

Letter to the Editor

Lower levels of hsa-mir-15a, which decreases VEGFA, in the CD4⁺ T cells of pediatric patients with asthma

To the Editor:

Asthma is a chronic lung disease characterized by airway hyperresponsiveness, which causes recurrent episodes of wheezing, breathlessness, chest tightness, and coughing. Although tremendous evidence suggests that the T_H2 cytokines IL-4, IL-5, and IL-13 play important roles in the induction of the pathophysiologic features of asthma, the underlying mechanisms by which the T_H2 cytokines induce the features of this disease are not completely understood.

Recently, vascular endothelial growth factor-A (VEGF-A), a key regulator of angiogenesis,¹ has been shown to be overexpressed in cases of T_H2-mediated lung inflammation such as asthma and to play a role in the induction of not only angiogenesis and edema but also other features of asthma pathogenesis, such as eosinophilic inflammation, goblet cell metaplasia, airway remodeling, and airway hyperresponsiveness.² Thus, VEGF-A is now considered to be one of the key mediators in the pathogenesis of asthma.

As VEGF-A is overexpressed in the sputum and serum of both adult and pediatric patients with asthma,^{3,4} the dysregulation of VEGF-A may underlie the pathogenesis of asthma. However, the mechanism of VEGF-A regulation in the pathogenesis of asthma is still unclear. In this study, we focused on the posttranscriptional regulation of *VEGFA* by microRNAs (miRs) in pediatric patients with asthma. We revealed that hsa-mir-15a, a human miR that regulates *VEGFA* expression in peripheral blood CD4⁺ T cells, was present at significantly lower levels in pediatric patients with asthma than in healthy controls. To the best of our knowledge, this report is the first to describe the involvement of miRs in pediatric asthma.

Detailed information on the study subjects and methods is reported in this article's Online Repository at www.jacionline.org. As previously reported,⁵ we found that pediatric patients with asthma had significantly higher levels of serum VEGF-A (median, 607.4 pg/mL; range, 99.3–1224 pg/mL) than did healthy control children (median, 250.9 pg/mL; range, 53.1–416.7 pg/mL) in our preliminary experiment ($P < .0001$; see Fig E1, A, and Table E1 in this article's Online Repository at www.jacionline.org), even though all the pediatric patients with asthma had been assessed as "controlled" according to the guideline criteria for assessing asthma control of the Global Initiative for Asthma (GINA).⁶ We did not find any relationship between the levels of serum VEGF-A and the usage of inhaled corticosteroids (ICSs) (Fig E1, B). Because CD4⁺ T cells, especially the memory subset, are considered one of the major cellular sources of serum VEGF-A,⁷ we focused on the expression of *VEGFA* mRNA and miRNAs targeting *VEGFA* in the CD4⁺ T cells of pediatric patients with asthma.

We selected 12 miRs (see Table E2 in this article's Online Repository at www.jacionline.org) that were predicted to have a high potential to bind to the *VEGFA* 3'UTR by 3 independent software programs (TargetScan, miRanda, and RNAhybrid) and were also highly conserved among mammalian species. Then, we compared the expression levels of *VEGFA* mRNA and those

of the miRs in CD4⁺ T cells isolated from the peripheral blood among the atopic asthma, atopic control, and nonatopic control groups (see Table E3 in this article's Online Repository at www.jacionline.org). As we expected, the expression of *VEGFA* mRNA in the CD4⁺ T cells was significantly higher in the atopic asthma group than in the atopic control and the nonatopic control groups ($P < .05$, Fig 1, A), which suggests that *VEGFA* expression in CD4⁺ T cells may play a role in the pathogenesis of asthma. Of the 12 candidate miRs, hsa-mir-15a, hsa-mir-15b, and hsa-mir-20a exhibited significantly lower expression levels in CD4⁺ T cells in the atopic asthma group than in the atopic control and the nonatopic control groups ($P < .05$, Fig 1, C). However, we did not find differences in the expression of any of the other candidate miRs among the groups (see Fig E2 in this article's Online Repository at www.jacionline.org). Moreover, in the atopic asthma group, *VEGFA* mRNA expression levels were significantly and negatively correlated with the expression of hsa-mir-15a ($P < .05$, Fig 1, B) but not the expression of hsa-mir-15b or hsa-mir-20a (see Fig E3 in this article's Online Repository at www.jacionline.org). These results suggest that the lower expression of hsa-mir-15a may be a key feature of miR expression in the CD4⁺ T cells of pediatric patients with asthma, which leads to increased expression of *VEGFA* relative to controls. Thus, we focused on hsa-mir-15a in the subsequent experiments.

Hsa-mir-15a is a member of the miR-15/miR-16 family whose members share an miR seed region indispensable in miR-target interactions. Recently, it was shown that miR-15/miR-16 family members, including hsa-mir-15a, downregulate *VEGFA* mRNA expression.^{8,9} To determine whether hsa-mir-15a directly targets *VEGFA* mRNA, we performed biotinylated miR pull-down assays.¹⁰ As we expected, *VEGFA* mRNA, but not the mRNA of nontarget genes (*ACTA2* and *ORMDL3*), was significantly enriched among transcripts pulled down by biotinylated hsa-mir-15a relative to those pulled down by biotinylated control miR (see Fig E4 in this article's Online Repository at www.jacionline.org). To verify the suppressive effect of hsa-mir-15a on *VEGFA* mRNA expression *in vitro*, we performed a dual luciferase reporter gene assay. To generate the reporter construct, a portion of the *VEGFA* 3'UTR containing the putative binding site of hsa-mir-15a (intact *VEGFA* 3'UTR) or a mutated binding site (mutant *VEGFA* 3'UTR) (see Table E4 in this article's Online Repository at www.jacionline.org) was placed under the *firefly* luciferase gene in the pmirGLO vector (Promega, Madison, Wis). We cotransfected the mature hsa-mir-15a-expressing vector (hsa-mir-15a vector) or control null expression vector (control vector) and the intact *VEGFA* 3'UTR or mutant *VEGFA* 3'UTR into HEK293 cells. After 48 hours, *firefly* luciferase activity was measured and normalized to *renilla* luciferase activity. In the cells transfected with the intact *VEGFA* 3'UTR, the hsa-mir-15a vector significantly decreased the normalized luciferase activity relative to the controls ($P < .05$, Fig 2, A). In contrast, in the cells transfected with the mutant *VEGFA* 3'UTR, the hsa-mir-15a vector did not affect the normalized luciferase activity (Fig 2, B). These results suggest that hsa-mir-15a may target the *VEGFA* 3'UTR and downregulate *VEGFA* mRNA. Finally, to validate these results, we determined whether mature hsa-mir-15a double-stranded RNAs

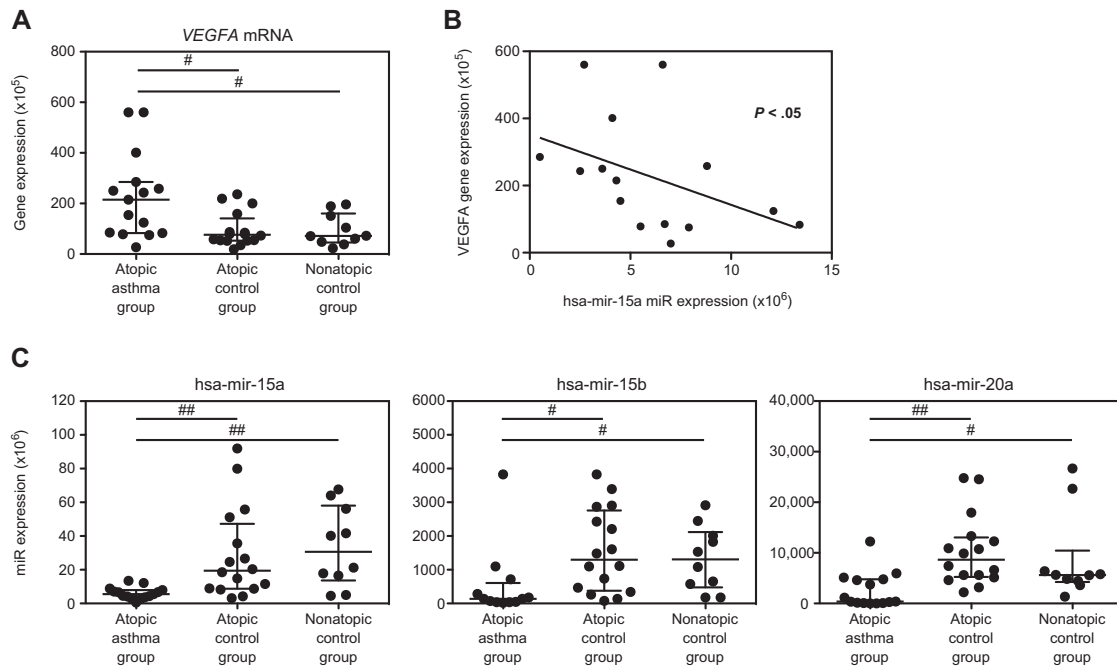


FIG 1. CD4⁺ T cells of pediatric patients with asthma contained higher levels of *VEGFA* and lower levels of hsa-mir-15a, hsa-mir-15b, and hsa-mir-20a. **A**, *VEGFA* mRNA expression in peripheral blood CD4⁺ T cells. **B**, The correlation between hsa-mir-15a miR expression and *VEGFA* mRNA expression in the peripheral blood CD4⁺ T cells of the atopic asthma group. **C**, miR expression in peripheral blood CD4⁺ T cells. The lines and error bars are the medians and 25% to 75% percentiles, respectively ($\#P < .05$, $\#\#P < .01$).

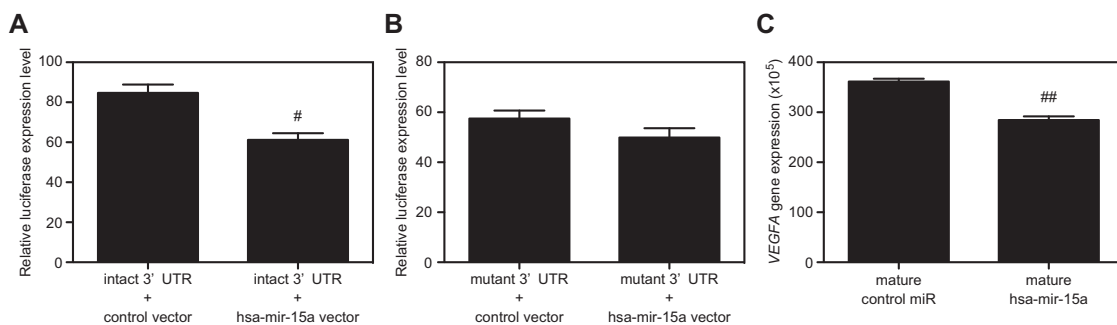


FIG 2. Hsa-mir-15a decreased *VEGFA* mRNA expression levels. **A** and **B**, A part of the *VEGFA* 3'UTR containing either the putative binding site of hsa-mir-15a (intact *VEGFA* 3'UTR) or a mutated binding site (mutant *VEGFA* 3'UTR) was placed under the *firefly* luciferase gene in the pmirGLO vector. Cotransfection with hsa-mir-15a significantly decreased the normalized luciferase activity in intact *VEGFA* 3'UTR-transfected HEK293 cells (Fig 2, **A**, $\#P < .05$, compared with control vector, $n = 3$) but not in the mutant *VEGFA* 3'UTR-transfected cells (Fig 2, **B**). **C**, Transfection of mature hsa-mir-15a but not mature control miR decreased endogenous *VEGFA* mRNA expression levels in HEK293 cells. The data are shown as the means $\pm$ SEM ($\#\#P < .01$, compared with mature control miR, $n = 4$).

downregulate endogenous *VEGFA* mRNA expression in HEK293 cells. As expected, *VEGFA* expression was significantly lower in cells transfected with mature hsa-mir-15a RNAs than in cells transfected with mature Cel-mir-67 RNAs (control mature miR) ($P < .01$, Fig 2, **C**).

The mechanism by which hsa-mir-15a expression was decreased in the CD4⁺ T cells of the atopic asthma group remains unclear. We did not find any relationship between the usage of ICS and the expression of hsa-mir-15a (see Fig E5, **B**, in this article's Online Repository at www.jacionline.org), levels of serum VEGF-A (Fig E1, **B**), or expression of *VEGFA* (Fig E5, **A**) among pediatric patients with asthma. These findings suggest that ICS

may not affect VEGF-A regulation through hsa-mir-15a in asthma pathogenesis. These data were consistent with those of previous studies.⁴ Because all our subjects were assessed as "controlled," we did not evaluate the relationship between severity of asthma and *VEGFA* and miR expression levels. Interestingly, the expression of hsa-mir-15a was identical between atopic controls and nonatopic control groups (Fig 1, **C**). In addition, we did not find any correlation between serum IgE levels and the expression of hsa-mir-15a (see Fig E6 in this article's Online Repository at www.jacionline.org). Because mir-15a has been shown to be associated with angiogenic or fibrotic processes in ischemia,¹¹ lupus,¹² and multiple sclerosis,¹³

the reduced expression of hsa-mir-15a in pediatric patients with asthma may be an independent feature of the T_H2-mediated immune response. Further studies are needed to clarify what initiates the reduced expression of hsa-mir-15a in pediatric asthma.

In conclusion, we found that pediatric patients with asthma have lower expression levels of hsa-mir-15a, which is related to the higher expression levels of *VEGFA*, in peripheral blood CD4⁺ T cells. Further studies focusing on the role of hsa-mir-15a in the pathogenesis of pediatric asthma should be performed.

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METHODS

Subjects

For the analysis of serum VEGF-A levels, we recruited 20 pediatric patients with asthma at the outpatient department of Chiba University Hospital and 19 control children at an elementary school attached to Chiba University. The median age of both groups was 12 years. A diagnosis of pediatric asthma was given by pediatric allergists at Chiba University Hospital on the basis of Japanese Pediatric Guideline for the Treatment and Management of Asthma 2008. The degree of asthma control for all the pediatric patients with asthma had been assessed as “controlled” according to GINA guidelines. Healthy control children were determined to not have asthma, allergic rhinitis, or atopic dermatitis by the ISSAC (International Study of Asthma and Allergies in Childhood) questionnaire. The characteristics of the subjects are shown in Table E1.

For analysis of *VEGFA* mRNA and miRs, we recruited 15 children with atopic asthma (atopic asthma group) from the outpatient department of Chiba University Hospital. The median age was 11 years (range, 8–15 years), and the diagnosis was given by pediatric allergists at Chiba University Hospital on the basis of Japanese Pediatric Guideline for the Treatment and Management of Asthma 2008. The degree of asthma control for all the pediatric patients with asthma had been assessed as “controlled” according to GINA guidelines. No subjects had been enrolled in our previous study measuring serum VEGF-A. As control subjects, 26 children aged 12 years who were determined to not have asthma by the ISSAC questionnaire when they were both 6 and 12 years old were recruited at an elementary school attached to Chiba University. The diagnosis of allergic rhinitis or atopic dermatitis was made in accordance with the ISSAC questionnaire. Control children were divided into 2 groups on the basis of their sensitivity to any one of *Dermatophagoides farinae*, house dust, or Japanese cedar pollen: the atopic control group (≥ 0.7 UA/mL, $n = 16$) and the nonatopic control group (< 0.7 UA/mL, $n = 10$). The characteristics of the subjects are shown in Table E3.

Ethical considerations

The study protocols were approved by the Committee on Human Research of Chiba University. Informed consent was obtained from each of the study subjects and their guardians.

In silico identification of miRNA binding to the VEGFA 3'UTR

Three consensus prediction programs were used to identify putative miRs that can bind to the *VEGFA* 3'UTR:

TargetScan: <http://www.targetscan.org>,

miRanda: <http://www.microrna.org/microrna/home.do>, and

RNAhybrid: <http://bibiserv.techfak.uni-bielefeld.de/mahybrid/>.

Purification of CD4⁺ T cells

PBMCs were isolated by using Ficoll density gradient centrifugation of the peripheral blood of the subjects. CD4⁺ T-cell isolation from the PBMCs was conducted by using CD4 microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany) according to the manufacturer's instructions. The purity of the CD4⁺ T cells was examined by using flow cytometry, and cells with more than 95% purity were used for the following experiments.

RNA extraction and real-time PCR

Total RNA was prepared from CD4⁺ T cells by using the miRCURY RNA Isolation kit (EXIQON, Vedbaek, Denmark), according to the manufacturer's instructions and stored at -80°C until further use.

To study the relative expression of *VEGFA*, *ACTA2*, and *ORMDL3*, real-time PCR (iQ SYBR Green Supermix; Bio Rad, Hercules, Calif) was performed and the results were normalized to *GAPDH* expression. To study the relative expression of the miRs, real-time PCR (miScript SYBR Green PCR kit; QIAGEN, Hilden, Germany) was performed and the results were normalized to *RNU6B* expression (a housekeeping gene for miRs). The following

primer sequences were used for the mRNA real-time PCR: *VEGFA* forward, 5'-CTCCACCATGCCAAGTGGTC-3'; *VEGFA* reverse, 5'-CTCGATTGGA TGGCAGTAGCT-3'; *ACTA2* forward, 5'-TCAATGTCCCAGCCATGTAT-3'; *ACTA2* reverse, 5'-CAGCACGATGCCAGTTGT-3'; *ORMDL3* forward, 5'-GCATCCCGTTTGTGAGTGTC-3'; *ORMDL3* reverse, 5'-CCATGTTGT GAATGAGGTTGGTG-3'; *GAPDH* forward, 5'-GAAGGTGAAGGTCG GAGTC-3'; and *GAPDH* reverse, 5'-GAAGATGGTGATGGGATTTC-3'. The PCR primers for the miRs were included with the reagents provided by the manufacturer, and the sequences are confidential.

ELISA assays

ELISA assays were performed by using the VEGF ELISA kit from R & D Systems (Minneapolis, Minn).

Luciferase assays

To verify the suppressive effect of hsa-mir-15a on *VEGFA* mRNA expression *in vitro*, we performed a dual luciferase reporter gene assay. A part of the *VEGFA* 3'UTR containing the putative binding site of hsa-mir-15a (intact *VEGFA* 3'UTR) or a mutated binding site (mutant *VEGFA* 3'UTR) (Table E4) was synthesized by a custom gene synthesis service (Integrated DNA Technologies, Coralville, Iowa). For the reporter construct, each synthesized DNA was placed under the firefly luciferase gene in the pmirGLO vector (Promega). The luciferase assays were conducted by using 1×10^4 HEK293 cells plated on a 96-well plate. HEK293 cells were cotransfected with 2 ng of the intact *VEGFA* 3'UTR or mutant *VEGFA* 3'UTR together with 80 ng of the hsa-mir-15a expression vector (Cell Biolabs, San Diego, Calif) or the control null expression vector (Cell Biolabs) by using Lipofectamine LTX reagent (Life Technologies, Carlsbad, Calif). Forty-eight hours after transfection, the cells were harvested and assayed for both firefly and renilla luciferase activity by using the Dual-Glo Luciferase Assay System (Promega) according to the manufacturer's instructions. The luminescence was measured by using a microplate reader (Infinite pro 200, Tecan, Männedorf, Switzerland). The firefly luciferase activity was measured and normalized to the renilla luciferase activity.

Functional assay for hsa-mir-15a

To determine whether hsa-mir-15a downregulates *VEGFA*, we transfected mature hsa-mir-15a double-strand RNAs into HEK293 cells and evaluated the effect on endogenous *VEGFA* mRNA expression. HEK293 cells in 24-well plates were transfected with 25 nM mature hsa-mir-15a double-strand RNAs (miRIDIAN hsa-mir-15a Mimic; Thermo Fisher Scientific, Waltham, Mass) or control mature miRs (mature Cel-mir-67, miRIDIAN microRNA Mimic Negative Control, Thermo Fisher Scientific) by using the siPORT NeoFX Transfection Agent (Life Technologies). The transfected cells were subsequently cultured in 10% FBS-Dulbecco modified Eagle medium. After 2 days of culture, *VEGFA* mRNA expression levels were analyzed by using real-time PCR.

Biotinylated miR pull-down assays

To determine whether *VEGFA* is a direct target gene of hsa-mir-15a, we performed a biotinylated miR pull-down assay. HEK293 cells (2×10^6) were transfected in quadruplicate with 200 pmol biotinylated hsa-mir-15a miRNA (Bi-hsa-miR-15a) or biotinylated cel-mir-67 miRNA (Bi-cel-miR-67) (Thermo Fisher Scientific) by using the siPORT NeoFX Transfection Agent (Life Technologies) before being cultured in 6-well plates. Twenty-four hours later, the cells from 4 wells were pelleted at 500g. After being washed twice with PBS, the cell pellets were resuspended in 0.7 mL of lysis buffer (20 mmol/L Tris-HCl [pH 7.4], 200 mmol/L sodium chloride, 2.5 mmol/L magnesium chloride, and 0.05 w/v% NP-40 substitute) (Wako Pure Chemical Industries, Osaka, Japan), 50 U of RNase OUT (Life Technologies), and a complete miniprotease inhibitor cocktail (Roche Applied Science, Indianapolis, Ind), and incubated on ice for 5 minutes. The cytoplasmic lysate was isolated by centrifugation at 10,000g for 10 minutes. Streptavidin-coated magnetic beads (Life Technologies) were blocked for 2 hours at 4°C in lysis buffer containing 1 mg/mL yeast tRNA and 1 mg/mL

BSA (Life Technologies) before being washed twice with 1 mL of lysis buffer. The cytoplasmic lysate was added to the beads and incubated for 4 hours at 4°C before the beads were washed 5 times with 1 mL of lysis buffer. RNA bound to the beads (pull-down RNA) or 10% of the extract (input RNA) was isolated by using Trizol LS reagent (Life Technologies, Carlsbad, Calif).

The levels of mRNAs in the Bi-hsa-miR-15a or Bi-cel-miR-67 control pull-down were quantified by using real-time PCR as described above, and mRNA levels were normalized to the mRNA levels of *GAPDH*. The fold enrichment of the pulled-down RNA relative to the input RNA levels was then calculated.

Data and statistical analysis

Differences in the expression levels of *VEGFA* mRNA or miRs between the study groups were determined by using a Kruskal-Wallis test followed by Dunn's multiple comparisons test. In the functional analysis and luciferase assays, a 2-tailed student *t* test was used. The statistical analyses were performed by using GraphPad Prism 5 (GraphPad Software, Inc, San Diego, Calif). *P* less than .05 was considered significant.

ISAAC questionnaire

Questionnaire for asthma.

1. Has your child ever had wheezing or whistling in the chest at any time in the past?

IF YOU HAVE ANSWERED "NO," PLEASE SKIP TO QUESTION 6.

2. Has your child had wheezing or whistling in the chest in the past 12 months?

IF YOU HAVE ANSWERED "NO," PLEASE SKIP TO QUESTION 6.

3. How many attacks of wheezing has your child had in the past 12 months?
4. In the past 12 months, how often, on average, has your child's sleep been disturbed because of wheezing?
5. In the past 12 months, has wheezing ever been severe enough to limit your child's speech to only 1 or 2 words at a time between breaths?
6. Has your child ever had asthma?
7. In the past 12 months, has your child's chest sounded wheezy during or after exercise?
8. In the past 12 months, has your child had a dry cough at night, apart from a cough associated with a cold or chest infection?

Questionnaire for allergic rhinitis. All questions are about problems that occur when you DO NOT have a cold or the flu.

1. Has your child ever had a problem with sneezing, or a runny or blocked nose when you DID NOT have a cold or the flu?

IF YOU HAVE ANSWERED "NO," PLEASE SKIP TO QUESTION 6.

2. In the past 12 months, has your child had a problem with sneezing, or a runny or blocked nose when you DID NOT have a cold or the flu?

IF YOU HAVE ANSWERED "NO," PLEASE SKIP TO QUESTION 6.

3. In the past 12 months, has this nose problem been accompanied by itchy-watery eyes?
4. In which of the past 12 months did this nose problem occur? (Please tick any that apply.)

January February March April May June

July August September October November December

5. In the past 12 months, how much did this nose problem interfere with your child's daily activities?

Not at all

A little

A moderate amount

A lot

6. Has your child ever had hay fever?

Questionnaire for eczema.

1. Has your child ever had an itchy rash that was coming and going for at least 6 months?

IF YOU HAVE ANSWERED "NO," PLEASE SKIP TO QUESTION 6.

2. Has your child had this itchy rash at any time in the past 12 months?

IF YOU HAVE ANSWERED "NO," PLEASE SKIP TO QUESTION 6.

3. Has this itchy rash at any time affected any of the following places:
the folds of the elbows, behind the knees, in front of the ankles, under the buttocks, or around the neck, ears, or eyes?
4. Has this rash cleared completely at any time during the past 12 months?
5. In the past 12 months, how often, on average, has your child been kept awake at night by this itchy rash?

Never in the past 12 months

Fewer than 1 night per week

One or more nights per week

6. Has your child ever had eczema?

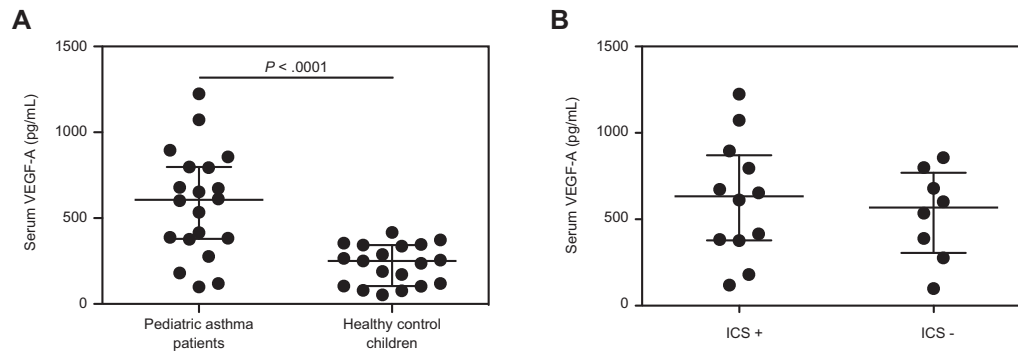


FIG E1. Serum VEGF-A levels in pediatric patients with asthma and healthy control children. **A**, Serum VEGF-A levels were significantly higher in pediatric patients with asthma than in healthy control children. **B**, There was no difference in serum VEGF-A levels between pediatric patients with asthma using ICSs (ICS+) and those not using ICSs (ICS-).

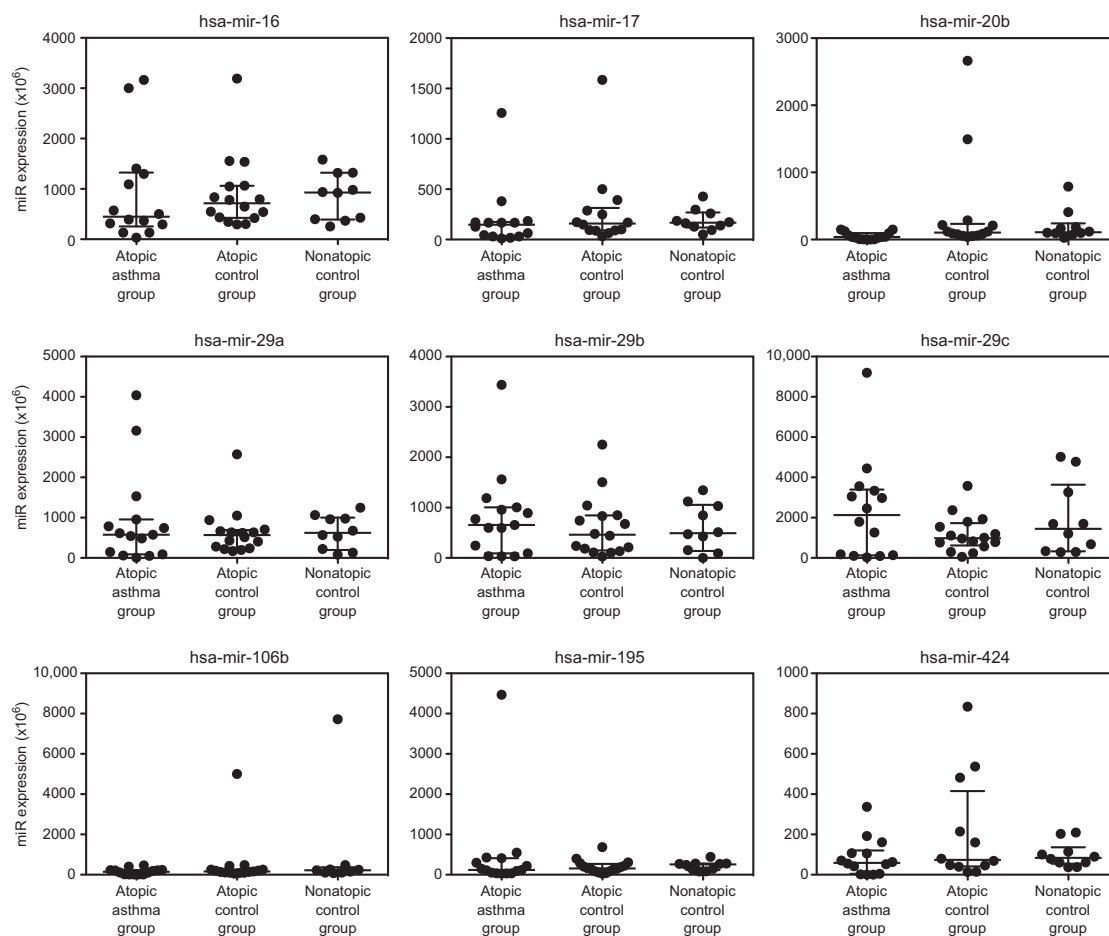


FIG E2. Expression of miRs that were predicted to bind *VEGFA* 3'UTR but were identically expressed in the atopic asthma group and the control groups. The expression levels of hsa-mir-16, -17, -20b, -29a, -29b, -29c, -106b, -195, and -424 were identical among the atopic asthma, atopic control, and nonatopic control groups.

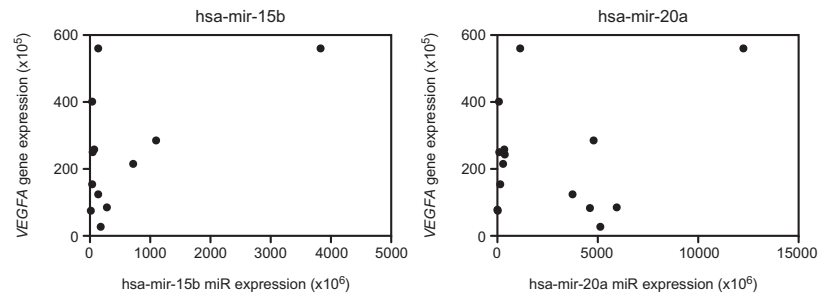


FIG E3. Correlation between hsa-mir-15b or -20a miR expression and *VEGFA* mRNA expression in the peripheral blood CD4⁺ T cells of the atopic asthma group. The expression levels of hsa-mir-15b and -20a were not significantly correlated with *VEGFA* mRNA expression in the peripheral blood CD4⁺ T cells of the atopic asthma group.

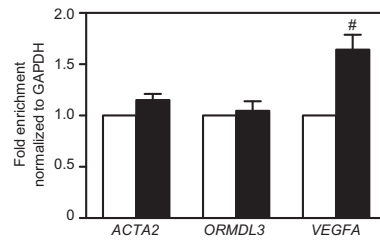


FIG E4. Biotinylated hsa-mir-15a pull-down of *VEGFA* mRNA. HEK293 cells were transfected in quadruplicate with Bi-hsa-miR-15a (*black*) or Bi-cel-miR-67 (*white*). Enrichment of *VEGFA* mRNA or nontarget mRNA (*ACTA2* and *ORMDL3*) was assessed by using quantitative RT-PCR relative to *GAPDH* mRNA. Values for the transcripts pulled down by Bi-hsa-miR-15a are shown as ratios to those pulled down by Bi-cel-miR-67.

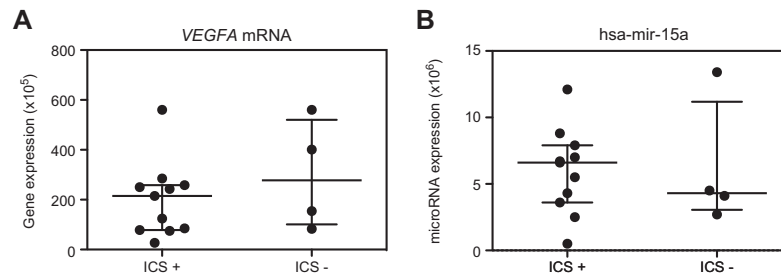


FIG E5. Expression of *VEGFA* and *hsa-mir-15a* in peripheral blood $CD4^+$ T cells was not associated with the usage of ICSs. There was no difference in the expression of *VEGFA* (**A**) and *hsa-mir-15a* (**B**) in peripheral blood $CD4^+$ T cells between pediatric patients with asthma using ICSs (ICS+) and those not using ICSs (ICS-).

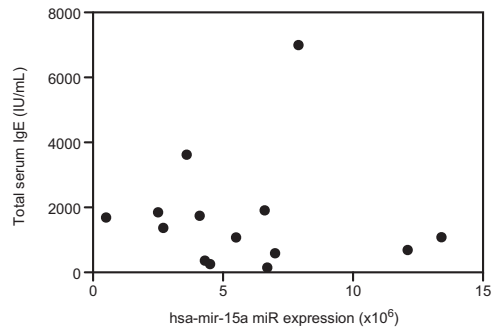


FIG E6. Correlation between hsa-mir-15a miR expression and total serum IgE in the atopic asthma group. Expression of hsa-mir-15a was not significantly correlated with total serum IgE in the atopic asthma group.

TABLE E1. Characteristics of subjects for measuring serum VEGF-A

Characteristics	Pediatric asthma patients	Healthy control children	P value
No. of subjects	20	19	
Age* (y)	12 (10-14)	12 (11-12)	NS
Male:Female	13:7	11:8	NS
Serum <i>Derf</i> -specific IgE* (U _A /mL)	74.65 (2.34-100<)	0 (<0.34-0.57)	<.05
Serum house dust-specific IgE* (U _A /mL)	100 (1.9-100<)	0 (<0.34-0.88)	<.05
Serum Japanese cedar pollen-specific IgE* (U _A /mL)	20 (<0.34-100<)	0 (<0.34-39.3)	<.05
Total serum IgE* (IU/mL)	1,240 (257-10,260)	53.3 (11.8-360)	<.05
Levels of asthma control,† no. of “controlled” subjects (%)	20 (100)		
Use of daily LTRA, no. of subjects (%)	14 (70)	—	—
Use of daily ICS, no. of subjects (%)	6 (30)	—	—
Use of daily ICS/LABA, no. of subjects (%)	6 (30%)	—	—

GINA, Global Initiative for Asthma; LABA, long-acting β_2 -agonists; LTRA, leukotriene receptor antagonists; NS, not significant.

*Data are shown as median (range).

†Assessment by GINA guideline.

TABLE E2. MiRs that were predicted to target *VEGFA* 3'UTR

	Position of the putative binding site in <i>VEGFA</i> 3'UTR	
	Start site	End site
hsa-mir-15a	292	299
hsa-mir-15b	292	299
hsa-mir-16	292	299
hsa-mir-17	193	199
hsa-mir-20a	193	199
hsa-mir-20b	193	199
hsa-mir-29a	1758	1765
hsa-mir-29b	1758	1765
hsa-mir-29c	1758	1765
hsa-mir-106b	193	199
hsa-mir-195	292	299
hsa-mir-424	292	299

TABLE E3. Characteristics of subjects for measuring the expression of *VEGFA* mRNA and miRs

	Atopic asthma group	Atopic control group	Nonatopic control group	P value
No. of subjects	15	16	10	
Age* (y)	11 (8-15)	12 (11-12)	12 (11-12)	.11
Male:Female	12:3	11:5	5:5	.28
Serum <i>Derf</i> -specific IgE* (U _A /mL)	100 (37.4-100<)	3 (<0.34-100<)	<.34	<.05
Serum house dust-specific IgE* (U _A /mL)	100 (20-100<)	4 (<0.34-100<)	<.34	<.05
Serum Japanese cedar pollen-specific IgE* (U _A /mL)	20 (0.6-100<)	3 (<0.34-69.4)	<.34	<.05
Total serum IgE* (IU/mL)	1372 (257-6998)	169 (11.8-1550)	48 (12.5-310)	<.05
Levels of asthma control,† no. of “controlled” subjects (%)	15 (100)			
Use of daily LTRA, no. of subjects (%)	15 (100)	—	—	—
Use of daily ICS, no. of subjects (%)	5 (33.3)	—	—	—
Use of daily ICS/LABA, no. of subjects (%)	6 (40)	—	—	—

GINA, Global Initiative for Asthma; LABA, long-acting β_2 -agonists; LTRA, leukotriene receptor antagonists.

*Data are shown as median (range).

†Assessment by GINA guideline.

TABLE E4. Sequence of intact *VEGFA* 3'UTR and mutant *VEGFA* 3'UTRIntact *VEGFA* 3'UTR

GCCGGGCAGGAGGAAGGAGCCTCCCTCAGGGTTTCGGGAACCAGATCTCTCACCAGGAAAGACTGATACAGAACGATCGATACAGAAACCAC
GCTGCCGCCACCACACCATCACCATCGACAGAACAGTCCTTAATCCAGAAACCTGAAATGAAGGAAGAGGAGACTCTGCGCAGAGCACTTT
GGGTCCGGAGGGCGAGACTCCGGCGGAAGCATTCCCGGGCGGGTGACCCAGCACGGTCCCTCTTGGAATTGGATTGCGCATTTTATTTTTC
TTGCTGCTAAATCACCGAGCCCGGAAGATTAGAGAGTTTTATTTCTGGGATTCCCTGTAGACACACCCACCCACATACATACATTT

Mutant *VEGFA* 3'UTR

GCCGGGCAGGAGGAAGGAGCCTCCCTCAGGGTTTCGGGAACCAGATCTCTCACCAGGAAAGACTGATACAGAACGATCGATACAGAAACCAC
GCTGCCGCCACCACACCATCACCATCGACAGAACAGTCCTTAATCCAGAAACCTGAAATGAAGGAAGAGGAGACTCTGCGCAGAGCACTTT
GGGTCCGGAGGGCGAGACTCCGGCGGAAGCATTCCCGGGCGGGTGACCCAGCACGGTCCCTCTTGGAATTGGATTGCGCATTTTATTTTTC
CACGACGATAATCACCGAGCCCGGAAGATTAGAGAGTTTTATTTCTGGGATTCCCTGTAGACACACCCACCCACATACATACATTT

Intact or mutated target site for hsa-mir-15a is shown in boldface.