

# Original article

## Thymic stromal lymphopoietin expression is increased in nasal epithelial cells of patients with mugwort pollen sensitive-seasonal allergic rhinitis

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**Keywords:** rhinitis, allergic; expression; thymic stromal lymphopoietin; mugwort pollen, pathogenesis

**Background** Excessive expression of thymic stromal lymphopoietin (TSLP) has been demonstrated in asthmatic airway epithelia and in nasal epithelia from animal models of allergic rhinitis (AR), but the evidence of expression of TSLP in nasal epithelial cells (NECs) of patients with AR is lacking. We aimed to investigate the expression of TSLP in NECs of patients with mugwort sensitive-seasonal AR and determine whether it is associated with severity of symptoms and the number of infiltrated eosinophils in nasal mucosa.

**Methods** NECs specimens were obtained by scraping with plastic curettes from the nasal inferior turbinates of patients with mugwort pollen sensitive-seasonal AR ( $n=22$ ) and nonallergic controls ( $n=11$ ) during last peak mugwort pollen season. The severity of nasal symptom was assessed using a Visual Analog Scale (VAS). In addition, serum mugwort pollen IgE levels were tested from each patient. *In situ* hybridization (ISH) was performed to test the messenger RNA (mRNA) of TSLP in the NECs. Furthermore, immunohistochemical staining (IHC) was scored to evaluate the expression of TSLP and eosinophil cell count was made by May-Grünwald/Giemsa staining. The correlation between expression of TSLP and all other parameters was analyzed in this study.

**Results** The mRNA level of TSLP was significantly increased in NECs of patients with AR compared with the nonallergic control group ( $P < 0.05$ ). In addition, IHC results showed that expression of TSLP in NECs from patients with AR was up-regulated which was correlated with VAS score ( $r=0.598$ ;  $P < 0.05$ ) and nasal eosinophils count ( $r=0.702$ ;  $P < 0.05$ ), but it was unrelated with mugwort pollen specific IgE level.

**Conclusions** These preliminary findings indicate a potential relationship between TSLP expression, severity of symptoms and nasal eosinophils count in pathogenesis of AR, but TSLP expression did not correlate with mugwort pollen specific IgE level. The elevated expression of TSLP might play a critical role in local atypical responses of AR. In the future, the TSLP has the potential to be one of the most important molecular markers for AR diagnoses and assessment.

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Allergic rhinitis (AR) and its impact on asthma (ARIA) has shown that AR and asthma are closely related based on evidences from etiology to pathogenesis. In 1997, this point of view was summarized by Jay Grossman as “one airway, one disease”.<sup>1</sup>

Recently, an epithelial cells derived cytokine, thymic stromal lymphopoietin (TSLP) has been widely studied in asthmatic airway, so we proposed to investigate whether it involved in allergic response of nasal epithelial cells (NECs) from AR patients.

TSLP was originally cloned from thymic stromal epithelium, which was structurally related to IL-7.<sup>2,3</sup> As far as we concerned, the sources which express TSLP including keratinocytes, thymic epithelial cells, mast cells activated by cross-linking their FcεR1, smooth muscle cells, fibroblasts and bronchial epithelial cells.<sup>3</sup> TSLP stimulates human myeloid dendritic cells (mDC) which uniquely express the heterodimeric TSLP receptor to promote Th2 polarization of inflammatory CD4<sup>+</sup> T cells and then express IL-4 and IL-5, which can prime CD4<sup>+</sup> T cells undergoing Th2 differentiation.<sup>4</sup> So TSLP is thought

to have a key role in network of regulation of asthma which is characterized as Th2 polarization. It has been proved that TSLP is elevated in asthma and triggers dendritic cell-mediated activation of Th2 inflammatory responses.<sup>5</sup> Several studies have revealed the close relationship between TSLP expression and Th2 cytokine regulation in asthmatic airways.<sup>6-8</sup> Although TSLP has been shown to be produced mainly by asthmatic airway epithelial cells, the expression and regulation of nasal epithelial TSLP of AR, which are also predominated with Th2 reaction, have not been extensively studied. It has been shown TSLP was expressed in nasal mucosa of animal model with allergic rhinitis.<sup>9</sup> However, it is unclear whether the expression of TSLP in human nasal epithelial cells of patients with allergic rhinitis correlates with clinical symptoms. Mugwort pollen is the most

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common outdoor allergen in northeast China. To further understand roles of TSLP in pathogenesis of AR, during the last peak mugwort pollen season, we examined the expression of TSLP in nasal mucosa from seasonal allergic rhinitis patients who were allergic to mugwort and evaluated whether its expression is associated with severity of symptoms, specific IgE level and the number of infiltrated eosinophils in nasal mucosa.

## METHODS

### Patients and clinical evaluation

Twenty-two patients were recruited from the clinics of the Department of Otorhinolaryngology, Head & Neck Surgery, China-Japan Union Hospital during 2008. Recruitment occurred in August and September, coinciding with mugwort pollen season. This study was approved by the Ethical Committee of the Jilin University of Medical Sciences, Changchun, China and informed consent was obtained from each patient before participation.

A detailed clinical history was taken. All patients were nonsmokers and had no concurrent significant illnesses. Patients with anatomical nasal abnormalities, or who had previously received allergen immunotherapy were excluded. Pregnant or lactating women were not eligible for the study. Patients who were treated with antihistamines, intranasal corticosteroids, oral corticosteroids, and leukotriene receptor antagonists had discontinued them for at least 2 weeks before enrollment.

According to ARIA guidelines, Visual Analog Scales can assess the severity of rhinitis grades. The severity of symptoms was objectively measured by a Visual Analogue Scale (VAS). A VAS ranging from 0 ("nasal symptoms, not at all bothersome") to 10 cm ("nasal symptoms, extremely bothersome") was used to assess the severity of combined nasal symptoms. We did not use a VAS for individual symptoms such as nasal obstruction, rhinorrhoea, sneezing and nasal pruritus.

All patients were evaluated by Skin-prick test (SPT) with mugwort pollen, house dust mite, animal dander and pollen were performed in parallel with standard extracts from ALK (Copenhagen, Denmark) for all patients. According to SPT responses, positive test result required a wheal diameter of at least 3 mm larger than the negative control or, alternatively, a wheal diameter of at least 2 mm larger than the negative control and an allergen wheal to histamine (10 mg/ml) ratio equal to or larger than 0.5.

The diagnosis of allergic rhinitis was made on the basis of a history of nasal symptoms and positive SPT results according to validated criteria.

The control group consisted of 11 volunteers with no allergic rhinitis symptoms, negative SPT responses, and specific IgE of less than 0.35 kU/L to latex. All patients

and volunteers were free from upper airway infections for at least four weeks preceding the study. Demographic characteristics of both patients group and control group, including sex and age, were described in Table 1. All patients suffered from seasonal allergic rhinitis caused solely by mugwort pollen. All of them had a positive skin prick history for at least two years previously.

**Table 1.** Characteristics of patients and control groups

| Variables                      | AR (n=22)          | Controls (n=11)      |
|--------------------------------|--------------------|----------------------|
| Gender (n)                     |                    |                      |
| M                              | 11                 | 5                    |
| F                              | 11                 | 6                    |
| Age (mean (SD), range (years)) | 39.8 (12.7, 17–58) | 32.7 (17.5, 12–42.6) |
| Allergic status                | 22                 | 0                    |

### Sample collections

Nasal scrapings were obtained according to previous report,<sup>10</sup> with some modification. In brief, nasal epithelia specimens were collected by scraping one third of inferiors with plastic curettes. Then plastic curettes were immersed, after the scraping, into a plastic tray with phosphate buffered saline (PBS). Control tissue samples were taken from nonallergic volunteers as the same procedure above. The nasal specimens were divided into single cell by incubating them for 1 hour at 4°C with PBS. The cell suspensions were centrifuged for 10 minutes at 3000r/min, 4°C and then the supernatant was discarded and the precipitated cells were smeared over each poly-L-lysine-coated slide, air-dried, and fixed in fresh 4% paraformaldehyde in PBS for 20 minutes. Cells were washed with PBS (0.1mol/L), and dehydrated through a graded series of alcohol, and stored at –70°C freezer.

### Nasal eosinophils count

The eosinophils count was made by May-Grünwald/Giemsa staining. The results were examined by using optic microscope (Olympus Microscope, 250×focus). The number of eosinophils was expressed as a mean of 10 fields. Slides were examined by two investigators masked to the identity of the samples.

### Specific IgE determination

Human IgE antibodies to mugwort pollen, house dust mite, animal dander and pollen were measured for patients by immunoblotting (AllergyScreen, MEDIWISS Analytic, Moers, Germany) according to the manufacturer's recommendations. Immunoblotting values > 0.35 kU/L were considered positive.

### In situ hybridization (ISH)

The antisense oligonucleotide probes were used to detect the TSLP mRNA (sequence of TSLP probe: 5'ATCTGAGTTTCCGAATAGCCTGGGCACC), which were labeled with digoxigenin (DIG) and DIG-labeled probe detection kits. *In situ* hybridization (ISH) was performed according to the manufacturer's instructions, with some modifications. Briefly, nasal epithelial cells on coverslips from patients were fixed with 4% paraformaldehyde in DEPC-phosphate buffer and rinsed,

**Table 2.** Characteristics of 22 patients with mugwort-sensitive allergic rhinitis (n=22)

| Variables                                              | Median | Range    |
|--------------------------------------------------------|--------|----------|
| Duration of rhinitis (years)                           | 5      | 2-9      |
| Average VAS scores                                     | 5.7    | 2.8-8.8  |
| Mugwort (mm)                                           | 6.25   | 4.0-8.5  |
| TSLP expression scores in nasal epithelial cells       | 2.5    | 1-4      |
| Eosinophils in nasal scraping specimens (cells/10 HPF) | 38.8   | 0-186    |
| Specific IgE level (kU/L)                              | 28.2   | 0.39-100 |

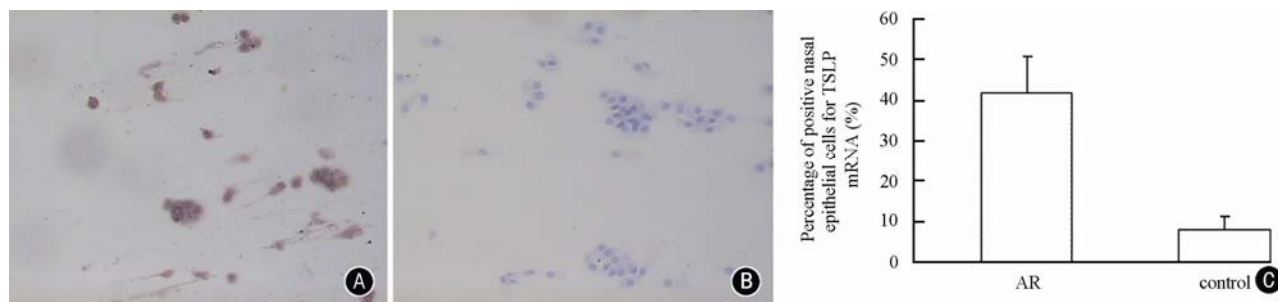


**Figure 1.** Expression of TSLP analyzed by using ISH in NECs of patients with AR and nonallergic subjects. Typical sample of ISH in NECs from AR patients (A) and nonallergic controls (B) (original magnification  $\times 400$ ). Occasionally, some NECs weakly stained by TSLP probes in control samples. However, in AR group, more cells were positively stained. The percentage of positive nasal epithelial cells for TSLP mRNA in patients with AR was higher than that in nonallergic control ( $P < 0.05$ , C).

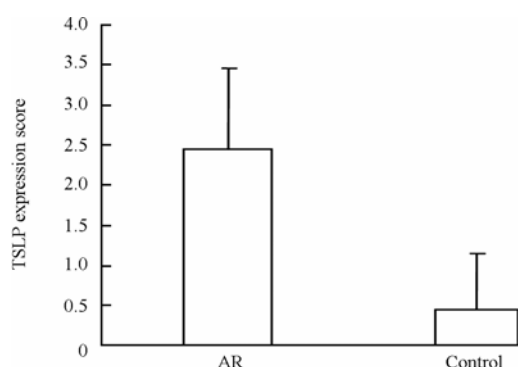


**Figure 2.** Expression of TSLP analyzed using IHC in NECs of patients with AR and nonallergic subjects. TSLP expression on nasal epithelial cells had been scored, according to a four-point rating scale, from 0 to 4. The TSLP expression scores in NECs of patients with AR are higher than nonallergic subjects. NECs: nasal epithelial cells. AR: allergic rhinitis. IHC: immunohistochemistry; Control: nonallergic control group.

three times in  $4 \times \text{SSC}$ . The slides were then treated with  $1 \mu\text{g/ml}$  of proteinase K for 5 minutes at room temperature to expose nucleic acid fragments/passage. After pre-hybridization procedure above, probes were added at a final concentration of  $2 \mu\text{g/ml}$ . Hybridization was performed over night at  $60^\circ\text{C}$ . After that, the coverslips were incubated in biotinylated anti-DIG for 60 minutes at  $37^\circ\text{C}$ , before the addition of streptavidin- peroxidase. After washing, the coverslips were incubated with diaminobenzidine in  $0.03\% \text{H}_2\text{O}_2$  for 10 minutes, washed, and counterstained with hematoxylin. Two slides were treated in-parallel to the rest (i.e., no addition of probe and NECs pretreated by RNaseA) as negative controls.

### Immunohistochemistry (IHC)

Immunohistochemical analysis of the nasal epitheliums was performed by standard avidin-biotin immuno-

peroxidase method. The fresh sample was fixed in  $4\%$  paraformaldehyde, deparaffinized in xylol, and rehydrated in graded alcohol. Endogenous peroxidase activity was blocked with  $3\%$  hydrogen peroxide for 20 minutes. Upon incubation, the serum was removed from the slides, and the slides were incubated with primary antibody against TSLP and SCF respectively for 30 minutes. After washing, the slides were treated with biotinylated secondary anti-rabbit antibody (1:200). The preparations were then washed in PBS, and the staining immunoproducat was visualized with diaminobenzidine (DAB; DingGuo Bio. Tech. C., China) in PBS with  $0.01\% \text{H}_2\text{O}_2$ . The slides were counterstained with hematoxylin and embedded in the medium. Positive controls were used in each staining.

### Evaluation of the expression of TSLP

In each patient, two interval slides were selected and observed to evaluate the expression of TSLP for ISH and IHC analysis, respectively. Five visual fields were examined in each slide that contained at least 500 cells. The mean percentage of positive cells in ISH results was calculated as positive cells using the  $\times 20$  objective. TSLP expression on nasal epithelial cells had been scored, according to a four-point rating scale, from 0 to 4 (0=no positive cells, 1=mild positivity on  $25\%$  of epithelial cells, 2=mild positivity on  $75\%$  of epithelial cells, 3=intense positivity on  $75\%$  of epithelial cells, 4= very intense positivity on all epithelial cells).

### Statistical analysis

Data were analyzed using SPSS 11.0 version (SPSS, Inc., Chicago, USA). Positive cells of ISH results are expressed as mean percentage. IHC results of TSLP expression are expressed as mean scores. Although some of the variables were normally distributed, others were

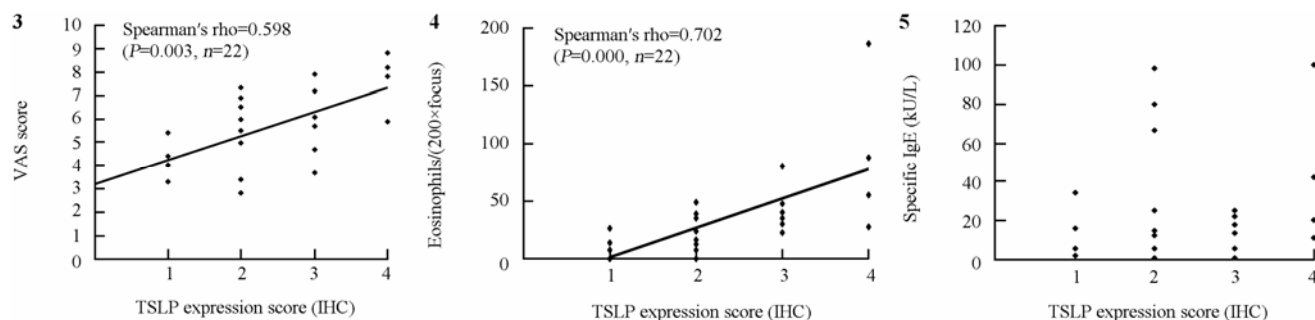


**Figure 3.** As expected, correlation between expression of TSLP and VAS score is significant ( $r=0.598$ ;  $P<0.05$ ). The Spearman's rank-order method was performed for statistical analysis. Values of  $P<0.05$  were considered statistically significant. NECs: nasal epithelial cells; VAS: Visual Analog Scales.

**Figure 4.** The expression of TSLP correlated with the number of infiltrated eosinophils in allergic nasal mucosa ( $r=0.702$ ;  $P<0.05$ ). The Spearman's rank-order method was performed for statistical analysis. Values of  $P<0.05$  were considered statistically significant.

**Figure 5.** There is no evidence that expression of TSLP in allergic nasal mucosa and mugwort specific IgE levels were correlated. The Spearman's rank-order method was performed for statistical analysis. Values of  $P<0.05$  were considered statistically significant. Specific IgE: mugwort pollen specific IgE level (kU/L).

not, we therefore chose to use nonparametric statistics for all the variables. The Mann-Whitney tests were used for intergroup comparisons and Spearman's rank-order method for correlation coefficients analysis, respectively. Differences were considered statistically significant at  $P<0.05$ .

## RESULTS

Twenty-two patients with mugwort pollen-sensitive allergic rhinitis (11 men and 11 women) were enrolled in this study. The patients' characteristics are listed in Table 2.

In our studies, we excluded 3 cover slides from patients (number 11, 18 and 20) because there were two heavy secretions and fewer cells. We resample after irrigation of nasal cavity. ISH results revealed the expression of TSLP in NECs of patients with AR (Figure 1A) was higher than that in control group (Figure 1B) at mRNA level ( $P<0.05$ ; Figure 1C). Levels of TSLP expression were evaluated by immunocytochemistry in all 22 patients and the eosinophils counts from nasal scraping specimens could be made by Giemsa staining (20/22 patients). The IHC results confirmed excessive expression of TSLP in NECs of patients with AR and the TSLP expression scores were higher in AR patients than that in nonallergic control group ( $P<0.05$ ; Figure 2). Furthermore, the expression of TSLP in NECs from patients with AR correlated with the nasal symptoms on VAS ( $r=0.598$ ,  $P=0.003$ ; Figure 3) and nasal eosinophils count ( $r=0.702$ ,  $P=0.000$ ; Figure 4). However, there was no correlation in expression between TSLP and specific IgE level (Figure 5).

## DISCUSSION

Emerging data have shown that the expression of TSLP was elevated in asthmatic airway.<sup>7,8</sup> Besides, many studies have revealed in animal model, the up-regulation of TSLP in nasal mucosa.<sup>9</sup> Our study demonstrates that the excessive expression of TSLP in NECs of patients with AR. TSLP is of particular interest because it appears

to be produced at the epithelial interface, and may represent a mechanism whereby environmental stimuli initiate Th2 responses to allergen, and through chemokine production retaining or attracting in asthmatic airways.<sup>8</sup>

According to the ARIA guidelines, VAS can assess the severity of rhinitis graded with a quantitative analysis.

The correlation between expression of TSLP in nasal epithelial cells and severity of allergic rhinitis is not well understood. So we investigated the relationship among the expressions of TSLP in nasal mucosa, VAS and the specific IgE level. Our results show that there is no correlation between expression of TSLP and specific IgE level.

However, our finding proved a good correlation between TSLP and VAS score. To exclude the effects of multiple allergens to specific IgE levels and clinical symptoms, patients who are only allergic to mugwort pollen were enrolled in this study. Our unpublished results show that specific IgE level does not well correlate with severity of clinical symptoms in AR patients. This may be the reason why specific IgE does not correlate with expression of TSLP in nasal epithelial cells from patients with AR. The associations of TSLP expression with VAS suggested TSLP might have an influential role in pathogenesis of AR, which may reflect severity of local allergic response, thus have impact on nasal symptoms.

AR is characterized by a polarized Th2 reaction that eosinophils recruited in allergic nasal mucosa.<sup>11,12</sup> To further investigate whether TSLP is associated with eosinophil infiltration in local response of nasal mucosa from AR patients who were allergic to mugwort pollen, Giemsa staining was performed to evaluate the numbers of eosinophils in each nasal epithelium slides. Our study proved the correlation between the numbers of eosinophils in nasal mucosa and TSLP expression for the first time. This results gave the evidence that epithelial cells sourced TSLP may have a critical role as

chemotactic factor in eosinophils infiltration. While the relationship between TSLP and local eosinophils responses deserved to further study. Besides, roles of TSLP in survival and recruitment of mast cells in allergic airway inflammation is also interesting, which are needed to be further investigated in the future.

Mou et al<sup>13</sup> reported that there was overexpression of TSLP in nasal mucosa of AR patients who were allergic to common allergen, such as house dust mite, mold, and animal dander. But they did not report whether the patients recruited in their study were sensitized to single allergen or not. The allergen may have influence on the VAS scores. So in our study, we excluded the patients who were allergic to multiple allergens. Although in our study the specific IgE level did not correlate to the expression of TSLP, the specific IgE levels in patients were allergic to the single allergen which were independent and meaningful factors.

In conclusion, we report TSLP expression in nasal epithelial cells of patients with mugwort pollen sensitive-seasonal AR correlates with VAS score and the number of infiltrated eosinophils in nasal mucosa but not specific IgE level. The expression of TSLP in allergic nasal epithelial cells may reflect the severity of allergic rhinitis. TSLP is a potential biological marker of allergic rhinitis, which may have similar roles just as in allergic asthma.<sup>14</sup>

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