Original Article

Elevated Serum Levels of Thymic Stromal Lymphopoietin in Wheezing Children Infected with Human Metapneumovirus

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SUMMARY: Our aim was to study the epidemiology and clinical manifestations of human metapneumovirus (hMPV) infection in children in the Suzhou area, China, and to investigate the effect of thymic stromal lymphopoietin (TSLP) on wheezing in hMPV-infected children. The study included 13,533 children with respiratory tract infections who were admitted to the Children's Hospital of Soochow University between January 2009 and December 2014. Clinical data were recorded. Plasma levels of TSLP, interleukin (IL)-4, and IL-2 were compared among 35 wheezing children with single hMPV infection, 15 non-wheezing children with hMPV infection, and 35 wheezing children with other viral infections. The annual rates of hMPV infection from 2009 to 2014 were 2.33%, 6.41%, 3.29%, 3.52%, 0.23%, and 0.64%, respectively, peaking in 2010. The number of CD3⁺/CD16⁺/CD56⁺ cells was significantly lower in inpatients with hMPV infection than that in patients with other viral infections. Serum levels of TSLP and IL-4 in wheezing children with hMPV infection were significantly higher than the levels in the other 2 groups. hMPV is a common viral pathogen that causes respiratory infections in children in Suzhou, but annual rates have declined since 2010. Infection with hMPV may affect cell-mediated immunity. Serum TSLP levels were elevated after hMPV infection.

INTRODUCTION

In 2001, a novel respiratory virus was isolated from nasopharyngeal aspirates of young children with respiratory tract diseases in the Netherlands and was named human metapneumovirus (hMPV). This virus was later identified in the rest of Europe, North America, and Asia (1–3). hMPV infection primarily affects children younger than 3 years, most often in winter or spring (3–5). Our previous study found that in Suzhou, China, hMPV infection occurred throughout the year, with peaks in late winter and early spring. Climatic factors, in particular the average monthly temperature may facilitate the spread of the virus (3).

Studies have shown that hMPV is the second most reported cause of bronchiolitis and pneumonia following respiratory syncytial virus (RSV), and it accounts for 10–21% of respiratory infections in children (6–8). It also contributes to the development and occurrence of asthmatic disease in children. This important pathogen triggers wheezing in infants and also causes persistent or recurrent wheezing in older children (3,9).

It is unclear how hMPV infection causes wheezing in children. A recent study showed that thymic stromal lymphopoietin (TSLP) may be involved in the development of inflammation during hMPV infection (10).

TSLP is an epithelial cell-derived interleukin (IL)-7-like cytokine that contributes to mucosal immunity induced by microbes. It is currently the focus of much attention because there may be a link between asthma and viral infections (11,12).

In the present study, hMPV-infected infants and young children hospitalized with acute respiratory tract infections between January 2009 and December 2014 were enrolled. We investigated the epidemiological and clinical characteristics of hMPV. Serum levels of TSLP, IL-2, and IL-4 in hMPV-infected children were examined. We also discuss the potential involvement of TSLP in wheezing.

MATERIALS AND METHODS

Study group: Between January 2009 and December 2014, 13,533 inpatients, aged from 1 month to 14 years, who received diagnoses of acute respiratory disease at the Department of Respiratory Medicine at the Children's Hospital of Soochow University were enrolled in this study. For each patient, tests were performed on sputum to determine the pathogens present. Clinical manifestations and laboratory results were recorded. Plasma cytokine levels were evaluated in 35 wheezing patients with hMPV infection, 15 non-wheezing children with hMPV infection, and an additional 35 wheezing patients with other viral infections, including RSV and human bocavirus (HBoV) those were randomly selected upon admission, and blood samples were collected. There was no statistical difference in ratio of age and sex between these 3 groups.

The Ethics Committees of Soochow University approved this study, and the parents of all the recruited children provided informed consent.

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Sample collection: Nasal aspirate samples were obtained from each patient within 24 h after admission using a sterile plastic catheter, which were briefly inserted into the lower pharynx via the nasal cavity. These samples were used for detection of common microorganisms, such as RSV, adenovirus, influenza viruses A and B, parainfluenza viruses 1, 2, and 3, hMPV, HBoV, *Mycoplasma pneumoniae*, and bacteria.

For the cytokine study, blood samples were collected from 3 study groups shown above.

Detection of hMPV infection by reverse transcription (RT)-PCR: The hMPV nucleoprotein (N protein) gene sequence (GenBank database) was used as a reference to design primers for a 213-bp fragment (nucleotide positions 562–774) (sense, 5'-AACCGTGTAAGTGA TGAAGTC-3'; antisense, 5'-CATGTGTTGACCGGCC CCATAA-3'). The primers were synthesized by Shanghai Sangon Biological Engineering (Shanghai, China). Viral RNA from blood was extracted with TRIzol reagent (Invitrogen, Carlsbad, CA, USA). Reverse transcription was performed using random hexamer primers. PCR assays were conducted in an automatic PCR cycler (Perkin Elmer, Foster City, CA, USA). PCR consisted of 45 cycles of denaturation at 94°C for 30 s, annealing at 55°C for 30 s, and extension at 68°C for 30 s.

Ten positive PCR amplicons were randomly selected and purified from agarose gels for sequencing. The resultant sequences were compared with hMPV sequences available from GenBank using Chromas software.

Other microbiological analyses: The nasal aspirate suspension was centrifuged at 1,000 rpm for 10 min. Cell pellets were collected for a cell smear. The following viruses were detected by immunofluorescence assays (D³ Ultra DFA Respiratory Virus Screening & ID Kit; Diagnostic Hybrids, Athens, OH, USA): RSV, adenovirus, influenza viruses A and B, and parainfluenza viruses 1, 2, and 3. A result was considered positive if ≥ 5 inclusion bodies were observed under the fluorescence microscope; otherwise, it was considered negative. *M. pneumoniae* and HBoV infections were determined via PCR (Nucleic Acid Amplification Fluorescent Reagent Kit; DaAn gene, Guangdong, China). Bacterial infection was determined by inoculating blood agar plates. The plates were checked following 18–20 h incubation. A positive result was defined as bacterial growth $> 10^4$ CFU/mL.

Enzyme-linked immunosorbent assay (ELISA) for cytokines: Plasma levels of TSLP, IL-4, and IL-2 were measured by ELISA in accordance with the manufacturer's recommendations (R&D systems, Minneapolis, MN, USA).

Flow cytometry for cell-mediated immunity: Peripheral blood mononuclear cells were isolated from patients' venous blood using the Ficoll gradient method (Ficoll; TBD Science, Tianjin, China). Cell-mediated immunity was determined according to the percentages of CD3+, CD3+/CD4+, CD3+/CD8+, CD3-/CD19+, CD3-/CD16+/CD56+, and CD19+/CD23+ cells. Flow cytometry (Beckman Coulter, Brea, CA, USA) was performed according to the manufacturer's instructions (Immunotech, Marseille, France). All antibodies were purchased from Biolegend (San Diego, CA, USA).

Statistical analysis: Cell counts were reported as mean $\pm$ standard deviation. The differences between 2 groups were compared using the nonparametric Mann-Whitney 2-sample *U* test for unpaired data. Differences between 3 or more groups were compared using the nonparametric Mann-Whitney *U* test. Enumeration data were reported as rate, determined by the chi-squared test and Fisher's exact test. SPSS ver. 18.0 software (Chicago, IL, USA) was used for data analysis. A *P*-value < 0.05 was considered significant.

RESULTS

Spectrum of viral infections: Between January 2009 and December 2014, 13,533 specimens from patients aged between 1 month and 14 years were collected, 3,438 (25.4%) of which were positive for at least one virus. The 5 most commonly detected viruses were as follows: RSV; parainfluenza virus 3; HBoV; hMPV; and influenza virus A (12.41%, 4.26%, 3.95%, 2.75%, and 1.66%, respectively).

Annually, from 2009 to 2014, the positive rates of hMPV detection were as follows: 2.33% (52/2,234) in 2009, 6.41% (138/2,154) in 2010, 3.29% (79/2,401) in 2011, 3.52% (84/2,384) in 2012, 0.23% (5/2,162) in 2013, and 0.64% (14/2,198) in 2014. The rate of hMPV infection was peaked in 2010.

Seasonal distribution of hMPV infection: Detection rates of hMPV infection varied significantly according to season (Figs. 1 and 2). The detection rate was highest in spring (6.04%, 207/3,425), followed by winter (3.09%, 106/3,429). The detection rate for hMPV fluctuated greatly from month to month. The peak detection rate occurred between the months of February and May each year. The highest rate (20.4%) was recorded for April 2010.

Age and sex distribution of hMPV infection: Among inpatients with hMPV infection, 231 were boys (62.1%) and 141 were girls (37.9%). The man-to-woman ratio was 1.64:1. Ages ranged from 1 month to 10 years, with an average age of 1.62 ± 1.71 years (Fig. 3). A high rate of hMPV infection was observed among children younger than 5 years. It was highest in children aged between 6 months and 1 year (3.73%) and lowest among those older than 5 years (1.18%). There was a

Fig. 1. Seasonal distribution of hMPV-positive rates among 13,533 children aged from 1 month to 14 years with acute respiratory tract infections from January 2009 to December 2014.

Fig. 2. Monthly distribution of hMPV-positive rates among 13,533 children aged from 1 month to 14 years with acute respiratory tract infections from January 2009 to December 2014.

Fig. 3. Age distribution of hMPV-positive rates among 13,533 children aged from 1 month to 14 years with acute respiratory tract infections from January 2009 to December 2014.

significant difference in hMPV prevalence based on age ($\chi^2 = 23.402$, $P < 0.001$).

Clinical characteristics of hMPV infection: Among the 372 inpatients with hMPV infection, 191 had hMPV infection only. The most common clinical manifestations of hMPV-infected patients were cough (98.4%), wheezing (44.0%), and fever (43.5%). Elevated levels of C-reactive protein were detected in 17.3% of the inpatients with hMPV infection only (Table 1). In 181 children co-infected with other pathogens, the most common clinical manifestations were also cough (98.3%), wheezing (44.8%), and fever (44.2%). There was no significant difference in clinical manifestations between children with hMPV infection only and those with co-infections (data not shown).

The clinical features of patients with hMPV infection only were compared with those of patients infected with a single other virus (from the same batch of patients). One notable finding was the low percentage of CD3⁺/CD16⁺/CD56⁺ cells in inpatients with hMPV infection (Table 1 and Fig. 4), suggesting that

hMPV infection may affect cell-mediated immunity, especially natural killer (NK) cells.

Plasma levels of TSLP, IL-2, and IL-4 in hMPV infected children with or without wheezing: There was no significant difference in terms of age or sex between hMPV infected children with wheezing and those without wheezing. The plasma levels of TSLP and IL-4 in wheezing children with hMPV infection were 270.09 ± 39.51 ng/L and 194.26 ± 45.35 ng/L, respectively, which were significantly higher than those of hMPV infected children without wheezing ($P = 0.008$ and $P = 0.043$, respectively). Furthermore, the plasma levels of IL-2 in wheezing children with hMPV infection were significantly lower than those of hMPV infected children without wheezing (301.14 ± 95.84 ng/L vs. 364.06 ± 97.27 ng/L, $P = 0.035$) (Table 2).

Plasma levels of TSLP, IL-2, and IL-4 in wheezing children with hMPV and other virus infections: There was no significant difference in terms of age and sex between children with hMPV and those with other viral infections. The plasma levels of TSLP and IL-4 in wheezing children with hMPV infection were 270.09 ± 39.51 ng/L and 194.26 ± 45.35 ng/L, respectively, which was significantly higher than those of wheezing children with other viral infections ($P < 0.01$ and $P = 0.049$, respectively). There was no significant difference in the level of IL-2 between the 2 groups (Table 3).

DISCUSSION

In this study, specimens were collected from 13,533 pediatric patients with respiratory infections over a 6-year period. These specimens were tested for hMPV using RT-PCR. The detection rates for the years 2009–2014 were 2.33%, 6.41%, 3.29%, 3.52%, 0.23%, and 0.64%, respectively. In our previous study we showed that hMPV infection rates in inpatients with respiratory infections for the years 2006–2008 were 8.4%, 7.6%, and 12.6%, respectively (3), it appears that hMPV infection rates have steadily declined in recent years. This may be explained by the epidemiolo-

Table 1. Clinical characteristics of hMPV and other virus infections¹⁾

		hMPV	RSV	Pinf 3	HBoV	Inf A	P
Subject, <i>n</i>		191	919	266	218	78	
Age, yr		1.41 ± 1.55	0.60 ± 0.79	1.79 ± 2.21	3.36 ± 2.52	1.21 ± 1.62	<0.001
Man		122 (63.9)	666 (72.5)	198 (74.4)	115 (52.8)	49 (62.8)	<0.001
Clinical manifestation	Fever	83 (43.5)	248 (27.0)	140 (52.6)	131 (60.1)	77 (98.7)	<0.001
	Cough	188 (98.4)	908 (98.8)	248 (93.2)	206 (94.5)	76 (97.4)	<0.001
	Nasal obstruction	71 (37.2)	424 (46.1)	97 (36.5)	81 (37.2)	16 (20.5)	<0.001
	Wheezing	84 (44.0)	616 (67.0)	142 (53.4)	75 (34.4)	12 (15.4)	<0.001
	Dyspnea	7 (3.7)	66 (7.2)	1 (0.4)	6 (2.8)	5 (6.4)	<0.001
	Tachypnea	18 (9.4)	187 (20.3)	35 (13.2)	44 (20.2)	8 (20.5)	<0.001
	Cyanosis	1 (0.5)	50 (5.4)	0 (0)	5 (2.29)	4 (5.1)	<0.001
	Lung rales	171 (89.5)	880 (95.8)	222 (83.5)	162 (74.3)	49 (62.8)	<0.001
Hemogram	WBC (× 10 ⁹ /L)	9.44 ± 4.56	8.77 ± 3.60	7.70 ± 2.77	9.66 ± 3.61	6.89 ± 2.65	0.019
	CRP (elevated)	33 (17.3)	100 (10.9)	55 (20.7)	46 (21.1)	5 (6.4)	<0.001
	Platelets (× 10 ⁹ /L)	351.4 ± 102.4	407.0 ± 114.3	358.2 ± 110.0	350.36 ± 90.86	252.3 ± 85.5	<0.001
Cellular immunity, %	CD3 +	59.15 ± 10.46	57.84 ± 12.54	59.83 ± 12.16	59.86 ± 10.10	56.82 ± 9.22	0.727
	CD3 + /CD4 +	35.52 ± 10.03	37.67 ± 12.12	38.12 ± 10.39	32.86 ± 9.21	28.00 ± 5.27	0.001
	CD3 + /CD8 +	20.17 ± 7.18	17.31 ± 6.97	18.54 ± 5.52	22.61 ± 5.12	23.13 ± 8.12	<0.001
	CD3 - /CD19 +	27.68 ± 10.16	24.49 ± 10.96	24.70 ± 10.27	27.90 ± 8.77	26.10 ± 12.05	0.040
	CD3 - /CD16 + /CD56 +	10.03 ± 5.82	16.39 ± 10.33	13.86 ± 10.72	10.77 ± 8.16	13.70 ± 7.74	<0.001
	CD19 + /CD23 +	9.84 ± 5.45	12.10 ± 5.70	13.31 ± 5.59	12.74 ± 4.49	7.99 ± 5.75	<0.001

¹⁾: Reported as *n* (%), unless noted otherwise.

Pinf 3, parainfluenza virus 3; Inf A, influenza virus A; WBC, white blood cells; CRP, C-reactive protein.

Table 2. Plasma level of TSLP, IL-2, and IL-4 in hMPV infected children with or without wheezing

	Children with wheezing	Children without wheezing	Statistic	P
Subject (<i>n</i>)	35	15		
Age (yr)	0.99 ± 1.38	1.32 ± 1.58	-0.740	0.459
Sex (M/W)	24/11	11/4	0.113	1.00
TSLP (ng/L)	270.09 ± 39.51	219.58 ± 62.99	-2.668	0.008
IL-4 (ng/L)	194.26 ± 45.35	159.48 ± 59.21	-2.022	0.043
IL-2 (ng/L)	301.14 ± 95.84	364.06 ± 97.27	-2.107	0.035

Table 3. Plasma level of TSLP, IL-2, and IL-4 in wheezing children with hMPV and other virus infections

	hMPV	Other virus	Statistic	P
Subject (<i>n</i>)	35	35		
Age (yr)	0.99 ± 1.38	1.07 ± 0.95	-0.742	0.458
Sex (M/W)	24/11	22/13	0.254	0.802
TSLP (ng/L)	270.09 ± 39.51	177.81 ± 45.44	-6.16	<0.01
IL-4 (ng/L)	194.26 ± 45.35	173.09 ± 44.29	-1.97	0.049
IL-2 (ng/L)	301.14 ± 95.84	298.67 ± 81.17	-0.094	0.925

gical features of hMPV. Different hMPV infection rates in different years have also been reported in the literature. In Italy, rates of hMPV infection also varied over a 3-year study period, with 37% in 2000, 7% in 2001, and 43% in 2002 (13). In the United States, the hMPV infection rate was 1.5% in 2000, significantly lower than the rate of 7% in 2001 (14). In the present study, the highest hMPV infection rate was detected in spring, followed by winter, peaking between February

and May every year. The infection rate was high in children younger than 5 years, with the highest rate in those aged 6 months to about 1 year. These results are consistent with reports from Italy, the Netherlands, and New York (13,15,16).

Cough, fever, and wheezing were the most common clinical manifestations in hMPV-infected patients (3,17,18). In the present study, almost all patients with hMPV had cough, and almost half of them had wheezing. It has been previously reported that clinical manifestations of hMPV infection are indistinguishable from those of other respiratory viruses such as RSV, influenza virus, or parainfluenza virus (11–17). Our study here provides further evidence for this conclusion. hMPV infection may affect the cells of the immune system. Our study showed that in children with bronchiolitis caused by hMPV infection, the percentages of CD19+ /CD23+ and CD4+ CD25+ cells were significantly elevated, while the percentage of CD3- /CD16+ /CD56+ cells was reduced (19). In a study conducted in mice, hMPV appeared to be a potent inducer of NK cell activity (20). In the present study, the percentage of CD3- /CD16+ /CD56+ in inpatients with hMPV infection only was low. Further studies are needed to clarify the relationship between hMPV infection and cellular immunity.

It is not clear how hMPV infection causes wheezing. hMPV infection of airway epithelial cells is cytopathic, and the resultant cell injury triggers a series of inflammatory reactions. On the other hand, an unbalanced T helper (Th)1/Th2 immune response to hMPV infection may occur, which would release a series of inflammatory mediators, cytokines, and chemokines, and cause wheezing (21–23). Studies have shown that TSLP, a newly discovered cytokine, may aggravate excessive

Fig. 4. Flow cytometry based profiles of cellular immunity (CD3+, CD3+/CD4+, CD3+/CD8+, CD3-/CD16+/CD56+, CD3-/CD19+, and CD19+/CD23+) in blood of 1,672 children with single virus infection from January 2009 to December 2014, including 191 cases with hMPV, 919 cases with RSV, 266 cases with parainfluenza virus 3, 218 cases with HBoV, and 78 cases with influenza A. The differences between 2 groups were compared using the nonparametric Mann-Whitney 2-sample *U* test for unpaired data.

pulmonary inflammation after hMPV infection. Lay et al. (24) found that hMPV infection promoted TSLP expression in both human airway epithelial cells and in lungs in mouse models; studies using TSLP-receptor deficient mice (TSLPR^{-/-}) demonstrated reduced lung inflammation and hMPV replication. In our study, we found that the plasma levels of TSLP in wheezing children with hMPV infection were significantly higher than those in wheezing children with other virus infections. This suggested that TSLP may participate in the inflammatory response after hMPV infection in humans.

Studies have shown that TSLP expression is closely associated with wheezing. Chauhan et al. (25) reported that children with asthma had elevated serum levels of TSLP, which correlated negatively with the results of asthma control tests and number of regulatory T cells.

Salter et al. (26) studied patients with allergic asthma and found that basophil activation during early- and late-phase responses to inhaled allergens may be driven, at least in part, by TSLP. Moreover, wheezing caused by elevated TSLP levels may be associated with Th2 activation. Akasaki et al. (27) examined the roles of TSLP and IL-33 in acute and chronic allergic rhinitis models, and found that TSLPR-deficient mice showed defective Th2 activation and nasal eosinophilia even after chronic ragweed exposure, which suggested that TSLPR contributes to Th2 responses in chronically allergen-exposed mice. Other experiments in vitro also showed that TSLP can promote Th2 cytokine-mediated inflammation and facilitate production of IL-4, IL-5, IL-13, and tumor necrosis factor- α , which can aggravate airway inflammation leading to difficulty in breathing (23,28). In our present study, the plasma

levels of TSLP and IL-4 in wheezing children with hMPV infection were significantly higher than those in non-wheezing children with hMPV infection and those in wheezing children with other viral infections. Elevated expression of TSLP may promote the Th2 immune response, which may cause airway inflammation and hyper-responsiveness. Such chain of reactions could trigger wheezing.

A recent study showed that hMPV infection activated the TSLP pathway that augmented excessive pulmonary inflammation and viral replication in mice (24). Experiments in airway inflammation mouse models suggested the involvement of TSLP in airway inflammation leading to airway hyper-responsiveness, airway reconstruction, and mucus secretion (29,30). TSLP can also promote infiltration of inflammatory cells, including neutrophils and eosinophils, into the lungs (30,31). If the link between TSLP and wheezing is confirmed, TSLP production could be blocked to control Th2-mediated airway inflammation, alleviating this symptom of hMPV infection.

In conclusion, our study showed that hMPV is a common viral pathogen in children with respiratory infection in Suzhou. However, hMPV infection rates have steadily declined over the last 5 years. The most common clinical manifestations in hMPV-infected patients are cough, wheezing, and fever. Elevated plasma levels of TSLP following hMPV infection may be related to wheezing caused by hMPV infection.

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Conflict of interest None to declare.

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