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Association of thymic stromal lymphopoietin gene polymorphisms with atopic status and pulmonary function in a Japanese adult asthmatic population

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Running title: **TSLP gene polymorphisms and pulmonary function in asthma**

Abstract

Single-nucleotide polymorphism (SNP) of thymic stromal lymphopoietin (TSLP) gene has been reported to have susceptibility to bronchial asthma. We analyzed the relationship between SNP of TSLP (rs2289276, rs3806933) and the atopic status, pulmonary function (PF) and airway hyperresponsiveness (AHR) in asthmatics among never smokers, ex-smokers and current smokers.

We recruited 302 asthmatics to the current study who visited the asthma out-patient clinic in Iwate Medical University Hospital from 2006 to 2012. Subjects were genotyped using rs2289276 and rs3806933 by 7500 Fast Real-Time PCR System. PF and AHR to methacholine were examined.

Genotyping of rs2289276 revealed 165 C/C, 115 C/T and 19 T/T genotypes among asthmatics. Atopic status and house dust mite (HDM) sensitization between C/C and

C/T+T/T was significantly different (Fisher's Exact test). There was a significant difference in FEV_1 % predicted between C/C and C/T+T/T in never smokers with asthma (p=0.015, Mann-Whitney U-test).

SNP (rs2289276) in TSLP was associated with atopic status and HDM sensitization in the present asthmatic population. It was also associated with FEV_1 % predicted in the never smokers of this population. These results suggested that TSLP was involved in the immune response induced by allergens and modification of immune reaction by smoking.

Key words: thymic stromal lymphopoietin (TSLP), Single-nucleotide polymorphism (SNP), atopic status, house dust mite (HDM) sensitization, smoking, asthma

Ⅰ. Introduction

Asthma is an inflammatory disorder of the conducting airways that has strong association with allergic sensitization. The disease is characterized by a polarized Th-2 (T-helper-2)-type T-cell response and the levels of IL-4, IL-5 and IL-13 are upregulated in the airways of patients with bronchial asthma [1)]. Genetic polymorphisms of these Th-2 cytokines have been reported to be associated with the occurrence of asthma and pulmonary functions in asthma [2)]. Recently, cytokines involved in innate immunity have been reported to play a key role in the pathogenesis of asthma [3, 4)].

Among these cytokines, thymic stromal lymphopoietin (TSLP) is thought to play critical roles in the pathogenesis of allergic diseases. TSLP is stored in keratiocytes and epithelial cells and is released by various stimuli including microorganisms, allergens,

chemicals, physical stimuli, etc. TSLP was initially thought to be a thymic epithelial cell-derived cytokine, but is now known to be produced by epithelial cells in the lungs, gut, skin, fibroblasts, ASM, endothelial cells, mast cells, macrophages, granulocytes and are revealed more recently, by dendritic cells (DC) [5-11]. The released TSLP molecules influenced dendritic cells to induce Th2 cells. TSLP plays a key role in Th2 type inflammation of skin in atopic dermatitis [12]. Harada et al reported that TSLP gene polymorphisms were associated with forced expiratory volume within one second % predicted (FEV_1 % pred.) in Japanese adult asthmatics [13]. They also reported that the single-nucleotide polymorphisms (SNP) of the TSLP gene, rs2289276 and rs3806933 are located in the promoter region of the TSLP gene, and influence promoter activity.

TSLP molecules are released from airway epithelial

cells by smoking stimuli and may induce allergic reaction among some population. Hizawa et al. reported that there was a significant difference in the pattern of allergen sensitization between never smokers and ex-smokers/current smokers [14].

In this regard, to evaluate the association between TSLP gene and pulmonary functions in asthma, it is important to estimate separately the genetic influence in smokers and ex-smokers/current smokers.

In the present study, we examined the effect of a polymorphism in TSLP (rs2289276) on the atopic status, house dust mite (HDM) sensitization, pulmonary functions, airway hyperresponsiveness (AHR) and asthma severity in a Japanese asthmatic population also evaluated for smoking history.

Ⅱ. Methods

1. Subjects

The asthmatic subjects were recruited from the outpatient clinic of the Division of Pulmonary Medicine, Allergy and Rheumatology, Department of Internal Medicine, Iwate Medical University School of Medicine. The patients had been treated for at least one year. The diagnosis of asthma was made and its severity was defined according to the Global Initiative for Asthma guidelines. All subjects provided written informed consent to participate in the study. The study protocol was approved by the Ethics Committee of Iwate Medical University School of Medicine.

Subjects were assessed for age, sex, age of asthma onset, history of smoking, spirometry, HDM sensitization and IgE concentration in the blood.

To estimate the asthma severity in the patients, we evaluated the therapeutic step of each patient according to the Japanese asthma guideline published in 2009 [15].

2. Analysis of polymorphism of the TSLP gene

DNA was isolated from lymphocytes using standard procedures. Subjects were genotyped using rs2289276 and rs3806933) (7500 Fast Real-Time PCR System, Applied Biosystems, Foster City, CA, USA).

3. Atopic status

Atopic status was estimated by determination of specific allergen sensitization by a prick test or specific serum IgE titer for allergens, total serum IgE concentration and comorbidity of allergic diseases, including allergic rhinitis and atopic dermatitis.

4. House dust mite (HDM) sensitization

HDM sensitization was judged by a prick test or specific serum IgE titer for dust mite allergens.

5. Smoking history

Nonsmokers were defined by a smoking history of fewer than 6 packs/year.

Current smokers were subjects who currently smoke and had a history of smoking more than 6 packs/year.

Ex-smokers had quit smoking more than one year and had a smoking history of more than 6 packs/year.

6. Pulmonary function

Spirometry was performed on the first visit in all subjects. VC, FVC, FEV_1, %VC, %FVC, $\%FEV_1$, $FEV_1\%$, $FEF_{50}\%$ and $FEF_{75}\%$ were evaluated by CHESTAC-8800 (Chest, Tokyo, Japan) and expressed as the % of predicted values.

7. **Airway Hyperresponsiveness (AHR)**

AHR to methacholine was measured by Astograph; Jupitor 21(Chest, Tokyo, Japan) as previously reported [22]. Briefly, AHR was tested by directly recording a the dose-response curve of Rrs (cmH2O/L/sec) during continuous inhalation of methacholine in two-fold incremental concentrations (49 to 25,000 g/ml) under tidal breathing from nebulizers with an output of 0.15 ml/minute [16]. The asthmatic condition of subjects was stable for 3 months at the time of the assessment. If bronchodilators were being used, their use was suspended 24 hours before methacholine inhalation. Briefly, after we recorded the baseline Rrs during inhalation of physiologic saline for 1 minute, the patients inhaled methacholine at 1-minute intervals, starting with the lowest concentration. The index of airway sensitivity that we adopted was D min, the cumulative dose of inhaled methacholine at the

inflection point where Rrs began to increase continuously. One unit of D min is equivalent to inhalation of 1 mg/ml methacholine for one minute.

8. Statistics

Comparisons of data among the three groups were performed using one-way ANOVA. All statistical analyses were performed using SigmaStat (Systat Software Inc., San Jose, CA). In post-hoc analysis, comparisons of the data of two groups were performed by Mann-Whitney U-test. The data are expressed as the mean ± SEM. A *P*-value of less than 0.05 was considered statistically significant. Comparison of the atopic status and HDM sensitization between the two genotype groups was analyzed using Fisher's Exact test.

Ⅲ. Results

1. Characteristics of the asthmatics in the present

study

We studied 302 asthmatics whose demographic data are shown in Table 1. They were 64.61 ± 1.21 year old (mean ± SEM) and 179 of the 302 were men.

59.8% of them were atopic and 47.6% were sensitized with house dust mites. Their asthma severity ranged from Step 1 (mild intermittent) to Step 4(severe)for the Japanese asthma guideline 2009 [15].

2. Distribution of TSLP gene polymorphism among asthmatics

In the distribution of SNP of TSLP (rs2289276), C/C was 56.6%, C/T was 37.7% and T/T was very low as 0.056%. In the case of other SNP of TSLP (rs3806933), the pattern of the distribution (C/C, C/T, T/T) was quite similar to that of rs2289276.

3. Association of TSLP polymorphism with atopic status

and pulmonary functions

The ratio of atopic asthmatics with C/T and T/T was statistically higher than that of those with C/C ($p<0.01$) (Figure 1, Table 3). The asthmatics with C/T and T/T showed a higher ratio of HDM sensitization compared to those with C/C (Figure 2). However there was no significance 1n the serum IgE concentration or the therapeutic steps corresponding to asthma severity among TSLP SNPs (rs2289276). TSLP SNP (rs2289276) was not associated with either airway hyperresponsiveness or pulmonary functions.

4. Association of TSLP SNP with pulmonary functions in never smokers with asthma

FEV_1 % predicted in asthmatics with C/T+T/T was significantly lower than that in those with C/C

Ⅳ. Discussion

The present study revealed that TSLP gene polymorphism (rs2289276) was associated with the atopic status and HDM sensitization in the current population of patients with bronchial asthma. In addition, there was a significant difference in FEV_1 % predicted in the asthmatics with never smoking history. These results suggested that TSLP was involved in immunologic reactions in the pathogenesis of asthma.

TSLP is a member of the 4-helix bundle cytokine family, and a distant paralog of IL-7 [17]. Recent evidence has accumulated suggestting that Th2-type CD4+ T cells play a triggering role in the activation and/or recruitment of IgE antibody-producing B cells, mast cells and eosinophils, in allergic inflammation. TSLP influences dendritic cells to prime naïve CD+ T cells into an inflammatory Th2 phenotype, producing

IL-4, IL-5 and IL-13 [18]. It has recently been shown that TSLP is also a potent growth and survival factor for Th2 effector cells [19]. In this regard, TSLP has been demonstrated to play a critical role in the atopic diseases of asthma, atopic dermatitis, and allergic rhinitis has been established [10, 18, 20]. The present study determind that SNP of TSLP was associated with the atopic status in the asthmatics, presumably due to complication with ofallergic diseases in which TSLP is involved.

Various studies have suggested a primary role for TSLP in the sensitization/priming stage of allergic airway disease. TSLP produced by activated human-derived lung cells stimulated human DCs to prime CD4+ TH2 cell development and mast cell production of TH2-associated cytokines [6, 21]. Iijima et al reported that SNP of TSLP was associated with HDM sensitization in the never smokers of 2 Japanese residential areas

with different circumstances in terms of pollen [14]. The present study found that the asthmatics with C/T+T/T in the TSLP SNP (rs2289276) were more likely sensitized with HDM than those with C/C. HDM is a candidate in-door allergen in Japan thought to influence the occurrence and severity of asthma in both children and adults. In this regard, information of TSLP gene polymorphism is thought to be very important to prevent the occurrence and advancing in severity of asthma.

A potential role for TSLP in airway inflammation was first suggested by the finding that TSLP mRNA was present in human lung fibroblasts and bronchial epithelial and smooth muscle cells [18]. The human TSLP gene is located on chromosome 5q22.1 next to the atopic cytokine cluster on 5q31 [22]. Harada et al reported that the promoter polymorphisms rs3806933 and rs2289276 in the TSLP gene were significantly associated with

disease susceptibility in both childhood atopic and adult asthma [13]. They found that these SNP were located in the promoter region of the TSLP gene and created a binding site for the transcription factor activating protein (AP)-1. In their report, the variant enhances AP-1 binding to the regulatory element, and increases the promoter--reporter activity of TSLP in response to polyinosinic- polycytidylic acid stimulation in normal human bronchial epithelium. According to their results, the subjects with C/T+T/T in the TSLP SNP (rs2289276) might produce more TSLP molecules in response to stimuli compared to those with C/C. They also reported that TSLP SNP (rs2289278) was correlated with pulmonary function irrespective of the smoking history. The present study demonstrated that there was a significant difference in FEV_1 % predicted in the asthmatics with never smoking history. Masuko et al. also reported that the FEV_1 in non-COPD and

nonasthmatic subjects was associated with TSLP genotypes [23]. Smoking itself is a strong factor in the reduction of pulmonary function in both healthy subjects and asthmatics [24]. In our data, by eliminating the impairment of pulmonary function by smoking, the association of the TSLP gene polymorphism with pulmonary function became more clear.

Bo passive smoking and active smoking have been reported to be involved in the occurrence, acute exacerbation and severity of asthma [15, 25]. Bouzigon et al. reported that the increased risk of early-onset of asthma by early-life exposure to environmental tobacco smoke was associated with 17q21 genetic variants. Cigarette smoking may induce the release of TSLP molecules from airway epithelial cells and the increased production of TSLP from airway smooth muscle cells [27]. When we try to understand the effects of smoking on the airway of asthmatics, 2 major effects

should be considered. One is the immunological effects *via* innate immunity including TSLP. The other is the toxic and destructive effects induced by chronic cigarette smoke-exposure as demonstrated by the emphysematous lesions often seen in COPD patients. We speculate that TSLP released by smoking may be associated with the occurrence and acute exacerbation of asthma by the augmentation of Th2-type allergic airway inflammation. However smoking does not always induce Th2-type allergic airway inflammation in healthy subjects, COPD patients and asthmatics. The effects of TSLP molecules on the airway seem to depend on whether the subject has a predisposing immunological response. This point needs to be elucidated to understand the action of TSLP molecules.

In conclusion, TSLP gene polymorphism (rs2289276) was associated with the atopic status and HDM sensitization in asthmatics. It was also associated

with FEV_1 % predicted in the never smokers of the asthmatics. These results suggest that TSLP is involved in the immune response induced by allergens and the modification of the immune reaction by smoking.

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Declaration of Interest

The authors report no conflicts of interest.

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Figure Legends

Fig. 1. TSLP gene polymorphism (rs2289276) influences atopic status of asthmatics. Closed columns: C/C, Open columns: C/T+T/T. Fisher Exact test: $p<0.01$

Fig. 2. TSLP gene polymorphism (rs2289276) influences HDM sensitization in the asthmatics. Closed columns: C/C, Open columns: C/T+T/T. Fisher Exact test: $p<0.01$

Fig. 3. TSLP polymophism associated with FEV1 % pred. in never smokers with asthma. Bars shows standard deviation.