CLINICAL RESEARCH ARTICLE

Phenotypes of the inflammatory cells in the induced sputum from young children or infants with recurrent wheezing

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BACKGROUND: To identify inflammatory cell types by phenotypic analysis of the inflammatory cells in the induced sputum.
METHODS: This retrospective study included 1232 children and infants, who were assigned into mild/moderate groups (326) and severe group (602) by clinical symptom scores. Phenotypes of sputum inflammatory cells were analyzed using liquid-based thin-cytologic test and eosinophil-derived neurotoxin (EDN) was quantified by ELISA.
RESULTS: Blood eosinophil count, serum total IgE level, and allergen detection rate were significantly higher in the severe group. In the 905 cases of qualified sputum, 526 cases exhibited at least one type of inflammatory cells, including neutrophil (343, 65.2%), eosinophil (161, 30.6%), and mixed granulocytes (22, 4.2%). Levels of neutrophils and eosinophils were significantly higher in the severe group than mild/moderate group, and eosinophil was predominant in the severe group. Serum EDN was 104.8 ± 39.4 µg/l in the eosinophil phenotype group, 112.6 ± 41.2 µg/l in the mixed group, 88.2 ± 36.6 µg/l in the neutrophil phenotype group, and 60.9 ± 34.6 µg/l in the paucigranulocytic phenotype group.
CONCLUSION: Induced-sputum inflammatory cell count may be used to determine phenotype of wheezing. The criteria of classifying adult asthma could be applicable for children and infants.

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INTRODUCTION

Wheezing in young children represents a common disorder characterized by airway obstruction.[1] Recurrent wheezing has a significant morbidity in preschool-age children.[2] The most frequent causes of wheezing in preschool children are bronchiolitis and asthma,[3] both of which cause narrowing and spasms (bronchospasms) in the small airways.[4]

Respiratory virus infection has been demonstrated to be closely associated with wheezing and asthma in children.[5–7] Due to the complex mechanism of viral infection and asthma-induced airway inflammation, it is difficult for clinicians to predict the prognosis of the recurrence wheezing. Phenotypes of the inflammatory cells have shown a clinical significance in the diagnosis and early intervention of asthma and wheezing in children/infants.

Clinical treatments, based on the two cell types (eosinophil and non-eosinophil), have accomplished great achievements in adults.[8–10] However, due to the complex mechanisms of asthma in young children/infants, clinical treatment in asthmatic children is distinct from that in adult. It has been revealed that monotherapy may not achieve clinical success because of complexity of asthma with recurrent respiratory tract infections (RRTI) in children.[11] Thus, determination of clinical phenotype of asthma and wheezing in children/infants may provide potential therapeutic targets and personalized treatments.[12]

Recently, the sputum inflammatory cell types have been well studied. Based on the proportion of sputum eosinophil and neutrophil, airway inflammation in asthma could be categorized into four inflammatory subtypes: eosinophilic asthma, neutrophilic asthma, mixed granulocytic asthma, and paucigranulocytic asthma.[13] This classification is helpful to determine therapeutic strategies for asthma in children and infants. Therefore, we hypothesized that children with recurrent wheezing may exhibit airway inflammation and the inflammatory cell types, which is similar to that in asthmatic airway inflammation. To test this hypothesis, we employed a liquid-based thin-cytologic method combined with EDN ELISA and spirometer to confirm the inflammatory cell types in induced sputum from young children/infants with recurrent wheezing.

MATERIALS AND METHODS

Study design

A total of 1232 young children or infants (age from 6 months to 3 years old) with recurrent wheezing were enrolled into this study at the Tianjin Children's Hospital between January 2009 and December 2013. All children were followed up for 2 years. Inclusive criteria were: (1) less than 3 years old; (2) more than 4 times of recurrent wheezing; and (3) have not received a long-term intervention therapy. The patients with congenital heart disease, congenital lung disorders, and/or immune disorders were excluded from this study. The participants were assigned into three groups (mild, moderate, and severe) according to the clinical symptoms (Table 1). A written informed consent was obtained from all participants' parents or legal guardians after they had completely understood the details of the study.

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Table 1. The scoring system of clinical symptoms

	1	2	3
Wheezing	Mild	Moderate	Severe
Cough	Occasionally	Regularly	Constantly
Exercise-induced wheezing and cough	Can run for short distance, can clime 3 stairs, ingest normally	Can walk, cannot constantly ingest	Cannot walk, cannot ingest
Sleep	Sleeping well, occasional wheezing or cough	Waking up 2–3 times with wheezing or cough	Hardly fall asleep, constantly wheezing or cough

Sputum induction
Sputum was induced by nebulizing 0.9% normal saline followed by inserting a thin nasal tube into deep airway. The procedure was conducted by an experienced nurse. If the infants or child had wheezing or asthmatic attack, this procedure was avoided.

Quantification of serum total IgE, inhaled allergen IgE, food allergen IgG, blood eosinophil, and neutrophil
Blood samples (3 ml) were collected on the admission day and serum was stored at $-20\,°C$ until used. Serum total IgE concentrations were determined by fluorescent enzyme immunoassay kit following the manufacturer's instruction (Mediwiss Analytic, Germany). IgE and IgG antibodies against a panel of relevant inhaled allergens and food allergens were analyzed, respectively. Eosinophil and neutrophil were counted using an automatic blood cell counter (DxH500 Hematology Analyzer, Beckman Coulter, CA).

Slide preparation and cell count
Induced sputum was mixed well with 2 times concentrated tissue preservative liquid containing 0.01% Dithiothreitol. The mixed solution was further diluted with 4 ml PBS followed by centrifugation at 1500 rpm for 5 min. Supernatant was discarded and pellet was re-suspended in 1 ml PBS. Solution was then transferred into TCT slide and centrifuged at 800 rpm for 3 min. Slides were stained with H&E.

Quantification of EDN by ELISA
Serum EDN was quantified using an ELISA kit following the manufacturer's instruction (MBL Life Science). Briefly, 50 µl serum was mixed with 200 µl sample dilution buffer followed by adding 100 µl into each well of pre-coated 96-well ELISA plate, and incubated for 1h at room temperature. After washing, 100 µl per well of HRP-conjugated antibody was added and incubated at room temperature for one hour. After washing, color was developed by adding 100 µl substrate and optimal density value was obtained at 450 nm using an ELISA reader. Minimal detection of the kit was 0.62 ng/ml.

Statistical analysis
Statistical analyses were performed using SPSS16.0. Mean values were compared using one-way ANOVA followed by Bonferroni corrections among three or more groups. The significance of categorical variables was examined by χ^2 tests. All data were presented as mean $\pm$ SEM. P value <0.05 was considered as significant.

RESULTS
Clinical characteristics of the children
As shown in Table 2, average age of the children was 11.78 ± 4.24 months, 826 males and 406 females with a male/female ratio of 1.1:1. Majority of the children (942) were living at urban area. Allergic rhinitis was present in 20.0% of the children and 8.3% of the direct relatives of the participants. Seasonal wheezing and atopic dermatitis were present in 3.2% and 39.9% of the children, respectively. Exercise-induced wheezing and cold-induced

Table 2. Clinical characteristics of the children, N (%)

	Mild	Moderate	Severe	N	P
Gender					
Male	630 (67.45)	117 (62.23)	79 (71.82)	826	0.205
Female	304 (32.55)	71 (37.77)	31 (28.18)	406	
Location					
Urban	717 (76.77)	144 (76.60)	81 (73.64)	942	0.764
Rural	217 (23.23)	44 (23.40)	29 (26.36)	290	
Allergic rhinitis					
No	859 (91.97)	78 (41.49)	48 (43.64)	985	<0.001
Yes	75 (8.03)	110 (58.51)	62 (56.36)	247	
Family history					
No	853 (91.33)	173 (92.02)	102 (92.73)	1128	0.856
Yes	81 (8.67)	15 (7.98)	8 (7.27)	104	
Seasonal wheezing					
No	915 (97.97)	176 (93.62)	102 (92.73)	1193	<0.001
Yes	19 (2.03)	12 (6.38)	8 (7.27)	39	
Atopic dermatitis					
No	565 (60.49)	110 (58.51)	66 (60.00)	741	0.879
Yes	369 (39.51)	78 (41.49)	44 (40.00)	491	
Wheezing after exercise					
No	913 (97.75)	180 (95.74)	104 (94.55)	1197	0.072
Yes	21 (2.25)	8 (4.26)	6 (5.45)	35	
Allergen					
Negative	723 (77.49)	105 (55.85)	47 (42.73)	875	<0.001
Positive	210 (22.51)	83 (44.15)	63 (57.27)	356	
Serum IgE					
Increased	36 (3.85)	18 (9.57)	5 (4.55)	59	0.004
Normal	898 (96.15)	170 (90.43)	105 (95.45)	1173	
IgE to inhaling allergen					
Negative	672 (87.73)	123 (79.35)	54 (64.29)	849	<0.001
Positive	94 (12.27)	32 (20.65)	30 (35.71)	156	
IgE to food allergen					
Negative	616 (80.42)	86 (55.48)	33 (39.29)	735	<0.001
Positive	150 (19.58)	69 (44.52)	51 (60.71)	270	
Cells in induced sputum					
Negative	925 (99.04)	185 (98.40)	109 (99.09)	1219	0.699
Positive	9 (0.96)	3 (1.60)	1 (0.91)	13	
Sputum cell types					
Paucigranulocyte	119 (12.74)	12 (6.38)	9 (8.18)	140	<0.001
Mixed granulocyte	154 (16.49)	61 (32.45)	54 (49.09)	269	
Eosinophil	136 (14.56)	68 (36.17)	41 (37.27)	245	
Neutrophil	525 (56.21)	47 (25.00)	6 (5.45)	578	

wheezing (longer than 3 weeks) were present in 2.8% and 91.5% of the children, respectively. In addition, 64.7% of the children had 2 or higher clinical score determined by clinical scoring system as shown in Table 1.

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Table 3. Inflammatory cell levels in young children or infants with recurrent wheezing

Variables	Group	N	Mean±SD	Median (Q1, Q3)	Range (Min, Max)	Analysis method	Statistics	P
Age (month)	Mild	934	11.21 ± 5.71	11 (7,15)	36 (0, 36)	Kruskal–Wallis test	3.37	0.185
	Moderate	188	11.99 ± 5.97	12 (8,16)	35 (1, 36)			
	Severe	110	11.7 ± 6.06	12 (7,17)	25 (0, 25)			
Blood eosinophil	Mild	934	0.96 ± 1.95	0 (0,1)	11 (0, 11)	Kruskal–Wallis test	3.852	0.146
	Moderate	188	1.18 ± 1.93	0 (0,2)	9 (0, 9)			
	Severe	110	1.01 ± 1.94	0 (0,2)	14 (0, 14)			
Blood Neutrophil	Mild	934	64.13 ± 16.94	67.5 (53,77)	92 (2.5, 94.5)	Kruskal–Wallis test	26.926	<0.001
	Moderate	188	59.26 ± 18.5	59.75 (50,75)	82.5 (5.5, 88)			
	Severe	110	57.25 ± 16.6	60.25 (48,69)	76.5 (9.5, 86)			
Sputum eosinophil	Mild	934	2.01 ± 2.04	1.5 (0.5,3)	12 (0, 12)	Kruskal–Wallis test	179.812	<0.001
	Moderate	188	3.65 ± 2.43	3.5 (2,5)	10 (0, 10)			
	Severe	110	4.76 ± 2.49	4.5 (3,6)	12 (0, 12)			
Sputum macrophage	Mild	934	2.73 ± 6.53	0 (0,0)	53 (0, 53)	Kruskal–Wallis test	2.116	0.347
	Moderate	188	2.97 ± 6.53	0 (0,0)	52 (0, 52)			
	Severe	110	3.57 ± 8.22	0 (0,0)	50 (0, 50)			
Sputum epithelial cell	Mild	934	12.79 ± 7.16	12 (7,18)	36 (0, 36)	Kruskal–Wallis test	9.204	0.01
	Moderate	188	13.04 ± 7.12	13 (7.5,18)	32 (0, 32)			
	Severe	110	14.78 ± 6.99	16 (11,19)	32 (0, 32)			
Sputum lymphocyte	Mild	934	18.31 ± 13.87	14 (9,23)	83 (1, 84)	Kruskal–Wallis test	0.707	0.702
	Moderate	188	21.01 ± 17.28	15 (8,28.5)	74 (2, 76)			
	Severe	110	19.59 ± 15.61	14 (9,23)	67 (3, 70)			
Sputum other cell	Mild	934	0.03 ± 0.39	0 (0,0)	7 (0, 7)	Kruskal–Wallis test	0.63	0.730
	Moderate	188	0.06 ± 0.53	0 (0,0)	5 (0, 5)			
	Severe	110	0.05 ± 0.48	0 (0,0)	5 (0, 5)			
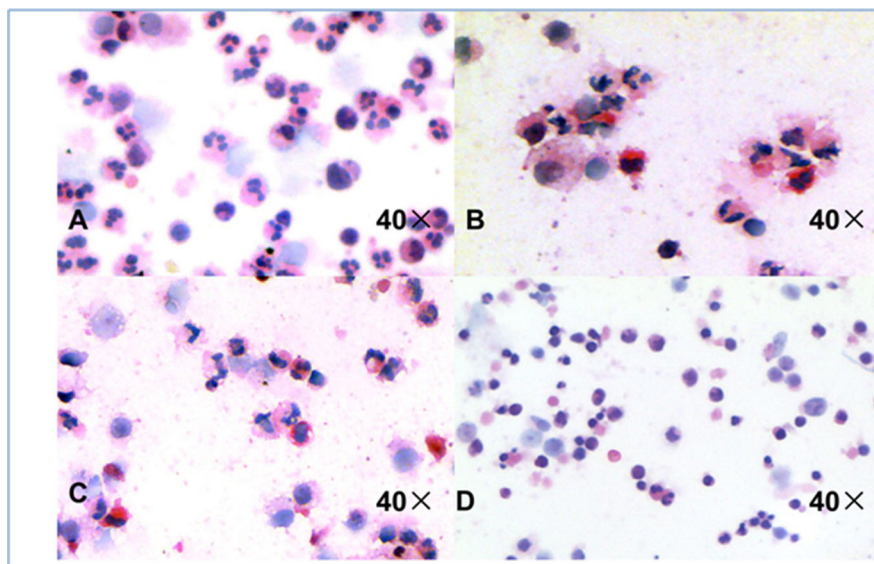
A. Neutrophil; B: Eosinophil; C: Mixed; D: Paucigranulocytic type

Fig. 1 Morphology of induced sputum inflammatory cells. **a** Eosinophilic type of induced sputum; **b** neutrophilic type of induced sputum; **c** mixed granulocytic type of induced sputum; **d** paucigranulocytic type of induced sputum. Magnification: ×100

Phenotypes of the induced sputum inflammatory cells from these subjects were determined using a liquid-based thin-cytologic test method. As shown in Table 2, 245 (19.9%) of the 1232 children displayed an increased level of eosinophil, which was found in 136 (14.6 %) of the mild group, 68 (36.2%) of the moderate group, and 41 (37.3%) of the severe group. An increased level of serum IgE was present in 59 (4.8%) of the children.

Allergens were positively detected in 356 (28.9%) of the children, and specifically, 156 (12.7%) of the children were positive IgE to inhaled allergens, and 270 (22.0 %) were positive IgE to ingested allergens. Furthermore, children with severe clinical manifestation showed a significantly higher level of eosinophil, serum total IgE and positive allergens compared with that of children with mild or moderate symptom.
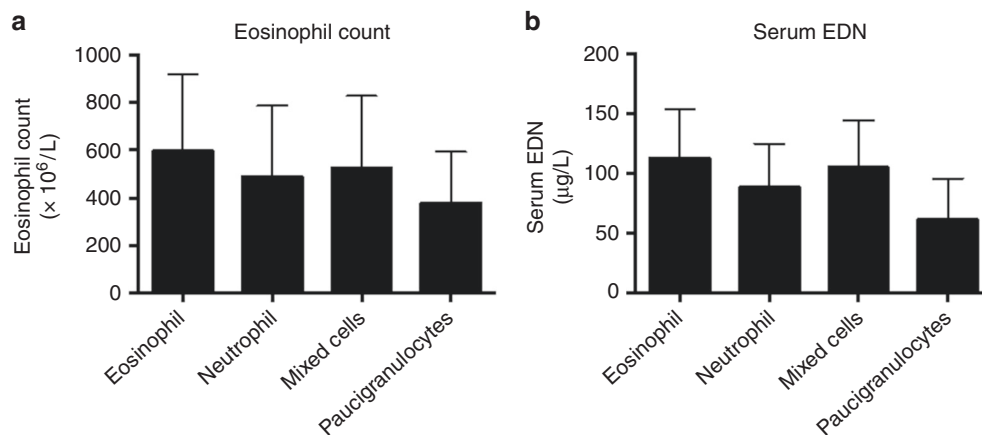
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Fig. 2 Comparison of eosinophil and EDN among the different cell type groups. **a** Eosinophil count. Sputum eosinophils were collected and counted as described in the Materials and methods. Vertical axis: number of eosinophil ($\times 10^6$/l); horizontal axis: groups of different cell type. **b** Serum EDN concentration. EDN was quantified by ELISA as described in Materials and methods. Vertical axis: serum EDN concentration (µg/l); horizontal axis: groups of different cell type

As shown in Table 3, blood eosinophil in mild, moderate, and severe wheezing children was 0.96 ± 1.95, 1.18 ± 1.93, and 1.01 ± 1.94, respectively, and they were not significantly different ($P = 0.146$). Blood neutrophil, however, was significantly different between the three groups (mild: 64.13 ± 16.94; moderate: 59.26 ± 18.5; severe: 57.25 ± 16.6, $P < 0.01$). Sputum eosinophil count was gradually increased in the order of mild (2.01 ± 2.04), moderate (3.65 ± 2.43), and severe (4.76 ± 2.49), and they were significantly different ($P < 0.001$). Similarly, sputum epithelial cell count was also significantly increased in moderate (13.04 ± 7.12) and severe (14.78 ± 6.99) groups compared to that in mild group (12.79 ± 7.16, $P = 0.01$). Other types of cells including macrophage and lymphocytes were not significantly different between the three groups ($P > 0.05$).

Phenotypes of the induced-sputum inflammatory cells
Under the light microscope, four types of inflammatory cells were identified in the induced-sputum (Fig. 1). Of the 905 successfully induced-sputum samples, 34.5% were from the children with mild/moderate, and 65.5% were from the children with severe wheezing. At least one type of inflammatory cell was detected in 526 samples, and of which, 343 were neutrophil only, 161 were eosinophil only, and 22 samples were both neutrophil and eosinophil. Inflammatory cells were identified in 117 out of the 312 children with mild/moderate symptom, and of which, 100 were neutrophil only, 13 were eosinophil only, and 4 were both neutrophil and eosinophil. In contrast, in the children with severe wheezing, 243 were neutrophil only, 148 were eosinophil only, and 18 were both neutrophil and eosinophil. Overall, neutrophil was the predominant inflammatory cell type in the children with wheezing. Nevertheless, eosinophil was higher in the children with severe wheezing compared with that in the children with mild/moderate symptoms. Total number of the inflammatory cells, both neutrophil and eosinophil, was significantly higher in the children with severe wheezing than that in the children with mild/moderate symptoms.

Quantification of the EDN, and correlation of EDN and tidal volume
As shown in Fig. 2, serum EDN level was highest in the eosinophil type group (112.6 ± 41.2 µg/l) compared to that in the mixed granulocytic type group (104.8 ± 39.4 µg/l), neutrophil type group (88.2 ± 36.6 µg/l), or paucigranulocytic type group (60.9 ± 34.6 µg/l). However, there was no significant difference between any two
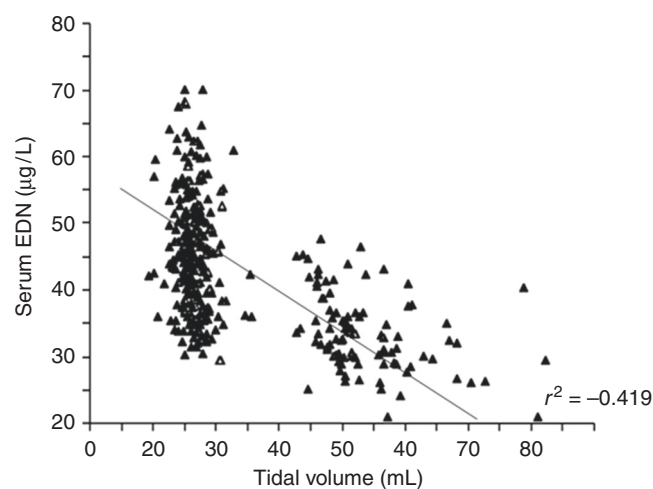

Fig. 3 Correlation of serum EDN concentration and tidal volume

groups ($P > 0.05$). Interestingly, serum EDN level was inversely correlated with tidal volume ($r^2 = -0.419$, $P < 0.05$, Fig. 3).

DISCUSSION
The development of individualized treatment based on the respite clinical phenotype becomes the top priority in optimal clinical control of wheezing or asthma. Previous studies have shown that sputum inflammatory cell counts, T-lymphocyte subpopulations and inflammatory cytokines did not differ between asthmatic adults and children, suggesting that the airway inflammatory mechanisms associated with childhood asthma or adult asthma are similar and that the immunopathogenic pathways might begin and continue from childhood into adulthood.[14] However, the detail pathogenic mechanism of inflammatory cell subpopulations in children with asthma has not been well clarified.

This study demonstrated that induced sputum could be used for assessment of airway inflammation in children with asthma. These children had intense airway inflammation with heterogeneous cell populations, including eosinophil and neutrophil. Activation of eosinophil leads to secretion of several types of cytotoxic cationic proteins, including eosinophil peroxidase (EPO), eosinophil cationic protein (ECP), major basic protein (MBP), and eosinophil-derived

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neurotoxin (EDN). Previous studies have shown that EDN is closely related to eosinophil activation and degranulation, which can serve as a marker for determining the etiology of asthma.[15] Findings of the current study indicated that serum EDN level was significantly increased as a result of increased number of eosinophil in children with asthma. Although the eosinophil counts were not different among the four groups, serum EDN level was significantly different in the four groups with highest in the eosinophil type group (112.6 ± 41.2 μg/l) followed by mixed granulocytic type group (104.8 ± 39.4 μg/l), neutrophil type group (88.2 ± 36.6 μg/l), and paucigranulocytic type group (60.9 ± 34.6 μg/l). Moreover, analysis on the relationship of EDN and tidal volume indicated that the level of EDN was negatively associated with tidal volume of children with wheezing. These finding suggested that EDN could be a sensitive marker for airway inflammation and could be used for the diagnosis of children with wheezing.

Our results indicated that at least one type of inflammatory cell was found in 526 of 905 (58.1%) children's sputum, and of which, neutrophil was found in 343 (65.2%) children, eosinophil was found in 161 (30.6%) children, and mixed granulocytes were found in 22 (4.2%) children. In the current study, inflammatory cells were not found in 41.9% of children with severe wheezing and in 47.8% of children with mild or moderate wheezing, which was different from adult asthma.[16] Moreover, in the children with severe wheezing, eosinophil was the most abundant inflammatory cell type (25%), while in the children with mild or moderate wheezing, eosinophil accounted only for 4.2%, suggesting that eosinophil could be a biomarker for severe recurrent wheezing in children and infants.

Previous studies reported that the level of eosinophil and neutrophil, which was used for determining the phenotype of induced-sputum inflammatory cells, were different in the wheezing children or infants from that in adult.[12,17] The level of eosinophil (2.5%) for determining the phenotype of induced-sputum inflammatory cells was performed in 72 normal children.[16] This eosinophil level was also considered to be the upper limit for normal children and infants. In the study of adult asthmatics, there were also multiple standards for the increased level of eosinophil, that is, 1.9% or 3.0%. For children and infants, while it was difficult to establish the standard level for neutrophil due to lacking the normal control data, a previous study suggested neutrophil level in children and infants was 54%.[18] In a study of adult asthmatics, 61 and 65.3% were used to determine the phenotype of neutrophils. But in our study, we found that phenotypic changes were mainly due to the enlargement of inflammatory cells, 12 children would be re-categorized, which would not affect our result. Of note, this study was not focused on the re-determining the level of inflammatory cells, but rather evaluating whether the determination method for adult asthma was applicable for wheezing children and infants.

AUTHOR CONTRIBUTIONS
Y.Z. contributed to the study design, Y.G. and J.Z. prepared the manuscript, J. Li and J. Liu performed the data analysis, C.M. searched references, X.J. and L.Z. collected the data. All the authors made the final approval.

DATA AVAILABILITY
The datasets supporting the conclusions of this article are included within the article.

ADDITIONAL INFORMATION
Competing interests: The authors declare no competing interests.

Ethical approval: The study protocol was approved by the Ethic Committee of Tianjin Children's Hospital and conducted in accordance with Helsinki's Declaration. All the patients gave their written information consent.

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