

LncRNA ANRIL Promotes Autophagy Activation Through miR-16-5p/TLR4 Axis in Allergic Rhinitis

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Abstract

Background: Allergic rhinitis (AR) is an allergic disease of nasal mucosa. LncRNAs are key modulators affecting AR development. Nevertheless, the impact of LncRNA ANRIL in AR is not clear.

Objective: This work decided to study the mechanism underlying the impact of ANRIL on TLR4 expression through targeting miR-16-5p during autophagy and epithelial barrier dysfunction in the progression of AR.

Methods: Human nasal epithelial cells were exposed to TNF- α to establish AR cell model, AR mice model was constructed by ovalbumin (OVA) treatment. QRT-PCR or western blot assays were applied to measure the levels of mRNA and proteins. Dual-luciferase reporter gene detection and RIP assay were conducted to verify the association between ANRIL and miR-16-5p. Autophagy flux assessment by mRFP-GFP-LC3 method was performed to detect autophagy level.

Results: AR progression could induce the autophagy, and the expressions of tight junction proteins were downregulated in AR cell model. Moreover, knockdown of ANRIL reversed the effect of AR on autophagy-related protein and tight junction proteins. MiR-16-5p was found to be bound with ANRIL and miR-16-5p inhibitor could reverse ANRIL knockdown-induced downregulation of autophagy-related proteins and epithelial barrier dysfunction. In addition, miR-16-5p directly targeted TLR4. Furthermore, knockdown of ANRIL reversed miR-16-5p and TLR4 expression, autophagy level, and tight junction protein levels in nasal mucosa of AR mice.

Conclusion: This study illustrated that ANRIL acted as a promotion factor in AR induced autophagy and epithelial barrier dysfunction by enhancing the expression of TLR4 via interacting with miR-16-5p.

Keywords

allergic rhinitis, ANRIL, miR-16-5p, TLR4, LncRNAs

Introduction

Allergic rhinitis (AR) results from inflammatory response mediated by immunoglobulin E (IgE) which was induced by particles.^{1,2} AR patients may experience a variety of nasal symptoms after being stimulated by allergenic antigens, including sneezing, rhinorrhea, nasal congestion and so on.³ Previous studies have shown that nasal epithelial cells are the first barrier against allergen infiltration in AR.⁴ Autophagy is an important cellular mechanism that plays a “housekeeping” role by removing longevity protein from the cytoplasm, invading pathogens and damaged organelles for cellular renovation and homeostasis.⁵ Autophagy is involved inflammatory bowel disease, AR and airway remodeling.^{6,7} Expression of autophagosomes and autophagy markers were high in the upper respiratory tract of AR patients, and correlated with the corresponding changes of airway remodeling markers.⁷ All these contents suggested that autophagy might play a vital role in AR development.

LncRNAs (>200 nts) are involved in the regulation of plenty of disease processes.⁸ Antisense non-coding RNA in the INK4 locus (ANRIL), also known as CDKN2B-AS1, is a lncRNA encoded in the chromosome 9p21 region, which is related with risk increase, severity and inflammatory status of AR.⁹ Previous preliminary studies have found that AR progression was closely related with the cell autophagy,^{10,11} and ANRIL was reported to be related with cell autophagy.^{12,13} For instance, lncRNA ANRIL could aggravate the chemoresistance of pancreatic cancer cells to gemcitabine by targeting

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HMGB1-induced autophagy¹⁴; ANRIL could mitigate neonatal hypoxic-ischemic brain damage via promoting the autophagy.¹⁵ Meanwhile, studies have shown that ANRIL depletion could inhibit inflammatory cytokines and mucins in IL-13-stimulated human nasal epithelial cells. Thus, ANRIL might be a new target for AR therapy.^{16,17} In addition, silencing of ANRIL could alleviate the injury of H₂O₂-treated PC-12 cells by suppressing autophagy and apoptosis by activating mTOR/p70S6K modulated by miR-499a/PDCD4 axis,⁹ which provides a promising strategy for spinal cord repair. However, the molecular mechanism by which ANRIL mediating AR injury remains to be further studied.

MiRNAs (22 nts) are encoded by endogenous genes, which inhibit the translation of target genes or directly degrade target genes.¹⁸ miR-16-5p has been proved to be widely found in multiple species and plays a vital role in regulating physiological activities such as body development, cell self-renewal and differentiation.¹⁹ As demonstrated in previous reports, miR-16-5p attenuated the inflammation of AR by inactivating IKK β /NF- κ B in nasal epithelial cells stimulated by IL-13.²⁰ Therefore, it is urgent to explore the mechanism underlying the function of miR-16-5p in AR.

Toll-like receptor4 (TLR4) has been shown to be implicated in the pathogenesis of asthma or atopy.²¹ In the atopic dermatitis model, calycosin decreased TSLP/IL-33 secretion and attenuated the disruption of epithelial tight junctions thus maintaining epithelial barrier by inhibiting TLR4 mediated NF- κ B.²² It has been reported that the expression of TLR4 was elevated in AR and can promote the progression of AR.²³ Besides, BPA could aggravate OVA-induced pulmonary inflammation by activating autophagy via mTOR and TLR4/NF- κ B.²⁴ However, whether the activation of autophagy plays a role through TLR4 signaling in the pathogenesis of AR is not clear. Consequently, it is of great value to assess the function of TLR4 implicated in autophagy activation during AR process.

This work aimed to study the mechanism underlying the function of ANRIL in AR. We hope this research would provide new strategies against AR.

Materials and Methods

Cell Culture

The human nasal epithelial cell line RPMI 2650 cells were bought from ATCC (USA). Cells were cultured in DMEM (Thermo Fisher Scientific) with supplementation of FBS (10%, Life Technologies, USA), penicillin (100 IU/mL, Gibco-BRL, USA) and streptomycin (100 μ g/mL, Gibco-BRL) and incubated in the condition of 37 °C and 5% CO₂. Cells were added with 10 ng/mL TNF- α for 48 h to construct the AR cell model.

Cell Transfection

The cell lines were inoculated overnight and Lipofectamine 2000 reagent was applied to transfect cells. For ANRIL

knockdown, sh-ANRIL and sh-NC were cloned into the vector (pSicoR). miR-16-5p mimics/inhibitor and mimics/inhibitor NC were bought from RiboBio (Guangzhou, China).

Dual-Luciferase Reporter Gene Detection

The 3'-UTR of ANRIL and TLR4 supplemented with the potential binding sites of miR-16-5p were bought from Beyotime (Shanghai, China). The wild-type ANRIL 3'-UTR sequence (ANRIL-WT) and the mutant-type ANRIL 3'-UTR sequence (ANRIL-MUT) were cloned into vector (pmirGLO, GenePharma). RPMI 2650 cells were transfected with ANRIL-WT/MUT and miR-16-5p mimics/inhibitor by the Lipofectamine 2000. Fragment of TLR4 with the predicted miR-16-5p binding site or mutant was also cloned into pmirGLO vector. RPMI 2650 cells were co-transfected with miR-16-5p mimics/inhibitor using Lipofectamine 2000. Dual-luciferase reporter system (Promega, USA) was applied to assess the luciferase activity, and the regulatory results were measured by calculating the ratio of Rluc/Luc ratio.

RNA Immunoprecipitation (RIP) Assay

RIP assay was carried out using a Magna RIP kit. After cells were rinsed, buffer (20 mM Tris-HCL pH7.5, 2 mM EDTA, 150 mM KCL, 1 mM NaF, 0.5% Nonidet, 0.5 mM DTT, RNase, phosphatase inhibitors and protease) was applied to lyse the cells. 10% of the lysates was kept as the input and sepharose beads conjugated with anti-AGO2 (Sigma, USA) or anti-IgG (Sigma, USA) was applied to incubate the remaining lysates for co-precipitation at 4 °C for 4 h. After digestion with DNase I and proteinase K, RNase-free water was applied to dissolve the purified RNA for qRT-PCR analysis.

Autophagy Flux Assessment

RPMI 2650 cells were transfected with tandem fluorescent adenoviral vector mRFP-GFP-LC3 (HanBio, China) following the manufacturer's instrument. The cells were cultured in DMEM with FBS (10%) for 24 h and incubated with mRFP-GFP-LC3 for 6 h, then grown in fresh DMEM with FBS (10%) for another 36 h. Afterwards, cells were observed under a confocal fluorescence microscopy (Zeiss, Germany). Autophagic flux was measured by evaluating the number of GFP, RFP and merged points (dots or cells were counted). 5 mmol/L 3-methyladenine (3-MA) was used in this study to block autophagy.

Establishment of AR Mice Model

Ethics Committee of the Second Affiliated Hospital of Nanchang University approved our work. BALB/C mice (n = 40, female, 5 weeks old) were purchased from

the Animal Research Center of Nanjing University. ANRIL knockdown adenoviruses (GenePharma, China) sh-ANRIL was injected into mice through tail vein, and then the mice were treated with OVA to establish AR mice model. 0.5 mg/ml OVA and 20 mg/ml Al(OH)₃ (1:1) in sensitization fluid containing were injected into mice three times a week. OVA (2 mg/ml) was After two weeks, 50 μ l of OVA solution (2 mg/ml) was applied to be inhaled in mice for 30 min once a week. Normal mice were treated with 0.9% normal

saline. The mice were then treated to collect the nasal mucosal tissues.

qRT-PCR

TRIZOL reagent was applied to extract total RNA from tissues and cells. PrimeScript RT reagent kit was applied to reverse transcribe total RNA into cDNAs. SYBR Premix Ex Taq II kit (Takara) was applied in qRT-PCR assay on Applied PRISM 7500 Sequence Detection System. U6 and

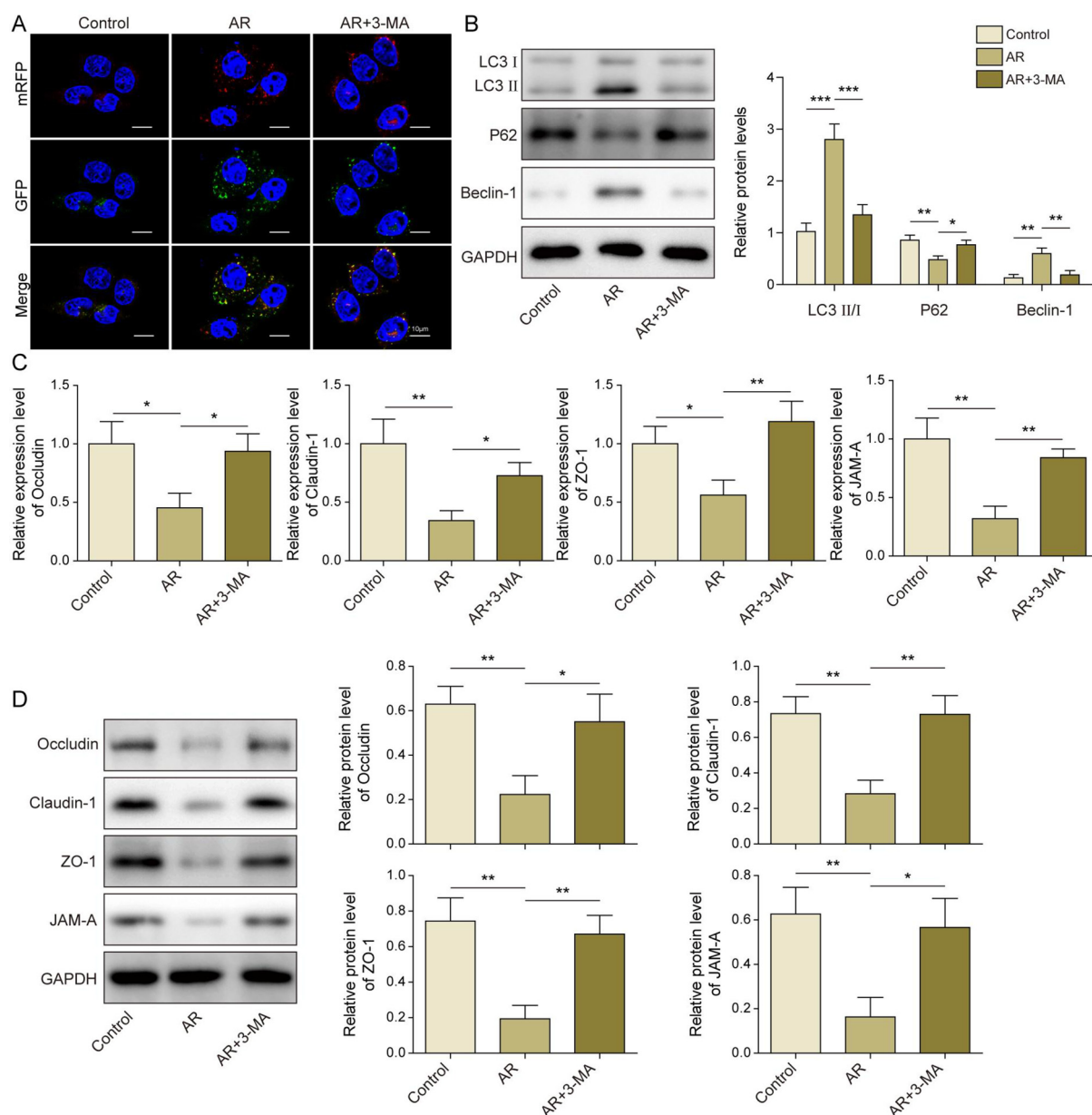


Figure 1. The epithelial barrier dysfunction and autophagy of nasal epithelial cells involved in AR. (A) Autophagy level of nasal epithelial cells in AR cell model was detected by mRFP-GFP-LC3 method. (B) p62, LC3 II/I and Beclin-I levels were assessed by western blot. (C–D) Claudin-I, Occludin, JAM-A and ZO-I levels were assessed by qRT-PCR and western blot. Mean $\pm$ SD was applied to express the data. * P < 0.05, ** P < 0.01, *** P < 0.001.

GAPDH were considered as the internal reference. The sequences were listed as follows:

Occludin (F): 5'- TTGCGGCGAGCGGATTG -3';
Occludin (R): 5'- CTCCTGGAGGAGAGGTCCAT -3';
Claudin-1 (F): 5'-TTGACTCCTTGCTGAATCTGAG-3';
Claudin-1 (R): 5'-TTCTGCACCTCATCGTCTTC-3';
ZO-1 (F): 5'- CCGCTCAAGAGGAAGCTGT-3';
ZO-1 (R): 5'- CCTCCATTGCTGTGCTAGTG-3';
JAM-A (F): 5'- ATCCCACAACAGGAGAGCTG -3';
JAM-A (R): 5'- CTCGGCTATAGGCAAACCAG -3';
ANRIL (F): 5'- TGCTCTATCCGCCAATCAGG -3';
ANRIL (R): 5'- GCGTGCAGCGGTTTAGTTT -3';

TLR4 (F): 5'- AGAAACTGCTCGGTCAGACG-3';
TLR4 (R): 5'- AATGGAATCGGGGTGAAGGG-3';
miR-16-5p (F): 5'- GCCGTAGCAGCACGTAAATA -3';
miR-16-5p (R): 5'- GTCGTATCCAGTGCAGGGTC
CGAGGTATTCGCACTGGATACGAC CGCCAA -3';
U6 (F): 5'- CTCGCTTCGGCAGCAC-3';
U6 (R): 5'- AACGCTTCACGAATTTGCGT-3';
GAPDH (F): 5'- CCAGGTGGTCTCCTCTGA -3';
GAPDH (R): 5'- GCTGTAGCCAAATCGTTGT -3'.

Western Blot

Tissues and cells were dissolved by the cold RIPA and protein concentrations were quantified by bicinchoninic acid kit

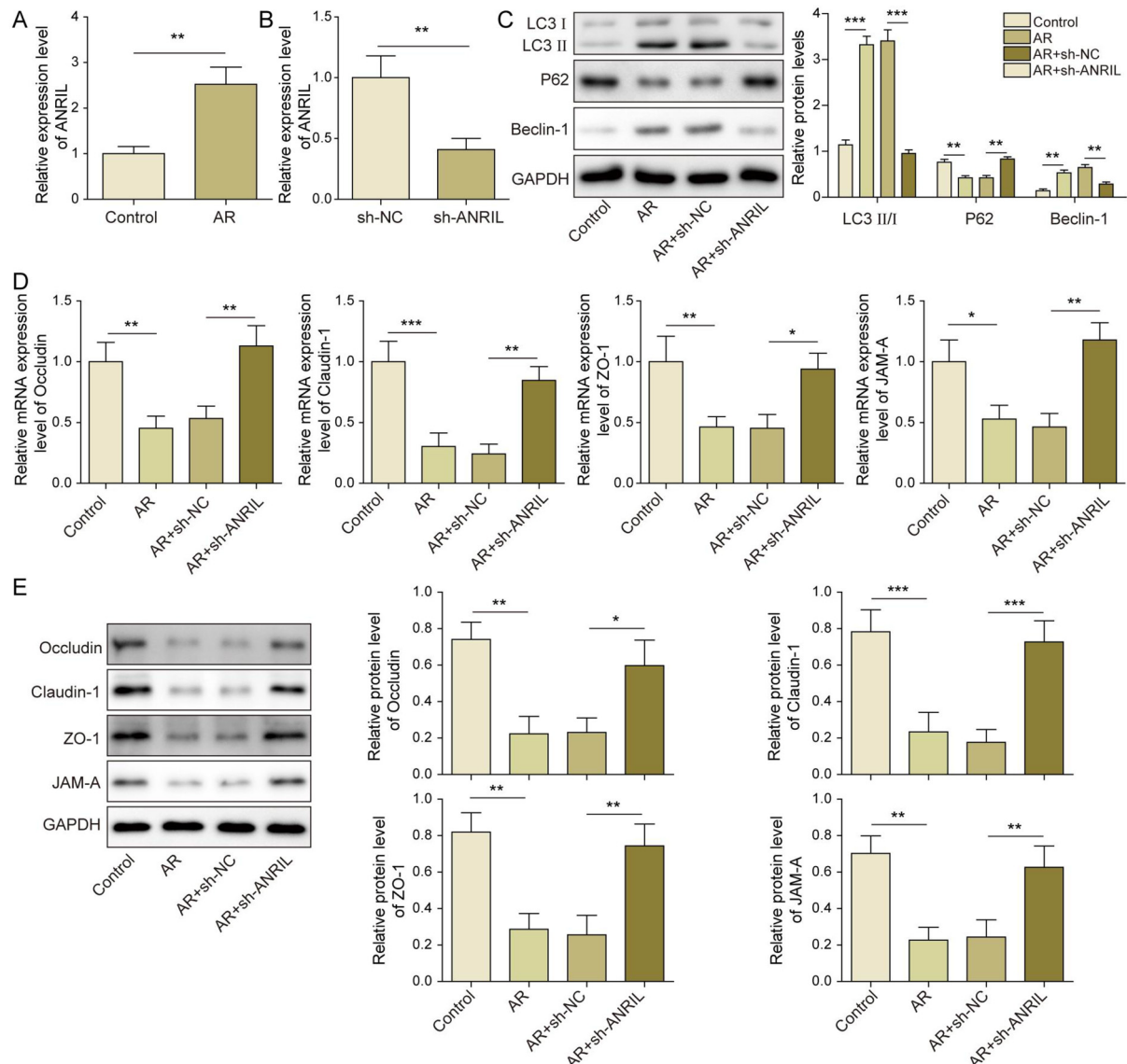


Figure 2. Knockdown of ANRIL reversed autophagy and epithelial barrier dysfunction in nasal epithelial cell AR model. (A) ANRIL level in AR cell model was detected by qPCR. (B) The knockdown efficiency of ANRIL was detected by qPCR. (C) LC3 II/I, p62 and Beclin-1 levels in RPMI 2650 cells with ANRIL knockdown were assessed by western blot assay. (D-E) Claudin-1, Occludin, JAM-A and ZO-1 levels in RPMI 2650 cells with ANRIL knockdown were assessed by qRT-PCR and western blot. Mean $\pm$ SD was applied to express data. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

could induce the increase of autophagy level, while 3-MA treatment contributed to autophagy suppression (Figure 1(A)). As shown in Figure 1(B), Beclin-1 and LC3 II/I levels were increased, while the level of p62 was decreased in AR cell model; however, 3-MA could reverse the effect of AR induction. In addition, the levels of Claudin-1, Occludin, JAM-A and ZO-1 were decreased in AR cell model, while this phenomenon was reversed by 3-MA (Figure 1(C) and (D)). Taken together, AR could

induce the dysfunction of epithelial barrier by inducing autophagy.

Knockdown of ANRIL Inhibited the Autophagy and Epithelial Barrier Dysfunction of AR-Induced Nasal Epithelial Cells

To verify the effect of ANRIL on AR induced epithelial barrier dysfunction, RPMI 2650 cells were transfected with sh-ANRIL.

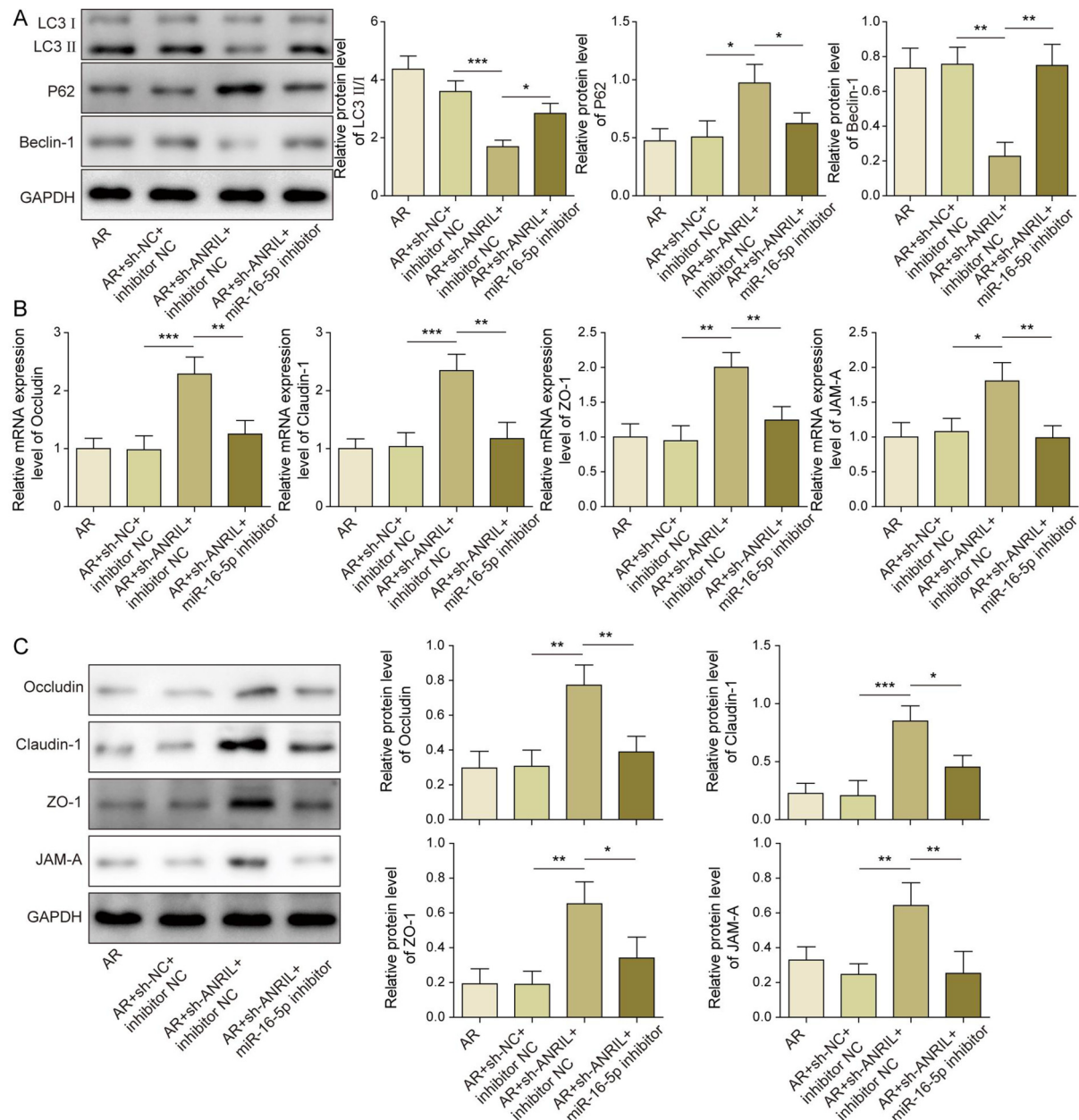


Figure 4. ANRIL regulated autophagy and epithelial barrier dysfunction through miR-16-5p in nasal epithelial cell AR model. (A) LC3 II/I, p62 and Beclin-1 levels in nasal epithelial cells were assessed by western blot. (B-C) Claudin-1, Occludin, JAM-1 and ZO-1 levels in nasal epithelial cells were assessed by qRT-PCR and western blot. Mean $\pm$ SD was applied to express data. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

In Figure 2(A) and (B), the expression of ANRIL was elevated in AR cell model, and the expression of ANRIL was decreased by sh-ANRIL. Besides, the levels of LC3 II/I and Beclin-1 were elevated, while p62 expression was decreased in AR cell model; nevertheless, knockdown of ANRIL restored the effect of AR induction (Figure 2(C)). Moreover, the levels of Claudin-1, Occludin, JAM-A and ZO-1 were decreased in AR cell model, while knockdown of ANRIL abolished this phenomenon (Figure 2(D) and (E)). To sum up, ANRIL knockdown could reverse AR-induced autophagy and epithelial barrier dysfunction.

ANRIL Bound with miR-16-5p

StarBase database was used to search for potential targets of ANRIL. In Figure 3(A), ANRIL had a putative binding site with miR-16-5p. The level of miR-16-5p was upregulated by miR-16-5p mimics, and it was markedly reduced by downregulation of miR-16-5p (Figure 3(B)). Furthermore, miR-16-5p negatively regulate luciferase activity in ANRIL-WT, while it did not affect the luciferase activity in ANRIL-MUT group (Figure 3(C)). ANRIL and miR-16-5p were specifically enriched in AGO2 precipitation. (Figure 3(D)) Moreover, the level of miR-16-5p was enhanced by ANRIL knockdown (Figure 3(E)). All data revealed that ANRIL might target miR-16-5p in AR.

ANRIL Regulated Autophagy and Epithelial Barrier Dysfunction Through Mediation of miR-16-5p in AR

To further determine whether ANRIL could induce autophagy and epithelial barrier dysfunction through

mediation of miR-16-5p, RPMI 2650 cells were co-transfected with sh-ANRIL and miR-16-5p inhibitor. The data showed that knockdown of ANRIL could inhibit the levels of Beclin-1, LC3 II/I and upregulated the level of p62 in AR-induced cells, while depletion of miR-16-5p partially reversed this phenomenon (Figure 4(A)). Meanwhile, knockdown of ANRIL increased the expressions of Occludin, Claudin-1, ZO-1 and JAM-A, while miR-16-5p downregulation markedly abolished these phenomena (Figure 4(B) and (C)). Thus, ANRIL might aggravate AR-induced autophagy and epithelial barrier dysfunction via binding with miR-16-5p.

miR-16-5p Targeted TLR4 in Nasal Epithelial Cells

As shown in Figure 5(A), there was interaction site between miR-16-5p and TLR4. MiR-16-5p negatively modulate the luciferase activity in TLR4-WT, while there was no obvious change in TLR4-MUT group (Figure 5(B)). Furthermore, the level of TLR4 was reduced after transfection with miR-16-5p mimics and elevated by miR-16-5p downregulation (Figure 5(C) and (D)). Thus, miR-16-5p might target TLR4.

Knockdown of ANRIL Relieved in Mice Reverses the Nasal Epithelial Cells Autophagy and Epithelial Barrier Dysfunction in Mice with AR

To assess the impact of ANRIL on AR-induced epithelial barrier dysfunction, nasal mucosal tissues of mice were

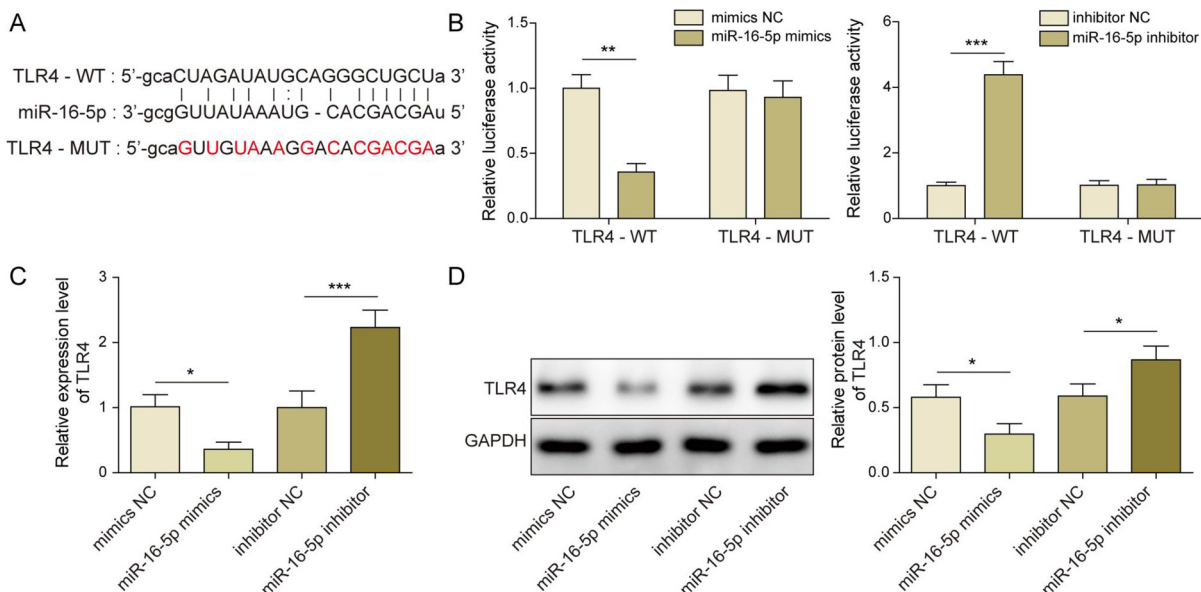


Figure 5. miR-16-5p targeted TLR4 in nasal epithelial cells. (A) StarBase software was applied to predict the association between TLR4 and miR-16-5p. (B) The dual-luciferase assay was applied in nasal epithelial cells for verifying the association between TLR4 and miR-16-5p. (C–D) TLR4 levels in nasal epithelial cells were assessed by qRT-PCR and western blot. Mean $\pm$ SD was applied to express data. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

(Figure 6(A)). Moreover, the levels of Beclin-1 and LC3 II/I were elevated in AR mice, while p62 level was decreased (Figure 6(B)). Meanwhile, sh-ANRIL injection restored this phenomenon (Figure 6(B)). Claudin-1, Occludin, ZO-1 and

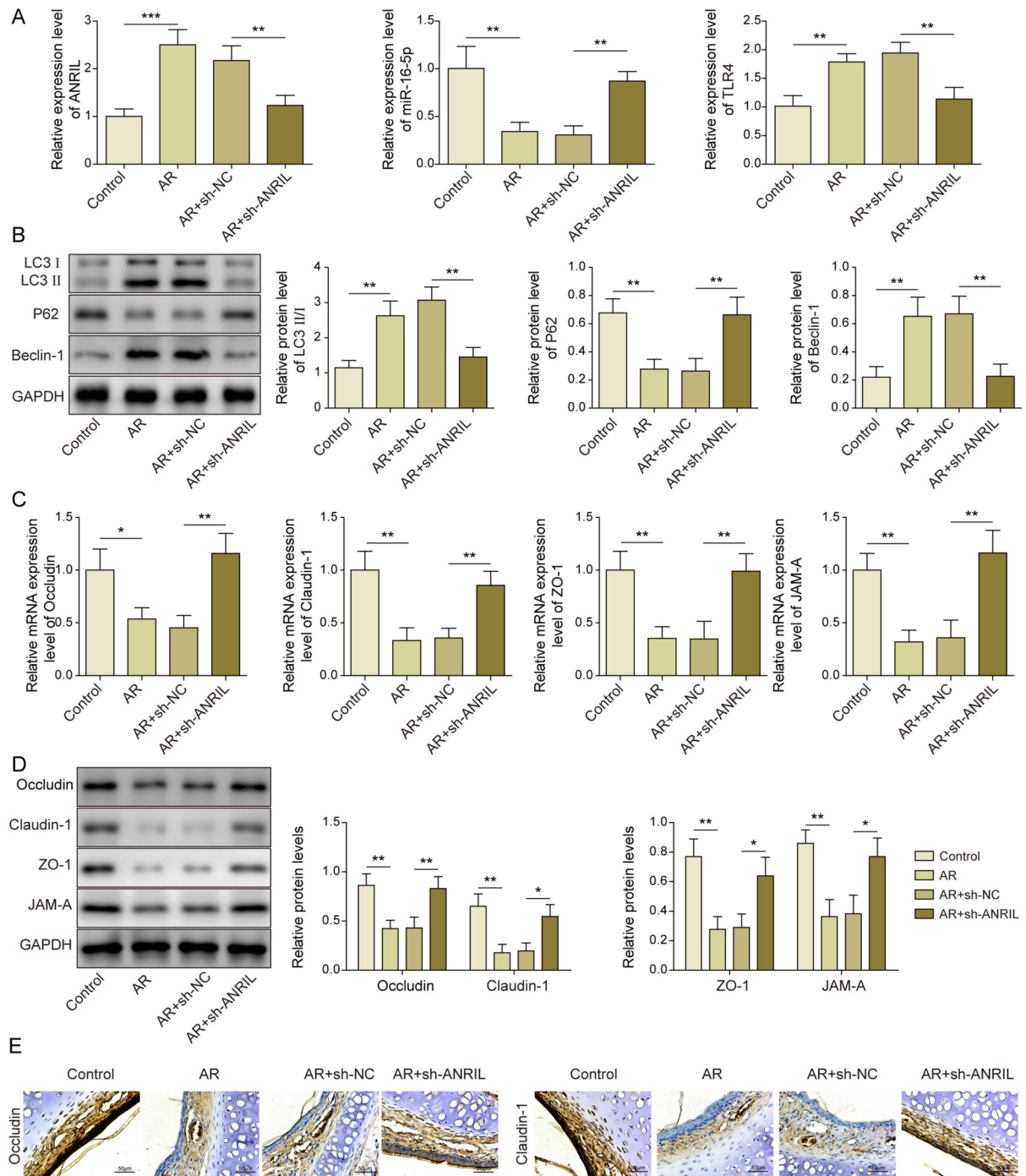


Figure 6. Knockdown of ANRIL relieved in mice reverses the nasal epithelial cells autophagy and epithelial barrier dysfunction in mice with AR. (A) ANRIL, miR-16-5p and TLR4 levels in AR mice were assessed by qRT-PCR. (B) P62, LC3 II/I and Beclin-1 levels in AR mice were assessed by western blot. (C–D) Claudin-1, Occludin, JAM-A and ZO-1 levels in AR mice were assessed by qRT-PCR and western blot. Mean $\pm$ SD was applied to express data. (E) The protein levels of Claudin-1 and Occludin were tested by IHC staining. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

JAM-A levels were decreased in AR mice, and their expression was reversed after ANRIL knockdown (Figure 6(C) and (D)). Furthermore, AR-induced downregulation of Claudin-1 and Occludin was reversed by ANRIL silencing (Figure 6(E)). Our work revealed a vital role of ANRIL knockdown in AR-induced epithelial barrier dysfunction.

Discussion

The incidence of AR has risen sharply in recent years and now affects about 10% to 20% of the people all over the world.²⁵ Although many experimental therapies have been developed to improve outcomes in patients with AR, the outcomes remain not ideal.²⁶ Therefore, it is necessary to explore specific biomarkers against AR. In our study, lncRNA ANRIL modulated the development of AR via mediation of miR-16-5p/TLR4. LncRNAs play essential roles in programmed cell death, DNA damage and inflammatory responses.^{27,28} LncRNAs participate in numerous human immune diseases, such as rheumatoid arthritis, T-cell acute lymphoblastic leukemia, AR.^{29–29} Ma Z, et al proved that lncRNAs were differentially expressed in AR patients.³² The inhibition of lncRNA ANRIL might lead to the suppression of ulcerative colitis development via modulating the miR-323b-5p/TLR4.³³ As demonstrated in previous study, ANRIL knockdown could exert anti-inflammatory effect in IL-13-induced cells by regulating miR-15a-5p.¹⁷ In addition, this work firstly showed that knockdown of ANRIL could reverse AR-induced autophagy and epithelial barrier dysfunction.

The miRNA microarray analysis conducted by Yu S, et al identified miRNAs which were differentially expressed in AR.³³ For example, miR-146a could enhance the inhibitory effect of OVA specific immunotherapy on nasal mucosal AR inflammation by inducing the antigen-specific regulatory T cells.³⁴ MiR-16 level was reduced in AR, as well as in IL-13 induced human nasal epithelial cells JME/CF15, and over-expression of miR-16 could suppress inflammation by inactivating IKK β /NF- κ B.²⁰ In our research, ANRIL directly targeted miR-16-5p. Furthermore, miR-16-5p downregulation abolished ANRIL depletion-induced upregulation of autophagy-related proteins and tight junction proteins, indicating that ANRIL might aggravate AR-induced autophagy and epithelial barrier dysfunction via targeting miR-16-5p. Similarly, miR-16 repressed cell autophagy and promoted apoptosis of human umbilical vein endothelial cells by directly targeting ATG14 in coronary heart disease.³⁵ It have been reported that TLR4 and ATG14 were key mediators in cell autophagy.^{36,37} Thus, the similar function between TLR4 and ATG14 might result in this similarity.

The TLR4 gene polymorphisms might be associated with the forms of severe disease in patients with atopic asthmatics and AR.³⁸ miR-146a mimic exerted anti-inflammatory effects by inhibiting the expression of proteins related to TLR4/TRAF6/NF- κ B signaling pathway, thereby

attenuating murine AR.³⁹ In this work, it was identified that miR-16-5p might target TLR4. Additionally, knockdown of ANRIL reversed miR-16-5p and TLR4 expression, autophagy level, and autophagy-related proteins and tight junction proteins in nasal mucosa of AR mice suggesting that ANRIL knockdown in mice reversed the epithelial barrier dysfunction caused by AR-induced autophagy level increase via miR-16-5p/TLR4 axis. These results provided theoretical evidence for the involvement TLR4 in autophagy regulation, which were in line with the research by Qin C, et al that TLR4 deletion could protect white matter against cerebral hypoperfusion induced injury through blocking autophagy.⁴⁰

Furthermore, Liu et al found that silencing of ANRIL could suppress the production of inflammatory cytokines and mucin 5AC in nasal epithelial cells via mediation of miR-15a-5p/JAK2 axis.⁹ MiR-15a-5p was found to be bound with ANRIL in the study of Liu et al.⁹ MiR-15a and miR-16 belong to the same cluster in miRNAs.^{41,42} Therefore, miR-15a and miR-16 might exert the same regulatory function in AR.

Previous studies have reported that lncRNA ANRIL was related to itching and congestion symptoms.⁴³ However, there was no significant difference between rhinorrhea and sneezing symptoms. But from the results of previous study, we can see that although there is no significant difference, as the sneeze score increases, the expression of lncRNA ANRIL is up-regulated, probably because of the limited sample size, and further research is needed.

Our study found that knockdown of ANRIL exerted an inhibition effects in AR-induced epithelial barrier dysfunction via mediation of miR-16-5p/TLR4 axis. Thus, our study might shed new lights on exploring new strategies against AR.

Declaration of Conflicting Interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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Research Ethics and Patient Consent

Ethics Committee of the Second Affiliated Hospital of Nanchang University approved our work.

Ethical Approval

Not applicable, because this article does not contain any studies with human or animal subjects.

Informed Consent

Not applicable, because this article does not contain any studies with human or animal subjects.

Trial Registration

Not applicable, because this article does not contain any clinical trials.

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