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Original Article

miroRNA-31 affects the expression of asthma-related cytokines via regulation of CD44

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Abstract: MiroRNAs (miRNAs) play a crucial role in inflammatory development and the progression of asthma. In this study, we aimed to identify miRNAs that regulate gene expression of CD44, an asthma-related inflammatory factor, by targeting CD44 promoter elements and to analyse the role of miRNAs in asthma. Bioinformatic analysis was performed to predict miRNAs that potentially regulate gene expression of CD44 by binding to the CD44 promoter. The expression of these miRNAs was detected in epithelial cells and plasma in both asthma patients and healthy controls. We then transfected the relevant miRNA mimic/inhibitor into human bronchial epithelial cells; measured the expression of CD44 using real-time quantitative polymerase chain reaction (qPCR), immunoblotting (Western blot) and cellular immunofluorescence; and detected asthma-related cytokine (IL-6, IL-8 and ICAM) expression levels by ELISA. We first identified that miR-31 expression was enhanced in the epithelial cells and plasma of asthma patients. In vitro, our data indicated that overexpression of miR-31 induced the expression level of CD44 and enhanced asthma-related cytokines in BEAS-2B cells, while knockdown of endogenous miR-31 decreased CD44 and asthma-related cytokine levels. Further studies demonstrated that miR-31 regulated the progression of asthma by directly binding the promoter region of the CD44 gene and that re-suppression and restoration of CD44 expression reversed the effects of miR-31 on expression of asthma-related cytokines. Taken together, our findings indicate that miR-31 may play a valuable role in the asthma-related inflammatory response and may be a promising interventional therapeutic target for asthma.

Keywords: microRNA-31, asthma, cytokines, CD44

Introduction

Asthma is a chronic airway inflammatory disease that seriously threatens human health and is characterized by airway inflammation, exaggerated bronchial airway hyperresponsiveness (AHR), and variable airflow obstruction in response to inhaled antigens [1, 2]. According to the World Health Organization (WHO), it is estimated that there are approximately 150-200 million asthma patients around the world [3]. Although great advances have been made in studies of immunologic and inflammatory mechanisms of asthma, its molecular mechanism has yet to be definitively characterized.

miroRNAs (miRNAs) are endogenous non-coding small RNA molecules with a length of 21 to 24 nucleotides [4, 5] that play an important role in the processes of cell differentiation, proliferation and apoptosis and regulate the body's growth, development and disease development process [6]. Studies have confirmed that miRNAs can inhibit mRNA translation or lead to mRNA degradation by binding to complementary sequences in the 3'-untranslated region (3'-UTR), 5'-UTR or open reading frame of target genes [7-9]. In addition, miRNAs have been proven to mediate gene activation via binding to the target gene promoter region [10, 11]. Despite deeper progress in the understanding of miRNA biological functions, regulatory mechanisms still need to be elucidated.

CD44, a transmembrane glycoprotein, belongs to the unclassified adhesion molecule group. It

is involved in activating lymphocytes, facilitating specific adhesion between cells and substrates, increasing airway reactivity, promoting the accumulation of inflammatory cells and stimulating a variety of cell proliferation activities [12]. Our previous study identified that the expression of CD44 is high in asthmatic rat lung tissue [13]. However, the relationship between CD44 and asthma is not clear, and a specific miRNA for the expression and regulation of CD44 may play a critical role. To explore whether miRNAs play a role in CD44-mediated airway inflammation, the levels of endogenous miRNAs were measured inunaffected controls and asthma patients. We then transfected miR-31 mimic/inhibitor into BEAS-2B cells to investigate the relationship between miR-31 and inflammatory mechanisms of asthma. The results reveal that miR-31 playa potentially significant role in regulating asthma by the direct regulation of CD44.

Materials and methods

Patients and tissue samples

Patients with asthma (n = 10) and healthy controls (n = 10) were recruited from the Department of Respiratory Medicine, Nanjing Children's Hospital affiliated with Nanjing Medical University between 2013 and 2014. Diagnoses of asthma were verified by a respiratory physician in our hospital according to the diagnostic criteria of asthma. Healthy control subjects had no respiratory symptoms. None of the subjects had ever smoked or received inhaled or oral corticosteroids or leukotriene antagonists. For each subject, blood and bronchial epithelial brushing samples were collected. All samples were obtained with informed consent, and the study protocol was approved by the Ethics Committee of Nanjing Medical University.

Cell culture

The BEAS-2B cell line was a gift from paediatrics professor Zhou Guoping of the Fourth School of Clinical Medicine of Nanjing Medical University. The cells were grown in Dulbecco's modified Eagle's medium (DMEM, Invitrogen, Carlsbad, CA, USA) with 10% foetal bovine serum (Invitrogen, Carlsbad, CA, USA) at 37°C and 5% CO_2 in a humidified chamber.

Cell transfection

BEAS-2B cells were plated in 6-well and 24-well plates and incubated overnight until the cells were 50-70% confluent. miRNAs and CD44 siRNA were purchased from Dharmacon (Austin, TX, USA) and ThermoFisher (Shanghai, China). The cells were transfected with a miRNA mimic (50 nM) or siRNA (25 nM) using Lipofectamine™ 2000 reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. The cells were counted and photos were taken under a green fluorescent inverted microscope to detect BEAS-2B cells with a FAM label after transfection.

Real-time quantitative polymerase chain reaction (qPCR)

qPCR was performed to verify the expression of miRNAs and CD44 in BEAS-2B cells. After transfection for 48 h, RNA was extracted from BEAS-2B cells by TriZOL. qPCR was performed using the SYBR PCR Master Mix (Applied Biosystems, Foster City, CA, USA) on an ABI 7500 Fast system (Applied Biosystems). Primers were synthesized from Changzhou Bo Hong Biological Engineering Co., LTD (Jiangsu, China). The miRNA primer sequences were as follows: miR-21, 5'-TAGCTTATCAGACTGATGTTGA-3'; miR-31, 5'-CGGAGGCAAGATGCTGCATAGCT-3'; miR-141, 5'-ATCTTTACCAGACAGTGTTATT-3'; U6 was used as an internal reference. Primers for CD44 analysis were as follows: CD44 forward, 5'-GAGAGCTGGCCAAGTCTTCA-3', reverse, 5'-GTCAAGCTGTGCTTCCAGAGTTA-3'; β-actin was selected as an internal reference. The reactions were performed using SYBR Premix Ex TaqTM II (Qiagen, Austin, TX), with conditions as follows: pre-degeneration at 95°C for 30 sec and 40 cycles of denaturation at 95°C for 5 sec and annealing/extension at 60°C for 34 sec. The expression levels of miR-21, miR-31, miR-141 and CD44 mRNA were calculated by the $2^{-\Delta\Delta Ct}$ method.

Western blots

Protein was extracted by RIPA lysis according to the instructions. The protein concentration was detected using the BCA Kit (Pierce, IL, USA), and 20 μg of each protein sample was separated by 10% SDS-polyacrylamide gel electrophoresis (PAGE) and transferred onto nitrocellulose (NC) membranes. The membranes were then blocked with 5% skim milk at room temperature for 1 h and washed in TBST for 5 min three times. Primary antibodies to CD44 (Cell Signaling Technology, Boston, USA, Catalogue NO. #3570; final dilution, 1:1,000; mouse anti-

human monoclonal antibody) and β-actin (Cell Signaling Technology, Boston, USA, Catalogue NO. #3700; final dilution, 1:1,000; mouse anti-human monoclonal antibody) were incubated at 4°C overnight. The HRP-labelled goat anti-mouse IgG (Cell Signaling Technology, Boston, USA, Catalogue NO. #7072; final dilution, 1:2,000; mouse anti-human monoclonal antibody) was incubated at room temperature for 1 h. Then, the bands were visualized with an enhanced chemiluminescence detection reagent usingthe ChemiDoc XRS system (Bio-Rad, Hercules, CA, USA).

Immunofluorescence

The cells were washed with PBS and fixed in 4% paraformaldehyde. Membranes were permeabilized with 0.2% Triton, and cells were blocked in normal fluid (1% BSA, 1% goat serum) for 30 min at room temperature. Subsequently, cells were washed with PBS and incubated with the primary antibody to CD44 (Cell Signaling Technology, Boston, USA, Catalogue NO. #3570; final dilution, 1:1,000; mouse anti-human monoclonal antibody) at 4°C in a humid chamber overnight. This was followed by incubation with FITC-labelled goat anti-mouse IgG for 1 h at room temperature and then by analysis using a confocal laser fluorescent microscope (Zeiss 710; Carl Zeiss, Oberchoken, Germany).

ELISA analysis

Culture supernatant was collected and analysed according to the ELISA kit instructions. The specimens and standard, respectively, were added to IL-6 monoclonal antibody-coated enzyme panels at different concentrations (100 μl/hole) and were incubated for 2 h at room temperature, followed by incubation with the HRP-labelled anti IL-6 monoclonal antibody (100 μl/hole) for 1 h at room temperature. Then, the plate was washed four times and a chromogenic agent was added; then, the plates were incubated away from light at room temperature for 10-30 min. The reaction was stopped with stop solution. The optical density value was measured by microplate reader (Varioskan Flash 3001, Thermo, USA). Detection of the IL-8 and ICAM levels were conducted according to the kit instructions as described above.

Plasmid construction

To construct a luciferase reporter vector, the 1-kb transcriptional start region of CD44, as well as the mutant sequence of CD44, was synthesized by PCR. The primers used contained the following restriction sites: CD44 forward, 5'-AACTCCCCACCCCTCACTCCC-3', reverse, 5'-CCTCGCCCA AAACTGCGCGC-3'; and Mut CD44 forward, 5'-TCCTGTGAAACCAAGAGATCGGGCTC-3', reverse, 5'-GAGCCCGATCTCTTGGTTTCACAGGA-3'. The PCR product was cloned into the SpeI and HindIII restriction sites downstream of the luciferase open reading frame in the pMIR-REPORT vector (Ambion, Carlsbad, CA, USA). The CD44-overexpressing plasmid was amplified by PCR with the following primers: forward, 5'-ATGGACAAGTTTTGGTGGCAC-3' and reverse, 5'-TTACACCCCAATCTTCATGTCC'. The PCR amplicons of CD44 were cloned into the T vector (Promega, Madison, WI, USA).

Luciferase reporter assays

For the luciferase assay, BEAS-2B cells were grown to 70-80% confluence in 24-well plates and co-transfected with a firefly luciferase reporter vector containing the seed sequence or its mutant sequence and miRNA mimics or inhibitors (50 nM) using Lipofectamine™ 2000 reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. The luciferase activity assay was performed 48 h after co-transfection using the Dual Luciferase Assay System (Promega, Madison, WI, USA), and the values were normalized with Renilla luciferase activity.

Statistical analysis

Statistical analyses were carried out using SPSS 15.0 software (SPSS, Inc., Chicago, IL, USA). All data are shown as the mean ± standard deviation (SD). The statistical significance between groups was analysed using Student's t-test. $P < 0.05$ for the difference was considered statistically significant.

Results

The expression of miR-31 is up-regulated in asthma patients

To determine the potential miRNA stargeting CD44, we analysed the sequence of the CD44

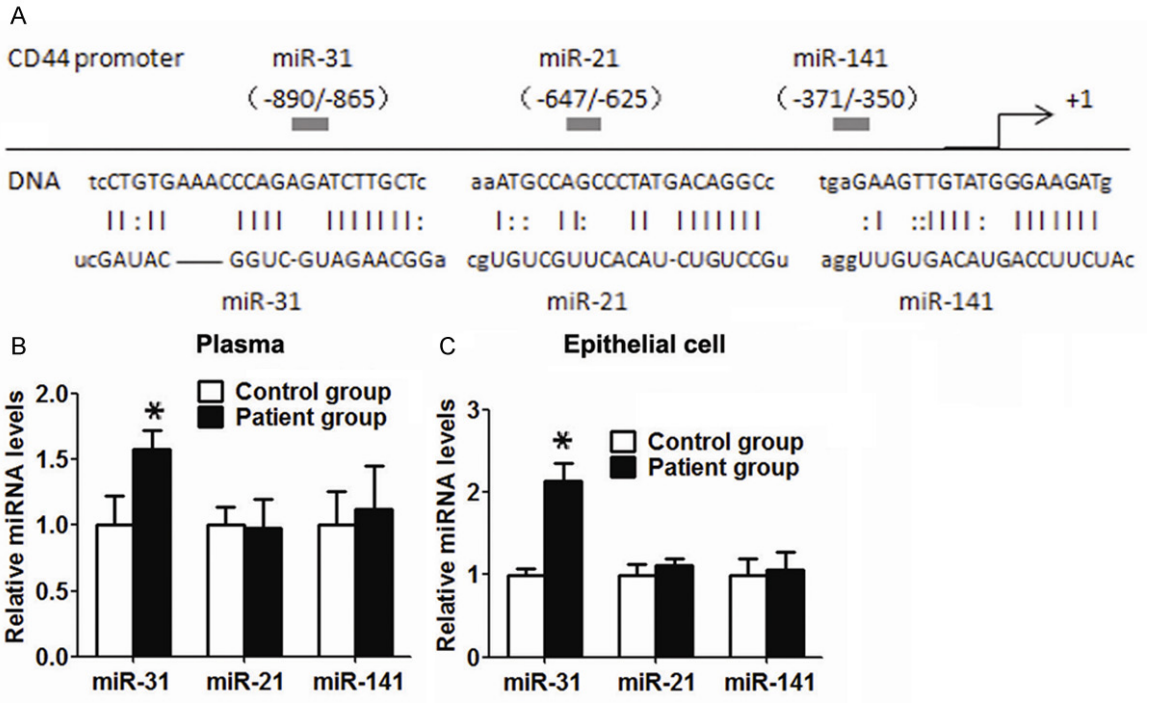

Figure 1. Enhanced miR-31 expression in asthma patients. A: The sequence of the CD44 promoter transcription start site was analysedvia bioinformatics using the miRNA target prediction software programs miRBase, TargetScan and RegRNA. B, C: Plasma and epithelial cell miR-31 expression was determined by quantitative PCR in subjects with asthma and in healthy controls. Data are expressed as the mean ± SD. *, $P < 0.05$, $n = 10$.

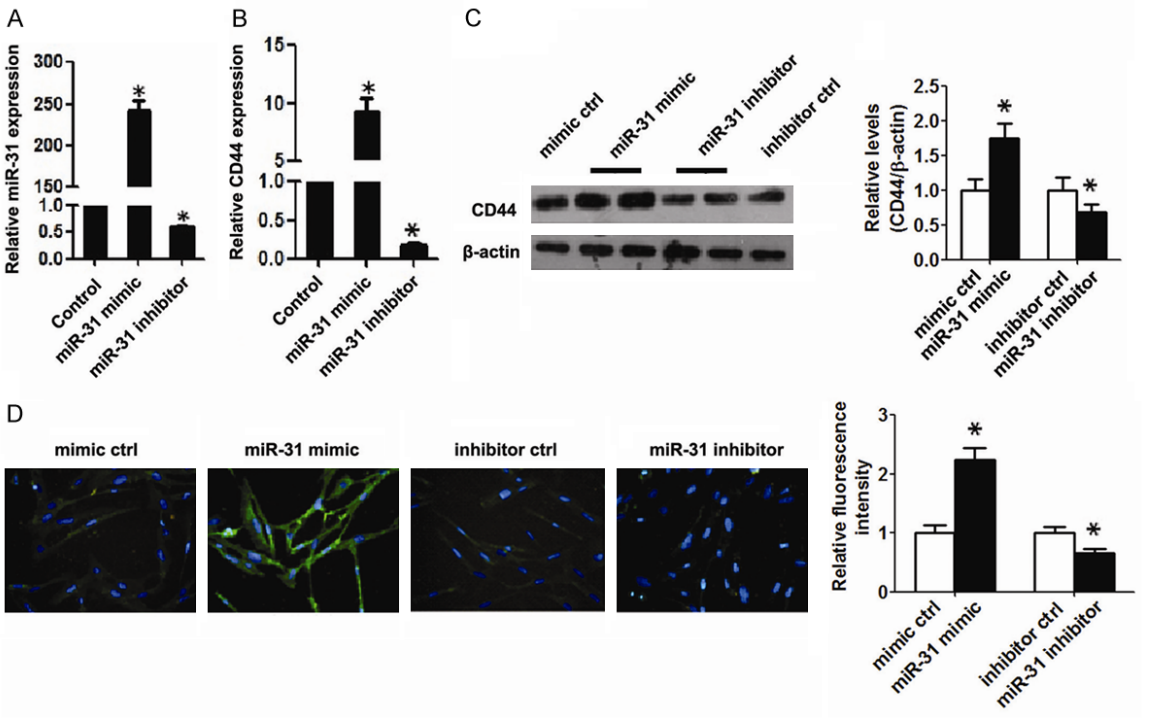

Figure 2. miR-31 regulates CD44 expression in BEAS-2B cells. BEAS-2B cells were transfected with control or a miRNA mimic/inhibitor for 48 h. A: Validation of miR-31 expression in BEAS-2B cells by RT-PCR analysis. B: CD44 mRNA level was detected by qPCR. C: Western blot analysis was performed to detect CD44 protein expression in BEAS-2B cells. Relative expression of CD44 was calculated based on densitometric analysis of band intensities. D:

Representative images of CD44 immunofluorescence in BEAS-2B cells (scale bar = 50 μm). Relative expression of CD44 was calculated based on fluorescence intensity. Data are expressed as the mean ± SD. *, $P < 0.05$, n = 5.

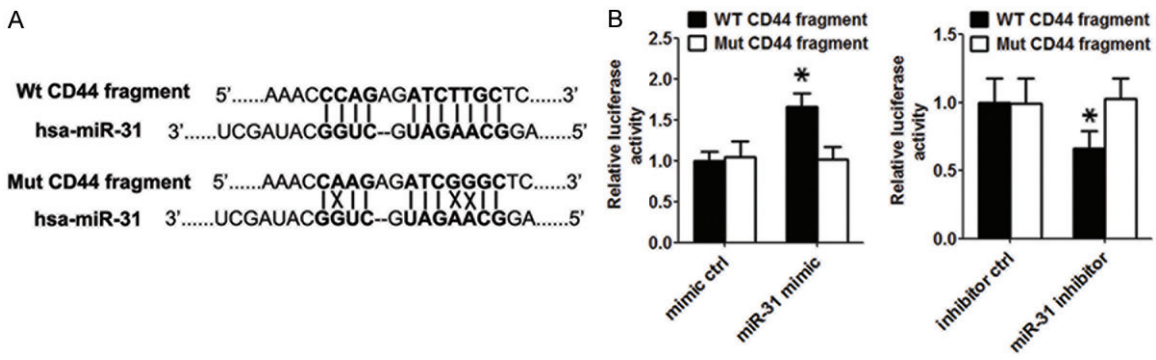

Figure 3. miR-31 targets CD44 by directly binding the transcriptional start region. A: Potential miR-31 target sequences in the transcriptional start region of CD44 are shown (solid lines indicate matching base pairs and crosses represent non-matching base pairs). B: The effect of miRNA mimic or inhibitor on luciferase intensity controlled by the wild-type or mutant fragment was determined with a luciferase assay. Data are expressed as the mean ± SD. *, $P < 0.05$, n = 5.

Table 1. The levels of IL-6, IL-8 and ICAM (pg/ml)

Group	IL-6	IL-8	ICAM
Control mimic	5.52±0.25	5.13±0.25	2.48±0.21
miR-31 mimic	6.63±0.84*	6.45±0.67*	3.56±0.25*
miR-21 mimic	5.53±0.90	5.05±0.27	2.10±0.27
miR-141 mimic	5.50±0.25	4.60±0.82	2.55±0.51
Control inhibitor	5.41±0.35	5.10±0.87	2.55±0.33
miR-31 inhibitor	4.35±0.75#	4.10±0.50#	1.97±0.40#
miR-21 inhibitor	5.20±0.76	5.60±0.60	2.71±0.36
miR-141 inhibitor	5.59±0.36	4.98±0.90	2.60±0.37

*, $P < 0.05$ vs. Control mimic group; #, $P < 0.05$ vs. Control inhibitor group.

promoter transcription start region and conducted a bioinformatic analysis using the miRNA target prediction software programs miRBase, TargetScan and RegRNA, screening miRNAs that may regulate the CD44 promoter. Three miRNAs (miR-31, miR-21 and miR-141) were predicted to bind to the CD44 promoter region (**Figure 1A**). We detected the expression level of miRNAs by qPCR in the plasma and epithelial cells of asthma patients. Compared with the control group, the expression level of miR-31 was obviously higher in asthma patients (**Figure 1B** and **1C**). However, there were no significant differences observed in the miR-21 and miR-141 levels.

miR-31 regulates CD44 expression in BEAS-2B cells

To demonstrate whether miR-31 regulated CD44 expression in BEAS-2B cells, we up-regulated and down-regulated miR-31 expression in BEAS-2B cells by miR-31 mimic/inhibitor transfection. Compared with the control group, the expression of miR-31 was obviously increased after transfection with the mimic and was decreased after transfection with the inhibitor (**Figure 2A**). qPCR and western blot results demonstrated that the CD44 mRNA and protein expression levels were significantly elevated by miR-31 mimic transfection, but significantly reduced by miR-31 inhibitor transfection (**Figure 2B** and **2C**). Immunofluorescence analysis also showed similar results, that miR-31 overexpression increased CD44 expression, while the inhibition of miR-31 expression inhibits CD44 protein expression in BEAS-2B cells (**Figure 2D**).

miR-31 directly regulates CD44 gene expression via targeting its promoter region

To verify whether CD44 is a direct target of miR-31 in BEAS-2B cells, we cloned wild-type and mutant CD44 seed fragments into a luciferase reporter gene system (**Figure 3A**). Wild-type or mutant binding sequence constructs were co-transfected with miR-31 or control mimic/inhibitor into BEAS-2B cells, followed by measurement of luciferase activity. The luciferase reporter assay indicated that miR-31 expression led to activation of the wild-type seed sequence, whereas knockdown of miR-31 decreased wild-type luciferase activity. In contrast, it had no effect on the luciferase intensity controlled by the mutant sequence (**Figure 3B**).

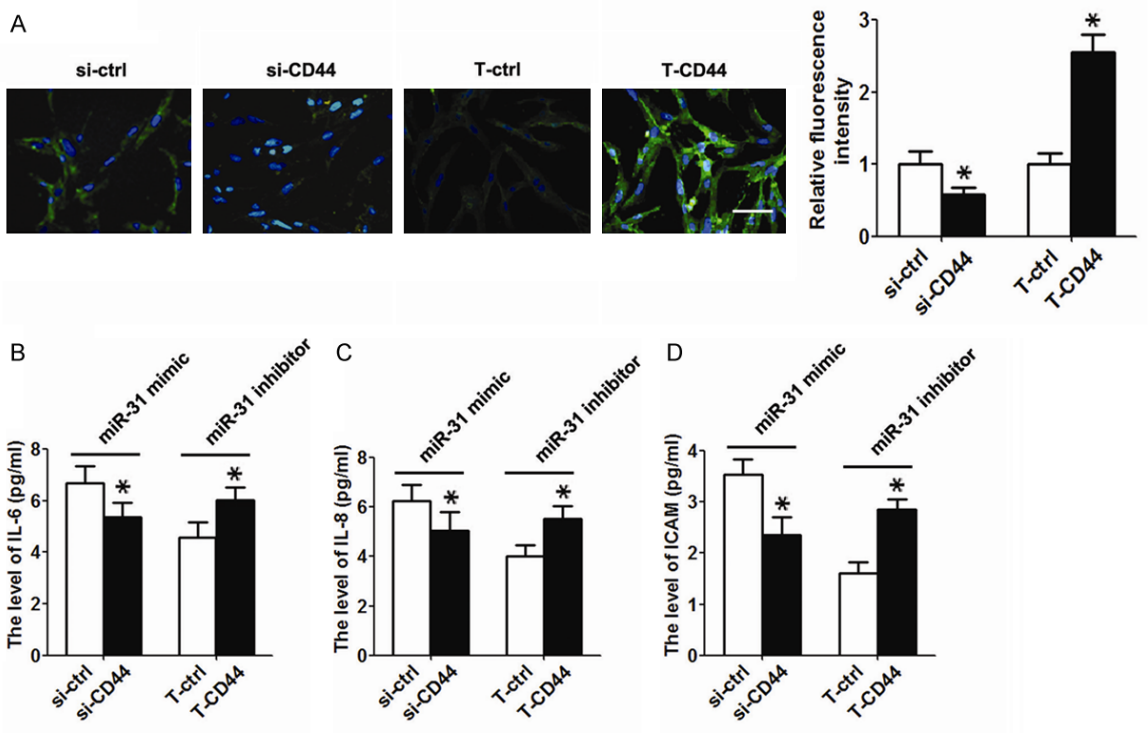

Figure 4. miR-31 controls asthma-related cytokinesvia CD44. BEAS-2B cells were co-transfected with the CD44 siRNA/CD44 recombinant plasmid and miR-31 mimic/miR-31 inhibitor for 48 h. A: Representative images of CD44 immunofluorescence in BEAS-2B cells (scale bar = 50 μm). Relative expression of CD44 was calculated based on fluorescence intensity. B-D: The levels of IL-6, IL-8 and ICAM were analysed by ELISA. The data are expressed as the mean ± SD, n = 5.

miR-31 elevates asthma-related cytokine expression by targeting CD44

To explore the effect of miR-31 on asthma, we detected the expression of asthma-related cytokines by ELISA after miRNA transfection. ELISA showed that overexpression of miR-31 increased the levels of IL-6, IL-8 and ICAM. However, inhibition of miR-31 significantly reduced the IL-6, IL-8 and ICAM levels. The expression of IL-6, IL-8 and ICAM did not change in the presence of the miR-21 or miR-141 mimic/inhibitor (**Table 1**). Moreover, we re-suppressed and restored the CD44 expression in BEAS-2B cells by transfecting CD44 siRNA and a recombinant plasmid. Immunofluorescence analysis revealed decreased expression of the CD44 protein after CD44 siRNA transfection, while increased CD44 protein expression was found after CD44 recombinant plasmid transfection (**Figure 4A**). Functionally, inhibition of CD44 expression abrogated the effect of miR-31, resulting in significantly attenuated levels of asthma-related cytokines. In contrast, restoration of CD44 expression re-induced asthma-related cytokine expression (**Figure 4B-D**).

Discussion

Current studies imply that the mechanism of asthma primarilyoccurs through chronic airway inflammation and airway remodelling, in which chronic airway inflammation is related to eosinophil, mast cell and lymphocyte infiltration and the release of inflammatory factors [14, 15]. A previous study suggests that CD44 participates in eosinophil and lymphocyte activation, increases airway reactivity, promotes a variety of cell proliferation activities, and is closely associated with the onset of asthma [16]. In addition, excessive expression of CD44 in lung tissue is closely related to the process of asthma, and participates in the onset of asthma at an early stage [17]. CD44 expression is also associated with eosinophil and lymphoid cell chemotaxis and infiltration, resulting in the inflammation of airway tissue in asthma [18, 19]. However, the transcriptional regulation mechanism of the CD44 gene remains unclear.

In the diagnosis and treatment of disease, miRNA-mediated target gene transcriptional activation also has great advantages in target specificity and the flexibility of target genes. Recent studies have focused on the regulatory mechanism of miRNAs in asthma. Some studies indicated that miRNAs mediate target gene transcriptional activation by binding to the promoter region of the target gene (by partially complementary pairing) in the nucleus [20, 21], suggesting that the recognition of gene promoters targeted by miRNAs may be a natural and general mechanism for gene transcriptional regulation [22]. Although miRNA binding sequencesare highly conserved in different species, even with good homologues, a bioinformatically-predicted target may not be the real target. In this study, we used bioinformatics software to analyse 1 kb upstreamof the CD44 transcriptional start site and identified three miRNAs (miR-31, miR-21, miR-141) that were highly complementary with the CD44 promoter region. Furthermore, we then identified that miR-31 expression was enhanced in the plasma and epithelial cells of asthma patients relative to unaffected people.

In vitro, we demonstrated that overexpression of a miR-31 mimic in BEAS-2B cells led to high expression of CD44 and asthma-related cytokines (IL-6, IL-8 and ICAM). However, knockdown of miR-31 expression in BEAS-2B cells decreased the CD44 and asthma-related cytokine levels. In addition, we confirmed that CD44 was a direct target gene of miR-31, and we found that miR-31 positively regulated CD44 expression by directly targeting the promoter region of the CD44 gene in BEAS-2B cells. Further studies will verify whether miR-31 plays a regulatory role in an animal asthma model in vivo. The role of miRNA-mediated gene transcriptional activation in the pathogenesis of asthma is still in its infancy, and many problems still need to be solved.

In summary, our results are the first to indicate that miR-31 affects the expression of asthma-related cytokines by up-regulation of CD44. It is expected that miR-31 will become a new target for diagnosis and treatment of asthma in the future.

Disclosure of conflict of interest

None.

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