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Original Article

Lncrna NEAT1 Regulates Th1/Th2 in Pediatric Asthma by Targeting Microrna-217/GATA3

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Abstract

Background: The imbalance of immune response between helper Th1 and Th2 cells is the direct cause of asthma. It was closely related to abnormal expression of lncRNAs. However, whether lncRNAs can regulate Th1/Th2 balance in pediatric asthma remains to be investigated.

Methods: Peripheral blood samples were collected from children with asthma and normal volunteers at the Children's Hospital of Shaanxi Provincial People's Hospital (Xi'an, China) in 2020. The qRT-PCR was used to detect the expression of lncRNA NEAT1, miR-217 and GATA3 in peripheral blood samples. The effects of lncRNA NEAT1, miR-217, and GATA3 on CD4+T cell population were detected in vitro. Meanwhile, the regulatory effect of lncRNA NEAT1/miR-217/GATA3 was evaluated through the dual luciferase report assay, functional assays and animal experiments.

Results: We investigated that lncRNA NEAT1 and GATA3 was significantly up-regulated in CD4+T cells in peripheral blood of children with asthma ($P<0.001$). Knockdown of lncRNA NEAT1 or GATA3 significantly reduced Th2-related cytokines ($P<0.05$), but had no effect on Th1 cells. Importantly, the interactions of lncRNA NEAT1 with miR-217 and miR-217 with GATA3 were confirmed by dual luciferase report assay. Meanwhile, functional assays and animal experiments demonstrated that lncRNA NEAT1 regulated GATA3 expression through sponge miR-217, thereby regulating Th1/Th2 balance in CD4+T cells in pediatric asthma.

Conclusion: lncRNA NEAT1/miR-217/GATA3 axis may reveal the immunological mechanism of pediatric asthma, which has potential clinical application value in the future.

Keywords: Pediatric asthma; Long noncoding RNAs; Immunology; Proteins

Introduction

Asthma is a chronic airway inflammation caused by the combination of genetic, lifestyle and environmental factors, which is characterized by increased airway responsiveness, airway remodeling, and reversible airway obstruction (1). Asthma not only affects the health and life of adults, but also significantly affects the physical and mental health of growing children (2). Asthma has caused millions of children's death and morbidity worldwide, and increases year by year (3). With the current upward trend, pediatric asthma is likely to become a growing public health problem.

CD4+ T cells are divided into helper T cells (including Th1 cells, Th2 cells and Th17 cells) and

regulatory T cells (Treg cells) according to their biological functions (4). Accumulating evidence indicates that the imbalance of immune response between helper Th1 and Th2 cells is the direct cause of asthma, with the decrease of Th1 and increase of Th2 (5). The pathogenesis of pediatric asthma is also closely related to immune dysfunction of Th1/Th2 cytokines released by CD4+ T cells (6).

Long noncoding RNAs (lncRNAs), a kind of gene transcripts with more than 200 nt in length, fail the function of encoding proteins or only encode short multi-classes of RNA molecules. LncRNAs have been considered as potential biomarkers for childhood asthma (7). There is growing evidence that lncRNAs and miRNAs act as competing endogenous RNA (ceRNAs) to inhibit each other, which form an accurate regulatory network and regulate the target genes of miRNAs (8). The regulatory network composed of lncRNAs and miRNAs plays a vital role in the occurrence and development of childhood asthma. For instance, the pro-inflammatory and profibrotic role of lncRNA RMRP in pediatric asthma through targeting miRNA-206/CCL2 axis (9). Liang et al. demonstrated the heightened expression of lncRNA MALAT1 in asthma, which targeted miR-155 to promote Th2 differentiation, thus aggravating the occurrence of asthma (10).

GATA3, a member of GATA family of zinc-finger proteins, is a T-helper specific transcription factor (11), which can promote the function and development of Th2 cells and inhibit the formation of Th1 cells (12). Therefore, GATA3 can affect Th1/Th2 balance. However, the upstream pathway of GATA3 in the process of pediatric asthma has not been fully elucidated. However, during the development of pediatric asthma, it is still unclear whether lncRNAs regulate specific miRNAs in the form of ceRNAs to indirectly affect GATA3 expression and ultimately induce Th1/Th2 imbalance. Thus, we demonstrated that the lncRNA NEAT1/miR-217/GATA3 axis played a key role in regulating Th1/Th2 balance in children with asthma.

Methods

Participants

We recruited 10 pairs of peripheral blood samples from childhood asthma patients and healthy children with an average age of 8 years in the Children's Hospital of Shaanxi Provincial People's Hospital (Xi'an, China) in 2020. The children with asthma had no liver and kidney dysfunction, respiratory tract infection, or heart failure in the past 3 months. The study protocol was approved by the Children's Hospital of Shaanxi Provincial People's Hospital (approval number 2021-100). The written consent was obtained from each participant.

Separation of CD4+T cells

CD4+T cells were isolated from peripheral blood according to previous studies (13). In brief, about 5 ml of peripheral blood was collected from subjects and then stored in heparin sodium pretreated tubes. Peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll-Hypaque solution (No.#L6115, Biocoll, Germany) based on density gradient centrifugation method. Then, PBMCs were incubated with magnetic beads precoated with anti-CD4 antibody to separate CD4+T cells. The purity of CD4+T cells was detected by flow cytometry. The isolated CD4+T cells (purity>90%) were routinely stored in human T cell culture medium (OpTmizer CTS T-Cell Expansion SFM, Gibco, USA) at 37 ℃ and 5% CO_2.

Cell transfection

All transfection vectors from GenePharma (Shanghai, China) were transfected into CD4+T cells using Lipofectamine 2000 (Invitrogen, USA). si-NEAT1: 5'-CCGTGGTGTGTGTTGTGGAATCTGT-3' si-GATA3-1: 5'-GGCCAGGCAAGAUGAGAAA-3', si-GATA3-2: 5'-CATCGACGGTCAAGGCAAC-3', miR-217 mimic: 5'- CAUCAGUUCCUAAUGCAU-UGCCUU-3', and miR-217 inhibitor: 5'-AAGGCAAUGCAUUAGGAACUGAUG-3'.

ELISA

ELISA was used to detect Th1-associated factor (INF-γ) and Th2-related cytokines (IL-4 and IL-10) in supernatant of CD4+T cell culture medium using ELISA kits (R&D Systems, Minneapolis, USA) according to the instructions (14-16). The OD value at 450 nm wavelength was measured using the BioTek Microplate reader system (Quant, Biotek, Germany).

RNA extraction and qRT-PCR

We used TRIZOL reagent to extract total RNA from CD4+T cells. The total RNA was reverse transcribed into cDNA through a reverse transcription kit (TaKaRa, Tokyo, Japan). Gene amplification was performed under the guidance of the fluorescent quantitative RT-PCR kit (SYBR Green, Bio-Rad, United States) and detected with fluorescence quantitative PCR instrument (Analytick Jena A G, Germany). U6 and GAPDH were used as the internal controls, respectively. All data were analyzed by $2^{-\Delta\Delta CT}$ method.

The primer sequences are as follows: lncRNA NEAT1 forward, 5'-GCTCATAGTGTGACGCTTTTT-3', and reverse, 5'-AAAGCGTCACACTATGAGCTT-3'; hsa-miR-217 forward, 5'-ACACTCCAGCTGGGTACTGCATCAG-GAACTG-3', and reverse, 5'-CTCAACTGGTGTCGTGGAGTCGG-CAATTCAGTTGAGTCCAATCA-3'; GATA3: forward, 5'-CTCCTTTTTGCTCTCCTTTTC-3' and reverse, 5'-AAGAGATGAGGACTGGAGTG-3'; INF-γ: forward, 5'-CGTCTTGGTTTTGCAGCTC-3' and reverse, 5'ACTCCTTTTCCTCTTCCTTA-3'; IL-4: forward, 5'-CTTGCTGTCACCCTGTTC-3' and reverse, 5'CATGGAAGTGCAGGACTGCA-3'; IL-10: forward, 5'-TGCCTTCAGTCAAGTGAAGACT-3' and reverse, 5'-AAACTCATTCA-TGGCCTTGTA-3'; GAPDH forward, 5'-AGCCACATCGCTCAGACAC-3', and reverse, 5'-GCCCAATACGACCAAATCC-3'; U6 forward, 5'-CTCGCTTCGGCAGCACA-3' .

Western blot

Western blotting was used for investigation of protein expression (17). In brief, The cells were performed using RIPA lysis buffer on ice for 30 min and centrifuged x12,000 g at 4 °C for 15 min. The concentration of proteins was examined by the BCA protein Kit (Beyotime, China). Approximately 50 μg of proteins were firstly placed on 10% SDS-PAGE, and then shifted to PVDF membranes. The proteins were incubated at 4 °C for 16 h with the following primary antibodies GATA3 (1:2000, SBI, USA) and GAPDH (1:5000, Abcam, USA). The following day, the membrane was incubated with the HRP-conjugated (IgG H&L) secondary antibodies. Protein bands were quantified with Image J Software.

Dual-Luciferase reporter assay

CD4+T cells were seeded in a 24 well plate until reaching 60% confluence. According to the instructions, using Lipofectamine 2000 (Thermofisher, USA), cells were co-transfected with luciferase reporter plasmid (0.5 μg lncRNA NEAT1 WT/MUT or GATA3 WT/MUT) and miR-217 mimic (100 pmol). After 48 hours transfection, the luciferase activity was measured by Dual-Luciferase Reporter Assay (Promega, Shanghai, China), and normalized to the Renilla signal.

Flow cytometry

The percentage of Th1 or Th2 cells was assessed by flow cytometry. The collected CD4+ T cells were stimulated with PMA/Ionomycin and BFA/Monensin for 4 h. The cells were treated with the fixation buffer and permeating buffer, and stained with anti-IFN-γ-APC and anti-IL-4-PE. The flow cytometry was performed in a Guava easyCyte Cytometer system (Merck Millipore, Germany). The data were analyzed by Cytometer system.

Animal experiment

Forty C57BL/6J mice, 3-4 weeks of age, weighed about 10-13 g. All animal experiments were approved by the Xi'an Jiaotong University (approval number: XJTULAC2010-1230). The mice were randomly divided into five groups (8 mice in each group): Control group, OVA group, NEAT1 KD group, miR-217 KD group, and GATA3 KD group. As described previously, the OVA mice model was used as an experimental model for asthma (18). In brief, mice were intraperitoneally injected with 100 μg ovalbumin (Sigma, USA) emulsified in 1 mg aluminum hydroxide with a total volume of 0.2 ml on days 0-14. OVA mice were challenged via the airway with 5% ovalbumin by ultrasonic nebu-lizer for 30 min each day on the next 15-22 days. When the mice appeared restlessness, fecal incontinence, abdominal spasm, respiratory acceleration and other reactions, it was judged as successful OVA model. Subsequently, NEAT1-siRNA, miR-217 inhibitor or GATA3-siRNA were intraperitoneally injected into the OVA mice, respectively. Finally, the mice were anesthetized and blood was taken from mouse tail vein for subsequent experiments. Lung tissues were cut into slices (3 μm) for hematoxylin and eosin (H&E) staining.

Statistical analyses

All of the data were presented as the mean ± SD for at least three independent experiments. Statistical analyses were performed by SPSS17.0 (Chigoe, IL, USA). Significant differences between groups were analyzed by the student's t-test, and were assigned as $*P<0.05$; $**P<0.01$; $***P<0.001$, respectively.

Results

Effect of lncRNA NEAT1 on Th1 and Th2 balance

qRT-PCR showed that lncRNA NEAT1 was significantly overexpressed in CD4+T cells of pediatric asthma patients ($P<0.001$, Fig. 1A). Therefore, lncRNA NEAT1 was selected as the follow-up research object to explore whether it was involved in regulating Th1/Th2 imbalance in children with asthma. We used siRNA to inhibit lncRNA NEAT1 expression in CD4+T cells and its knockdown efficiency was verified by qRT-PCR (Fig. 1B). ELISA assay showed that knockdown of lncRNA NEAT1 significantly reduced Th2-related cytokines IL-4 and IL-10 ($P<0.01$, Fig. 1C-1D), but had no effect on Th1-associated cytokine INF-γ (Fig. 1E). Additionally, flow cytometry results showed that knockdown of lncRNA NEAT1 resulted in the decreased percentage of Th2 cells, but had no effect on Th1 cells (Fig. 1F-1G). In conclusion, the data showed that knockdown of lncRNA NEAT1 inhibited Th2 cell differentiation.

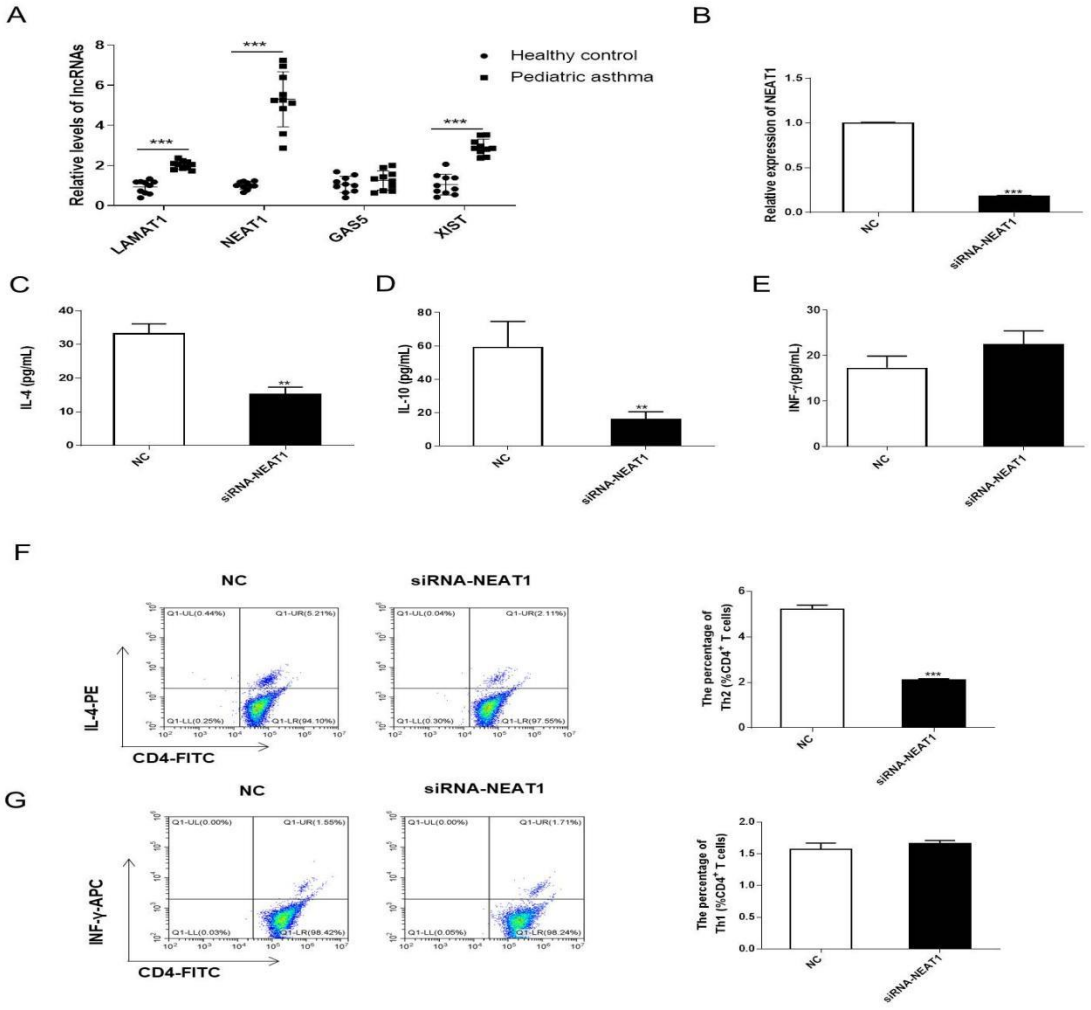

Fig. 1: Effect of downregulation of lncRNA NEAT1 on Th1/Th2 balance in pediatric asthma.
(A) qRT-PCR detected lncRNA LAMAT1, lncRNA NEAT1, lncRNA-GAS5 and lncRNA XIST expression in CD4+T cells from 10 pairs of peripheral blood samples from pediatric asthma patients and normal children. (B) NEAT1 expression in transfected CD4+T cells were examined using qRT-PCR. (C-E) Th2-related cytokines (IL-4 and IL-10) and Th1-associated cytokine (INF-γ) were detected using ELISA assay. (F-G) The percentage of Th1 and Th2 cell were measured by flow cytometry. Data are shown as mean ± SD (n=3). ***P*<0.01; ****P*<0.001

LncRNA NEAT1 negatively regulated expression of miR-217

miR-217 was down-regulated in CD4+T cells from pediatric asthmatic by qRT-PCR (Fig. 2A). Subsequently, we overexpressed miR-217 in CD4+T cells by transfecting miR-217 mimic (Fig. 2B). ELISA showed that overexpression of miR-217 decreased IL-4 and IL-10 (Fig. 2C-2D). However, it also had no effect on INF-γ (Fig. 2E). Additionally, flow cytometry results showed that miR-217 mimic decreased the percentage of Th2 cells, but showed no effect on the percentage of Th1 cells (Fig. 2F-2G). Interestingly, the effects of miR-217 mimic and lncRNA NEAT1 knockdown on Th1/Th2 cells were surprisingly consistent. Therefore, we speculated a competitive regulation mechanism between lncRNA NEAT1 and miR-217. To determine whether lncRNA NEAT1 can target miR-217, we predicted their binding site based on RNAhybrid database (Fig. 2H). Dual-luciferase report assay further verified the interaction between lncRNA NEAT1 and miR-217 (Fig. 2I). Next, based on qRT-PCR assay, knockdown of lncRNA NEAT1

resulted in significant high-expression of miR-217, while miR-217 mimic resulted in low expression of lncRNA NEAT1 (Fig. 2J-2K). Together, miR-217 and lncRNA NEAT1 could inhibit the expression of each other by binding to each other.

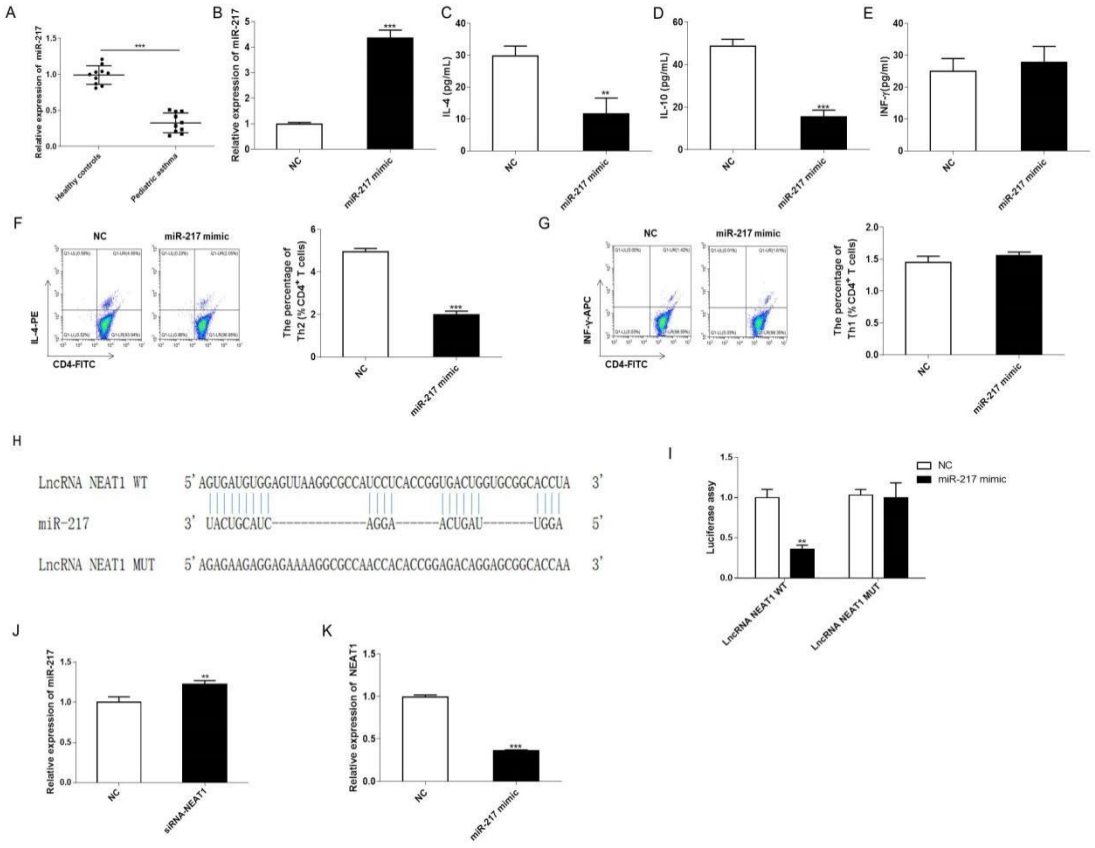

Fig. 2: LncRNA NEAT1 negatively regulates expression of miR-217.

(A) qRT-qPCR detected miR-217 expression in CD4+T cells from pediatric asthma patients and normal children. (B) miR-217 expression in CD4+ T cells transfected with miR-217 mimic were examined using qRT-PCR. (C-E) Th1/2-related cytokines were detected by ELISA assay. (F-G) Flow cytometry was used to detect the effect of miR-217 mimic on the percentage of Th1 and Th2 cells. (H) The binding site between NEAT1 and miR-217 was predicted by RNAhybrid database. (I) Dual luciferase reporter assay validated the target relationship between NEAT1 and miR-217. (J) The qRT-qPCR analysis of miR-217 expression in CD4+T cells transfected with siRNA-NEAT1. (K) qRT-qPCR analysis of NEAT expression in CD4+T cells transfected with miR-217 mimic. Data are shown as mean ± SD (n=3). ***P*<0.01; ****P*<0.001

miR-217 affected Th1/Th2 cells by regulating GATA3 expression

GATA3 expression was significantly higher in CD4+T cells from pediatric asthma patients than healthy children (*P*<0.001, Fig. 3A). To further explore the role of GATA3 in pediatric asthma, we down-regulated GATA3 expression by siRNA in CD4+ cells, and verified the knockdown efficiency by qRT-PCR. The results showed that si-GATA3-1 knockdown had a better inhibitory effect on GATA3 expression (Fig. 3B), which was used in next experiments. Knockdown of GATA3 significantly reduced IL-4 and IL-10 (*P*<0.001, Fig. 3C-3D), while it had no effect on INF-γ (Fig. 3E). Additionally, flow cytometry results showed that knockdown of GATA3 resulted in a decrease of Th2 cells rather than Th1 cells (Fig. 3F-3G). The target binding sites be-

tween miR-217 and GATA3 were predicted by RNAhybrid database (Fig. 3H) and further confirmed by dual luciferase reporting assay (Fig. 3I). Combined with our previous results, lncRNA NEAT1 was negatively correlated with miR-217, and GATA3 was the direct target of miR-217. Therefore, we thought lncRNA NEAT1 could up-regulate the expression of GATA3 through sponge miR-217. Next, functional complementation assay were used to confirm this assumption. Knockdown of lncRNA NEAT1 significantly decreased GATA3 expression, but co-transfection with miR-217 inhibitor rescued GATA3 expression (P<0.001, Fig. 3J).

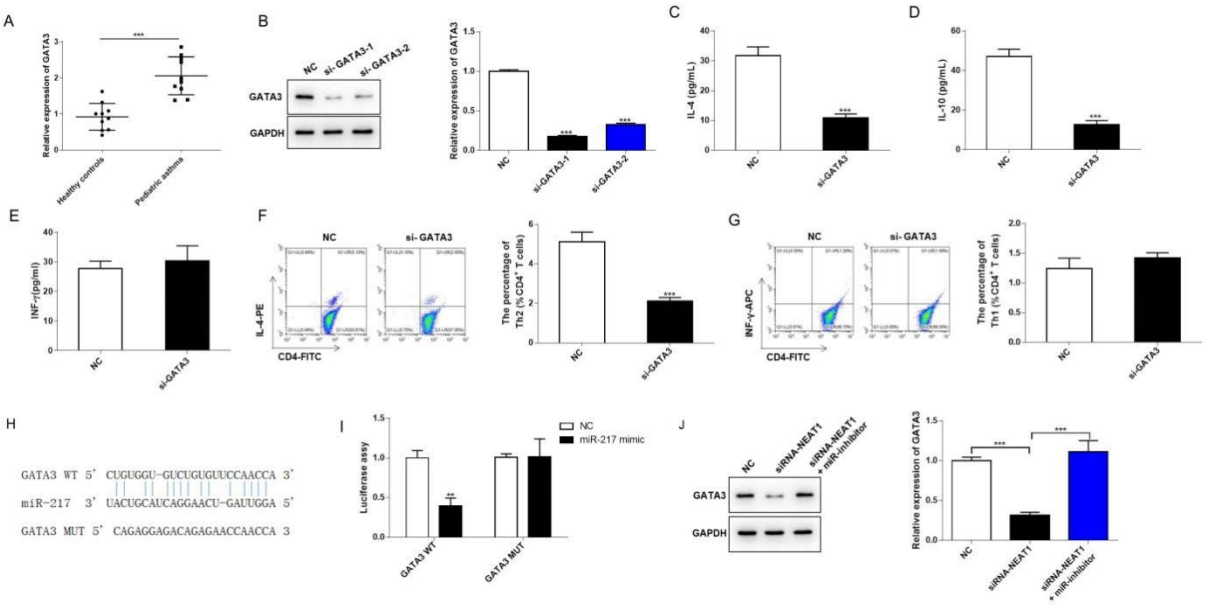

Fig. 3: miR-217 affects Th1/Th2 cells by regulating GATA3 expression.

(A) qRT-qPCR detected GATA3 expression in CD4+T cells from pediatric asthma patients and normal children. (B) GATA3 expression were examined using western blot after CD4+T cells transfected with si-GATA3-1/2. (C-E) Th1/2-related cytokines were detected by ELISA assay. (F-G) Flow cytometry was used to detect the effect of GATA3 knockdown on Th1 and Th2 cell percentages. (H) The binding site between miR-217 and GATA3 was predicted by RNAhyrid website. (I) The Dual luciferase reporter assay to validate target relationship between miR-217 and GATA3. (J) The RT-qPCR analysis of miR-217 expression in CD4+T cells transfected with siRNA-NEAT1 and siRNA-NEAT1+miR-217 inhibitor. Data are shown as mean ± SD (n=3). **P<0.01; ***P<0.001

lncRNA NEAT1 and miR-217 competitively regulated GATA3

Compared with Si-NC, the level of IL-4 and IL-10 was significantly reduced in CD4+ T cells transfected with Si-NEAT1 (P<0.001, Fig. 4A-4B). Further rescue experiments showed that miR-217 inhibitor effectively reversed the inhibitory effect of Si-NEAT1 on IL-4 and IL-10 expression. Importantly, we found that the above series of activities could be partly cancelled by Si-GATA3 (Fig. 4A-4B). However, the above functional complementation assay did not affect the expression of Th1-associated cytokine INF-γ (Fig. 4C). Subsequent flow cytometry of Th2 percentage showed the same trend with Fig. 4A-4B, however there was no effect on Th1 percentage (Fig. 4D-4E). The above results suggested that lncRNA NEAT1 can cause upregulation of GATA3 expression by inhibiting miR-217, which in regulating Th1/Th2 balance.

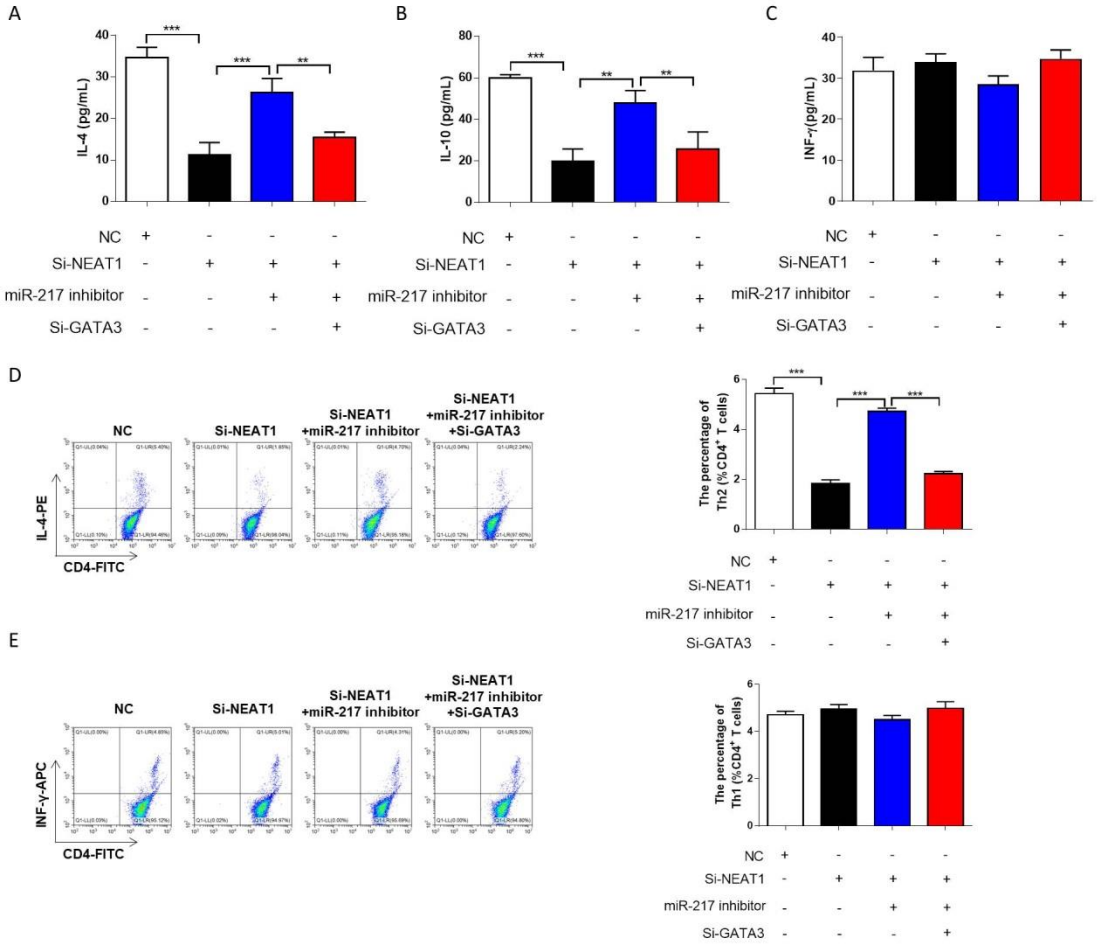

Fig. 4: lncRNA NEAT1 and miR-217 competitively regulated GATA3

CD4+T cells were transfected with Si-NC, Si-NEAT1, Si-NEAT1+miR-217 inhibitor, and Si-NEAT1+miR-217 inhibitor+Si-GATA3, respectively. (A-C) ELISA assay of Th1/2-associated cytokine. (D-E) The percentage of Th1 and Th2 cells in CD4+T cells using flow cytometry. Data are shown as mean ± SD (n=3). ***P*<0.01; ****P*<0.001

lncRNA NEAT1 regulated Th1/Th2 balance by targeting microRNA-217/GATA3 in vivo

The levels of IL-4 and IL-10 in serum was significantly increased in OVA mice compared with control mice (*P*<0.001), which was reduced when the OVA mice were treated with Si-NEAT1 or Si-GATA3. However, IL-4 and IL-10 levels were significantly increased when the model group mice were treated with miR-217 inhibitor (*P*<0.001, Fig. 5A-5B). These treatments did not affect INF-γ level (Fig. 5C). Compared with control group, the number of Th1 and Th2 cells in CD4+T cells of OVA group was increased. The percentage of Th2 cells was decreased by Si-NEAT1 or Si-GATA3, and increased by miR-217 inhibitor, while these treatments had no significant effect on Th1 cells (Fig. 5D-5E). Finally, based on H&E staining, there was inflammatory cell infiltration in lung tissue of OVA mice, and Si-NEAT1 or Si-GATA3 treatment could alleviate inflammatory infiltration, while miR-217 inhibitor had a negative effect (Fig. 5F).

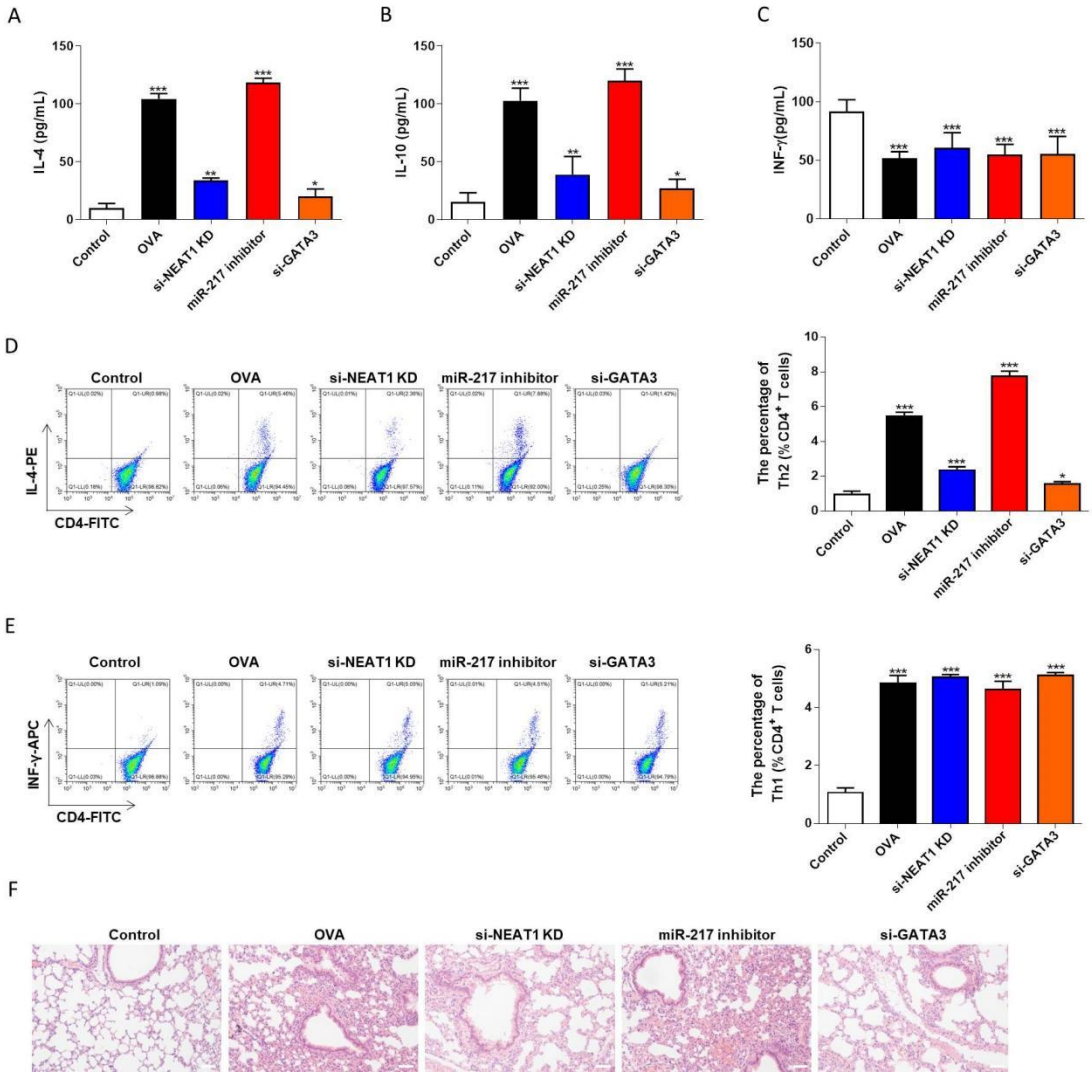

Fig. 5: lncRNA NEAT1 regulates Th1/Th2 balance in pediatric asthma by targeting miR-217/GATA3 in vivo.

A mouse asthma model was constructed by OVA, followed by intraperitoneal injection of si-NEAT1, miR-217 inhibitor or si-GATA3, respectively. At the end of experiment, the peripheral blood of mice was collected to isolate CD4+T cells. (A-C) Th1/2-related cytokines were detected by ELISA. (D-E) The percentage of Th1 and Th2 cells detected using flow cytometry. (F) H&E staining of mouse lung tissues in each group (magnification: 200X). Data are shown as mean ± SD (n=3). **P*<0.05; ***P*<0.01; ****P*<0.001

Discussion

Childhood asthma is a common chronic respiratory disease, which seriously affects children's health and growth (19). CD4+T cells are major participants in asthma related inflammation (20). Th1/Th2 imbalance is considered to be the immunological pathogenesis of asthma, that is, T cells tilt toward Th2 cells, resulting in the disorder of Th1 and Th2 cytokines, so as to promote the onset and progression of asthma (21). However, the mechanism of Th1/Th2 imbalance in childhood asthma needs to be further elucidated. LncRNAs are reported to be involved in activation of CD4+T cells to maintain immune homeostasis in asthma (22). According to a previous study (23), lncRNA-LAMAT1, lncRNA NEAT1, lncRNA-GAS5 and lncRNA-XIST were signifi-

cantly overexpressed in CD4+T cells from peripheral blood of adult patients with severe asthma. Therefore, we isolated CD4+T cells from 10 pairs of peripheral blood samples from pediatric asthma patients and normal children to study the above differentially expressed lncRNAs. Thus, our results proved that lncRNA NEAT1 was significantly up-regulated in CD4+T cells in peripheral blood of children with asthma. Studies have shown that lncRNA NEAT1 overexpression is associated with increased exacerbation risk, severity, and inflammation, as well as decreased lung function through the interaction with miRNA-124 in asthma (24). Furthermore, lncRNA NEAT1 is closely associated with Th1/Th2 balance in PBMCs of patients with systemic lupus erythematosus (25). However, the correlation between lncRNA NEAT1 expression and Th1/Th2 balance in CD4+T cells in asthmatic children has not been explored. In the study, we found that knockdown of lncRNA NEAT1 significantly reduced the production of Th2-related cytokines (IL-4 and IL-10) and the percentage of Th2 cells, but had no effect on Th1 cells.

Increasing evidence has indicated that lncRNAs are mostly act as miRNAs sponge and form lncRNA-miRNA-mRNA axis, thereby playing their biological role in gene regulation (26). For instance, lncRNA NEAT1 regulated MMP-16 by targeting miR-200a/b to aggravate inflammation in asthma (27). In our study, miR-217 was significantly under-expressed in CD4+T cells in peripheral blood of children with asthma. Interestingly, the effects of miR-217 mimic and lncRNA NEAT1 knockdown on Th1/Th2 cells are surprisingly consistent. Therefore, we speculated that there might be a competitive regulation mechanism between lncRNA NEAT1 and miR-217.

There is a lot of evidence that targeted regulation of GATA3 is an effective therapeutic strategy for asthma (28, 29). GATA3 is currently considered to be a key transcription factor affecting Th2 differentiation and function (30). In addition, GATA3 can dependently activate Th2 lymphocyte in airway hyperresponsiveness (31). In our study, GATA3 is highly expressed in CD4+T cells from pediatric asthma patients, which selectively activated Th2 cells, but its upstream regulatory mechanism is not fully understood. Elevation of NEAT1 was corresponded to diminish GATA3 expression in breast cancer progression (32). The dual luciferase assays confirmed that miR217 targeted to GATA3 and inhibited its expression. Based on this, we explored the underlying ceRNA network between lncRNA NEAT1 and GATA3 in pediatric asthma. We observed that lncRNA NEAT1 negatively regulates the expression of miR-217, while miR-217 negatively regulates the expression of GATA3. Importantly, functional assays and animal experiments demonstrated that lncRNA NEAT1 regulated GATA3 expression through sponge miR-217, thereby regulating Th1/Th2 balance in CD4+T cells in asthma in children.

Conclusion

lncRNA NEAT1 in CD4+T cells can act as a ceRNA to inhibit miR-217, thereby regulating GATA3 expression, and ultimately affecting Th1/Th2 balance in childhood asthma. lncRNA NEAT1/miR-217/GATA3 axis may reveal the immunological mechanism of childhood asthma, which has potential clinical application value.

Journalism Ethics considerations

Ethical issues (Including plagiarism, informed consent, misconduct, data fabrication and/or falsification, double publication and/or submission, redundancy, etc.) have been completely observed by the authors.

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No funding was received in this study.

Conflict of Interest

The authors declare that there is no conflict of interest.

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