



## Original article

## LncRNA MALAT1 promotes proliferation and migration of airway smooth muscle cells in asthma by downregulating microRNA-216a

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## ARTICLE INFO

## Article history:

Received 14 November 2020

Revised 29 March 2021

Accepted 30 March 2021

Available online 9 April 2021

## Keywords:

Asthma

Airway smooth muscle cells

LncRNA MALAT1

MicroRNA-216a

## ABSTRACT

Asthma is a difficult chronic airway inflammation, if it cannot be treated and relieved in time, it will seriously affect the health and quality of life of patients. Airway remodeling is relevant to asthma, but there is currently no effective treatment for airway remodeling. Regulating the biological function of airway smooth muscle cells (AMSCs) may be an important method to inhibit airway remodeling. LncRNA MALAT1 and microRNA-216a are involved in the regulation of AMSCs respectively, but there is no research to prove that they can regulate airway remodeling of asthma through mutual combination. Hence, the aim of the present study was performed to investigate the function of lncRNA MALAT1 and microRNA-216a on AMSCs in asthma. The relationship between lncRNA MALAT1, microRNA-216a and AMSCs was studied by MTT, qPCR, Western blot, Transwell and flow cytometry. The results revealed that lncRNA MALAT1 was up-regulated and microRNA-216a was down-regulated in asthma. lncRNA MALAT1 inhibited microRNA-216a targetedly. Whether downregulating lncRNA MALAT1 or upregulating microRNA-216a, cell proliferation, migration and invasion were reduced and apoptosis increased. Therefore, it is believed that lncRNA MALAT1 promotes proliferation and migration of asthma AMSCs by downregulating microRNA-216a. Since lncRNA MALAT1 and microRNA-216a take part in asthma by jointly regulating the proliferation of airway smooth muscle cells and other biological functions, it would be interesting to study if they become biomarkers of asthma, and relationship between the two in asthma diagnosis and poor prognosis.

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## 1. Introduction

Asthma is a chronic airway inflammation (Lambrecht et al. 2019), usually manifested as cough, pant, short of breath and chest tightness (Mattiuzzi and Lippi, 2020). If the above symptoms cannot be treated and relieved in time, the patients may die. In the process of asthma, abnormal proliferation of airway smooth muscle cells leads to tracheal remodeling (Yan et al. 2019), while tracheal remodeling directly leads to changes in airway structure of patients (Zhang and Li, 2011). At present, asthma treatment lacks

an effective treatment for airway remodeling (McAlinden et al. 2019). Therefore, understanding the molecular mechanism of abnormal proliferation of tracheal smooth muscle cells is helpful to solve this dilemma. Apparent regulation is one of the popular genetic mechanisms that have received extensive attention in recent years. microRNA (miRNA), as an apparent regulator, is closely linked to much gene expression. microRNA-216a is located on human chromosome 2 at 28, 779, 949–28, 780, 037bp. Non-coding RNA such as microRNA-216a is different from coding RNA, which realizes its function on genetic mechanism by combining with downstream sequences. According to the results of many studies (Kara et al., 2019; Olena et al., 2015; Kong et al., 2020; Menghini et al., 2014), microRNA-216a causes downstream target genes to change at post-transcriptional level through abnormal expression, and this change directly leads to cell life process disorder (Kato et al., 2010; Chen et al., 2020; Ji et al., 2017). The disorder of cell life process will inevitably lead to pathological changes in tissues or organs and damage to body functions. There are many regulatory factors upstream of miRNA that affect miRNA. Long-chain non-coding RNA (lncRNA) is one of them. lncRNA metastasis associated lung adenocarcinoma transcript 1 (MALAT1) can change

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Peer review under responsibility of King Saud University.



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the miRNA expression level through “sponge adsorption” on miRNA sequences, and induce downstream abnormal cellular biological events through this change (Wang et al., 2018; El-samahy et al., 2018; Safaralizadeh et al, 2019). Many studies have shown that lncRNA MALAT1 participates in the occurrence and development of breast cancer, diabetic retinopathy, liver cancer and other diseases by regulating downstream miRNA. It has become a potential therapeutic target for many diseases due to its active expression in the disease process (Kim et al., 2018; Biswas et al, 2018; Malakar et al, 2017). In this paper intends to construct asthmatic rats and isolate airway smooth muscle cells (ASMCs) to understand the relationship between lncRNA MALAT1, microRNA-216a and asthma. LncRNA MALAT1 and microRNA-216a are involved in the regulation of ASMCs respectively, but there is no research to prove that they can regulate airway remodeling of asthma through mutual combination. Based on this, we speculate that lncRNA MALAT1 and microRNA-216a may be involved in asthma through some connection. Hence, the aim of the present study was performed to investigate the function of lncRNA MALAT1 and microRNA-216a on ASMCs in asthma.

## 2. Methods

This work was supported and approved by institutional review board of QingDao Chengyang District People’s Hospital, Qingdao, Shandong 266600, PR China (IRB/No. 81972349).

### 2.1. Isolation of ASMCs from asthmatic rats

There were 30 65 g–85 g SD rats in total. The research was allowed by the Animal Protection and Use Committee and it followed the

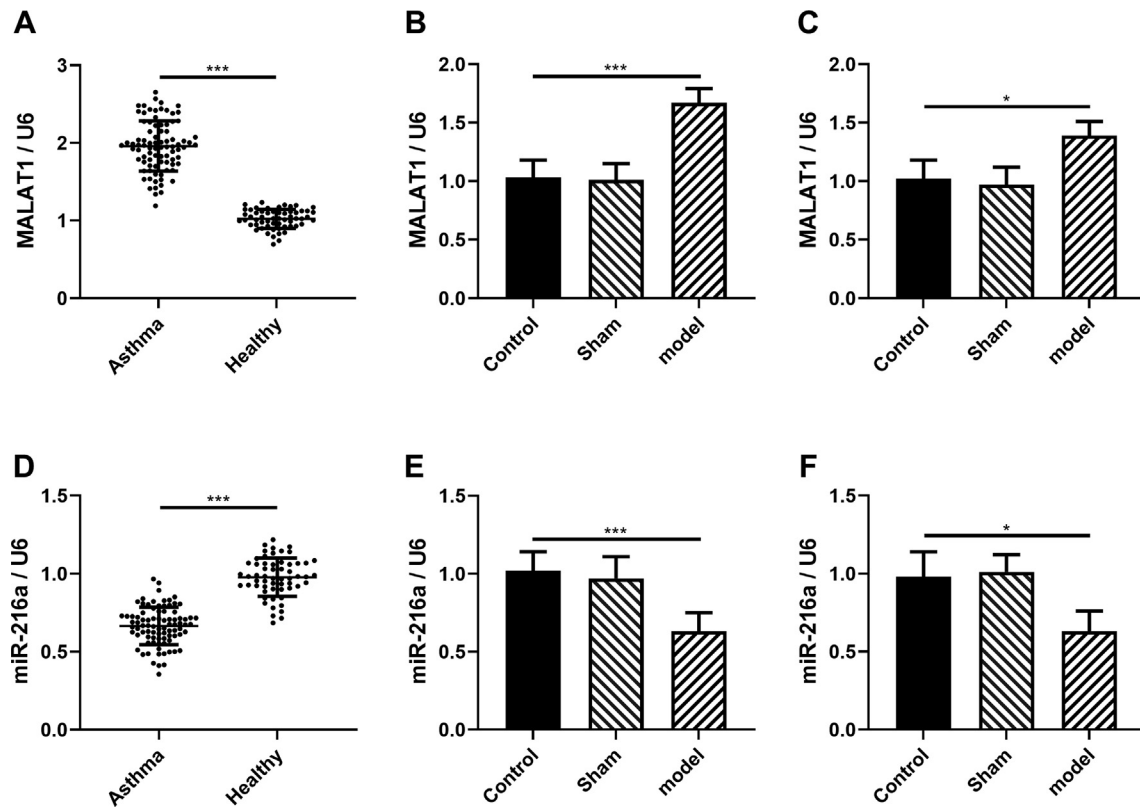
guidelines for animal protection and use in the laboratory of the National Institutes of Health. The construction method of asthma model and airway smooth muscle cells referred to studies of Lin et al. (2019a), Lin et al. (2019b). The separated AMSCs was cultured in DMEM basic medium containing 10% fetal bovine serum and 1% double antibody, and placed in an animal cell incubator at 37°C, 5% CO<sub>2</sub>. During transfection, 1 × 10<sup>5</sup> cells per well were seed into cell culture plate (size 6 wells). lncRNA MALAT1 siRNA, microRNA-216a mimics, microRNA-216a inhibitor and negative control vectors were all acquired from Sangon Biotechnology, Shanghai. Cell lines were transfected with Lipofectamine 2000 transfection kit (Invitrogen, USA). The procedures referred to the kit instructions. Eight hours after transfection, fresh culture medium was changed to avoid poisoning cells. The experiment was repeated three times.

### 2.2. qPCR

Serum RNA was extracted based on the miRNeasy Serum/Plasma kit (QIAGEN, Germany, 217184) kit instructions, and total RNA of ASMCs was extracted by Trizol method. The purity of total RNA was analysed by micro-spectrophotometer. Total RNA samples were reversely transcribed and amplified via TaqMan One Step RT-qPCR kit (Solarbio, China). miR-23a and lncRNA MALAT1 primers were designed and synthesized by Tiangen (Beijing). The qPCR reaction system was referenced from the kit manual. The internal reference genes were U6 and GAPDH, which are standardized by 2<sup>-ΔΔCt</sup> method. The experiment was repeated three times.

### 2.3. Western blot (WB)

AMSCs was washed 3 times with PBS buffer, then RIPA lysis buffer (Solarbio, China) was supplemented to lyse cells. Lysis solution



**Fig. 1. Expression of lncRNA MALAT1 and microRNA-216a in asthma.** (lncRNA MALAT1 and microRNA-216a were collected from human and rat serum samples respectively, and the relative expression was calculated by 2<sup>-ΔΔCt</sup> method. \*\*\* indicates P < 0.001. A: lncRNA MALAT1 up-regulates in asthmatic patients; B: lncRNA MALAT1 up-regulates in asthmatic rats; C: lncRNA MALAT1 up-regulates in asthma ASMCs; D: microRNA-216a is decreased in asthmatic patients; E: microRNA-216a is decreased in asthmatic rats; F: microRNA-216a is decreased in asthma ASMCs.).

was dissociated at 4°C in precooling centrifuge ( $1.6 \times 10^4$  g, 20 min), the precipitate was discarded, and the concentration of protein in supernatant was analysed by BCA kit (Thermo Fisher Scientific). The sample protein was separated by SDS-PAGE electrophoresis. The protein was transferred to polyvinylidene fluoride membrane (EMD millipore). Subsequently, the protein to be detected and primary antibody were supplemented and left to stand at 4°C all night long. Polyvinylidene fluoride membrane was cleaned three times by PBS buffer, then, secondary antibody was supplemented (1: 10000). Finally, the protein blots were visualized by ECL luminescent solution. All antibodies were purchased from Abcam. The experiment was repeated three times. Dilution concentration of primary antibody: Caspase 3, 1: 2000; Bax, 1: 2000; E-cadherin, 1: 10000; Bcl-2, 1: 1000; N-cadherin, 1: 5000;  $\beta$ -catenin, 1: 1000;  $\beta$ -actin, 1: 5000.

2.4. MTTxxx

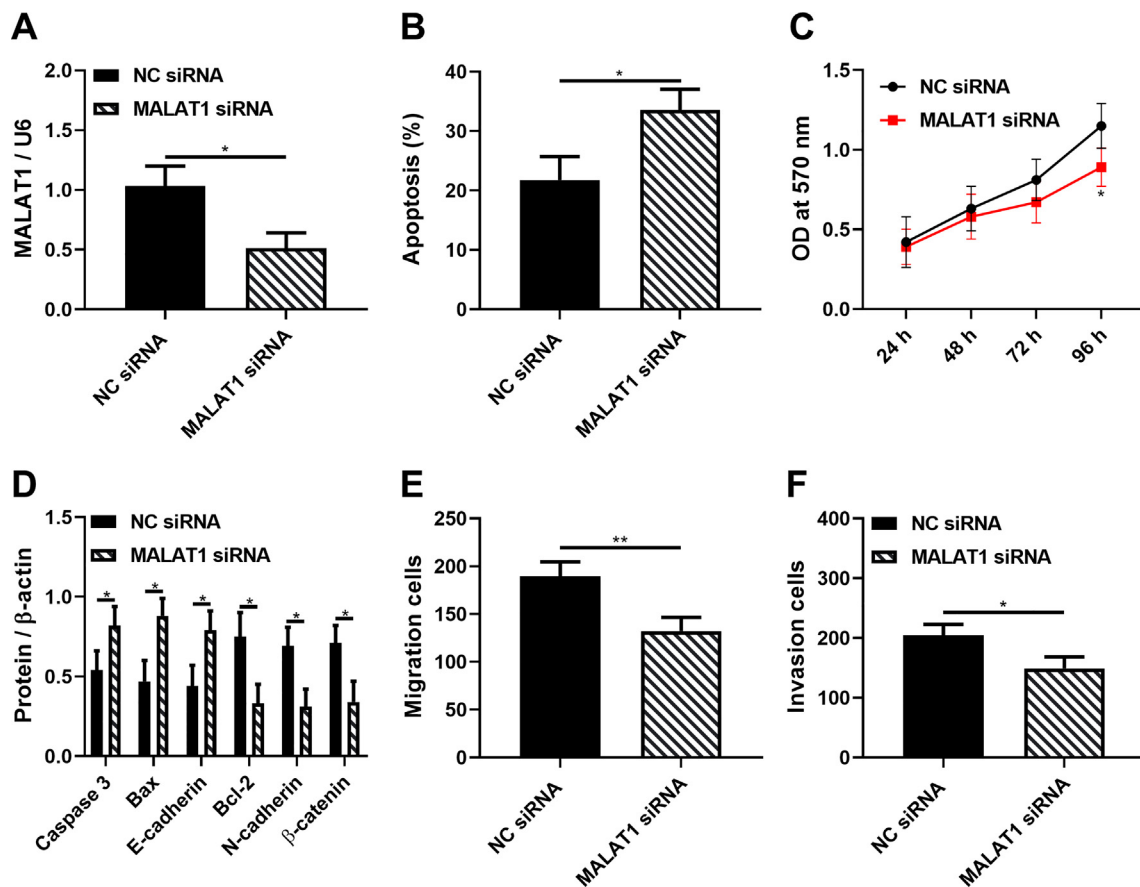
We took 96-well plates, selected 3 wells to inoculate cells,  $3 \times 10^3$  cells per well, and inoculated 4 well plates in total. Cells were cultured at 37 °C/5%CO<sub>2</sub>. One well plate was taken out 24, 48, 72 and 96 h after inoculation respectively. Then, 10  $\mu$ L MTT solution was supplemented. Four hours later, 100  $\mu$ L dimethyl sulfoxide (Solarbio) was supplemented. Enzyme reader evaluated the OD value at 570 nm. The experiment was repeated three times.

2.5. Transwell

Transwell protocol was followed as per earlier published article by Kong et al. 2020. In brief, Cells were inoculated into the migration upper chamber with  $2 \times 10^4$  cells/well, and 500  $\mu$ L DMEM medium was supplemented to the lower chamber. The transfer cell was cultured 24 h at 37 °C, 5%CO<sub>2</sub>, and then the upper chamber liquid was removed and the cell wall was wiped off. Cells of Transwell chamber were immobilized 20 min through 4% paraformaldehyde. Transwell chamber was stained 15 min by crystal violet and cleaned by PBS buffer solution. The cell number was calculated by 3 fields of view that was randomly selected. The experimental conditions of invasion were similar to migration, but an extra layer of 8% Matrigel was needed, and the number of inoculations were increased to  $5 \times 10^4$ . The experiment was repeated three times.

2.6. Dual-luciferase reporter gene

The sequence information of lncRNA MALAT1 and microRNA-216a was analyzed by Starbase2.0, predicting whether the two had binding sites. Cells were inoculated into 12-well plates and cultured until they grew well. PmirGLO-MALAT1-wt (including binding sites) and pmirGLO-MALAT1-mut (binding site mutation) were constructed according to the predicted sites and pmirGLO was set as the control group. AMSCs were co transfected with NC mimcs and microRNA-216a mimcs respectively with the above



**Fig. 2. Effect of lncRNA MALAT1 on airway smooth muscle cells.** (\* indicates  $P < 0.05$ . A: siRNA down-regulates lncRNA MALAT1; B: Down-regulation of lncRNA MALAT1 causes decrease of Caspase 3, Bax, E-cadherin and increase of Bcl-2, N-cadherin and  $\beta$ -catenin; C: Down-regulation of lncRNA MALAT1 leads to increased apoptosis; D: Down-regulation of lncRNA MALAT1 leads to reduction of cell migration; E: Down-regulation of lncRNA MALAT1 leads to reduction of cell invasion; F: Down-regulation of lncRNA MALAT1 leads to reduction of cell activity.)

three. pmirGLO-MALAT1-wt and pmirGLO-MALAT1-mut were designed and synthesized by Tiangen (Beijing). The luciferase intensity was detected by dual-luciferase reporter gene assay system (Promega). Relative luciferase intensity = firefly luciferase intensity/sea kidney luciferase intensity. The experiment was repeated three times.

2.7. MS2-RNA binding protein co-immunoprecipitation (MS2-RIP)

Co-immunoprecipitation protocol was followed as per earlier published article by Lin et al. (2019a), Lin et al. (2019b). MS2-MALAT1-wt containing MS2 hairpin structure (with microRNA-216a binding site) and MS2-MALAT1-mut (without microRNA-216a binding site) were constructed. The above vector was transfected into AMSCs. Forty-eight hours later, co-immunoprecipitation was performed using Magna RIP™ RNA binding protein co-immunoprecipitation kit (Millipore). RNA purification was then carried out and qPCR quantified microRNA-216a. MS2-MALAT1-wt and MS2-MALAT1-mut were repeated three times.

2.8. Flow cytometry

An earlier published protocol was followed for below mentioned protocol (Kong et al. 2020). In brief, Cell suspension with cell number  $1 \times 10^6$  was prepared. The cells were fixed 30 min at 4 °C in 70% ethanol ice-cold solution. We removed the ethanol solution, added Annexin V-FITC/PI-A mixed solution into the cells,

and incubated them 30 min at room temperature. FACSscan flow cytometry (Becton Dickinson, USA) was applied to determine apoptosis date.

2.9. Statistics and analysis

The experiment was repeated 3 times, and the measurement results were expressed as Mean ± SD (standard deviation). Data differences were analyzed by SPSS 20.0 and pictures were drawn by GraphPad 8.0. The differences between the two groups were compared by independent-samples *T* test. The differences among groups were analyzed by one-way analysis of variance, and post hoc pairwise comparison was under LSD-*t* test. The correlation between lncRNA MALAT1 and microRNA-216a was compared by Pearson analysis. All comparisons were two-tailed tests. We took 95% as its confidence interval. The difference between group was marked in statistics When  $p < 0.05$ .

3. Results

3.1. lncRNA MALAT1 and microRNA-216a in asthma

Serum samples of 86 asthmatic patients and 58 healthy people were collected. qPCR quantified the lncRNA MALAT1 and microRNA-216a expression levels. Fig. 1A and 1C manifested that lncRNA MALAT1 increased and microRNA-216a decreased in asthmatic patients compared with healthy people. After asthmatic rats were constructed, their expression levels in serum and AMCSs

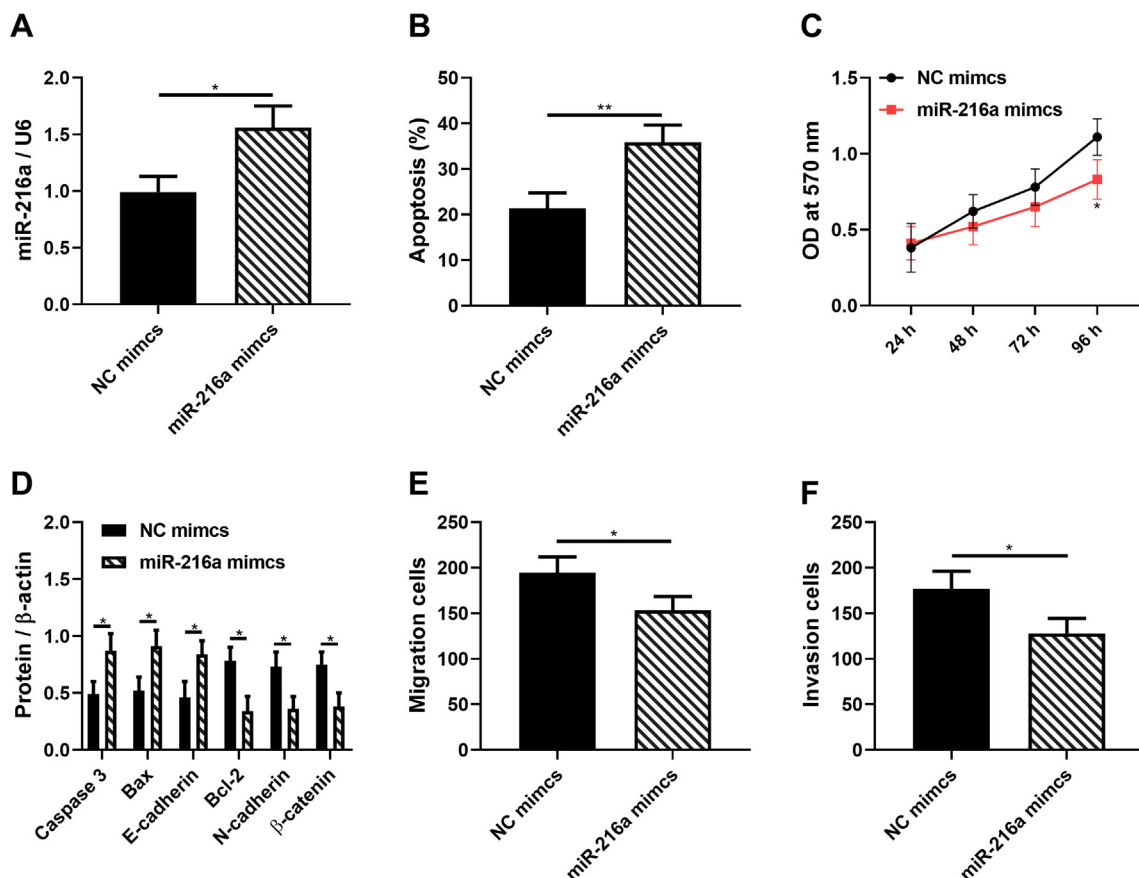


Fig. 3. Effect of microRNA-216a on airway smooth muscle cells. (\* indicates  $P < 0.05$  and \*\* indicates  $P < 0.01$ ). A: mimics upregulates microRNA-216a; B: Up-regulation of microRNA-216a causes decrease of Caspase 3, Bax and E-cadherin and increase of Bcl-2, N-cadherin and -catenin; C: Up-regulation of microRNA-216a increases apoptosis; E: Down-regulation of microRNA-216a leads to reduction of cell migration; E: Up-regulation of microRNA-216a leads to reduction of cell invasion; F: Up-regulation of microRNA-216a leads to reduction of cell activity.)

were quantified by qPCR. Fig. 1B, 1D, 1E and 1F manifested that lncRNA MALAT1 was up-regulated and microRNA-216a was down-regulated in serum and AMSCs of asthmatic rats. The above results signified that lncRNA MALAT1 was up-regulated and microRNA-216a was down-regulated in asthma.

### 3.2. lncRNA MALAT1 promotes proliferation, migration and invasion of AMSCs

Because lncRNA MALAT1 up-regulated in asthma might be tied to AMSCs, the relationship between lncRNA MALAT1 and AMSCs was observed by down-regulating lncRNA MALAT1. Cell proliferation was detected by MTT assay, apoptosis was checked by flow cytometry, protein level was detected by WB assay, and cell migration and invasion were tested by Transwell assay. Caspase 3, Bax and Bcl-2 are proteins related to apoptosis, while E-cadherin, N-cadherin and -catenin are relevant to cell migration and invasion. Hence, the specific effect of lncRNA MALAT1 on cell biological function can be evaluated based on the level changes of the above proteins. Fig. 2 shows that the down-regulated lncRNA MALAT1 leads to increased apoptosis, decreased cell activity, decreased cell migration and invasion, and up-regulated Caspase 3, Bax, E-cadherin and down-regulated N-cadherin, Bcl-2 and -catenin. The above results indicate that lncRNA MALAT1 up-regulated in asthma promotes proliferation, migration and invasion of AMSCs and promotes apoptosis.

### 3.3. microRNA-216a inhibits proliferation, migration and invasion of AMSCs

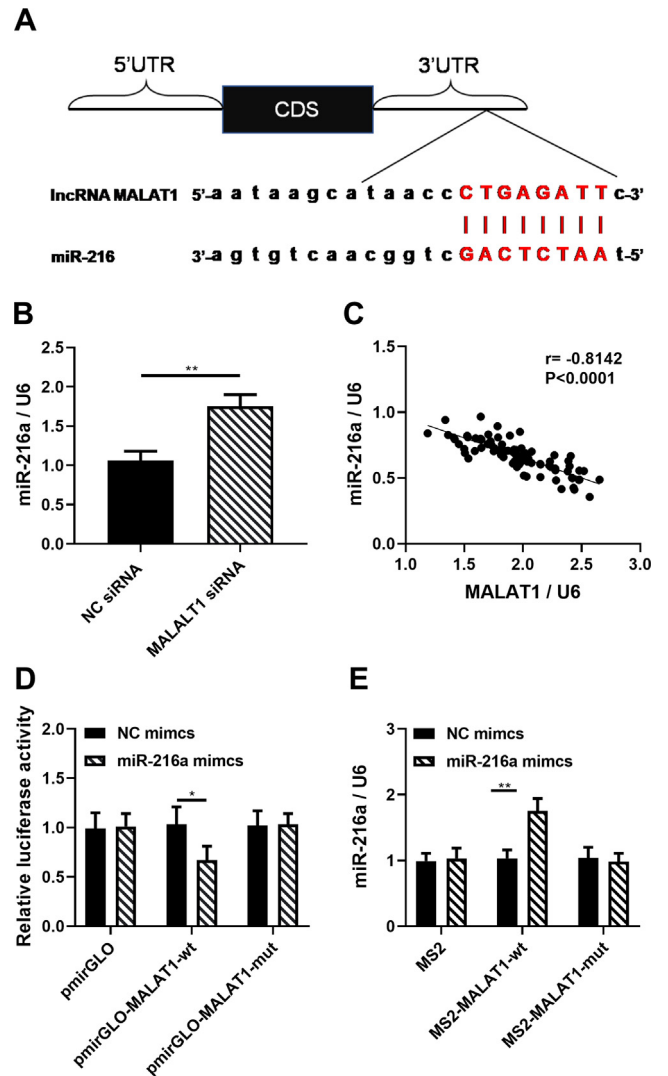
Since miR216a down-regulated in asthma may be related to AMSCs, the relationship between microRNA-216a and AMSCs was observed by up-regulating microRNA-216a. Fig. 3 shows that up-regulation of microRNA-216a leads to increased apoptosis, decreased cell activity, decreased cell migration and invasion, and up-regulation of Caspase 3, Bax and E-cadherin and down-regulation of N-cadherin, Bcl-2 and -catenin. The above results reveal that microRNA-216a inhibits proliferation, migration and invasion of AMSCs and promotes apoptosis by regulating Caspase 3, Bax, E-cadherin, N-cadherin, Bcl-2 and -catenin.

### 3.4. Targeted inhibition of microRNA-216a by lncRNA MALAT1

Starbase2.0 predicted the presence of a sequence site that could bind microRNA-216a in the 3' noncoding region (3'UTR) of lncRNA MALAT1 (Fig. 4A). When lncRNA MALAT1 was down-regulated, microRNA-216a was up-regulated (Fig. 4B). Pearson analysis also manifested that lncRNA MALAT1 was negatively correlated with microRNA-216a (Fig. 4C). According to the prediction results, pmirGLO-MALAT1-wt (including binding site) and pmirGLO-MALAT1-mut (mutation of binding site) were constructed and co-transfected into AMSCs in NC mimics and microRNA-216a mimics respectively. Fig. 4D shows a statistical change in luciferase intensity after co-transfection of microRNA-216a mimics and MALAT1-wt into AMSCs. In this paper, MS2-MALAT1-wt and MS2-MALAT1-mut are also constructed according to the predicted sites. MS2-RIP results signify that microRNA-216a is enriched in MS2-MALAT1-wt. The above results manifest that lncRNA MALAT1 can target to inhibit microRNA-216a.

### 3.5. Rescue experiment

AMSCs were taken and divided into two groups. One group was transfected with MALAT1 siRNA, the other group was transfected with MALAT1 siRNA and microRNA-216a inhibitor. Apoptosis, proliferation, migration and invasion of cells in both groups were

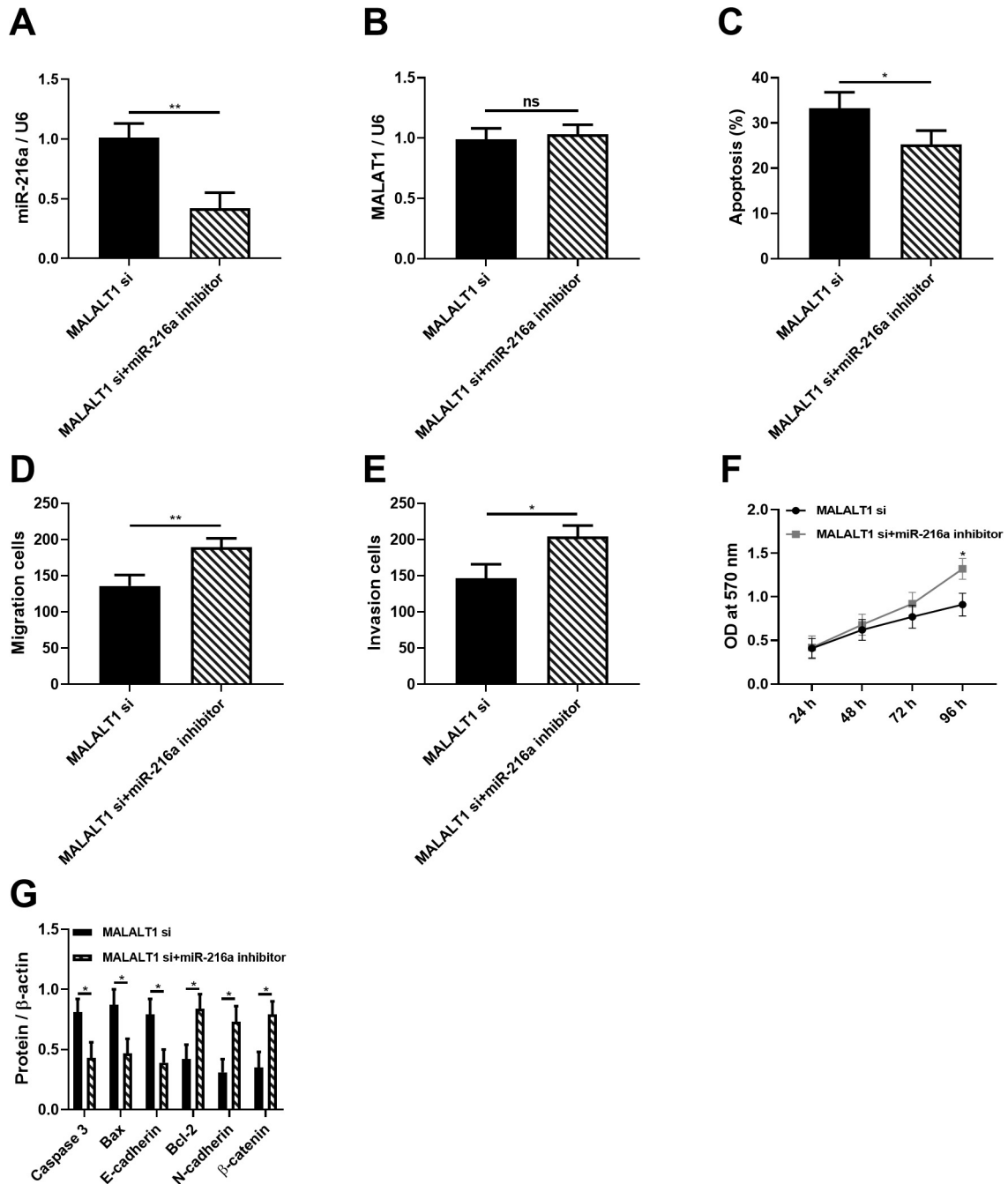


**Fig. 4. lncRNA MALAT1 has a targeting relationship with microRNA-216a.** (5' UTR: 5' non-coding region, 3' UTR: 3' non-coding region, CDS: protein coding region, \* indicates  $P < 0.05$  and \*\* indicates  $P < 0.01$ . A: 3'UTR of lncRNA MALAT1 has a sequence fragment binding to microRNA-216a, and the red part is the binding site; B: Down-regulation of lncRNA MALAT1 causes up-regulation of microRNA-216a; C: lncRNA MALAT1 is negatively correlated with microRNA-216a; D: dual-luciferase reporter gene. E: MS2-RIP).

observed. Fig. 5 shows that downregulation of microRNA-216a counteracts cell proliferation, migration, invasion reduction and apoptosis increase caused by downregulation of MALAT1, and counteracts up-regulation of Caspase 3, Bax, E-cadherin and down-regulation of Bcl-2, N-cadherin and -catenin caused by downregulation of MALAT1. The above results reveal that lncRNA MALAT1 promotes AMSCs hyperproliferation, migration and invasion and inhibits apoptosis by downregulating microRNA-216a.

## 4. Discussion

Tracheal remodeling brings about changes in tracheal structure, thus causing asthma and immune system disorders. Unfortunately, although airway remodeling is currently believed to have a causal relationship with asthma, there is currently no more preferred treatment for asthma airway remodeling. The existing viewpoint (Fehrenbach et al., 2017) holds that airway remodeling caused by hyperproliferation of airway smooth muscle cells can aggravate the severity of asthma. From the perspective of airway smooth



**Fig. 5. Rescue experiment.** (\* indicates  $P < 0.05$ , \*\* indicates  $P < 0.01$ , and there was no statistical difference. A: Down-regulation of microRNA-216a counteracts up-regulation of microRNA-216a caused by down-regulation of MALAT1; B: Down-regulation of microRNA-216a does not affect lncRNA MALAT1; C: Down-regulation of microRNA-216a counteracts changes in apoptotic proteins caused by down-regulation of MALAT1; D: Down-regulation of microRNA-216a counteracts the increase in apoptosis caused by down-regulation of MALAT1; E: Down-regulation of microRNA-216a counteracts cell migration reduction caused by down-regulation of MALAT1; F: Down-regulation of microRNA-216a counteracts cell invasion reduction caused by down-regulation of MALAT1; G: Down-regulation of microRNA-216a counteracts the decrease in cell activity caused by down-regulation of MALAT1.).

muscle cells, if it can inhibit cell hyperproliferation, it may alleviate or improve airway remodeling. Cell proliferation is regulated in many ways, and the common regulation of lncRNA and miRNA is one of the more prominent regulatory mechanisms. At the moment, many studies (Fan et al., 2019; Liu et al., 2020) manifest that the biological function of airway smooth muscle cells can be regulated by changing lncRNA or miRNA. These studies make it possible to improve airway remodeling (Li et al, 2019; Masoumi et al., 2019).

Interestingly, when comparing the serum samples of asthmatic patients and healthy people, we found that lncRNA MALAT1 is up-regulated and microRNA-216a is down-regulated in asthmatic patients, and the same results are obtained after comparing the serum samples of asthmatic and normal rats. Fan et al. (2019) and Lin et al. (2019a), Lin et al. (2019b) confirmed that microRNA-216a and lncRNA MALAT1 participated in the regulation of airway smooth muscle cells, respectively, but there was no research showing that the two could involve in asthma together. Pearson correlation results signified that lncRNA MALAT1 was

negatively correlated with microRNA-216a, and then we found that it could bind with microRNA-216a through starbase2.0. This article speculated that lncRNA MALAT1 might participate in asthma by down-regulating microRNA-216a. Therefore, dual-luciferase reporter gene and MS2-RIP experiment were employed to verify this binding site. Fig. 4D and 4E reveal that lncRNA MALAT1 targetedly inhibits microRNA-216a expression.

Next, the relationship between lncRNA MALAT1, microRNA-216a and airway smooth muscle cells was studied by regulating the two. Cell proliferation was detected by MTT method, apoptosis was detected by flow cytometry, cell migration and invasion were detected by Transwell method, and apoptosis proteins (Caspase 3, Bax, Bcl-2) and migration invasion proteins (E-cadherin, N-cadherin and -catenin) were tested by WB method. Whether down-regulating lncRNA MALAT1 or up-regulating microRNA-216a, cell proliferation, migration and invasion reduce, apoptosis increases, Caspase, Bax and E-cadherin are up-regulated, and Bcl-2, N-cadherin and -catenin are down-regulated. In addition, down-regulation of lncRNA MALAT1 causes up-regulation of microRNA-216a. Rescue experiments show that down-regulation of microRNA-216a can offset cell changes caused by down-regulation of lncRNA MALAT1. According to the above results, it is speculated that lncRNA MALAT1, which is up-regulated in asthma, promotes Bcl-2, N-cadherin and -catenin by down-regulating microRNA-216a, and inhibits Caspase, Bax and E-cadherin. However, this regulation will cause excessive proliferation, migration and invasion of cells, decrease of apoptosis, and ultimately greatly improve the viability of airway smooth muscle cells.

Although the expression levels of lncRNA MALAT1 and microRNA-216a in serum samples of healthy people and asthma patients were compared, those of the two in clinical samples airway smooth muscle cells were not studied. With regard to the airway remodeling in asthma, this research found that lncRNA MALAT1 could promote airway remodeling by down-regulating microRNA-216a, causing airway obstruction and asthma. This finding suggests that lncRNA MALAT1 or microRNA-216a are potential targets for the treatment of airway remodeling in asthma, which can be clinically controlled to improve airway remodeling and relieve asthma. Interestingly, since lncRNA MALAT1 and microRNA-216a take part in asthma by jointly regulating the proliferation of airway smooth muscle cells and other biological functions, can they become biomarkers of asthma? What is the relationship between the two and asthma diagnosis and poor prognosis? What target gene does downstream of microRNA-216a regulate airway smooth muscle cells? These problems are worth discussing in the future research. Overall, it is found that lncRNA MALAT1, which is up-regulated in asthma, promotes proliferation, migration and invasion of airway smooth muscle cells and inhibits apoptosis through sponge action on microRNA-216a, which may lead to airway obstruction and asthma. Down-regulation of lncRNA MALAT1 or up-regulation of microRNA-216a may be a potential direction for tracheal remodeling in asthma.

#### Acknowledgements

None.

#### Funding

None.

#### Declaration of Competing Interest

Authors do not have anything to disclose and declare not conflict of interest.

#### Authors' contributions

*JunQian Huang and Fang Wang* - Concepts, Design, Data analysis, Statistical analysis, Manuscript preparation, Manuscript review, Guarantor.

*LongTao Wang and YongMei Li* -Definition of intellectual content, Literature search, Experimental studies, Data acquisition, Manuscript editing.

*Junli Lu and JianYou Chen* -Data analysis, Statistical analysis, Manuscript preparation, Literature search, Experiment + al studies, Data acquisition.

#### Availability of data, data sharing and accessibility

All study related data are provided in this study and raw data can be requested to corresponding author. There is no data shared from other sources.

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