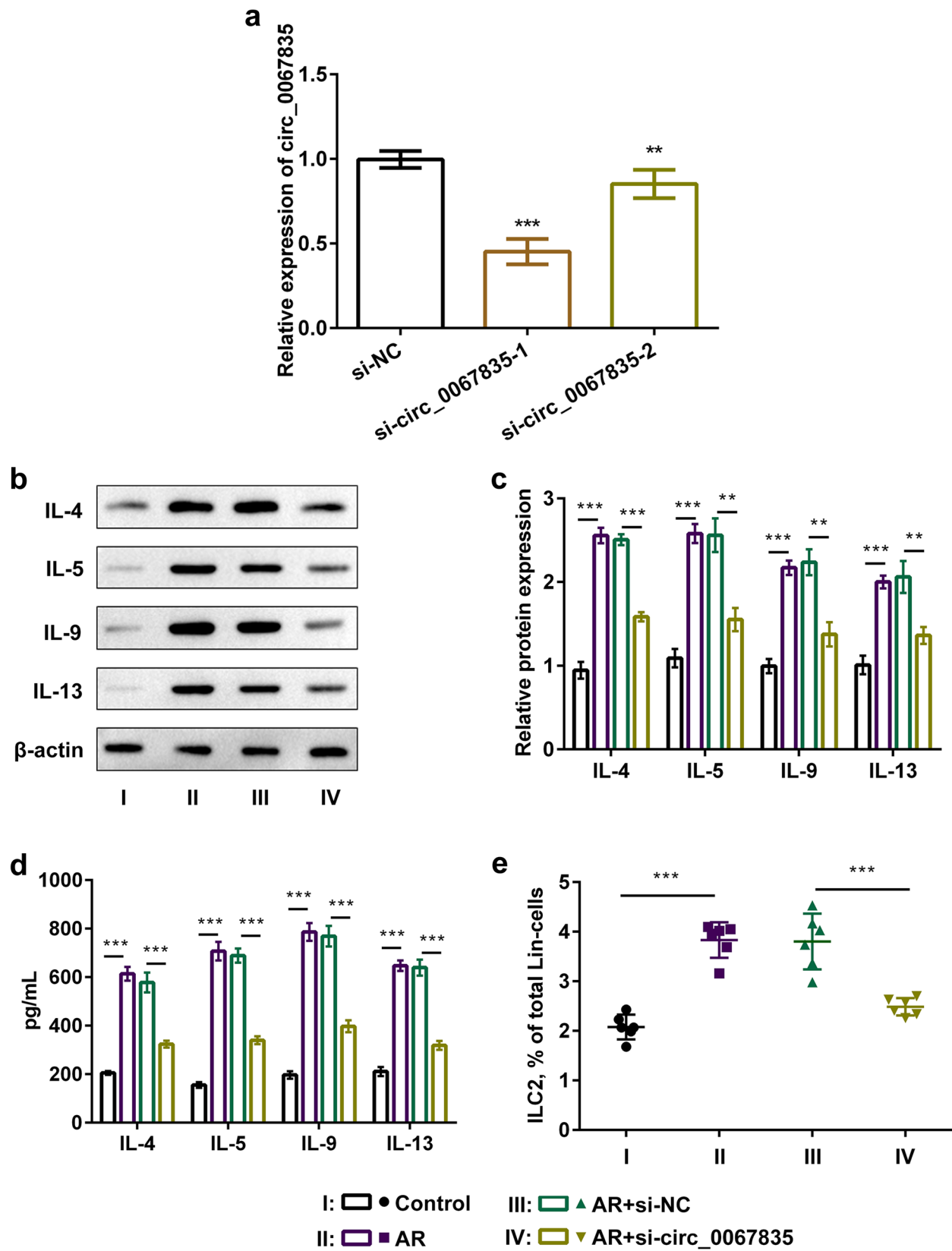


Fig. 2 Inhibiting circ_0067835 expression had an influence in Type 2 cytokine levels in AR mice. AR was induced by recombinant murine IL-33 (0.05 µg/ul) on days 1, 3, and 5. The lentiviral vectors carrying si-circ_0067835 or si-NC were injected into AR mice. Thus, mice were divided into control, AR, AR+si-NC and AR+si-circ_0067835 groups. **a** qRT-PCR assay showed the effect of the siRNA transfection on si-circ_0067835 expression. **b–c** IL-4, IL-5, IL-9 and IL-13 protein expression, **(d)** IL-4, IL-5, IL-9 and IL-13 supernatant concentration and **(e)** ILC2s level were detected exploiting western blot, ELISA and flow cytometry experiments. Data shown in A–B represent mean ± SD from three independent experiments, $n=3$. Data shown in B–E represent mean ± SD from three independent experiments, $n=6$. ANOVA followed by Tukey's test. $**P<0.01$ and $***P<0.001$

with hematoxylin and eosin (HE) for histopathological examination.

Flow cytometry analysis for ILC2s

The collected nasal mucosal cell suspension was incubated using following labeled antibodies at 4 °C for 30 min to test ILC2s expression. The lineage cocktail antibodies were labeled by PerCP-CyTM 5.5, including CD3e, CD11b, CD45R, Ly-76, and Ly-6C. In addition, CD45, KLRG1, and CD90.2 antibodies were, respectively, labeled by FITC, PE, and APC. It has been confirmed that the markers of ILC2s are $^{\text{CD45}^+}$ KLRG1⁺ and CD90.2⁺ in mice. Above all adopted antibodies were purchased from BD Bioscience (Franklin Lakes, NJ, USA). The number of cell and analysis of data were accomplished by FACSJazzTM (BD Biosciences, Franklin Lakes, NJ, USA) and FACSDiva software (BD Biosciences, Franklin Lakes, NJ, USA). The ratio of ILC2s in marker-positive cells and Lin⁻ cells was exhibited in mice.

Luciferase reporter assay

The targeted association between circ_0067835 and miR-155, as well as the association between miR-155 and GATA3 were predicted by circular RNA Interactome and validated using luciferase reporter assay. For the construction of luciferase reports, the sequence of circ_0067835 binding with miR-155 was inserted into pGL4 vector (Promega Corp., Madison, WI, USA) to form wide type of circ_0067835 (circ_0067835-WT), and this sequence of circ_0067835 was mutated and inserted into pGL4 vector to obtain mutant type of circ_0067835 (circ_0067835-MUT). Accordingly, the sequences of GATA3 3'-untranslated region (3'-UTR) binding with miR-155 was inserted into pGL4 vector (Promega Corp., Madison, WI, USA) to form wide type of GATA3 (GATA3-WT), and this sequence of GATA3 was similarly mutated and inserted into pGL4 vector to obtain mutant type of GATA3 (GATA3-MUT). MiR-155 mimic and its control (miR-NC) were severally transfected into HEK-293

cells with luciferase reporter vectors utilizing Lipofectamine 3000. After transfection of 48 h, luciferase activity was evaluated using a Dual Luciferase Reporter Assay Kit (Vazyme).

RNA immunoprecipitation (RIP) assay

NEpC cells (1×10^7) were lysed in the RIP lysis buffer and then subjected to RIP assay utilizing the EZ-Magna RIP Kit (Merck Millipore, Darmstadt, Germany) and AGO2 antibody (Cell Signaling Technology, Beverly, MA, USA). The negative control immunoglobulin G (IgG) (Cell Signaling Technology) was used as a control group. Cell lysate was incubated with bead-antibody complexes overnight at 4 °C. After isolating the RNA from protein–RNA-immunoprecipitated complex using Proteinase K, the RNA samples were subjected to qRT-PCR detection.

RNA pull down assay

The miR-155 sequences covering circ_0067835 or GATA3 target sites (wild type and mutant type) were biotin-tagged into Bio-miR-155-WT/MUT probes. Cell lysates were mixed with probes and beads overnight, and then, the retrieved RNA mixture was detected utilizing qRT-PCR assay.

Statistical analysis

All data processing was carried out via exploiting GraphPad Prism 6 (GraphPad Inc., La Jolla, CA, USA), and data were expressed as a manner of mean ± standard deviation (SD). The significance of difference relied on results of *student's t* test or ANOVA test followed by Tukey's test. $P<0.05$ denoted that the difference was statistical significant.

Results

The circ_0067835 expression was high in AR mice model

We collected AR mice and normal control mice nasal mucosa tissues, and circ_0067835 levels were examined by qRT-PCR assay. The results of qRT-PCR illustrated an increase of circ_0067835 in AR model ($P<0.001$, Fig. 1a). When si-circ_0067835 was effectively transfected, circ_0067835 expression was significantly declined ($P<0.001$). The behavioral symptom was quantified in different groups of mice, based on above established criteria. Symptom scores were directly proportional to anaphylaxis. The results in Fig. 1b suggested that AR mice had higher scores contrasted with normal mice ($P<0.001$) and si-circ_0067835-transfected AR mice have a decreased scores

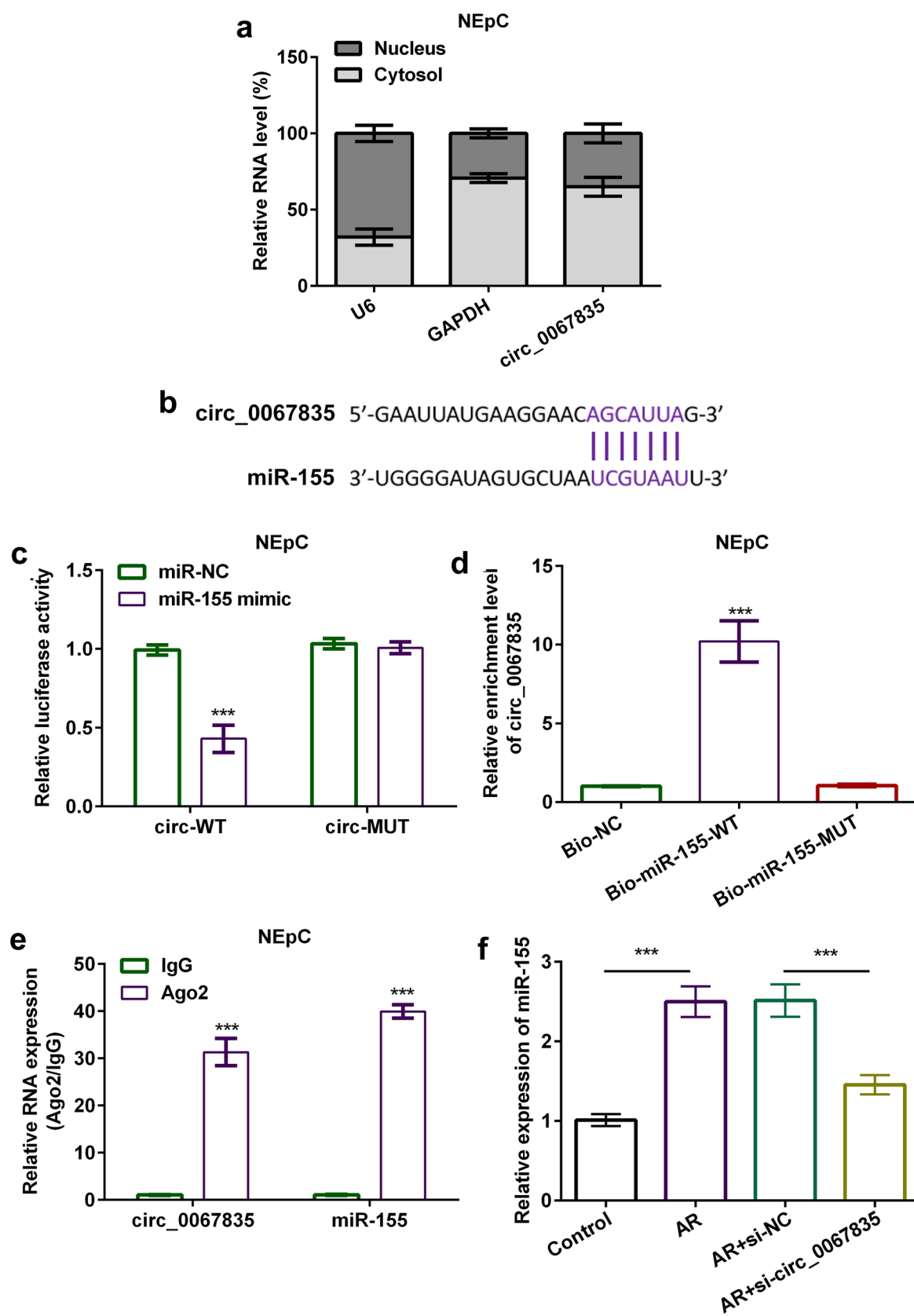


Fig. 3 Circ_0067835 targeted miR-155 and regulated miR-155 expression. **a** Subcellular localization of circ_0067835 in NEPC cells. **b** Combined sequences between circ_0067835 and miR-155 were assumed. **c** pGL4 vectors carrying circ_0067835-WT or circ_0067835-MUT and miR-155 mimic or miR-NC were co-transfected into NEPC cells, relative luciferase activity was inspected exploiting luciferase reporter assay. **d** RNA pull-down assay was applied to verify the enrichment of circ_0067835 in NEPC cells using bio-NC, bio-miR-155-WT or bio-miR-155-MUT probe. **e** Enrichment of circ_0067835 and miR-155 was confirmed using the AGO2-IP assay. **f** AR was induced by recombinant murine IL-33 (0.05 µg/µl) on days 1, 3, and 5. The lentiviral vectors carrying si-circ_0067835 or si-NC were injected into AR mice. Thus, mice were divided into control, AR, AR+si-NC and AR+si-circ_0067835 groups. Relative expression of miR-155 was tested exploiting qRT-PCR. Data shown represent mean ± SD from three independent experiments, $n=3$. ANOVA followed by Tukey's test. *** $P<0.001$

compared with si-NC-transfected AR mice ($P<0.001$). These results implied that inhibiting circ_0067835 expression could alleviate allergic symptom. This meant that circ_0067835 expression was involved in the process of AR.

Inhibiting circ_0067835 expression could remit Th2 cytokine levels in AR mice model

To explore role of circ_0067835 in Type2 immunity, two specific siRNA targeting circ_0067835 was used to reduce its expression in mice. As shown in Fig. 2a, si-circ_0067835-1 had a higher silencing efficiency than si-circ_0067835-2 in mice ($P<0.01$ or $P<0.001$). Next, we monitored the expression of Type2 cytokines (IL-4, IL-5, IL-9, and IL-13) in the next experiments. In Fig. 2b, c, protein expression levels of four cytokines were assessed by western blot experiment. It was found that AR induced four cytokines protein expression, while circ_0067835 silence inhibited their protein expression ($P<0.01$ or $P<0.001$). We also detected concentration of above four inflammatory factors in serum samples. The trends of concentration of cytokines were in keeping with protein expression in Fig. 2d (all $P<0.001$). As shown in Fig. 2e, we observed that the proportion of ILC2s in AR mice nasal mucosa tissues was elevated, compared with normal mice ($P<0.001$), but the silence of circ_0067835 could partly ease the ratio of ILC2s in AR mice ($P<0.001$). In all, the inhibition of circ_0067835 expression could relieve Th2 cytokine and ILC2s levels in AR mice nasal mucosa tissues.

Circ_0067835 targeted miR-155 and positively regulated miR-155 expression

To investigate the ceRNA mechanism of circ_0067835 in allergic rhinitis, cell fractionation assay was performed to confirm the subcellular localization of circ_0067835. It was found that circ_0067835 was expressed in both cytoplasm and nucleus of NEPC cells, and the expression level of

circ_0067835 was higher in cytoplasm (Fig. 3a). MiR-155 was searched for the downstream target of circ_0067835 through exploiting circular RNA Interactome. The predicted binding sequences were seen in Fig. 3b. To confirm the correction between circ_0067835 and miR-155, relative luciferase activity was analyzed via luciferase reporter assay. As shown in Fig. 3c, the co-transfection of circ_0067835-WT and miR-155 mimic dramatically weakened luciferase activity, compared with co-transfection of circ_0067835-WT and miR-NC ($P<0.001$). However, luciferase activity remained unchanged regardless of co-transfection with circ_0067835-MUT and miR-155 mimic or miR-NC ($P>0.05$). The result of RNA pull-down experiment also revealed that there was a binding complex between circ_0067835 and miR-155, as indicated by the circ_0067835 expression in the bio-miR-155-WT group, instead of bio-miR-155-MUT group ($P<0.001$, Fig. 3d). In addition, RIP assay was carried out to study the molecular interplay among circ_0067835 and miR-155 in NEPC cells. AGO2, a key protein in RNA-induced silencing complex (RISC) formed by circ_0067835 and miR-155, was captured by anti-AGO2 antibody. Both circ_0067835 and miR-155 was enriched in NEPC cells ($P<0.001$, Fig. 3e). These results indicated that miR-155 served as a downstream target of circ_0067835. MiR-155 expression levels were assessed in AR and si-circ_0067835-transfected mice nasal mucosa tissues. The results in Fig. 3f hinted that, AR promoted miR-155 expression, as well as transfection si-circ_0067835 decreased miR-155 expression (both $P<0.001$). This explained that there existed a positive regulatory relationship between circ_0067835 and miR-155. In a word, circ_0067835 targeted miR-155 and positively regulated miR-155 expression.

Circ_0067835 silence could relieve the level of Type2 cytokines through mediating the down-regulation of miR-155

For the functional mechanism of miR-155 in AR mice, we applied miR-155 mimic to transfect into AR mice. The results of symptom score in Fig. 4a declared that transfection of miR-155 mimic elevated symptom score decreased by circ_0067835 silence ($P<0.05$ or $P<0.001$), which implicated that circ_0067835 inhibition could mitigate allergic symptom through down-regulating miR-155 in AR mice. Afterwards, HE staining was performed on nasal mucosal tissues. The results of Fig. 4b showed that AR mice showed pathological changes in nasal mucosa, including hyperemia, edema and necrosis. There were normal histological structures in the control group with regular goblet cells in the epithelium (blue arrows indicate). Few eosinophilic and inflammatory cells were observed in the lamina propria. In contrast, the ciliated epithelium was damaged and discontinuous in the AR group. Numerous inflammatory cells,

including eosinophils, were observed in the submucosa (green arrows indicate). However, all of allergic inflammation was improved after circ_0067835 knockdown. Subsequently, after transfection of miR-155 mimic, Th2-secreted cytokines were again assessed in following experiments. Transfection of miR-155 mimic facilitated IL-4, IL-5, IL-9 and IL-13 protein expression inhibited by transfecting si-circ_0067835 in AR mice ($P < 0.01$ or $P < 0.001$, Fig. 4c, d). Moreover, transfection of miR-155 mimic also boosted IL-4, IL-5, IL-9, and IL-13 concentration of reduced by transfecting si-circ_0067835 in serum (all $P < 0.01$, Fig. 4e). In addition, overexpression of miR-155 obviously up-regulated ILC2s expression that was down-regulated by si-circ_0067835 transfection in AR mice ($P < 0.01$, Fig. 4f). These results demonstrated that circ_0067835 silence could relieve the level of inflammatory factors through mediating the down-regulation of miR-155.

GATA3 acted as a downstream target of miR-155 and adjusted by circ_0067835/miR-155 axis

The results of luciferase activity in Fig. 5a proved that GATA3 was a potential downstream target of miR-155, as co-transfection of GATA3-WT and miR-155 mimic lightened luciferase activity, contrasted with co-transfection of GATA3-WT and miR-NC ($P < 0.001$), and nothing has any change between co-transfection of GATA3-MUT and miR-155 mimic group and GATA3-MUT and miR-NC ($P > 0.05$). Subsequent data of RNA pull down assay demonstrated that GATA3 could be pulled down by biotinylated miR-155-WT but not by biotinylated miR-155-Mut ($P < 0.001$, Fig. 5b). Moreover, the results of RIP assays displayed that miR-155 and GATA3 was enriched by AGO2 antibody in NEpC cells ($P < 0.001$, Fig. 5c). Therefore, we guessed that GATA3 was regulated by circ_0067835/miR-155 axis. It was attested that the above assumption was correct. The results in Fig. 5d displayed that AR promoted GATA3 protein expression, and silence of circ_0067835 repressed GATA3 protein expression, as well as miR-155 mimic transfection increased GATA3 protein expression ($P < 0.05$, $P < 0.01$ or $P < 0.001$). In short, as a downstream target of miR-155, GATA3 was regulated by circ_0067835/miR-155 axis.

Discussion

AR is characterized by accumulated Type2 cytokines production. It was convinced that ILC2s was accumulated in the peripheral blood of AR patients [17]. On the other hand, ILC2s have been discovered to involve in Type2 immune response in AR individuals, secreting Type2 cytokines (IL-4, IL-5, IL-9 and IL-13) [18]. Our understanding of function of ILC2s provided potential therapeutic targets for

AR. In this work, our goal was finding one or more targets of decreasing ILC2s level or Type2 cytokines, to achieve the improvement or treatment of AR. First of all, we used IL-33 simulation to establish AR model, referring to a previous article [15]. From our experimental results, we further demonstrated that AR promoted the percentage of ILCs and Type2 cytokines (IL-4, IL-5, IL-9, and IL-13). It was a surprise that circ_0067835 expression was up-regulated in AR model. Furthermore, circ_0067835 silence could lower the level of ILCs and Type2 cytokines by means of down-regulation of miR-155.

Recent studies have uncovered that abnormal expression of miRNA is thought to participate in the pathogenesis of AR, including the differentiation of immune cells and Type2 cytokines levels [19, 20]. For instance, the expression of miR-202-5p was significantly increased in peripheral blood mononuclear cells and mucosal tissues during AR when compared with healthy control. MiR-202-5p obviously improved IL-4 and IL-17 expression, but reduced regulatory T cells (Tregs)-related cytokines (IFN- γ , IL-10, and TGF- β 1) expression [21, 22]. However, there has a research reported that miR-146a expression level is clearly declined in AR nasal mucosa tissues, and the overexpression of miR-146a exerts its anti-inflammatory role to improve clinical symptoms [23]. In the previous researches, there were a few of studies regarding the expression and function action of miR-155 in AR development. Up-regulated miR-155 occurred in some stimulated immune cells [24]. Moreover, there existed the enrichment of miR-155 and ILC2s in the nasal mucosa tissues of AR patients [25]. Our previous and current studies also further showed that miR-155 expression was up-regulated in nasal mucosa tissues of IL-33-stimulated AR mice.

Abnormal circRNA expression also took part in the occurrence and development of AR. In recent years, the studies about circRNAs in AR were fewer. In ovalbumin-induced AR mice model, circDdx17 expression level was lower, and overexpressing circDdx17 reduced miR-17-5p expression, and IL-4 and IL-5 levels through directly interacting with miR-17-5p [26]. On the side, highly expressed CircHIPK3 could attribute to aggravate nasal symptoms of AR mice [14]. Although circ_0067835 has been known as a promoting-factor in colorectal cancer, and circ_0067835 silence limits cancer cell growth [27], the expression and position of circ_0067835 in AR were not clear yet. Our study unclosed that circ_0067835 expression was up-regulated in AR mice nasal mucosa tissues. This result will provide potential targets for the remission or treatment of AR. CircRNA plays its role by acting on miRNA in most cases [28]. Circ_0067835 silence inhibited colorectal cancer cell proliferation through the down-regulation of miR-1236-3p [27]. Although lasted researches have elucidated that circRNA

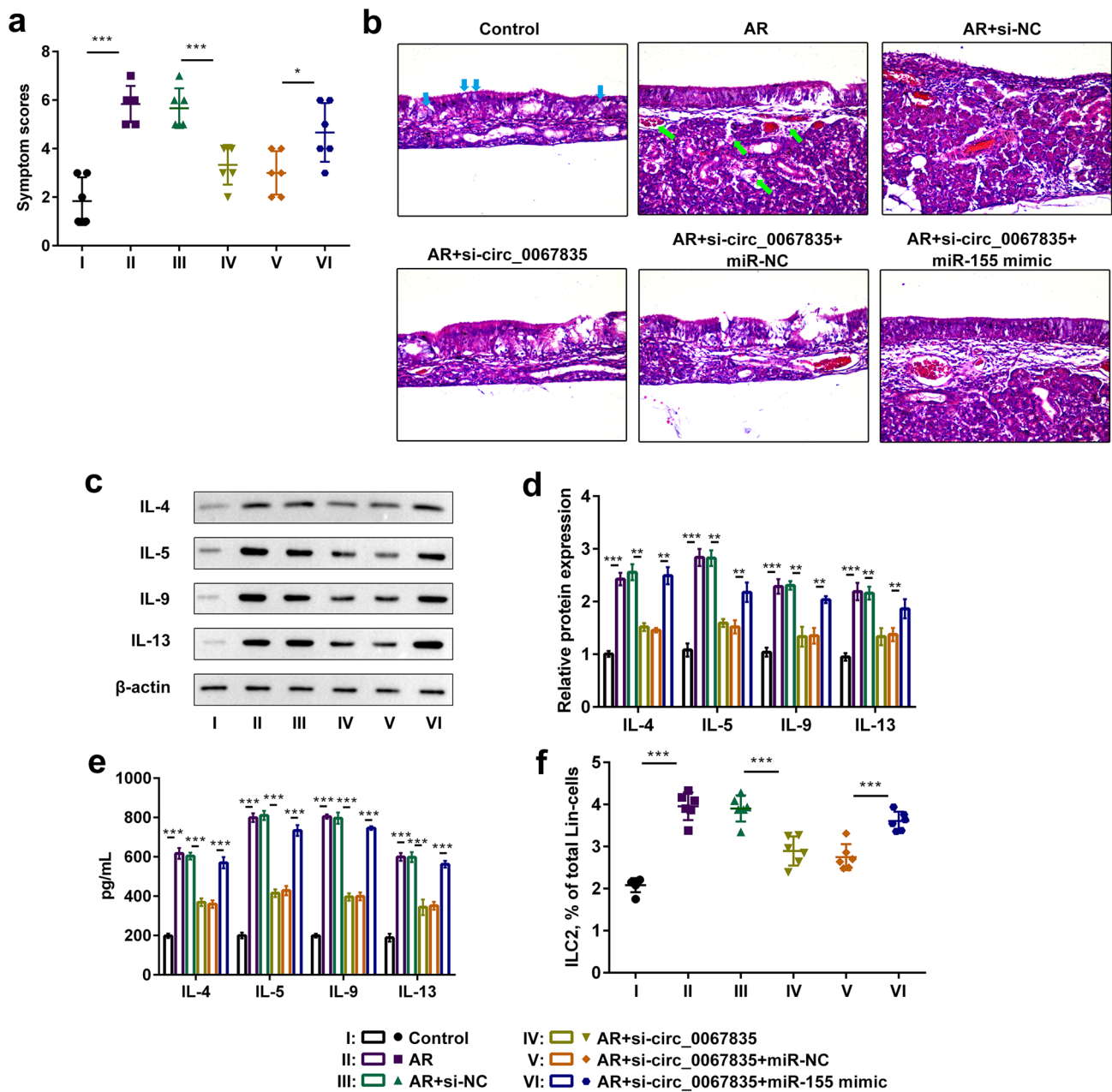


Fig. 4 Circ_0067835 had an influence in Type 2 cytokine levels through mediating the regulation of miR-155. AR was induced by recombinant murine IL-33 (0.05 μ g/ μ l) on days 1, 3, and 5. The lentiviral vectors carrying si-circ_0067835, miR-155 mimic, si-NC or miR-NC were injected into AR mice. Thus, mice were divided into control, AR, AR+si-NC, AR+si-circ_0067835, AR+si-circ_0067835+miR-NC and AR+si-circ_0067835+miR-155 mimic groups. **a** symptom scores, **(b)** pathological changes, **(c-d)** IL-4,

IL-5, IL-9 and IL-13 protein expression, **(e)** IL-4, IL-5, IL-9 and IL-13 supernatant concentration and **(f)** ILC2s level were monitored by behavioral tests, hematoxylin and eosin (HE) staining, western blot, ELISA and flow cytometry experiments. Data shown represent mean $\pm$ SD from three independent experiments, $n=6$. ANOVA followed by Tukey's test. Blue arrows indicate goblet cells, and green arrows indicate eosinophils. * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$

may have a great influence in the pathogenesis of various diseases through affecting miRNA, the circRNA-miRNA network in the process of AR is rarely studied. In this study, miR-155 was recognized as the downstream target of circ_0067835 through circular RNA Interactome and

actively regulated by circ_0067835. Fortunately, it was confirmed that silencing circ_0067835 suppressed the ratio of ILC2s and Type2 cytokines through the down-regulation of miR-155.

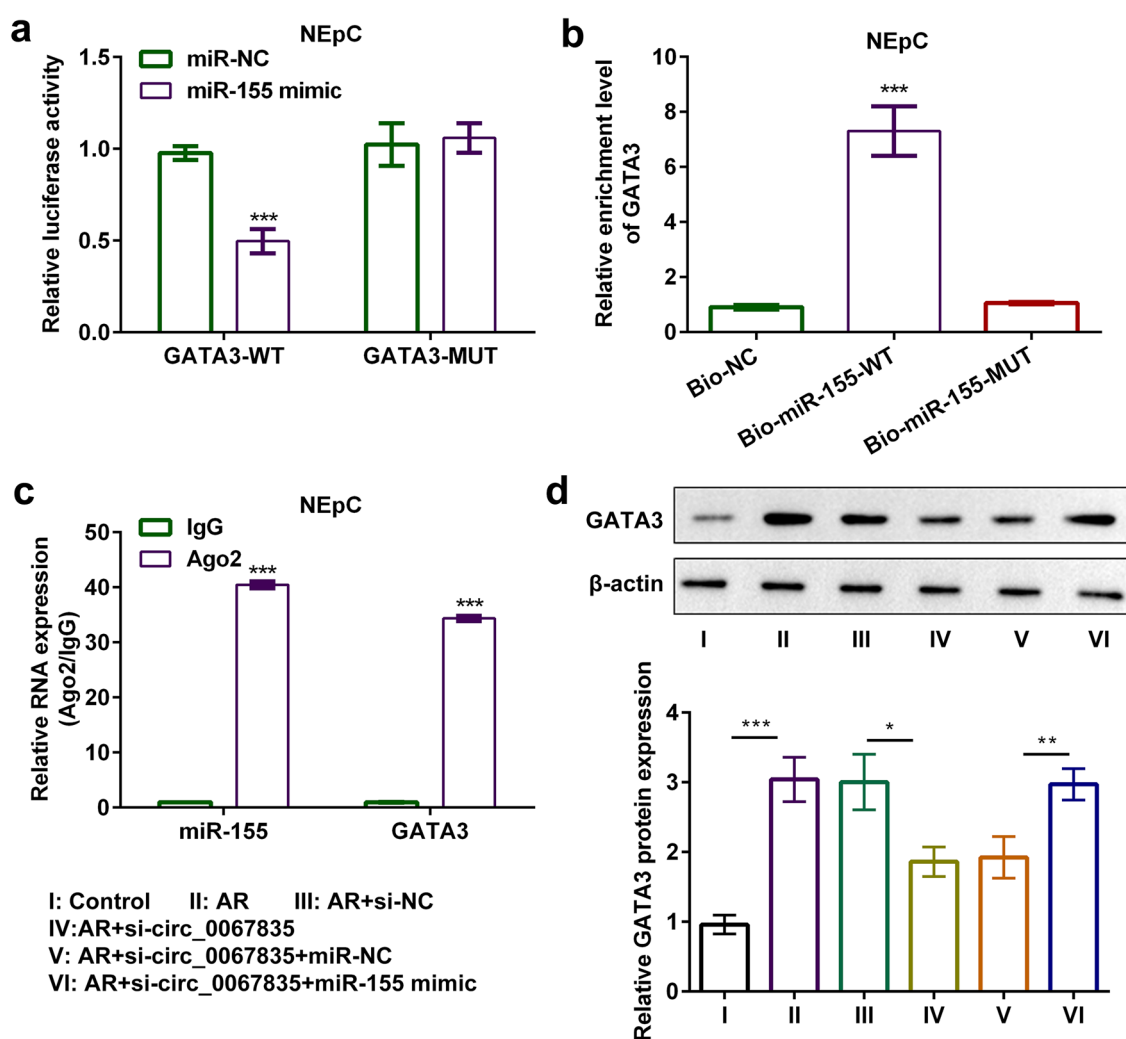


Fig. 5 GATA3 acted as a downstream target of miR-155 and adjusted by circ_0067835/miR-155 axis. **a** pGL4 vectors carrying GATA3-WT or GATA3-MUT and miR-155 mimic or miR-NC were co-transfected into NEpC cells, relative luciferase activity was inspected exploiting luciferase reporter assay. **b** RNA pull-down assay was applied to verify the enrichment of GATA3 in NEpC cells using bio-NC, bio-miR-155-WT or bio-miR-155-MUT probe. **c** Enrichment of GATA3 and miR-155 was confirmed using the Ago2-IP assay. **d** AR was induced

by recombinant murine IL-33 (0.05 μ g/ μ l) on days 1, 3, and 5. The lentiviral vectors carrying si-circ_0067835, miR-155 mimic, si-NC or miR-NC were injected into AR mice. Thus, mice were divided into control, AR, AR+si-NC, AR+si-circ_0067835, AR+si-circ_0067835+miR-NC and AR+si-circ_0067835+miR-155 mimic groups. Relative GATA3 protein expression was tested exploiting western blot. Data shown represent mean $\pm$ SD from three independent experiments, $n=3$. * $P<0.05$, ** $P<0.01$ and *** $P<0.001$

GATA protein family is a transcriptional regulator that can recognize and bind to the GATA motif and it generally has zinc finger structure [29, 30]. So far, six members of this family have been found, namely GATA1 ~6. As a member of family, GATA3 promotes differentiation and activation of ILC2, Th2 and Tc2 cells [31]. GATA3 was firstly found in T lymphocytes and embryonic brain in 1991. GATA3 gene is located on chromosome 10. GATA3 not only plays a key role in the differentiation and orientation of Th2 cells, but also is necessary for the transcription of all Type2 cytokines.

Furthermore, the circRNA-miRNA-mRNA network has been studied maturely. A study has put forward that circHIPK3 increases the expression of GATA3 via miR-495 expression, and the knockdown of circHIPK3 can relieve nasal symptoms and Th2 differentiation via miR-495/GATA3 pathway [14]. In addition to this, miR-155 knockout impaired GATA3 expression, which probably was a factor that contributed to the reduction of Type2 cytokines expression [32]. Our study found that in IL-33-simulated AR mice model, GATA3 was a downstream target of miR-155 and regulated by circ_0067835/

miR-155 axis. In future experiments, we focused on whether circ_0067835 influenced AR process through miR-155/GATA3.

In conclusion, our research proposed novel and potential targets during the development of AR treatment, which provided solid theoretical foundation for clinical application.

Acknowledgements This research was supported by National Natural Science Foundation of China (No.81760183), Jiangxi Provincial Natural Science Foundation (No. 20202ACBL206013), The Key Research and Development Program of Jiangxi Science and Technology Department (No. 20202BBGL73018) and the science and technology program of Jiangxi Provincial Education Department (GJJ200250).

Author contributions Guarantor of integrity of the entire study: CY. Study concepts: CY. Study design: definition of intellectual content: XJ. Literature research: JD. Clinical studies: HL. Experimental studies: TH. Data acquisition: KL. Data analysis: HZ. Statistical analysis: HZ. Manuscript preparation: XJ. Manuscript editing: XC. Manuscript review: CY.

Funding This research was supported by National Natural Science Foundation of China (No.81760183), Jiangxi Provincial Natural Science Foundation (No. 20202ACBL206013), The Key Research and Development Program of Jiangxi Science and Technology Department (No. 20202BBGL73018) and the science and technology program of Jiangxi Provincial Education Department (GJJ200250).

Declarations

Conflict of interest Authors declare that there is no conflict of interests.

Ethics approval The animal usage protocols were approved in advance by the Animal Care and Use Committees of the Second Affiliated Hospital of Nanchang University.

Informed consent Not applicable.

References

- Hon KL, Fung CK, Leung AK, Lam HS, Lee SL. Recent patents of complementary and alternative medicine for allergic rhinitis. *Recent Pat Inflam Allergy Drug Discov*. 2015;9(2):107–19. <https://doi.org/10.2174/1872213x10666151119144718>.
- Alsamarai AM, Abdulsatar M, Ahmed Alobaidi AH. Evaluation of topical black seed oil in the treatment of allergic rhinitis. *Anti-Inflam Antiallergy Agents Med Chem*. 2014;13(1):75–82. <https://doi.org/10.2174/18715230113129990014>.
- Brożek JL, Bousquet J, Agache I, Agarwal A, Bachert C, Bosnic-Anticevich S, et al. Allergic rhinitis and its impact on asthma (ARIA) guidelines-2016 revision. *J Allergy Clin Immunol*. 2017;140(4):950–8. <https://doi.org/10.1016/j.jaci.2017.03.050>.
- Seidman MD, Gurgel RK, Lin SY, Schwartz SR, Baroody FM, Bonner JR, et al. Clinical practice guideline: allergic rhinitis. *J Am Acad Otolaryngol-Head Neck Surg*. 2015;152(1 Suppl):1–43. <https://doi.org/10.1177/0194599814561600>.
- Nelson HS. Allergen immunotherapy now and in the future. *Allergy Asthma Proc*. 2016;37(4):268–72. <https://doi.org/10.2500/aap.2016.37.3966>.
- Kim HY, Kim J, Jeong HJ, Kim HM. Potential anti-inflammatory effect of Madi-Ryuk and its active ingredient tannic acid on allergic rhinitis. *Mol Immunol*. 2019;114:362–8. <https://doi.org/10.1016/j.molimm.2019.08.013>.
- Kouzegar S, Zamani MA, Faridhosseini R, Rafatpanah H, Rezaee A, Yousefzadeh H, et al. Immunotherapy in allergic rhinitis: its effect on the immune system and clinical symptoms. *Open Access Macedonian J Med Sci*. 2018;6(7):1248–52. <https://doi.org/10.3889/oamjms.2018.264>.
- Sanders NL, Mishra A. Role of interleukin-18 in the pathophysiology of allergic diseases. *Cytokine Growth Factor Rev*. 2016;32:31–9. <https://doi.org/10.1016/j.cytogfr.2016.07.001>.
- Beermann J, Piccoli MT, Viereck J, Thum T. Non-coding RNAs in development and disease: background, mechanisms, and therapeutic approaches. *Physiol Rev*. 2016;96(4):1297–325. <https://doi.org/10.1152/physrev.00041.2015>.
- Matsui M, Corey DR. Non-coding RNAs as drug targets. *Nat Rev Drug Discov*. 2017;16(3):167–79. <https://doi.org/10.1038/nrd.2016.117>.
- Han X, Huang S. LncRNA PTPRE-AS1 modulates M2 macrophage activation and inflammatory diseases by epigenetic promotion of PTPRE. *Sci Adv*. 2019;5(12):9230. <https://doi.org/10.1126/sciadv.aax9230>.
- Dissanayake E, Inoue Y. MicroRNAs in allergic disease. *Curr Allergy Asthma Rep*. 2016;16(9):67. <https://doi.org/10.1007/s11882-016-0648-z>.
- Wang T, Chen D, Wang P, Xu Z, Li Y. miR-375 prevents nasal mucosa cells from apoptosis and ameliorates allergic rhinitis via inhibiting JAK2/STAT3 pathway. *Biomed Pharma Biomed*. 2018;103:621–7. <https://doi.org/10.1016/j.biopha.2018.04.050>.
- Zhu X, Wang X, Wang Y, Zhao Y. The regulatory network among CircHIPK3, LncGAS5, and miR-495 promotes Th2 differentiation in allergic rhinitis. *Cell Death Dis*. 2020;11(4):216. <https://doi.org/10.1038/s41419-020-2394-3>.
- Zhu YQ, Liao B, Liu YH, Wang Z, Zhu XH, Chen XB, et al. MicroRNA-155 plays critical effects on Type2 factors expression and allergic inflammatory response in type-2 innate lymphoid cells in allergic rhinitis. *Eur Rev Med Pharmacol Sci*. 2019;23(10):4097–109. https://doi.org/10.26355/eurev_201905_17911.
- Yanagida N, Minoura T, Kitaoka S. Erratum: butter tolerance in children allergic to cow's milk. *Allergy Asthma Immunol Res*. 2016;8(2):178. <https://doi.org/10.4168/aa.2016.8.2.178>.
- Fan D, Wang X, Wang M, Wang Y, Zhang L, Li Y, et al. Allergen-dependent differences in ILC2s frequencies in patients with allergic rhinitis. *Allergy Asthma Immunol Res*. 2016;8(3):216–22. <https://doi.org/10.4168/aa.2016.8.3.216>.
- Diefenbach A, Colonna M, Koyasu S. Development, differentiation, and diversity of innate lymphoid cells. *Immunity*. 2014;41(3):354–65. <https://doi.org/10.1016/j.immuni.2014.09.005>.
- Liu Z, Zhang XH, Callejas-Díaz B, Mullol J. MicroRNA in united airway diseases. *Internat J Mole Sci*. 2016;17(5):8. <https://doi.org/10.3390/ijms17050716>.
- Lu TX, Rothenberg ME. Diagnostic, functional, and therapeutic roles of microRNA in allergic diseases. *J Allergy Clin Immunol*. 2013;132(1):3–13. <https://doi.org/10.1016/j.jaci.2013.04.039>.
- Wang L, Yang X, Li W, Song X, Kang S. MiR-202-5p/MATN2 are associated with regulatory T-cells differentiation and function in allergic rhinitis. *Hum Cell*. 2019;32(4):411–7. <https://doi.org/10.1007/s13577-019-00266-0>.
- Wang L, Liu X, Song X, Dong L, Liu D. MiR-202-5p promotes M2 polarization in allergic rhinitis by targeting MATN2. *Int Arch*

- Allergy Immunol. 2019;178(2):119–27. <https://doi.org/10.1159/000493803>.
23. Wang J, Cui Z, Liu L, Zhang S, Zhang Y, Zhang Y, et al. MiR-146a mimic attenuates murine allergic rhinitis by down-regulating TLR4/TRAF6/NF- κ B pathway. *Immunotherapy*. 2019;11(13):1095–105. <https://doi.org/10.2217/imt-2019-0047>.
 24. Ruggiero T, Trabucchi M, De Santa F, Zupo S, Harfe BD, McManus MT, et al. LPS induces KH-type splicing regulatory protein-dependent processing of microRNA-155 precursors in macrophages. *FASEB J Off Pub Feder Am Soc Exp Biol*. 2009;23(9):2898–908. <https://doi.org/10.1096/fj.09-131342>.
 25. Zhu YQ, Liu YH. [A review on group 2 innate lymphoid cells and miR-155 in allergic rhinitis] Lin chuang er bi yan hou tou jing wai ke za zhi. *J Clin Otorhinolaryngol Head Neck Surg*. 2017;31(24):1940–3. <https://doi.org/10.13201/j.issn.1001-1781.2017.24.019>.
 26. Liu J, Cao Z. Protective effect of circular RNA (CircRNA) Ddx17 in ovalbumin (OVA)-induced allergic rhinitis (AR) mice. *Med Sci Monitor Internat Med J Exp Clin Res*. 2020;26:e919083. <https://doi.org/10.12659/msm.919083>.
 27. Feng W, Gong H, Wang Y, Zhu G, Xue T, Wang Y, et al. circIFT80 functions as a ceRNA of miR-1236-3p to promote colorectal cancer progression. *Mole Therapy Nucleic Acids*. 2019;18:375–87. <https://doi.org/10.1016/j.omtn.2019.08.024>.
 28. Zhang F, Zhang R, Zhang X, Wu Y, Li X, Zhang S, et al. Comprehensive analysis of circRNA expression pattern and circRNA-miRNA-mRNA network in the pathogenesis of atherosclerosis in rabbits. *Aging*. 2018;10(9):2266–83. <https://doi.org/10.18632/aging.101541>.
 29. Fujiwara T. GATA transcription factors: basic principles and related human disorders. *Tohoku J Exp Med*. 2017;242(2):83–91. <https://doi.org/10.1620/tjem.242.83>.
 30. Caramori G, Lim S, Ito K, Tomita K, Oates T, Jazrawi E, et al. Expression of GATA family of transcription factors in T-cells, monocytes and bronchial biopsies. *Eur Respir J*. 2001;18(3):466–73. <https://doi.org/10.1183/09031936.01.00040701>.
 31. Jungraithmayr W, Ji L, Yang L, Weder W, Korom S, Hersberger M. Increased T-bet to GATA-3 ratio during acute allograft rejection in the rat lung. *Transpl Proc*. 2009;41(10):4316–20. <https://doi.org/10.1016/j.transproceed.2009.08.057>.
 32. Johansson K, Malmhäll C, Ramos-Ramírez P, Rådinger M. MicroRNA-155 is a critical regulator of type 2 innate lymphoid cells and IL-33 signaling in experimental models of allergic airway inflammation. *J Allergy Clin Immunol*. 2017;139(3):1007–16.e9. <https://doi.org/10.1016/j.jaci.2016.06.035>.

Publisher's Note Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.