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Diagnostic and Therapeutic Value of Hsa_circ_0002594 for T Helper 2-Mediated Allergic Asthma

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Keywords
Asthma · $CD4^+$ T cells · hsa_circ_0002594 · Th2

Abstract

Introduction: Circular RNAs (circRNAs) are an endogenous mircoRNA sponge that could act as potential biomarkers for the diagnosis and treatment of diseases. However, the role of circRNAs in asthma is far from clear. ***Objective:*** The aim of this study is to assess the diagnostic and therapeutic value of hsa_circ_0002594 for T helper (Th) 2-mediated allergic asthma. ***Methods:*** The expression profiles of hsa_circ_0002594 in $CD4^+$ T cells were revealed by circRNA microarray. Hsa_circ_0002594 expression was confirmed via quantitative real-time PCR (qRT-PCR) in asthmatic patients and healthy subjects. Hsa_circ_0002594 levels were compared between subgroups. The clinical diagnostic abilities and therapeutic response of hsa_circ_0002594 were evaluated. The analyses utilized included a student's *t* test, non-parametric tests, Spearman's rank-order correlation, Fisher's exact test, and the generation of receiver operating characteristic (ROC) curves. ***Results:*** Hsa_circ_0002594 was upregulated and positively correlated with fraction of exhaled nitric oxide while negatively correlated with methacholine dose producing a decrease of 20% from baseline in forced expiratory volume in the first second (PD20) in $CD4^+$ T cells of asthma. Furthermore, hsa_circ_0002594 expression was higher in subgroups with a family history, skin pricking test (SPT)-positive, or Th2-high. The hsa_circ_0002594-high subgroup was more frequently associated with Th2-high biomarker profiles and positive SPT. Hsa_circ_0002594 was decreased after inhaled corticosteroids (ICS) treatment. ROC curve analyses of hsa_circ_0002594 showed high area under the curve values in the presence of ICS or not. ***Conclusions:*** Our data suggested that hsa_circ_0002594 was upregulated in $CD4^+$ T cells and might have potential value in the diagnosis and treatment of Th2-mediated allergic asthma.

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Introduction

Asthma is a worldwide chronic lung disease characterized by recurrent bronchospasms, airway inflammation and hyperresponsiveness, mucus hypersecretion, and irreversible remodeling of the airways [1]. As a heterogeneous disease, both genetic and environmental factors are involved in the development of asthma. Therefore, it can be divided into several different phenotypes and subgroups that have varying therapeutic responses, especially following treatment with inhaled corticosteroids (ICS) [2, 3]. Allergic asthma with the generation of allergen-specific $CD4^+$ T cells as the initial event is the most common type of asthma. Naive T cells activated by antigen-presenting cells differentiate into T helper (Th) 2 cells [4, 5]. Many studies dedicated that Th2 cells play a vital role in allergic asthma via interleukin (IL)-4, IL-5, IL-13 secretion which was associated with the production of allergen-specific IgE by B cells, airway inflammation and hyperresponsiveness, and inflammatory cell infiltration [4, 6]. Virtually, allergic asthma is described as a Th2 inflammation of the airway [6, 7].

Epigenetics, such as DNA methylation, histone modification, and noncoding RNAs (ncRNAs) regulation, were involved in the pathogenesis of asthma [8, 9]. Recent studies have shown that ncRNAs might play an important role in Th2-mediated inflammatory of asthma, likely to be attractive noninvasively biomarkers or targets for the treatment of asthma. For instance, the lncRNAs-LNC_000127 functions in the Th2 inflammation pathway in eosinophilic asthma and targeting LNC_000127 may be effective for reducing Th2 inflammation [10]. The axis of miR-29c/B7-H3 participated in children with asthma through regulating Th2/Th17 cell differentiation and might provide new targets for the treatment of asthma [11]. Some studies also found that let-7e (previous ID of let-7e-5p) was downregulated in allergic rhinitis (AR) with asthma and emphasized the important role of let-7e in Th2-mediated allergic inflammation [12, 13].

CircRNAs as a new class of ncRNAs often function as molecular sponges to bind and inhibit microRNA (miRNA) transcription or activity, which subsequently affects the downstream mRNA expression [14]. Our previous study showed that circular RNA (circRNA) profiles were different and hsa_circ_0005519 regulated IL-13/IL-6 by sponging hsa-let-7a-5p in $CD4^+$ T cells from asthma [15]. However, to our knowledge, the study of circRNAs in the regulation of asthma was in its infancy, and the contribution of circRNAs to the pathogenesis of asthma, especially to the Th2 inflammation of asthma, remains unknown. Here we hypothesized that circRNAs acting as endogenous hsa-let-7e-5p sponges might be involved in asthma, particularly in Th2-mediated allergic asthma and have potential diagnostic and therapeutic values. To verify our conjecture, we further analyzed our circRNA array and selected the one with the most significant difference for clinical validation in $CD4^+$ T cells from asthmatic patients. We found that hsa_circ_0002594 in $CD4^+$ T cells played a proinflammatory role in Th2-mediated allergic asthma. Moreover, hsa_circ_0002594 expression was downregulated by treatment with ICS. These observations suggested that hsa_circ_0002594 was a proinflammatory factor in allergic asthma and that downregulation of hsa_circ_0002594 might partially account for the anti-inflammatory effect of ICS.

Materials and Methods

Subjects and Classification

156 subjects between 16 and 71 years in age were recruited from Tongji Hospital (Wuhan, China), including 102 asthmatic patients and 54 healthy subjects. None of the healthy subjects had a history of respiratory disease or evidence of bronchial hyperreactivity to methacholine challenge. All asthmatic patients had a doctor's diagnosis of symptomatic asthma and an accumulation dosage of methacholine provoking a 20% fall of forced expiratory volume in the first second (FEV_1, PD20) <2,505 mg or bronchodilator responsiveness (≥12% improvement in FEV_1% predicted and ≥200 mL improvement in FEV_1 following inhalation of 200 μg salbutamol) according to the Global Initiative for Asthma (GINA) guidelines. Among the 102 subjects with asthma, 83 had no course of any drug while 19 had a course of ICS at the first visit. The enrolled subjects were followed up for a second visit after treatment with ICS for 4 weeks. Written informed consent was obtained from all participants and all studies relevant to human subjects were approved by the Ethics Committee of Tongji Hospital, Huazhong University of Science and Technology. The composition of subjects included in the study was shown in online suppl. data 1 (for all online suppl. material, see www.karger.com/doi/10.1159/000511612).

$CD4^+$ T Cells Isolation and RNA Extraction

$CD4^+$ T cells were isolated using density centrifugation and magnetic beads as described in our previous study [15]. Briefly, the diluted peripheral blood was carefully layered onto a Ficoll-Hypaque gradient to obtain peripheral blood mononuclear cells. Then, $CD4^+$ T cells were isolated from peripheral blood mononuclear cells using a MagCellect Human $CD4^+$ T Cell Isolation Kit (R&D Systems, Minneapolis, MN, USA) according to the manufacturer's instructions. The isolated cells were frozen in TRIzol reagent (Takara, Dalian, China) at −80°C. The RNA purity and quantification were measured with a NanoDrop ND-2000 (Thermo, Waltham, MA, USA).

Microarray and Quantitative Analysis

$CD4^+$ T cells of 5 asthmatic patients and 5 healthy subjects were homogenized in TRIzol reagent. The sample preparation and microarray hybridization were performed based on the Arraystar's standard protocols. The microarray and quantitative analysis were

Table 1. Characteristics of subjects providing peripheral blood

	Asthmatic patients	Healthy subjects	Th2-high	Th2-low
Number	83	54	42	41
Sex (M/F)	29/54	23/31	16/26	13/28
Age, yr	42 (32, 54.5)	36 (28, 50)	43.5 (32.8, 54.3)	42 (31, 55)
BMI, kg/m^2	23.81±4.182	22.81±3.304	23.07±4.114	24.63±4.155
Lung function				
FEV_1, L	2.43±0.7257*	3.042±0.7752	2.38±0.7187	2.478±0.7383
FEV_1, % predicted	85.45±17.81*	97.1±9.575	81.40±17.11**	89.60±17.77
FVC, L	3.32 (2.97, 3.892)	3.47 (2.94, 4.408)	3.41 (3.02, 4.20)	3.26 (2.87, 3.767)
FVC, % predicted	103.4 (94.8, 113.7)	98.95 (93.28, 107.5)	103.0 (94.4, 111.8)	103.4 (94.58, 114.8)
FEV_1/FVC, %	68.8±12.6*	81.6±6.397	65.6±13.69**	72.05±10.59
Methacholine PD20, mg	0.29 (0.0676, 0.95)	na	0.12 (0.0351, 0.66)**	0.73 (0.2285, 1.70)
FeNO, ppb	44 (23, 103)	na	93 (54, 160)**	23 (19, 41)
Blood				
Eosinophil count, ×10^6/L	245 (92.5, 565)	na	500 (270, 710)**	100 (50, 195)
Eosinophil rate, *n* (%)	4.05 (1.3, 7.85)	na	7.50 (4.50, 11.6)**	1.3 (0.70, 2.25)
Neutrophil count, ×10^6/L	3,670 (2,863, 4,878)	na	3,260 (2,780, 4,020)**	4,490 (3,225, 5,530)
Neutrophil rate, *n* (%)	55.3 (50.6, 65.2)	na	51.6 (47.3, 58.9)**	61.2 (53.7, 70.55)
IgE, IU/mL	85.48 (33.98, 288.3)	na	248.4 (92.68, 475.7)**	43.35 (14.90, 84.18)
ACT	17.73±4.11	na	16.84±4.278	18.69±3.748
SPT (+/−)	27/22	na	23/6**	4/16
AR history (+/−)	35/42	0/54	23/17**	12/25
Family history (+/−)	24/53	na	13/27	11/26

Blood counts were missing in 7 asthmatic patients. Serum IgE measurements were missing in 1 asthmatic patients. SPT was missing in 34 asthmatic patients; AR history and family history were unknown in 6 asthmatic patients. We expressed as the mean±SD and used paired or unpaired *t* test for normally distributed data while we reported as medians with interquartile ranges and used nonparametric tests (Kruskal-Wallis test) for non-normally distributed data. Values are presented as mean±SD or median (interquartile range). FEV_1, forced expiratory volume in 1 s; FVC, forced vital capacity; na, not applicable; PD20, provocation dose resulting in 20% fall in baseline FEV_1; FENO, fraction exhaled nitric oxide; ACT, Asthma Control Test; SPT, skin prick test; AR, allergic rhinitis. * $p < 0.05$ compared asthmatic patients with healthy subjects. ** $p < 0.05$ compared Th2-high with Th2-low group.

described previously [15]. Quantile normalization and subsequent data processing were performed using the limma package [16]. The screening threshold was set to a fold change >2.0 or <2.0 with a value of $p < 0.05$.

Quantitative Reverse Transcription PCR (qRT-PCR)

The RNA was reverse transcribed into cDNA using Prime Script RT MasterMix (Takara, Dalian, China) following the previous reports [15]. qRT-PCR was performed to quantify circRNA levels using an ABI Prism 7900 Real-Time System (Applied Biosystems, Foster City, CA, USA) with SYBR Premix Ex Taq (Takara, Dalian, China) following the manufacturer's instructions. The PCR parameters were the same as before [15]: 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. The primers used in qRT-PCR are shown in online suppl. Table 2. β-Actin was used to normalize. The data were analyzed using the $2^{-\Delta\Delta Ct}$ method and reported as the relative variation (log_2 transformed).

Sanger Sequencing

To verify the specificity of the divergent primers, we amplified the cDNA around the junction site of hsa_circ_0002594 in A549, followed by Sanger sequencing. The PCR was carried out for 35 cycles: denaturation at 95°C for 30 s, annealing at 60°C for 30 s, and elongation at 72°C for 30 s. Sanger sequencing was performed by Geneseed Co. Ltd. (Guangzhou, China).

ICS Treatment

All subjects with asthma were placed on a standard ICS regimen (budesonide 200 μg BID) by their personal physicians. Asthmatic patients with no ICS history continued to use ICS throughout 4 weeks period from the first visit to the second. A questionnaire was conducted to collect information and clinical data for the second visit. Characteristics of asthmatic patients with ICS were listed in online suppl. Table 3.

Bioinformatics Analysis for hsa_circ_0002594

MiRNA response elements (MREs) of hsa_circ_0002594 were predicted by Arraystar's miRNA target prediction software based on TargetScan and miRanda. The screened candidate targets of hsa_circ_0002594 were the integration of 3 online algorithms (miRanda, TargetScan, and PicTar or DIANA-microT-CDS). The circRNA/miRNA/mRNA interaction network was illustrated using Cytoscape software.

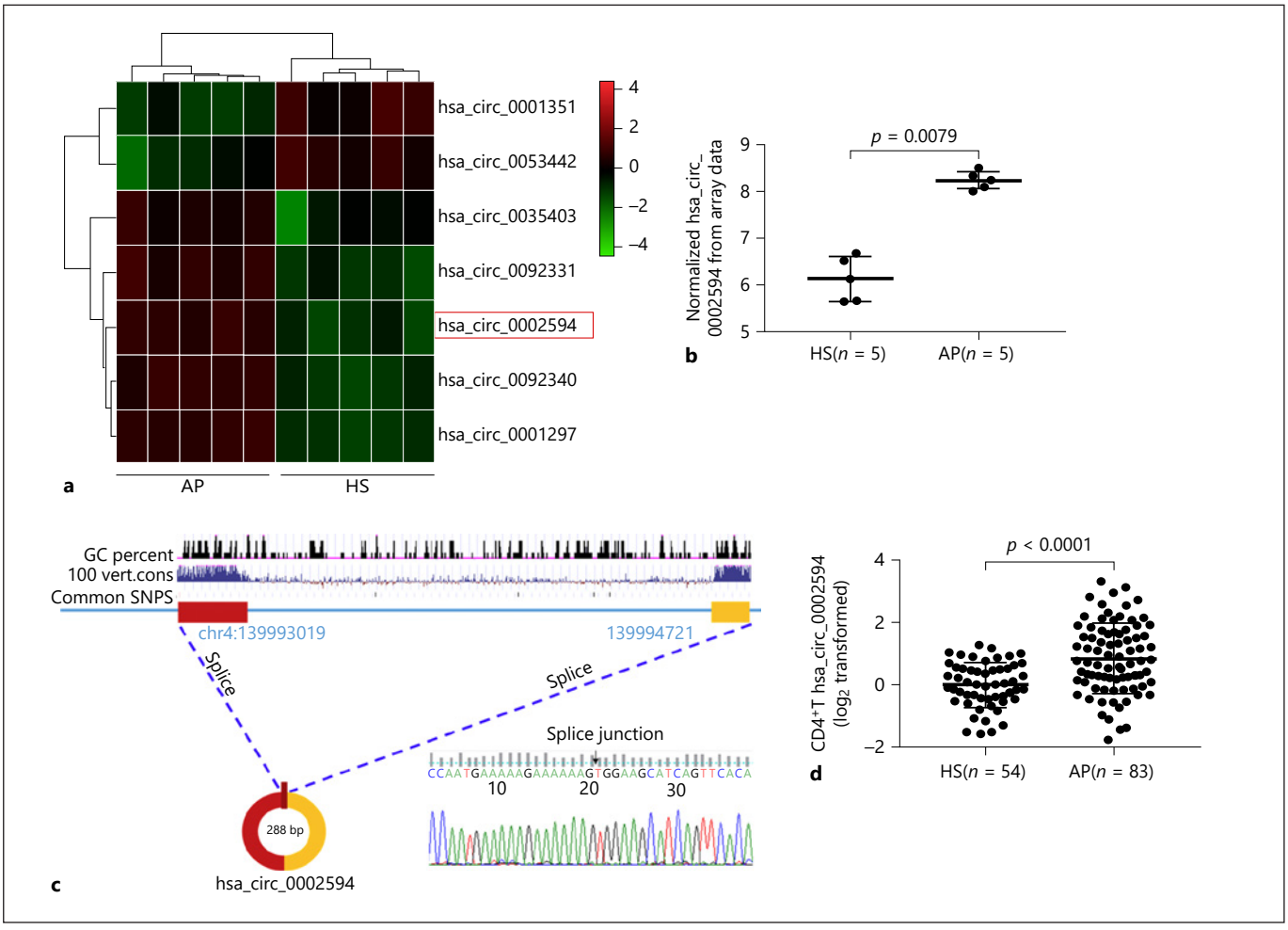

Fig. 1. Differential expression of circRNAs that could sponge hsa-let-7e-5p in CD4⁺ T cells from asthmatic patients. **a** Hierarchical clustering analysis of differentially expressed circRNAs that could sponge hsa-let-7e-5p between AP and HS; each group included 5 subjects (fold change >2; $p < 0.05$). Expression levels above and below the median expression level across all samples were represented in different colors. Pseudocolors indicate expression levels from low to high (green to red). **b** Hsa_circ_0002594 normalized expression levels were determined by the circRNA array in CD4⁺ T cells from AP ($n = 5$) and HS ($n = 5$). **c** Schematic illustration of the genomic location and splicing pattern of hsa_circ_0002594, which formed by head-to-tail splicing of 2 exons. Sanger sequencing depicted the splice junction. **d** The expression of hsa_circ_0002594 was analyzed in CD4⁺ T cells ($n = 83$ for AP; $n = 54$ for HS). Values were relative to the median value of healthy subjects and expressed as $\log_2$ transformed, normalized to β-actin. The values presented were the mean ± SD. p values were calculated by Kruskal-Wallis test. circRNAs, circular RNAs; HS, healthy subjects; AP, asthmatic patients without ICS.

Statistical Analysis

Statistical analyses were performed using SPSS 21.0 (IBM, SPSS, Chicago, IL, USA) and GraphPad Prism 7.0 (GraphPad, San Diego, CA, USA). We expressed as mean ± SD and used paired or unpaired *t* test for normally distributed data while we reported as medians with interquartile ranges and used nonparametric tests (Kruskal-Wallis test) for non-normally distributed data. Fisher's exact test was performed on analysis of categorical data and Spearman's rank-order correlation for correlation analysis. Receiver operating characteristic (ROC) was generated to determine the diagnostic value of hsa_circ_0002594. Differences were considered significant at a p value of <0.05.

Results

Overview of Asthmatic Patients without ICS and Healthy Subjects

A questionnaire was conducted to collect information and clinical data for 83 patients with asthma and 54 healthy subjects. Participants were excluded if they had a respiratory tract infection in the previous 4 weeks. Clinical data for asthmatic patients without ICS were shown in Table 1. There were no significant differences across asthmatic pa-

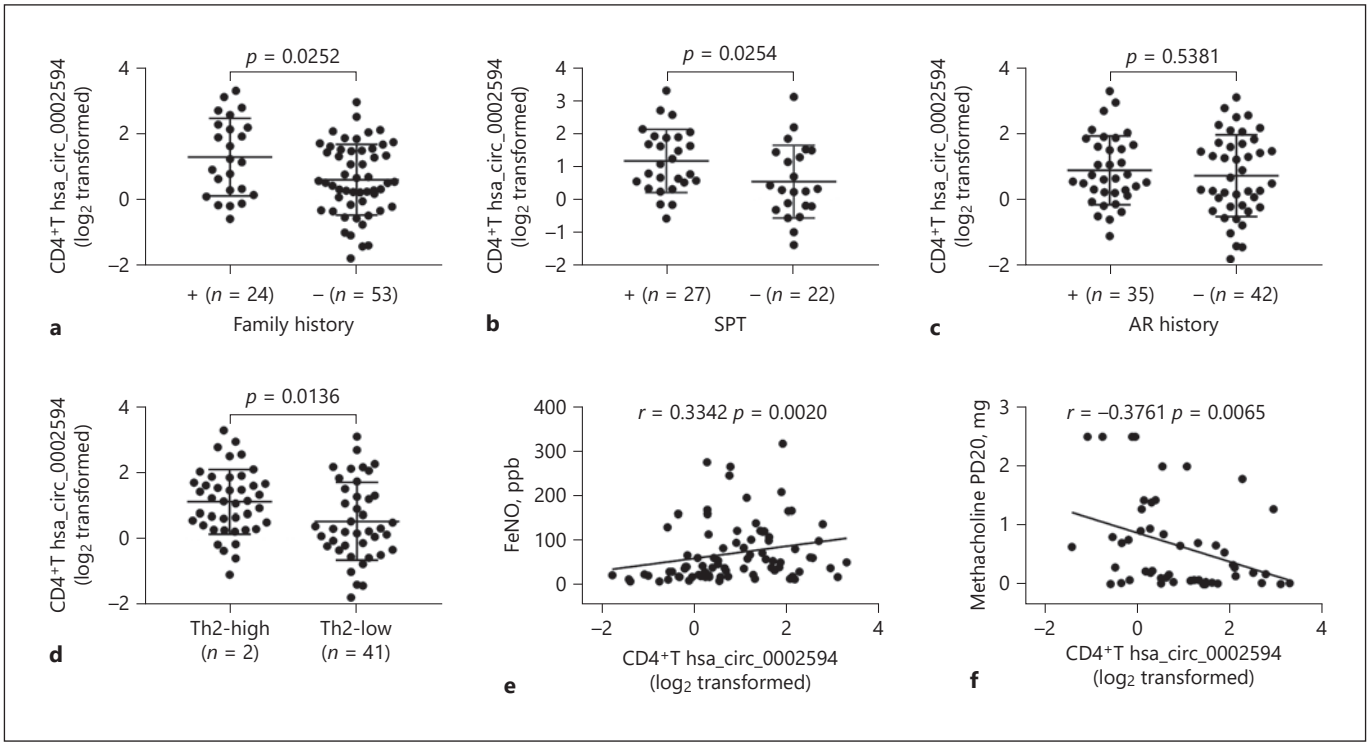

Fig. 2. Hsa_circ_0002594 expression in different subgroups and its contribution to Th2 immune activation of asthma. Hsa_circ_0002594 expressions in patients with or without a family history (**a**), patients with a positive or negative SPT (**b**), patients with or without AR (**c**), and patients with Th2-high or Th2-low (**d**). **e** Correlation assay between hsa_circ_0002594 levels and FeNO in asthmatic patients (n = 83). **f** Correlation assay between hsa_circ_0002594 levels and PD20 in asthmatic patients (n = 51). We used nonparametric tests (Kruskal-Wallis test) for non-normally distributed data. Fisher's exact test was performed on analysis of categorical data and Spearman's rank-order correlation for correlation analysis. SPT, skin pricking test; AR, allergic rhinitis; Th, T helper.

tients and healthy subjects in terms of sex distributions, age, and BMI. Eighty-three subjects were classified as Th2-high or Th2-low asthma based on biomarkers reflecting Th2 immune activation, including blood eosinophil count, IgE level, and fraction of exhaled nitric oxide (FeNO) concentration. Th2-high indicated an elevation in 2 or more Th2 biomarkers while Th2-low indicated an elevation in 1 or no Th2 biomarker. For each biomarker, cut points used to define a high level were as follows: eosinophil count ≥300/μL or eosinophil rate ≥3%, IgE ≥100 IU/mL, and FeNO ≥50 parts per billion [17, 18].

Expression of hsa_circ_0002594 in CD4⁺ T Cells of Asthmatic Patients

To identify the roles of circRNAs that could act as an endogenous hsa-let-7e-5p sponge in the pathogenesis of asthma, we found 7 differentially expressed circRNAs in our circRNA array of CD4⁺ T cell samples from 5 asthmatic patients and 5 healthy controls as mentioned in our previous study [15]. The 5 up- and 2 downregulated circRNAs were listed in online suppl. 4. The expression profiles of 7 selected circRNAs were shown by hierarchical clustering in Figure 1a, and our array data revealed that hsa_circ_0002594 (fold change ≥4.3) was one of the most upregulated circRNAs in CD4⁺ T cells of asthmatic patients (Fig. 1b). The cDNA around the junction site of hsa_circ_0002594 was detected by Sanger sequencing and the results were consistent with the circBase data (Fig. 1c). Another cohort study including 83 asthmatic patients and 54 healthy subjects further verified the results of the circRNA array that hsa_circ_0002594 levels were markedly increased in CD4⁺ T cells from asthmatics (Fig. 1d).

Hsa_circ_0002594 Contributed to Clinical Characteristics Especially Th2 Immune Activation of Asthma

We further analyzed the correlations between hsa_circ_0002594 expression and various clinical parameters

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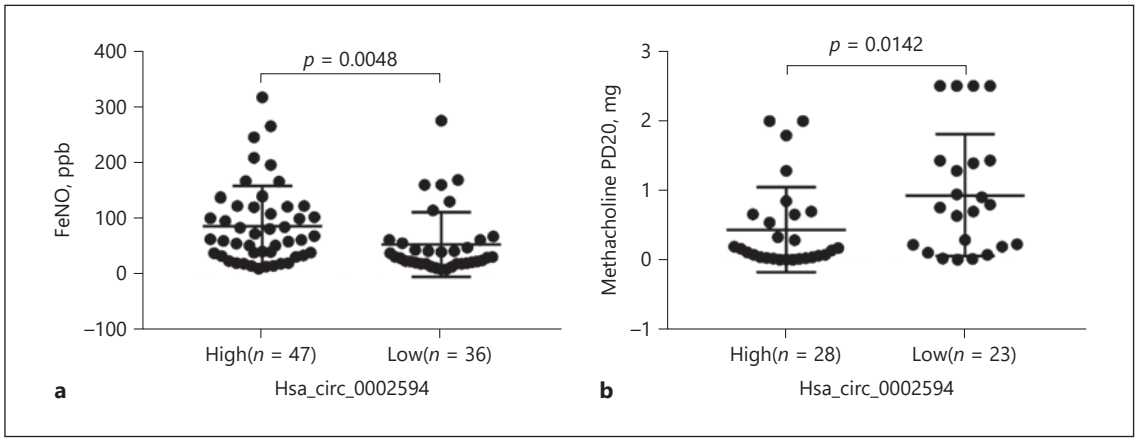


Fig. 3. Hsa_circ_0002594-high asthma was characterized by clinical indicators of allergic asthma. Hsa_circ_0002594-high subgroup seemed to be characterized by a higher FeNO (**a**) and a lower PD20 (**b**). *p* values were calculated by Kruskal-Wallis test.

Table 2. Th2-high and Th2-low and SPT frequencies in asthmatic patients with high or low expressions of hsa_circ_0002594

	Hsa_circ_0002594		OR (95% CI)	*p* value
	high	low		
Th2				
High	30	12	3.5 (1.42–8.80)	0.0079
Low	17	24		
SPT				
Positive	20	9	3.6 (1.09–11.86)	0.0234
Negative	7	13		

See Table 1 legend for expansion of abbreviations. $p < 0.05$ was considered statistically significant. Fisher's exact test was performed on analysis of categorical data. OR, odds ratio; CI, confidence interval; Th, T helper; SPT, skin prick test.

of asthma. In 83 asthmatic patients without ICS, hsa_circ_0002594 expression was found higher in patients with a family history or a skin pricking test (SPT)-positive group than those with no family history or a SPT-negative group (Fig. 2a, b). Allergic asthma was a Th2-dominated disease, with Th2 cells being prevalent in AR, a common comorbidity with asthma [13, 19]. However, hsa_circ_0002594 levels were comparable in the asthmatics with or without AR (Fig. 2c). Interestingly, when we divided asthmatic patients into Th2-high and Th2-low subgroups, Th2-high revealed higher expression of hsa_circ_0002594 (Fig. 2d). There was also a positive correlation between hsa_circ_0002594 levels and FeNO, a parameter of Th2 immune activation and a good predictor of corticosteroid response [17, 20] (Fig. 2e). We also observed that the expression of hsa_circ_0002594 was negatively correlated with methacholine PD20 in 51 asthmatic patients (Fig. 2f). Collectively, these findings suggested that elevated hsa_circ_0002594 levels might be correlated with Th2-mediated allergic asthma.

Hsa_circ_0002594-High Asthma Is Characterized by Multiple Clinical Indicators of Th2-Mediated Allergic Asthma

When we used the lower limit of the upper quartile of hsa_circ_0002594 expression level ($\log_2$ transformed = 0.53143125) of the healthy controls as the cutoff point, the asthmatic patients were divided into hsa_circ_0002594-low and hsa_circ_0002594-high subgroups. Compared with the hsa_circ_0002594-low group, the hsa_circ_0002594-high group had higher FeNO and lower PD20 (Fig. 3a, b). Moreover, in the hsa_circ_0002594-high subgroup, the frequencies of Th2-high and SPT-positive were both higher, 3.5 and 3.6 times more likely than non-Th2 inflammation and SPT-negative, respectively (Table 2). Correlations between hsa_circ_0002594-high asthma and clinical characteristics were listed in Table 3. To sum up, hsa_circ_0002594-high asthma was characterized by multiple clinical indicators of Th2-mediated allergic asthma.

Diagnostic Value and Therapeutic Response of hsa_circ_0002594

To identify the diagnostic value of hsa_circ_0002594, 83 asthmatic patients without ICS and 54 healthy subjects were analyzed to construct a ROC curve, with an area un-

Table 3. Comparison of patients in the clinical trials used to define hsa_circ_0002594-high and hsa_circ_0002594-low subgroups

	Asthmatic patients	Hsa_circ_0002594-high	Hsa_circ_0002594-low	*p* value
Number	83	47	36	
Sex (M/F)	29/54	16/31	13/23	1.0000
Age, yr	42 (32, 54.5)	42 (34, 54)	43 (29,57)	0.7063
BMI, kg/m^2	23.81±4.182	23.14±3.838	24.71±4.509	0.0985
Lung function				
FEV_1, % predicted	85.45±17.81	82.81±17.41	88.88±18.21	0.1246
FVC, % predicted	103.4 (94.8, 113.7)	101.9 (94.8, 109.5)	104.1 (94.52, 116.9)	0.2662
FEV_1/FVC, %	68.8±12.6	67.27±12.74	70.81±12.31	0.2067
Methacholine PD20, mg	0.29 (0.0676, 0.95)	0.1572 (0.03785, 0.663)	0.7611 (0.2, 1.43)	0.0142
FeNO, ppb	44 (23, 103)	63 (34, 122)	30.5 (19.25, 60.5)	0.0048
Blood				
Eosinophil count, ×10^6/L	245 (92.5, 545)	270 (110, 600)	220 (80, 405)	0.2009
Eosinophil rate, *n* (%)	4.05 (1.3, 7.85)	4.5 (1.7, 8.8)	3.1 (0.95, 5.9)	0.0915
Neutrophil count, ×10^6/L	3,670 (2,863, 4,878)	3,660 (2,820, 4,600)	3,810 (3,015, 5,415)	0.3989
Neutrophil rate, *n* (%)	55.3 (50.6, 65.2)	58.7 (50.9, 64.9)	53.8 (50.25, 69.3)	0.8299
IgE, IU/mL	85.48 (33.98,288.3)	118.9 (40.67, 330.1)	56.26 (20.54, 249.2)	0.1488
Th2 (high/low)	42/41	30/17	12/24	0.0079
ACT	17.73±4.11	17.44±4.595	18.13±3.329	0.4833
SPT (+/−)	27/22	20/9	7/13	0.0234
AR history (+/−)	35/42	21/21	14/21	0.4912
Family history (+/−)	24/53	16/26	8/27	0.2169

See Table 1 legend for expansion of abbreviations. *p* for comparison between hsa_circ_0002594-high and hsa_circ_0002594-low subgroups. $p < 0.05$ was considered statistically significant. *p* values were calculated by unpaired 2-sided Student's *t* test, Kruskal-Wallis test, or Fisher's exact test, as appropriate. Blood counts were missing in 4 hsa_circ_0002594-high and 3 hsa_circ_0002594-low subgroups. Serum IgE measurements were missing in 1 hsa_circ_0002594-low subgroup. SPT was missing in 19 hsa_circ_0002594-high and 15 hsa_circ_0002594-low subgroups. AR history and family history were unknown in 5 hsa_circ_0002594-high and 1 hsa_circ_0002594-low subgroups. FEV_1, FVC, forced vital capacity; PD20, provocation dose resulting in 20% fall in baseline FEV_1; FENO, fraction exhaled nitric oxide; Th, T helper; ACT, Asthma Control Test; SPT, skin prick test; AR, allergic rhinitis.

der the curve (AUC) value of 0.7278 ($p < 0.0001$, Fig. 4a). To explore the therapeutic response of hsa_circ_0002594, the levels of hsa_circ_0002594 in $CD4^+$ T cells originated from subjects with asthma after 4 weeks of ICS treatment were determined. Interestingly, hsa_circ_0002594 levels were obviously decreased when compared with their first visit ($n = 29$, Fig. 4b). The same results were observed in asthmatic subjects with ICS ($n = 48$) including another 19 subjects with a course of ICS when compared with 83 subjects without ICS at their first visit (Fig. 4c). When examining the diagnostic value of hsa_circ_0002594 in the presence of ICS, an AUC value of 0.7671 ($p < 0.0001$, Fig. 4d) was obtained.

Construction of circRNA-miRNAs-mRNAs Network

Importantly, circRNAs have been demonstrated to act as miRNA sponges to regulate gene expression. They could naturally function as competing endogenous RNA by competing with mRNAs for the same MREs [14, 21]. To further explore the functions of hsa_circ_0002594, we constructed the hsa_circ_0002594-miRNAs-mRNAs network based on bioinformatics and the mRNA profile published by our group previously [22]. First, the potential MREs of hsa_circ_0002594 predicted by Arraystar's miRNA target prediction software were hsa-let-7e-5p, hsa-miR-16-5p, hsa-miR-503-5p, hsa-miR-514a-3p, and hsa-miR-587 (Fig. 5a). Then, 222 screened candidate targets of miRNAs were the merged common targets of 3 online algorithms and mRNA profile (online suppl. 5). Finally, the entire network was illustrated using Cytoscape software (Fig. 5b).

Discussion

CircRNAs acting as miRNA sponges played a critical role in the occurrence and development of many diseases associated with abnormal immune response including systemic lupus erythematous, rheumatoid arthritis,

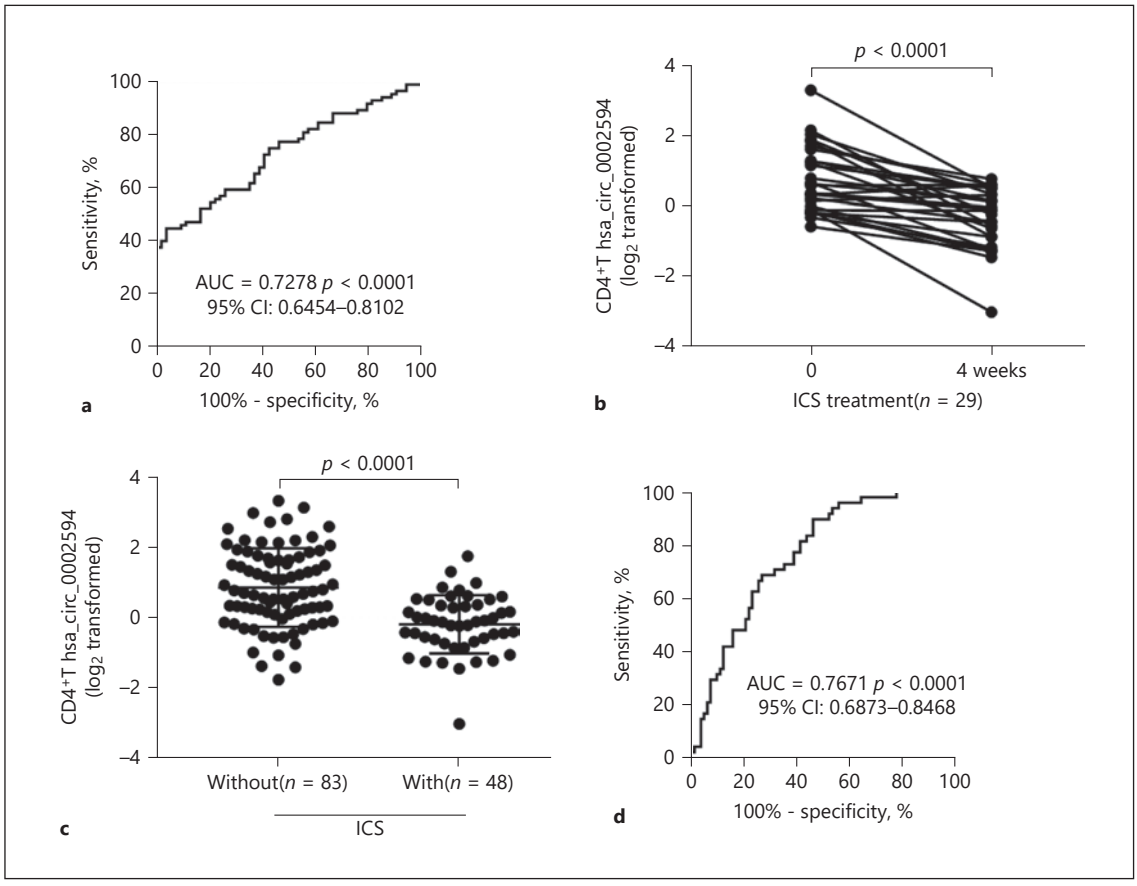
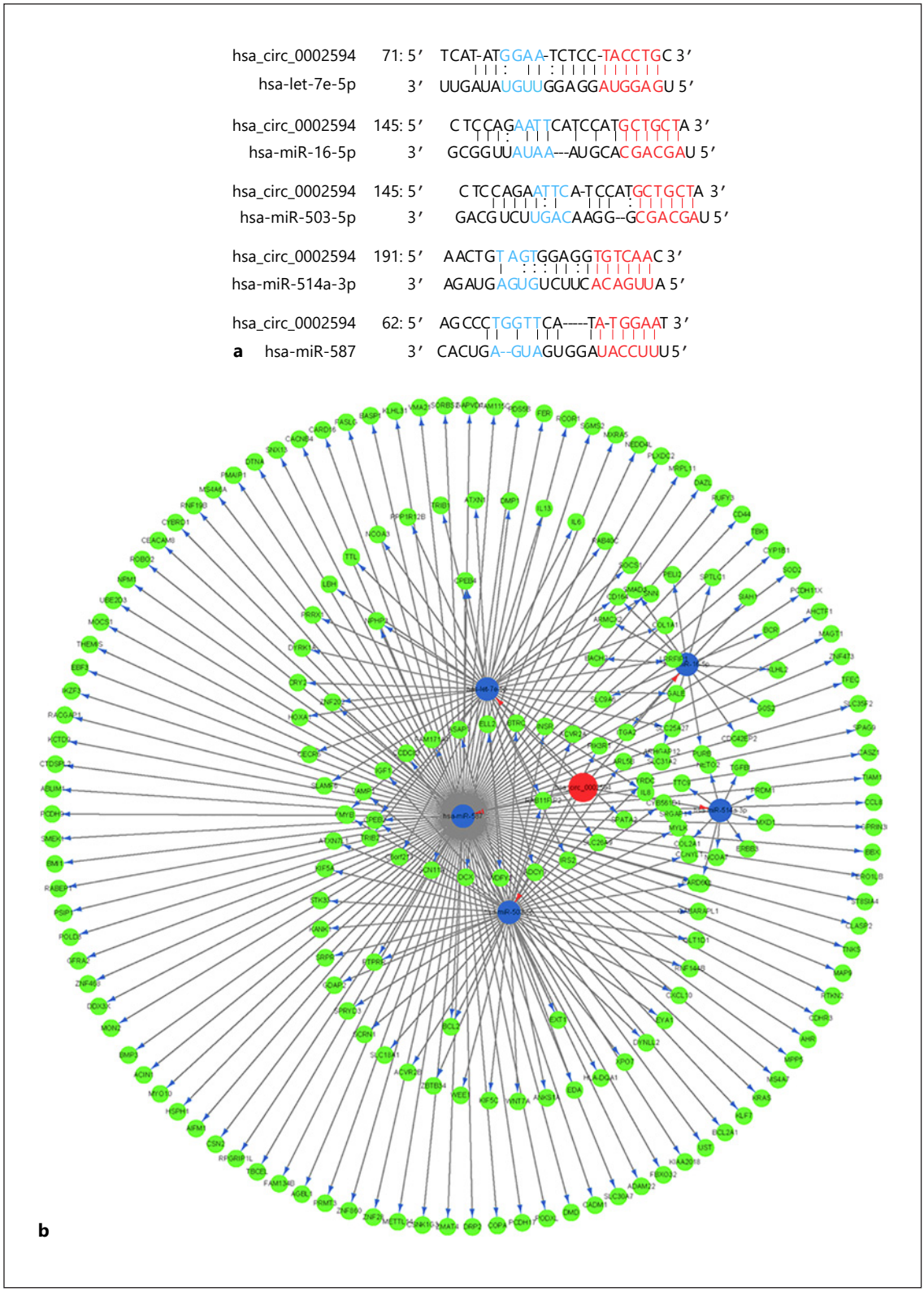


Fig. 4. Diagnostic and therapeutic value of hsa_circ_0002594 for Th2-mediated allergic asthma. **a** ROC curve analyses of hsa_circ_0002594 in 83 asthmatic patients without ICS. **b** Hsa_circ_0002594 levels were decreased after 4 weeks of ICS treatment ($n = 29$). p values were calculated by paired t test. **c** Hsa_circ_0002594 levels were lower in asthmatic subjects with ICS ($n = 48$). p values were calculated by Kruskal-Wallis test. **d** ROC curve analyses of hsa_circ_0002594 in the presence of ICS ($n = 48$). ROC, receiver operating characteristic; AUC, area under the curve; ICS, inhaled corticosteroids.

Crohn disease, and diabetes [23–26]. In our previous study, we demonstrated circRNAs profiles and hsa_circ_0005519 regulated IL-13/IL-6 by sponging hsa-let-7a-5p in $CD4^+$ T cells from asthma [15]. But the expression profiles and functions of circRNAs in asthma were still scarce. In the present study, we found 7 differentially expressed circRNAs that might act as an endogenous hsa-let-7e-5p sponge in our circRNA array [15], among which hsa_circ_0002594 was upregulated in another cohort $CD4^+$ T cells of allergic asthma for further verification.

Current studies have found that the pathogenesis of asthma was closely related to the imbalance of T cells especially the helper T cells subgroups (Th1, Th2, Th9, Th17, Treg, etc.), and Th2 played a crucial role in asthma allergic airway inflammation [27–31]. In previous study, Guan et al. [32] demonstrated that let-7e played a critical role in the differentiation of Th1, Th2, and Th17 cells and negatively regulated Th2 polarization as well as IL-10 production. In addition, let-7e was downregulated in AR with asthma and emphasized the important role of let-7e in Th2-mediated allergic inflammation [12, 13]. Given the roles of circRNAs as miRNA "sponges" to regulate gene expression [14], we conceived that some circRNAs could sponge let-7e to participate in Th2-mediated allergic asthma.

In this study, we found 7 differentially expressed circRNAs that could act as an endogenous hsa-let-7e-5p in our circRNA array, and hsa_circ_0002594 that exhibited the most significant change in the 7 circRNAs was selected for follow-up study. Subsequent qRT- PCR validation suggested that hsa_circ_0002594 was upregulated in $CD4^+$ T cells of asthma, matching with the microarray results. We also demonstrated that hsa_circ_0002594 ex-



Color version available online

Fig. 5. Bioinformatics analysis of hsa_circ_0002594. **a** Detailed annotation for the hsa_circ_0002594/miRNAs interaction. **b** The hsa_circ_0002594-miRNAs-mRNAs network. Red nodes: hsa_circ_0002594; blue nodes: miRNAs; green nodes: mRNAs. miRNAs, microRNA.

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pression was higher in subjects with a family history, SPT-positive, or Th2-high. Hsa_circ_0002594 levels were positively correlated with FeNO and negatively correlated with methacholine PD20. Hsa_circ_0002594-high asthma was characterized by multiple clinical indicators of allergic asthma such as FeNO, Th2-high phenotype, PD20, etc. These results indicated that hsa_circ_0002594 might be involved in Th2-mediated allergic asthma. Furthermore, hsa_circ_0002594 was decreased after ICS treatment. Moreover, hsa_circ_0002594 showed high ROC AUC values with or without ICS respectively, indicating its high potential diagnostic and therapeutic value for asthma. In conclusion, hsa_circ_0002594 might act as a promising diagnostic or therapeutic biomarker for Th2-mediated allergic asthma. In addition, to further explore the possible molecular mechanism of hsa_circ_0002594 in asthma, we identified that hsa_circ_0002594 might target hsa-let-7e-5p, hsa-miR-16-5p, hsa-miR-503-5p, hsa-miR-514a-3p, and hsa-miR-587 based on bioinformatics and thus competitively sequester miRNA activity.

Our study had some limitations. First, considering the limitations of our sample size, more samples from different regions were needed to confirm our results. Second, we hypothesized the possible mechanism by which hsa_circ_0002594 was involved in allergic asthma mainly based on bioinformatics analysis, but no in vitro studies were conducted. Third, circulating hsa_circ_0002594 was not detected to assess its potential as a noninvasive biomarker for the diagnosis and therapy of allergic asthma.

In conclusion, hsa_circ_0002594 was upregulated in $CD4^+$ T cells of allergic asthma. Our findings might enhance the knowledge regarding the role of circRNAs in asthma since there were few studies in this field. In addition, hsa_circ_0002594 might have value in the diagnosis and treatment of Th2-mediated allergic asthma. These observations should have potential clinical significance, and might provide new insight into the molecular mechanisms of allergic asthma, all of which merit further investigation.

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Statement of Ethics

The Ethics Committee of Tongji Hospital, Tongji Medical College, Huazhong University of Science and Technology approved this study (IRB ID: 20150503). All procedures performed in the study involving human participants were in accordance with the ethical standards of the institutional research committee.

Conflict of Interest Statement

The authors have no conflicts of interest to declare.

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Author Contributions

H.Z. recruited patients; collected, analyzed, and interpreted the data; and wrote the draft. F.B., Q.X., X.Y., M.Y., and W.G. recruited patients and collected data. W.Y., Z.M., and C.Y. analyzed and interpreted the data, and critically revised the manuscript. W.Y., Z.J., and X.W. conceived and designed the study, recruited patients, and drafted the manuscript for important intellectual content. X.W., X.J., and Z.J. provided overall supervision and critically revised the manuscript. All authors read and approved the final manuscript.

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