

Title: Hsa_circ_0005519 increases IL-13/IL-6 by regulating hsa-let-7a-5p in CD4⁺ T cells to affect asthma

Running head: Hsa_circ_0005519 and airway inflammation in asthma

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Abstract

Background: Circular RNAs (circRNAs) are a class of non-coding RNAs that could serve as novel biomarkers for the diagnosis and treatment of diseases. We hypothesized that circRNAs of CD4⁺ T cells are involved in asthma.

Objective: In this study, we investigated the circRNA expression profile and the possible mechanism by which hsa_circ_0005519 participates in asthma.

Methods: The expression profiles of circRNAs in CD4⁺ T cells were revealed by circRNA microarray. Hsa_circ_0005519 expression in CD4⁺ T cells was confirmed in asthmatic patients (n=65) and healthy subjects (n=30). Hsa-let-7a-5p, the target of hsa_circ_0005519, was predicted by online algorithms and verified by a dual luciferase reporter assay. Correlation assays

between the expression of hsa_circ_0005519 and hsa-let-7a-5p, the mRNA levels of interleukin (IL)-13 and IL-6 in CD4⁺ T cells, and the clinical characteristics of asthmatic patients were performed. The role of hsa_circ_0005519 in proinflammatory cytokine expression was investigated in CD4⁺ T cells from asthmatic patients *in vitro*. Hsa_circ_0005519 expression in PBMCs was determined in another cohort including 30 asthmatic patients and 24 controls. Correlation assays of hsa_circ_0005519 expressions between CD4⁺ T cells and PBMCs were performed.

Results: Hsa_circ_0005519 was up-regulated and negatively correlated with hsa-let-7a-5p expression in CD4⁺ T cells of asthmatic patients. Both the fraction of exhaled nitric oxide (FeNO) and the peripheral blood eosinophil ratio were positively correlated with hsa_circ_0005519 expression in CD4⁺ T cells. These outcomes were also different in asthmatic patients with low vs high hsa_circ_0005519 levels. Hsa_circ_0005519 expressions between CD4⁺ T cells and PBMCs were concordant in asthmatic patients. Mechanistically, hsa_circ_0005519 might bind to hsa-let-7a-5p and relieve suppression for IL-13/IL-6 in CD4⁺ T cells.

Conclusions and Clinical Relevance: Our data suggest that hsa_circ_0005519 may induce IL-13 and IL-6 expression by regulating hsa-let-7a-5p in CD4⁺ T cells to affect asthma. And hsa_circ_0005519 may be a potential biomarker of asthma.

Keywords

asthma, CD4⁺ T cells, hsa_circ_0005519, hsa-let-7a-5p, PBMCs

Introduction

Asthma is a worldwide respiratory disorder associated with airway inflammation(1, 2), in which CD4⁺ T cells play an important role, controlling immunoglobulin E (IgE) production against allergens(3), releasing proinflammatory mediators , mediating the migration and activation of inflammatory cells such as eosinophil, neutrophil and macrophages(4-6).

Several cytokines released from CD4⁺ T cells, including interleukin (IL)-4, IL-5, IL-6, IL-9 and IL-13(7, 8), are involved in the pathogenesis of asthma. Of these, IL-6 has a functional role in T cell differentiation and activation and modulating the balance between regulatory T cells (Treg) and effector Th17 cells(9). IL-13 increases eotaxin production to recruit eosinophils to the airway(10), contributes to IgE and mucus production(11, 12), induces airway hyperresponsiveness and promotes airway remodeling(11).

Non-coding RNAs (ncRNAs), such as long noncoding RNAs (lncRNAs) and microRNAs (miRNAs), participate in inflammatory processes in asthma by regulating multiple cell types such as T cells(13-16) , macrophages(17) and epithelial cells(18). Tsitsiou et al. observed widespread changes in the

expression of lncRNAs in CD8⁺ T cells from patients with severe asthma(13).

MiR-155 contributes to the regulation of Th2-mediated allergen-induced eosinophilic inflammation in the lung(14, 16). MiRNAs bind to the 3' -untranslated region (UTR) of their target mRNA ,leading to translational repression and/or mRNA degradation. The let-7 family participates in the development of inflammation by suppressing the expression of IL-13 and IL-6(19-21).

Circular RNAs (circRNAs) are a new class of ncRNAs that are characterized by a covalently closed loop with neither 5' to 3' polarity nor a polyadenylated tail(22, 23). They take part in various diseases, such as cardiac fibrosis(24), diabetes(25), and carcinomas(26). All these studies indicated that circRNAs function as miRNA sponges to regulate gene expression. However, the role of circRNAs in the pathogenesis of asthma is not yet known.

We therefore hypothesized that circRNAs in CD4⁺ T cells are involved in the pathogenesis of asthma by sponging miRNAs. To address this hypothesis, we profiled circRNA expression by microarray processing of CD4⁺ T cells from asthmatic patients and healthy subjects. We found that hsa_circ_0005519 (ID in circBase), which is derived from the *SNX13* gene, was up-regulated in asthma and might regulate the expression of the proinflammatory cytokines IL-13 and IL-6 in CD4⁺ T cells by targeting hsa-let-7a-5p. Further investigation showed that hsa_circ_0005519 expression was negatively correlated with hsa-let-7a-5p

expression. Both the fraction of exhaled nitric oxide (FeNO) and the peripheral blood eosinophil ratio were positively correlated with the expression of hsa_circ_0005519. Hsa_circ_0005519 expressions between CD4⁺ T cells and PBMCs were concordant in asthmatic patients.

Materials and methods

Subjects

Sixty-five asthmatic patients between 18 and 65 years in age were recruited from Tongji Hospital (Wuhan, China). Patients were diagnosed according to the Global Initiative for Asthma (GINA) guidelines. All subjects had a doctor's diagnosis of symptomatic asthma and an accumulation dosage of methacholine, provoking a 20% reduction in forced expiratory volume in the first second (FEV1 PD20) < 2.505 mg and/or bronchodilator responsiveness (> 12% improvement in FEV1% predicted following inhalation of 200 µg salbutamol). Participants were excluded if they had a course of oral or inhaled corticosteroids, or a respiratory tract infection in the previous 4 weeks. All studies relevant to human subjects were approved by the Ethics Committee of Tongji Hospital, Huazhong University of Science and Technology. Written informed consent was obtained from all participants. Clinical data for all subjects are shown in Table 1.

PBMCs and CD4⁺ T cell isolation

CD4⁺ T cells were isolated using density centrifugation and magnetic beads.

Briefly, the diluted blood was carefully layered onto a Ficoll-Hypaque gradient

after the peripheral blood was mixed with PBS. PBMCs layer was obtained

after centrifugation at 800 g for 25 min. CD4⁺ T cells were isolated from

PBMCs using a MagCelect Human CD4⁺ T Cell Isolation Kit (R&D Systems,

Minneapolis, Minnesota, USA) according to the manufacturer's instructions.

Flow-cytometric analysis showed that the purity of isolated CD4⁺ T cells was

more than 90% (Supplementary Fig. S1). The isolated cells were incubated at in

TRIZol reagent (Takara, Dalian, China) and frozen at -80 °C or cultured for

intervention experiments (transduction or transfection).

Cell culture

CD4⁺ T cells were cultured in RPMI 1640 medium (GIBCO, Grand Island, NY,

USA) with 1% penicillin-streptomycin (KeyGEN, Nanjing, China), 100 U/ml

recombinant hIL-2 (Boehringer Roche, Basel, Switzerland) and 10% fetal

bovine serum (FBS) (GIBCO, Grand Island, NY, USA). A549 cells were

cultured in RPMI 1640 medium and 10% FBS. 293T cells were maintained in

DMEM-high glucose (Gibco-BRL, Carlsbad, CA, USA) supplemented with

10% FBS. All the culture plates were incubated at 37 °C in a humidified

atmosphere containing 5% CO₂.

Microarray and quantitative analysis

CD4⁺ T cell samples (5 from asthmatic patients and 5 from healthy subjects) were homogenized in TRIzol reagent. The sample preparation and microarray hybridization were performed based on the Arraystar's standard protocols. The enriched circRNA was transfected into fluorescent circRNA using an Arraystar Super RNA Labeling Kit (Arraystar, USA). The labeled circRNAs were hybridized onto the Arraystar Human circRNA Array (6x7K, Arraystar). Then, the images were analyzed by Agilent Feature Extraction software. Quantile normalization and subsequent data processing were performed using the limma package(27). The screening threshold was set to a fold change > 2.0 or < 2.0 with a value of $P < 0.05$.

Bioinformatics analysis

Potential targets of miRNAs were predicted utilizing Arraystar's miRNA target prediction software based on both the TargetScan and miRanda databases. Then the miRNAs were selected in accordance with the high-complementary activity between miRNA response elements (MREs) of circRNA and "seed sequence" of miRNAs. The circRNA/miRNA/mRNA interaction network was depicted using Cytoscape software. To investigate the potential functions of the differentially expressed circRNAs, Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis was conducted (<http://www.kegg.jp>). The P-value here represents the significance of the pathways.

Adenovirus vectors transduced or agomir transfected in CD4⁺T cells

To knockdown hsa_circ_0005519, short hairpin RNAs (shRNAs) were synthesized and cloned into adenovirus vectors, which were provided by Hanbio Biotechnology Co. Ltd. (Shanghai, China). The knockdown efficiency was examined by qRT-PCR (Supplementary Fig. S2). CD4⁺ T cells in asthmatic patients were isolated and transferred to 24-well tissue culture plates at a density of 0.25×10^6 cells with 5 μ g/ml polybrene for 30 min, followed by shRNA transductions. Transductions were performed using an optimum multiplicities of infection (MOI, plaque-forming units per target cell) of 500 by thawing the titrated virus stocks at 37 °C. Then the plates were centrifuged for 1 hour at room temperature at 1000 *g*. After 6 hours of incubation at 37 °C, the cells were resuspended in fresh medium supplemented with 10% serum and hIL-2. A second transduction was performed 24h later. The cells were harvested 24 hours after the second transduction.

Cultured CD4⁺ T cells were transfected with hsa-let-7a-5p agomir (a novel class of chemically engineered miRNA mimics) (RiboBio, Guangzhou, China) or agomir-negative vehicle at a final concentration of 50 or 100 nM to overexpress hsa-let-7a-5p. After 6 hours of incubation at 37 °C, the cells were resuspended in fresh medium supplemented with 10% serum and hIL-2. A second transfection was performed 24h later. The cells were harvested 24 hours after the second transfection.

Quantitative reverse transcription polymerase chain reaction (qRT-PCR)

For quantitative PCR analysis of miRNAs, specific miRNAs were reverse-transcribed with the Bulge-Loop™ miRNA Primer Set (RiboBio, Guangzhou, China) according to the manufacturer's instructions. For quantitative PCR analysis of circRNAs and mRNAs, the cDNA was prepared from 500 ng total RNA using Prime Script RT MasterMix (Takara, Dalian, China) following the manufacturer's instructions. qRT-PCR was performed to quantify mRNA levels using an ABI Prism 7900 Real-Time System (Applied Biosystems, Foster City, California, USA) with SYBR Premix Ex Taq (Takara, Dalian, China) following the manufacturer's instructions. The PCR parameters were: 95 °C for 30 s, followed by 40 cycles of 95 °C for 5 s, 60 °C for 30 s and 72 °C for 30 s (Supplementary S1) . The primers used in qRT-PCR are shown in Table 2. β -actin and U6 were used to normalize. The data were analyzed using the $2^{-\Delta\Delta C_t}$ method and are reported as the relative variation (fold change).

Sanger sequencing

To verify the specificity of the primers, we amplified it in A549 (hsa_circ_0005519 is highly expressed in A549 cells based on the database). PCR was carried out for 35 cycles according to the protocol: denaturation at 95 °C for 30 s, annealing at 60 °C for 30 s, and elongation at 72 °C for 30 s. Sanger sequencing of PCR products was performed by Majorbio Co. Ltd. (Shanghai, China).

Quantification of IL-13 and IL-6 in cell culture supernatants

The concentrations of IL-13 and IL-6 were analyzed from supernatants of intervened CD4⁺ T cells in asthmatic patients *in vitro* by the V-PLEX Proinflammatory Panel1 (human) Kit (Mesoscale Discovery, Rockville, MA, USA) following the manufacturer's instructions.

Luciferase reporter assay

The full-length cDNA of hsa_circ_0005519 (with either wild-type or mutant miRNA-binding sites) amplified by PCR from 293T cells was cloned into the XhoI and NotI sites downstream of the luciferase reporter gene of the pmiR-RB-Report™ vector (RiboBio, Guangzhou, China), which were named hsa_circ_0005519-wt and hsa_circ_0005519-mut, respectively. Primers are listed in Table 2. 293T cells were co-transfected in 96-well plates with either miRNA mimics (RiboBio, Guangzhou, China) or negative control and hsa_circ_0005519-wt or hsa_circ_0005519-mut with Lipofectamine 2000 (Invitrogen, Foster city, CA) according to the manufacturer's protocol. After 24 h of incubation, luciferase activity was measured with a dual-luciferase reporter assay (Promega, Wisconsin, USA).

Statistical analysis

Statistical analyses were carried out using SPSS 20.0 (IBM, SPSS, Chicago, IL, USA) and GraphPad Prism (GraphPad, San Diego, CA, USA). Data are

expressed as the mean $\pm$ SD. For normally distributed data, we used parametric tests (one-way analysis of variance with Tukey's correction or an unpaired t-test). For non-normally distributed data, we calculated medians (with interquartile ranges) and used nonparametric tests. We analyzed correlations using Spearman's rank-order correlation. Fisher's exact test was performed on KEGG pathway results. Differences were considered significant at a P value of less than 0.05.

Results

Expression of circRNAs in CD4⁺ T cells of asthmatic patients

To identify the roles of circRNAs in the pathogenesis of asthma, we used microarray analysis to explore the global circRNA expression in CD4⁺ T cell samples from five asthmatic patients and five healthy controls. The differentially expressed circRNAs were shown by hierarchical clustering in Fig. 1A. Scatter and volcano plots were created to assess the variation in circRNA expression between the two patient groups. (Fig. 1B, C). Overall, 597 circRNAs in CD4⁺ T cells were differentially expressed between asthmatic patients and controls. Among these circRNAs, 264 were up-regulated and 333 were down-regulated in asthmatic patients. The top 20 up- and down-regulated circRNAs are listed in Supplementary Table S1. According to the circRNA microarray results, hsa_circ_0005519 was one of the most upregulated circRNAs (fold change ≥ 3.7). Based on the CircBase annotation,

hsa_circ_0005519 is spliced from the *SNX13* gene on chr7(-):17908029-17915413 and has a final length of 397 bp (Supplementary Table S2). Our array data revealed that hsa_circ_0005519 was up-regulated in CD4⁺ T cells in asthmatic patients compared with its expression in healthy subjects (Fig. 1D). The cDNA of hsa_circ_0005519 around the junction site was amplified in A549 cells using divergent primers (the definition was shown in Supplementary S2), followed by Sanger sequencing. The sequencing results were consistent with the CircBase data (Fig. 1E). qRT-PCR was used to validate the results of the circRNA array. As expected, hsa_circ_0005519 levels were markedly increased in CD4⁺ T cells from asthmatic patients (Fig. 1F).

Bioinformatics analysis of hsa_circ_0005519

CircRNAs naturally sequester and competitively suppress miRNA activity and influence the expression of their target mRNA by acting as an endogenous miRNA sponge(23). Thus, we speculated that hsa_circ_0005519 could target miRNAs to inhibit their activities.

We screened for targeted miRNAs by utilizing Arraystar's miRNA target prediction software based on TargetScan and miRanda. Then the miRNAs were selected in accordance with conserved seed-matching sequence, including hsa-miR-130b-5p, hsa-let-7a-5p, hsa-let-7f-5p, hsa-let-7g-5p, hsa-miR-605-5p, and hsa-miR-136-5p. To further explore the functions of hsa_circ_0005519, we constructed a hsa_circ_0005519/miRNAs/mRNAs network, in which the

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screened candidate targets of miRNAs were the merged common targets from at least three of four online algorithms (miRanda, PicTar, TargetScan and DIANA-microT-CDS) (Supplementary Table S3). Finally, the entire network was illustrated using Cytoscape software (Fig. 2A), suggesting that hsa_circ_0005519 may regulate genes expression through miRNA sponge mechanism. Based on the existing literature, we paid attention to three miRNAs, hsa-let-7a-5p, hsa-miR-605-5p and hsa-miR-136-5p. Their binding sites are displayed in (Fig. 2C). The type of MREs is 7mer-m8. Each miRNA mimic or negative control with a luciferase vector harboring either wild-type or mutant hsa_circ_0005519 into 293T cells. Intriguingly, the luciferase activity of hsa_circ_0005519-wild-type decreased only in the hsa-let-7a-5p mimic group by 32.2%. Remarkably, no perceptible difference in terms of luciferase activity was noted for hsa_circ_0005519-mutant, empty vector or the other two miRNAs (Fig. 2B, C). This indicates that hsa_circ_0005519 could interact with hsa-let-7a-5p.

Based on the relationship between hsa_circ_0005519 and hsa-let-7a-5p, we explored whether hsa_circ_0005519 could regulate hsa-let-7a-5p target mRNA. Given the conservative nature of the let-7 family, we conducted KEGG signaling pathway analysis on the target mRNA of the whole family when the target mRNA of hsa-let-7a-5p were not enriched successfully. The top 10 pathways were displayed and showed strong enrichment for the MAPK and JAK-STAT signaling pathways (Fig. 2E), which are strongly linked to T cell

function(28-31) (Supplementary Table S4). IL-13 and IL-6 are closely related to the pathogenesis of asthma (8-12, 32, 33). We employed the TargetScan to show the interaction between hsa-let-7a-5p and the 3'UTR of IL-13 and IL-6 mRNA (Fig. 2D). Intriguingly, previous studies have demonstrated that let-7a-5p (previous ID : let-7a, Supplementary S3) can regulate IL-13 and IL-6 expression by targeting 3'-UTR of IL-13 and IL-6(19, 21). Then we hypothesized that hsa_circ_0005519 might induce IL-13 and IL-6 expression through targeting hsa-let-7a-5p to affect T cell function in asthma.

Hsa_circ_0005519 may regulate the expression of IL-13 and IL-6 in CD4⁺ T cells by targeting hsa-let-7a-5p

To verify the hypothesis above, we next constructed an *in vitro* system. We analyzed the protein levels of IL-13 and IL-6 after transduction with shRNA to knock down hsa_circ_0005519 in CD4⁺ T cells from 12 asthmatic patients. After knockdown of hsa_circ_0005519, the protein levels of IL-13 and IL-6 were decreased, while there was no effect on the protein level of IFN- γ (Fig. 3A, B, C, D).

In contrast, when overexpressing hsa-let-7a-5p in CD4⁺ T cells from 9 asthmatic patients, the protein levels of IL-13 and IL-6 were also decreased, but there was nearly no change in IFN- γ levels (Fig. 3E, F, G, H).

Collectively, those data indicated that hsa_circ_0005519 might regulate the expression of IL-13 and IL-6 in CD4⁺ T cells by targeting hsa-let-7a-5p.

Hsa_circ_0005519 may increase IL-13 and IL-6 levels by regulating hsa-let-7a-5p in CD4⁺ T cells to affect asthma

To further verify the interrelationship among hsa_circ_0005519, hsa-let-7a-5p and IL-13 & IL-6, we tested the expression level of hsa-let-7a-5p in CD4⁺ T cells from asthmatic patients. The levels of hsa-let-7a-5p were decreased in asthmatic patients compared with those in healthy controls. Furthermore, hsa-let-7a-5p expression was negatively correlated with hsa_circ_0005519 levels in CD4⁺ T cells (Fig. 4A, B). These data also suggested that hsa_circ_0005519 might functionally relate with hsa-let-7a-5p.

We next analyzed the correlation between the mRNA levels of IL-13 and IL-6 and the hsa-let-7a-5p level in CD4⁺ T cells in asthmatic patients. There was an inverse correlation between the IL-13 mRNA level and the hsa-let-7a-5p levels, and the same was observed for IL-6. (Fig. 4C, D). We also ran a bivariate correlation analysis between the RNA levels of hsa_circ_0005519, IL-13 and IL-6 in CD4⁺ T cells from asthmatic patients. The results showed that both IL-13 and IL-6 were positively correlated with hsa_circ_0005519 expression (Fig. 4E, F). In general, these results suggested that hsa_circ_0005519 might regulate IL-13 and IL-6 by harboring hsa-let-7a-5p.

Correlation between hsa_circ_0005519 expression level and clinical characteristics

We further analyzed the correlations between hsa_circ_0005519 expression level and various clinical parameters of asthmatic patients. In 65 asthmatic patients, there was a positive correlation between hsa_circ_0005519 level and FeNO, a parameter of airway eosinophilia and a good predictor of corticosteroid response (34) (Fig. 5A). We also found that the level of hsa_circ_0005519 was positively correlated with the peripheral blood eosinophil ratio in 52 asthmatic patients who had routine bloodwork data (Fig. 5B). Interestingly, when we used the lower limit of the upper quartile of hsa_circ_0005519 expression level (fold change ≥ 1.788366) of the healthy controls as the cut-off point, the asthmatic patients were divided into low and high hsa_circ_0005519 expression. High hsa_circ_0005519 seemed to be correlated with lower hsa-let-7a-5p levels, higher FeNO and a higher blood eosinophil ratio (Fig. 5C, D, E). Altogether, our data indicate that hsa_circ_0005519 was associated with airway eosinophilic inflammation and might act as a promising biomarker for asthma detection.

Potential values of hsa_circ_0005519 as a biomarker for asthma

On the basis of study above, we had discovered the promising diagnostic value of hsa_circ_0005519. For further validation, we detected the CD4⁺ T cells and PBMCs levels of hsa_circ_0005519 in another cohort study

including 30 asthmatic patients and 24 controls. The clinical characteristics of the cohort were shown in Table 3. The expressions of hsa_circ_0005519 in both CD4⁺ T cells and PBMCs in asthmatic patients were increased compared to healthy subjects (Fig.6A, B). We analysed the association between CD4⁺ T cells and PBMCs expression of hsa_circ_0005519 and found hsa_circ_0005519 in PBMCs were significantly correlated with in CD4⁺ T cells in asthmatic patients (Fig.6C). Our data indicated that the level of hsa_circ_0005519 was also differentially expressed between asthmatic patients and healthy subjects in whole PBMCs. Moreover, we analysed the association between the level of hsa_circ_0005519 in PBMCs and FeNO and the peripheral blood eosinophil ratio in asthmatic patients. Consistent with the association between the level of hsa_circ_0005519 in CD4⁺ T cells and FeNO, the level of hsa_circ_0005519 in PBMCs was also positively correlated with FeNO but not with peripheral blood eosinophil ratio (Fig. 6D, E, F, G). This indicates that hsa_circ_0005519 in PBMCs may be used as a non-invasive biomarker for airway eosinophilic inflammation in asthma.

Discussion

In the present report, we conducted experiments to dissect the role of hsa_circ_0005519 in the pathogenesis of asthma. We demonstrated that hsa_circ_0005519 was up-regulated in CD4⁺ T cells from asthmatic patients and

was likely to directly target hsa-let-7a-5p to promote IL-13 and IL-6 expression in CD4⁺ T cells (Fig. 7). We also demonstrated that there was a negative correlation between hsa_circ_0005519 expression and hsa-let-7a-5p levels.

Furthermore, both FeNO and the peripheral blood eosinophil ratio were positively correlated with hsa_circ_0005519 level and were different in asthmatic patients with low vs high levels of hsa_circ_0005519.

Hsa_circ_0005519 might increase IL-13 and IL-6 levels by regulating hsa-let-7a-5p in CD4⁺ T cells to affect airway eosinophilic inflammation in asthma. In order to identify hsa_circ_0005519 as diagnostic biomarker for noninvasive diagnosis of asthma, we demonstrated that hsa_circ_0005519 in PBMCs were increased and correlated with in CD4⁺ T in asthmatic patients.

Hsa_circ_0005519 in PBMCs were correlated with FeNO but not with peripheral blood eosinophil ratio. These results not only provide novel insight into pathogenesis of asthma but also demonstrate that hsa_circ_0005519 could be a viable biomarker for the classification, diagnosis and treatment of asthma in clinical settings.

Although asthma is a notoriously heterogeneous disease, emerging evidence has suggested that CD4⁺ T cells, such as Th1, Th2, Th9, Th17 and Treg cells, play a crucial role in the development of asthma(7, 35-38). These cells can produce the majority of the known cytokines, such as IFN- γ from Th1 cells; IL-4, IL-5, IL-9, IL-6, and IL-13 from Th2 cells; and IL-17A and IL-17F from Th17 cells,

which comprise a complex network that regulates the intricate immune response in asthma(7, 8, 39).

CircRNAs, which form covalently closed continuous loops, often display complex cell-, tissue-, disease-, and stage-specific expression patterns in the eukaryotic transcriptome, revealing their particular regulatory functions in the expression of genes in diseases and development(40, 41). Recently, it was shown that circRNA-mcRasGEF1B induced by LPS participated in the immune response by regulating the stability of the mature intercellular adhesion molecule-1 (ICAM-1) mRNA, considered the most important adhesion molecule for leukocyte recruitment to inflamed areas(42, 43). CircRNAs serve as miRNA sponges in cardiac fibrosis(24), diabetes(25), and rheumatoid arthritis(44). All these studies have shown the potential implications for therapeutic and research applications of circRNAs in inflammation-related diseases based on their ability to target miRNAs. Accordingly, we hypothesized that circRNAs are involved in the mechanisms of asthma by acting as miRNA sponges. In the present study, hsa_circ_0005519 was aberrantly expressed in CD4⁺ T cells in asthmatic patients compared with its expression in healthy controls, and there were differences in clinical characteristics between asthmatic patients with low and those with high hsa_circ_0005519 levels.

Through bioinformatics analysis, we found six possible targeted miRNAs, which were hsa-miR-130b-5p, hsa-let-7a-5p, hsa-let-7f-5p, hsa-let-7g-5p, hsa-miR-605-5p, and hsa-miR-136-5p. We constructed an hsa_circ_0005519-miRNAs-mRNA network, which provided an important reference for the potential associations between circRNAs and their targets. The existing literature found that let-7 family miRNAs were abundant lung miRNAs, comprising 58% and 64% of total lung miRNAs from control and allergen-challenged lungs, respectively(21). It was also reported that let-7a play an role in allergic airway inflammation(20, 21). In our study, the luciferase reporter assay indicated that hsa-let-7a-5p might be a target of hsa_circ_0005519. We took advantage of KEGG analyses to point out that hsa_circ_0005519 could related to the MAPK and JAK-STAT signaling pathways in asthma. IL-6 and IL-13, which are important inflammatory mediators in asthma as mentioned above, play a role in T cell differentiation and activation by MAPK or JAK-STAT signaling pathways(45, 46). And accumulating evidence indicates that these two signaling pathways are involved in regulating cytokines such as IL-6 and IL-13 in asthma inflammation(28-31). Our study demonstrated that hsa_circ_0005519 specifically enhanced the expression of IL-13 and IL-6 in CD4⁺ T cells of asthmatic patients *in vitro*. A positive correlation was found between the expression levels of hsa_circ_0005519 and levels of IL-13/IL-6. Mechanistically, we demonstrated that hsa_circ_0005519 might function as an endogenous sponge for

hsa-let-7a-5p in CD4⁺ T cells. And the inhibitory role of hsa_circ_0005519 in hsa-let-7-5p may be associated with the binding of the target, not the degradation of hsa-let-7-5p. First, bioinformatics prediction and luciferase reporter assays revealed that hsa_circ_0005519 has identical hsa-let-7a-5p response elements and might bind to hsa-let-7a-5p. Second, hsa_circ_0005519 levels were negatively correlated with hsa-let-7a-5p expression in CD4⁺ T cells from asthmatic patients. Third, the expression of hsa-let-7-5p was no statistically significant difference when knocking down hsa_circ_0005519 *in vitro* (Data was not shown).

Our study has provided several lines of evidence to support the notion that hsa-let-7a-5p inhibits asthmatic airway inflammation by targeting IL-13 and IL-6. First, the *in silico* prediction indicated that IL-13 and IL-6 were two potential targets of hsa-let-7a-5p. Additionally, an hsa-let-7a-5p agomir inhibited IL-13 and IL-6 expression at the protein level in asthmatic cells *in vitro*, and there was a negative correlation between the expression level of hsa-let-7a-5p and the expression of IL-13 and IL-6. Our conclusions are partially supported by previous studies showing that IL-13 and IL-6 are targets of let-7a-5p in experimental models of asthma and oncogenesis, respectively (19, 21). Importantly, our study suggested that hsa_circ_0005519 might regulate the expression of IL-13 and IL-6 in CD4⁺ T cells by targeting hsa-let-7a-5p. However, further studies are needed to determine the exact mechanisms.

To verify the possibility of hsa_circ_0005519 as a biomarker, we calculated and analyzed the relevance of clinicopathological parameters and hsa_circ_0005519 expression in PBMCs in asthmatic patients, indicating the close relation with those in CD4⁺ T cells.

Our study has several shortcomings that should be acknowledged. Only a few samples were available, and more samples are needed to confirm our results and facilitate further development of the hsa_circ_0005519 profile as a diagnostic, classification and therapeutic biomarker in the future. Furthermore, in view of the difficulty in transduction or transfection into CD4⁺T cells, the co-transfection of sh-circ0005519 and anta-let-7a-5p seemed difficult to achieve in CD4⁺ T cells.

In conclusion, our study may provide new insight into circRNA regulatory networks in asthma. Hsa_circ_0005519 may increase IL-13/IL-6 by regulating hsa-let-7a-5p in CD4⁺ T cells to affect asthma. Additionally, hsa_circ_0005519 might have utility as a biomarker in the classification, diagnosis and treatment of asthma.

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Conflict of interests

The authors declare no conflict of interest.

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Table 1 Characteristics of subjects providing peripheral blood.

	Microarray analysis		Validation	
	Asthma n=5	Control n=5	Asthma n=65	Control n=30
Sex (M/F)	1/4	1/4	24/41	11/19
Age (y)	33(26.5, 44)	35(25,41.5)	42(32.5,51.50)	41(28.25,51.50)
BMI (kg/m ²)	24.47±2.045	23.67±3.110	22.62±4	23.45±3.334
Lung function				
FEV ₁ (L)	1.876±0.3836**	3.130±0.4899	2.431±0.7281***	3.085±0.7857
FEV ₁ % predicted	63.96±10.32***	104.4±2.676	83.56±19.32***	100.9±8.693
FVC (L)	3.092±0.5562	4.02±0.7413	3.549±0.791	3.843±1.005
FVC % predicted	92.2(78.65,101.5)**	116.4(109.7,118.3)	100.9(95.10,110.0)	105.4(98.9,111.6)
FEV1/FVC (%)	60.49±4.308*	78.2±2.512	68.46±12.32***	80.47±4.661
Blood eosinophils (%)	4.6 (2.75,12.85)	NA	3(1.3,6.4)	NA

FEV1: forced expiratory volume in 1 second; FVC: forced vital capacity.

Values are presented as mean ± SD or median (interquartile range). NA=not applicable; PD20=provocation dose resulting in 20% fall in baseline FEV1. *P<0.05 compared asthma with control in microarray analysis. **P<0.05 compared asthma with control in validation group.

Table 2. The sequence of primers used for experiments in this study.

Primer Name	Sequence
hsa_circ_0005519-F	CCTGGAGATTTCCAGAACAAGA
hsa_circ_0005519-R	CTCGTAAGTGTGTGCCAAAGTCA
IL-6-F	ACTCACCTCTTCAGAACGAATTG
IL-6-R	CCATCTTTGGAAGGTTTCAGGTTG
IL-13-F	CCTCATGGCGCTTTTGTTGAC
IL-13-R	TCTGGTTCTGGGTGATGTTGA
β -actin-F	AGAAAATCTGGCACCACACCT
β -actin-R	GATAGCACAGCCTGGATAGCA
hsa_circ_0005519-wt-F	GCGGCTCGAGCAGACTCTTCAAAACGCACT C
hsa_circ_0005519-wt-R	AATGCGGCCGCTTGCAAGTTAGAATCACGGA
hsa_circ_0005519-mut1-F	AAGTTGAATACCTCAAGGAGGTTTGCCGTGA T
hsa_circ_0005519-mut1-R	ACCTCCTTGAGGTATTCAACTTCAACTTCAA A
hsa_circ_0005519-mut2-F	TTCCTAAGCCTAAAGTGTGAGGTCTTACTAT A
hsa_circ_0005519-mut2-R	CCTCACACTTTAGGCTTAGGAATCCTTCTTC A
hsa_circ_0005519-mut3-F	TTTATTGCATGGAGCTGGAGATTTCCAGAAC A
hsa_circ_0005519-mut3-R	ATCTCCAGCTCCATGCAATAAATATAGTAAG A

Table 3. Characteristics of subjects providing peripheral blood

	Asthma n=30	Control n=24
Sex (M/F)	16/14	13/11
Age (y)	43(29.5,54.50)	48(34,5.58.75)
BMI (kg/m ²)	22.54±3.988	21.98±3.766
Lung function		
FEV ₁ (L)	2.6±0.6695	NA
FEV ₁ % predicted	84.06±17.79	NA
FVC (L)	3.885±0.9291	NA
FVC % predicted	3105.4±15.89	NA
FEV1/FVC (%)	66.89±10.52	NA
Blood eosinophils (%)	4.2(2.75,7.35)	NA

FEV1: forced expiratory volume in 1 second; FVC: forced vital capacity.

Values are presented as mean ± SD or median (interquartile range).

NA=not applicable; PD20=provocation dose resulting in 20% fall in baseline FEV1. *P<0.05 compared asthma with control in microarray analysis. **P<0.05 compared asthma with control in validation group.

Figure 1. Differential expression of circRNAs in CD4⁺ T cells from

asthmatic patients. (A) Hierarchical clustering analysis of circRNAs that were differentially expressed between AP and HS; each group included 5 individuals (fold change > 2; P < 0.05). Expression levels above and below the median expression level across all samples are represented in different colors.

Pseudo-colors indicate expression levels from low to high (blue to yellow). (B) Scatter plot of circRNAs to show variations in circRNA expression between AP and HS. The color-codes denote the expression levels of circRNAs. The values on the X and Y axes in the scatter plot are the normalized signal values of the samples (log₂- scaled). The green lines represent fold changes. The circRNAs

above the top green line and below the bottom green line indicate more than 2.0-fold changes between the two patient groups. (C) Volcano plots of circRNAs constructed using fold-change values and P values. The vertical lines correspond to P values, and the horizontal lines represent log₂ (fold change) up- and down-regulation between AP and HS. The significantly differentially expressed circRNAs are indicated by red points. HS=healthy subjects,

AP=asthmatic patients. (D) Hsa_circ_0005519 normalized expression levels were determined by a circRNA array performed with CD4⁺ T cells from AP (n = 5) and HS (n = 5). (E) Schematic illustration of the genomic location and splicing pattern of hsa_circ_0005519, which forms by head-to-tail splicing of four exons. Sanger sequencing depicts the splice junction. (F) The expression levels of hsa_circ_0005519 were analyzed via qRT-PCR in CD4⁺ T cells (n =

65 for AP; n = 30 for HS). Values are relative to the median value of healthy subjects and are expressed as $2^{-\Delta\Delta C_t}$, normalized to β -actin. The values presented are the mean $\pm$ SD. HS=healthy subjects, AP=asthmatic patients. *P < 0.05, **P < 0.001, ***P < 0.0001.

Figure 2. Bioinformatics analysis and the possible mechanism of

hsa_circ_0005519. (A) The hsa_circ_0005519-miRNAs-mRNAs network is predicted based on sequence-pairing prediction. Red nodes: hsa_circ_0005519; blue nodes: miRNAs; green nodes: mRNAs. (B) Luciferase reporter assay for the luciferase activity of Luc-circ-0005519 in 293T cells transfected with a library of 3 miRNA mimics to identify miRNAs that interact with hsa-circ-0005519. Hsa-let-7a-5p inhibited luciferase activity by 32.2% (> 30%). (C) Detailed annotation for the hsa_circ_0005519/miRNA interaction. Bottom: mutation in the hsa_circ_0005519 sequence to generate the mutant luciferase reporter construct. (D) Predicted binding sites of hsa-let-7a-5p matching to the sequences in the 3' UTRs of the IL-13 and IL-6 mRNAs. (E) KEGG pathway analysis displayed the top 10 pathways of hsa_circ_0005519-targeted genes. Data indicate mean $\pm$ SD, n = 3. *P < 0.05, **P < 0.01, ***P < 0.001.

Figure 3. Hsa_circ_0005519 may regulate the expression of the proinflammatory cytokines IL-13 and IL-6 in CD4⁺ T cells by targeting hsa-let-7a-5p. (A) hsa_circ_0005519 was analyzed by qRT-PCR in CD4⁺ T cells from 12 asthmatic patients transfected with sh-circ0005519 or sh-NC. The protein levels of IL-13 (B), IL-6 (C) and IFN- γ (D) were determined by MSD after transfection with sh-circ0005519 or sh-NC vector. (E) hsa-let-7a-5p was analyzed by qRT-PCR in CD4⁺ T cells from 9 asthmatic patients that overexpressed hsa-let-7a-5p via agomirs. The protein levels of IL-13 (F), IL-6 (G) and IFN- γ (H) were determined by MSD after overexpression of hsa-let-7a-5p. hsa-let-7a-5p agomir = Ago-let-7a-5p. The expression of the cytokines normalized by protein concentrations.

Figure 4. Hsa_circ_0005519 may increase IL-13 and IL-6 by regulating let-7a-5p in CD4⁺ T cells to affect asthma. (A) hsa-let-7a-5p was analyzed via qRT-PCR in CD4⁺ T cells in AP compared with HS (n = 65 for AP; n = 30 for HS). Values are relative to the median value of healthy subjects and are expressed as $2^{-\Delta\Delta C_t}$, normalized to U6. The values presented are the means $\pm$ SD. *P < 0.05, **P < 0.001, ***P < 0.0001. (B) Correlation assay between hsa_circ_0005519 levels and hsa-let-7a-5p levels in CD4⁺ T cells from asthmatic patients (n = 65). (C) Correlation assay between hsa-let-7a-5p levels and the mRNA level of IL-13 in CD4⁺ T cells from asthmatic patients (n = 65).

(D) Correlation assay between hsa-let-7a-5p and the mRNA level of IL-6 in CD4⁺ T cells from asthmatic patients (n = 65). (E) Correlation assay between hsa_circ_0005519 levels and the mRNA level of IL-13 in CD4⁺ T cells from asthmatic patients (n = 65). (F) Correlation assay between hsa_circ_0005519 levels and the mRNA level of IL-6 in CD4⁺ T cells from asthmatic patients (n = 65).

Figure 5. Correlation between hsa_circ_0005519 expression level and clinical characteristics. (A) Correlation assay between hsa_circ_0005519 levels and FeNO in asthmatic patients (n = 65). (B) Correlation assay between hsa_circ_0005519 levels and blood eosinophil ratio in asthmatic patients (n = 52). (C) Comparison of hsa-let-7a-5p levels in asthmatic patients with normal (n = 28) vs high hsa_circ_0005519 levels (n = 37). (D) Comparison of FeNO in asthmatic patients with normal (n = 28) vs high hsa_circ_0005519 levels (n = 37). (E) Comparison of blood eosinophil ratios in asthmatic patients with normal (n = 28) vs high hsa_circ_0005519 levels (n = 37). High hsa_circ_0005519 is defined as an expression level of hsa_circ_0005519 in CD4⁺ T cells that is 1.788366-fold (lower limit of upper quartile for healthy subjects). Values are relative to the median value of healthy subjects and are expressed as $2^{-\Delta\Delta C_t}$, normalized to β -actin. The values presented are the mean $\pm$ SD. Healthy subjects = 30. *P < 0.05, **P < 0.001, ***P < 0.0001.

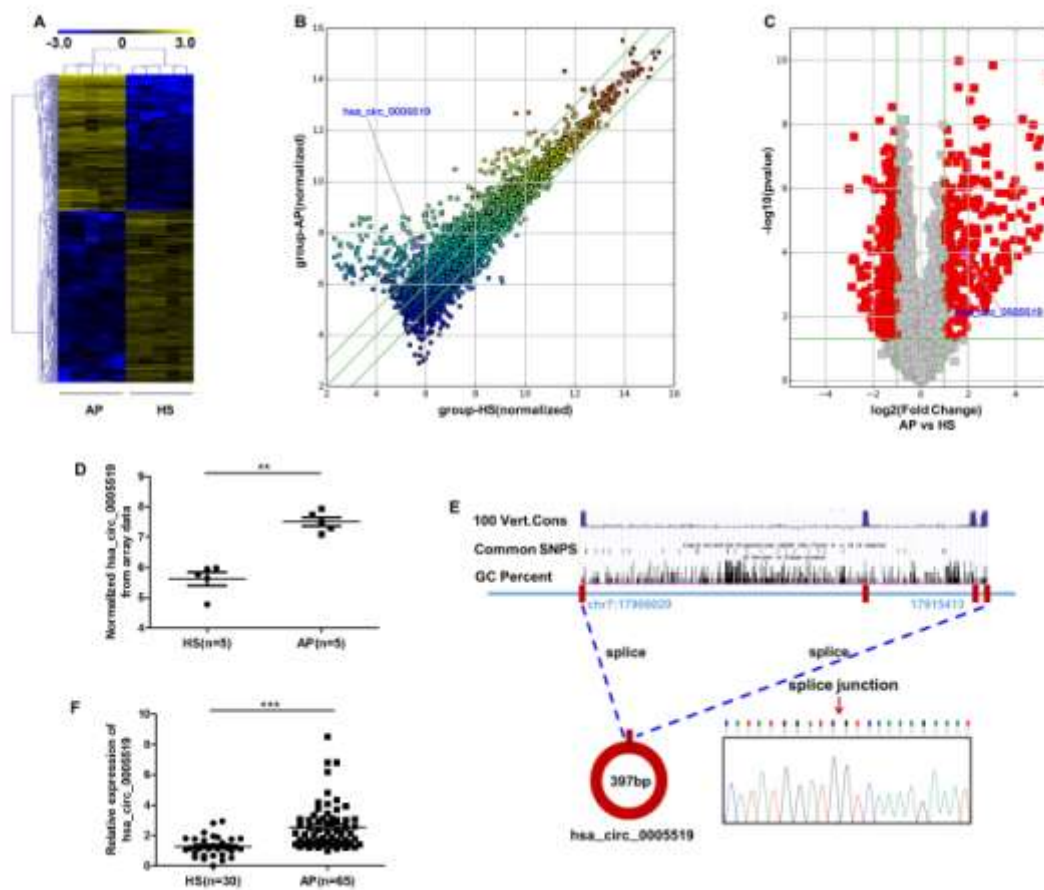
Figure 6. Potential value of hsa_circ_0005519 as a biomarker for asthma.

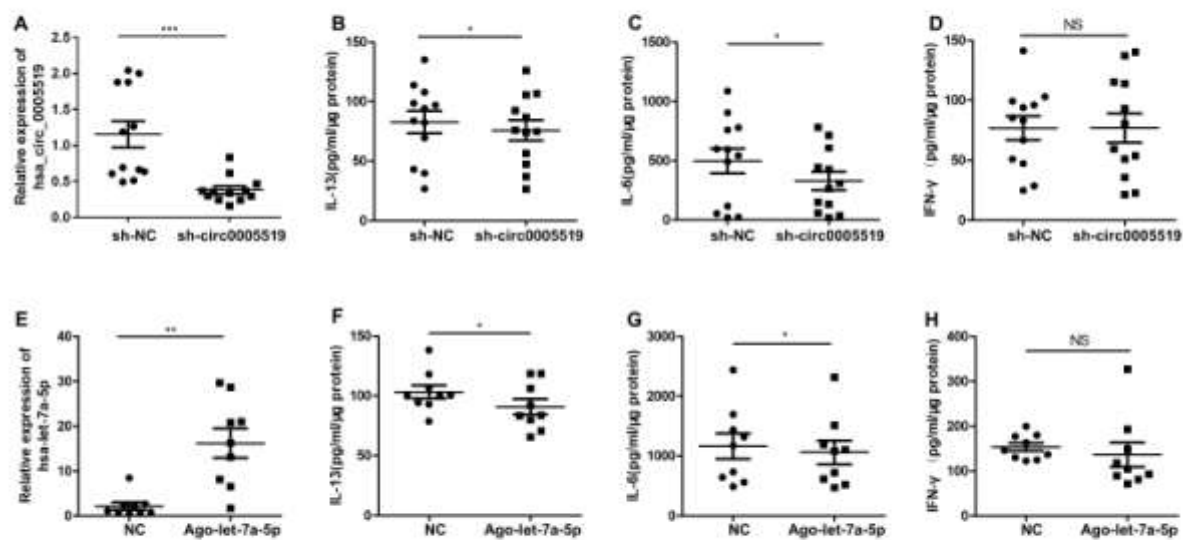
(A) The expression levels of hsa_circ_0005519 were analyzed via qRT-PCR in CD4⁺ T cells (n = 30 for AP; n = 24 for HS). (B) The expression levels of hsa_circ_0005519 were analyzed via qRT-PCR in PBMCs (n = 30 for AP; n = 24 for HS). (C) Correlation assay of hsa_circ_0005519 levels between CD4⁺ T cells and PBMCs in asthmatic patients (n = 30). (D, E) Correlation assay between hsa_circ_0005519 levels and FeNO in CD4⁺ T cells (D) and PBMCs (E) in asthmatic patients (n = 30). (F, G) Correlation assay between hsa_circ_0005519 levels and blood eosinophil ratio in CD4⁺ T cells (F) and PBMCs (G) in asthmatic patients (n = 30). Values are relative to the median value of healthy subjects and are expressed as $2^{-\Delta\Delta C_t}$, normalized to β -actin. The values presented are the mean $\pm$ SD. Healthy subjects = 24. *P < 0.05, **P < 0.001, ***P < 0.0001.

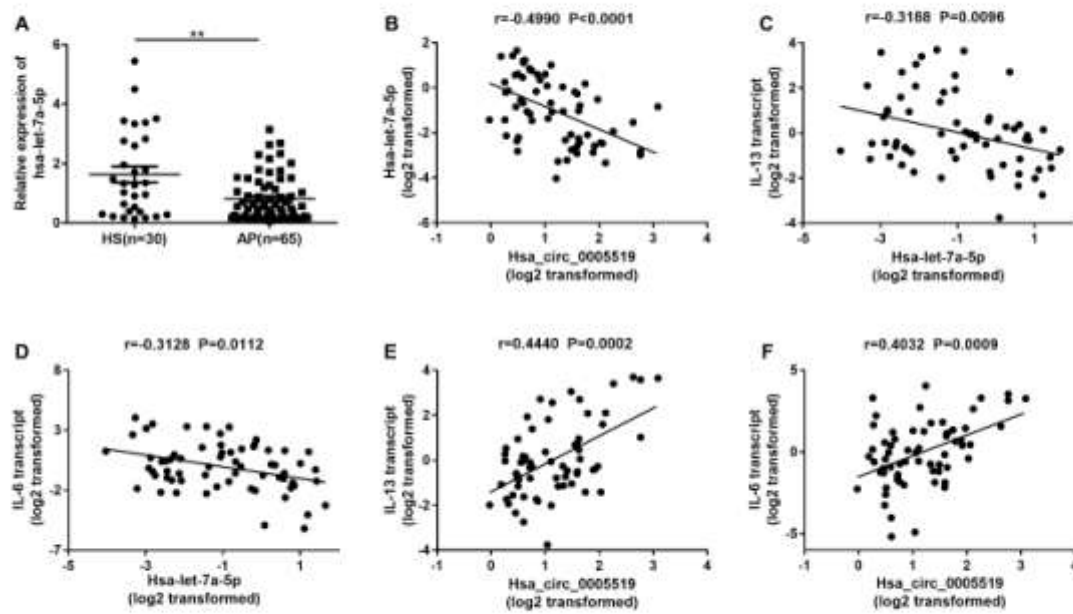
Figure 7. Schematic diagram of

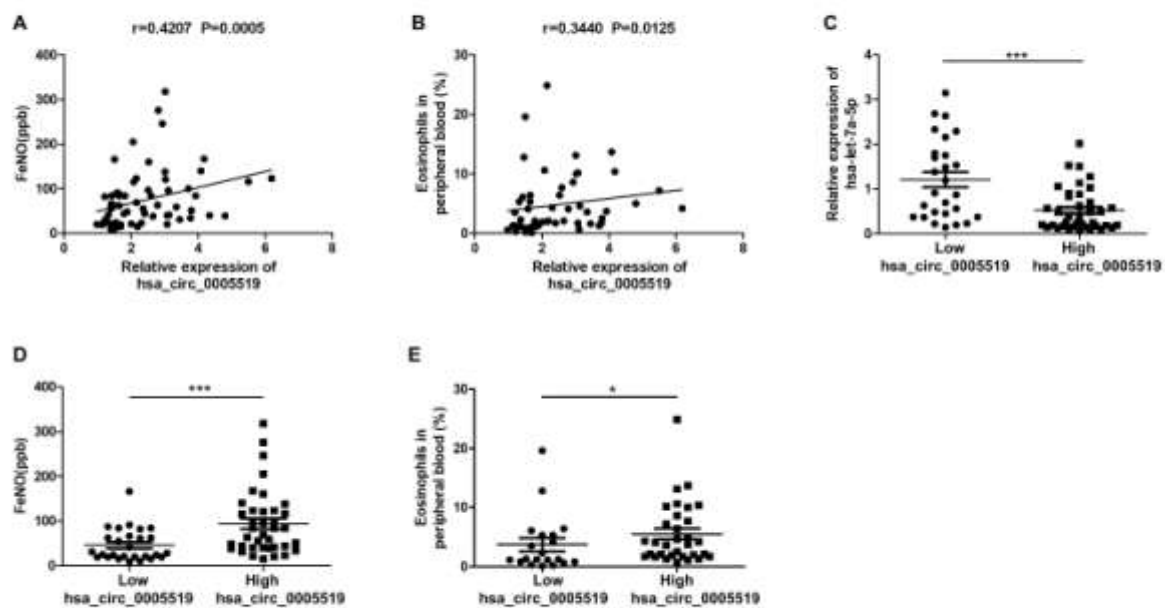
hsa_circ_0005519/hsa-let-7a-5p-associated network in CD4⁺ T cells.

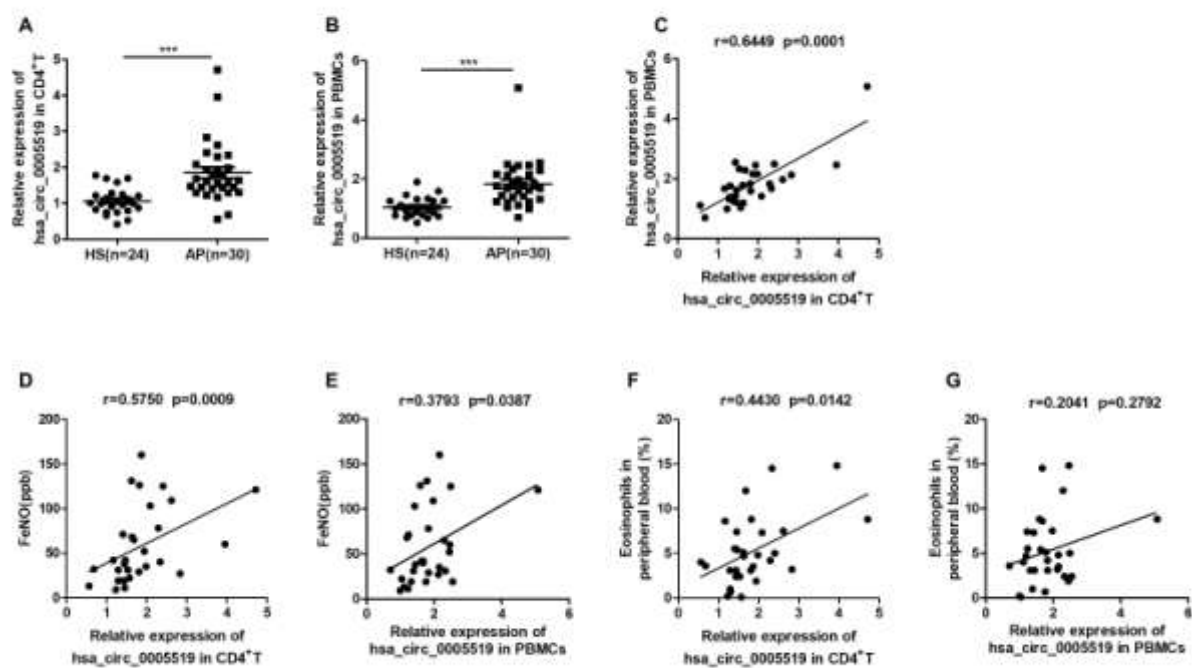
Hsa_circ_0005519 functions as an hsa-let-7a-5p sponge to regulate the hsa-let-7a-5p/IL-13/IL-6 pathway in CD4⁺ T cells. Increased hsa_circ_0005519 expression in asthma suppresses the activity of hsa-let-7a-5p, which up-regulates the expression of the cytokines IL-13 and IL-6, thereby promoting the development of asthma.

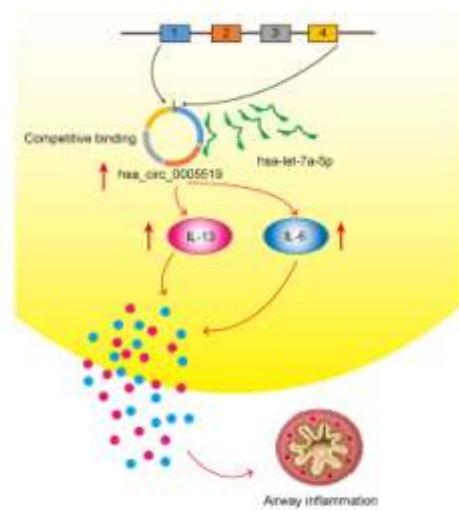


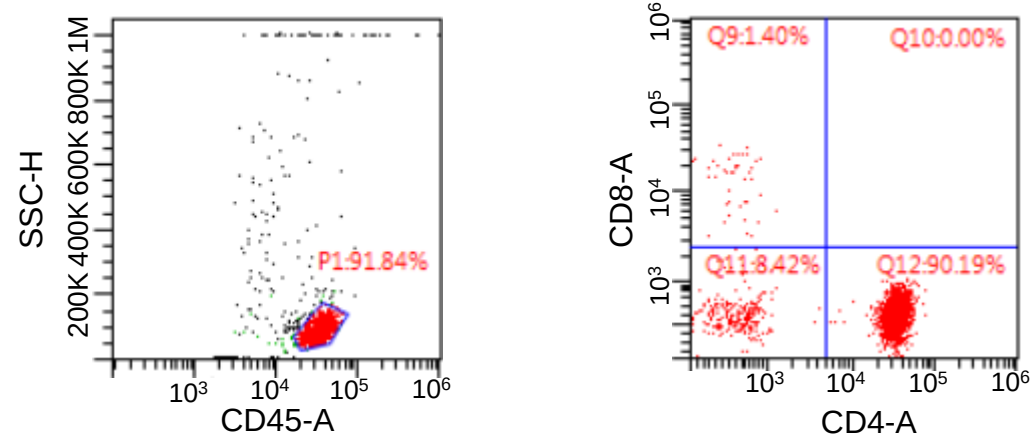




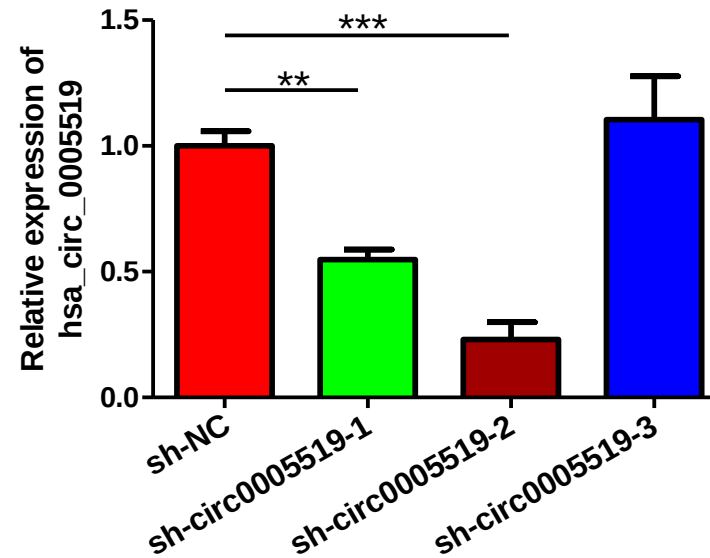








Supplementary Fig. S1. Flow-cytometric analysis showed that the purity of isolated CD4⁺ T cells



Supplementary Fig. S2. The relative expression levels of hsa_circ_0005519 in CD4⁺T cell transfected with three shRNAs detected by qRT-PCR

Table 1: The top 20 up-regulated and down-regulated circRNAs ranked by fold changes in microarray data

circRNA ID	<i>P</i> -value	Fold	circRNA		circRNA ID	<i>P</i> -value	Fold	circRNA	
		change	type	chrome			change	type	chrome
Up-regulated circRNAs					Down-regulated circRNAs				
hsa_circ_0008844	3.3E-10	44.124862	exonic	chr1	hsa_circ_0001801	1.005E-06	8.1859901	exonic	chr8
hsa_circ_0001783	2.443E-06	38.453166	intronic	chr8	hsa_circ_0066631	0.001169	7.481157	exonic	chr3
hsa_circ_0001720	2.398E-06	37.596541	intragenic	chr7	hsa_circ_0012938	0.0001682	7.4044347	exonic	chr1
hsa_circ_0074146	2.831E-08	33.147247	exonic	chr5	hsa_circ_0001924	2.327E-08	7.0327154	intragenic	chard
hsa_circ_0001482	1.988E-07	32.330796	antisense	chr5	hsa_circ_0004586	0.0001486	6.9918157	exonic	chr1
hsa_circ_0000493	1.743E-05	32.02203	antisense	chr13	hsa_circ_0000798	0.0001725	6.7937161	exonic	chr17
hsa_circ_0032261	4.761E-08	30.329678	exonic	chr14	hsa_circ_0017286	0.0003218	5.9257324	exonic	chr1
hsa_circ_0003071	3.18E-05	28.91615	exonic	chr2	hsa_circ_0001344	0.0005901	5.7655553	intragenic	chr3
hsa_circ_0008433	1.004E-08	26.567224	exonic	chr1	hsa_circ_0023233	0.0018993	5.6916266	exonic	chr11
hsa_circ_0072684	3.59E-06	25.566887	exonic	chr5	hsa_circ_0004968	0.0052528	5.6061577	exonic	chr3
hsa_circ_0046938	2.382E-08	24.533152	exonic	chr18	hsa_circ_0024457	0.0032257	5.0220785	exonic	chr11
hsa_circ_0031583	2.244E-05	24.158869	exonic	chr14	hsa_circ_0020028	0.0015699	4.9796494	exonic	chr10
hsa_circ_0077913	8.228E-05	22.422098	exonic	chr6	hsa_circ_0000826	5.069E-07	4.8787313	exonic	chr18
hsa_circ_0003198	8.527E-06	21.940264	exonic	chr1	hsa_circ_0018402	0.0005247	4.8625835	exonic	chr10
hsa_circ_0004622	1.147E-06	19.706931	exonic	chr17	hsa_circ_0008923	0.0046371	4.8569658	exonic	chr3
hsa_circ_0063809	6.64E-09	19.67584	exonic	chr22	hsa_circ_0001134	0.0053478	4.8023246	exonic	chr20
hsa_circ_0001928	4.232E-05	19.378977	intragenic	chard	hsa_circ_0086736	0.0002512	4.7352202	exonic	chr9
hsa_circ_0013171	5.762E-06	17.841144	exonic	chr1	hsa_circ_0019985	5.635E-05	4.7319971	exonic	chr10
hsa_circ_0028630	0.000125	17.076158	exonic	chr12	hsa_circ_0002607	0.0029154	4.3148521	exonic	chr2
hsa_circ_0092375	1.684E-06	16.271386	intronic	chr11	hsa_circ_0005932	0.0114566	4.2880534	exonic	chr6

Note: CircRNA ID: The circRNA ID is in circBase (<http://www.circBase.org/>). CircRNA type: The circRNAs are classified into 5 types: "exonic", "intronic", "antisense", "intragenic" and "intergenic". *P*-value: *P*-value calculated from paired t-test. Fold change: The absolute ratio (no log scale) of normalized intensities between two conditions

1.The quantitative reverse transcription polymerase chain reaction (qRT-PCR) protocol

Step	Temperature	Duration	cycles
Holding Stage	95°C	30s	1
Cycling Stage	95°C	5s	40
	60°C	30s	
	72°C	30s	

2.What is the divergent primers?

They face away from each other on the linear RNA, so that they can only amplify the circRNAs, and not the linear RNAs with the same sequence. The PCR amplicon for the detection of circRNAs using divergent primers spans the backsplice junction of circRNAs.

3. Let-7a was previous ID of let-7a-5p. It is highly conserved and the mature sequences of human and mouse are consistent. (miRbase : <http://mirbase.org/index.shtml>)

Mature sequence hsa-let-7a-5p	
Accession	MIMAT0000062
Previous IDs	hsa-let-7a
Sequence	6 - http://www.ncbi.nlm.nih.gov/nuclseq/ - 27 Get sequence
Deep sequencing	9604809 reads, 77 experiments
Evidence	experimental; cloned [1-3,5-8], Northern [1], Illumina [9]
Database links	RNAcentral: URS0000416056_9606
Predicted targets	TargetMiner: hsa-let-7a-5p TargetScanVert: hsa-let-7a-5p miRDB: hsa-let-7a-5p microrna.org: hsa-let-7a-5p

Mature sequence mmu-let-7a-5p	
Accession	MIMAT0000521
Previous IDs	mmu-let-7a
Sequence	13 - UGAGGAGGAGGAGGAGGAGU - 34 Get sequence
Deep sequencing	198064381 reads, 76 experiments
Evidence	experimental; cloned [1-4], Illumina [5,7]
Database links	RNAcentral: URS0000416056_10090
Predicted targets	TargetScanVert: mmu-let-7a-5p miRDB: mmu-let-7a-5p microna.org: mmu-let-7a-5p