—Original—

Preliminary Clinical Trial of Biomarkers to Predict Response to Sublingual Immunotherapy for Japanese Cedar Pollinosis

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Background: As part of the planning for a future multicenter study, this preliminary clinical trial used serum samples from patients to identify biomarkers for predicting the therapeutic effects of sublingual immunotherapy (SLIT) for Japanese cedar pollinosis (JCP).

Methods: This prospective study included patients undergoing SLIT for JCP at our hospital. All enrolled patients (N = 17) started SLIT between June and November of 2015. With informed consent from the patients, blood samples were obtained in January, March, and June of 2016, and patients completed the Japan rhino-conjunctivitis quality of life questionnaire (JRQLQ). On the basis of the JRQLQ results, the 6 patients with the best outcomes were included in the high-response group (HRG), and the 5 patients with the worst outcomes were included in the poor-response group (PRG). We then compared serum data between the two groups, to identify useful biomarkers.

Results: IL-12p70 and VEGF levels tended to be higher in the HRG than in the PRG in January, March, and June ($0.10 > p > 0.05$). In addition, the June IL-17 level was significantly higher ($p < 0.05$) in the HRG than in the PRG.

Conclusions: IL-12p70 and VEGF may be useful biomarkers for predicting the effects of SLIT. In addition, although IL-17 does not appear to be useful as a biomarker for evaluating treatment response at the start of SLIT, it may be useful as a biomarker after the beginning phase of SLIT.

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Key words: biomarkers, Japanese cedar, pollinosis, cytokine, sublingual immunotherapy

Introduction

Since 2014, sublingual immunotherapy (SLIT) has been covered by the national health insurance system in Japan as a treatment option for Japanese cedar pollinosis (JCP). A previous study reported that, even within the first year after the start of treatment, prominent remission was achieved in approximately 17% of patients who underwent SLIT for JCP[1]. Although complete remission may be achieved in some cases, some patients are nonresponders or poor responders. Treatment response is currently evaluated solely on the basis of clinical symptoms after the start of treatment. However, obvious effects may not be noticeable, even if treatment is prolonged, leading patients to discontinue treatment. Thus, an important concern with SLIT is the lack of an established biomarker for predicting the effects before the start of SLIT. There are also no useful biomarkers for evaluating treatment response after the start of SLIT.

Biomarkers have been examined in several studies that used cytobiological techniques at the laboratory level, e.g., by measuring the basophil activation marker CD203c level with flow cytometry[2] and apolipoprotein A by electrophoresis[3]. However, no simple method is available for clinical use, and measurement of serum cytokine levels has not been standardized for prediction of the effects of SLIT. To identify predictive biomarkers of re-

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Table 1 Patient characteristics of the high response and poor response groups

High response group				Poor response group			
Case number	Sex†	Age (years)	Positive antigens	Case number	Sex†	Age (years)	Positive antigens
1	F	40	Cedar, cypress	1	M	43	Cedar, cypress, Japanese alder, cat
2	F	72	Cedar, cypress, orchard grass	2	F	13	Cedar, cypress
3	M	44	Cedar, cypress, orchard grass	3	M	49	Cedar
4	F	41	Cedar, cypress	4	F	48	Cedar, cypress, dog
5	M	67	Cedar, cypress	5	F	50	Cedar, cypress
6	F	51	Cedar, cypress				
Average		52.5		Average		40.6	

F: female; M: male.

Table 2 Japan rhino-conjunctivitis quality of life questionnaire (JRQLQ) symptom scores

Nasal and eye symptoms	0: None	1: Mild	2: Moderate	3: Severe	4: Very severe
Runny nose	☐	☐	☐	☐	☐
Sneezing	☐	☐	☐	☐	☐
Stuffy nose	☐	☐	☐	☐	☐
Itchy nose	☐	☐	☐	☐	☐
Itchy eyes	☐	☐	☐	☐	☐
Watery eyes	☐	☐	☐	☐	☐

sponse to SLIT, we investigated the usefulness of serum variables by using blood samples obtained during routine outpatient care from patients undergoing SLIT for JCP.

Methods

Participants

Seventeen patients undergoing SLIT for JCP were enrolled in this study after written informed consent for a survey using the Japan Rhino-conjunctivitis Quality of Life Questionnaire (JRQLQ) and blood sampling was obtained from each patient. The patients started SLIT at Musashi Kosugi Hospital between June and November 2015. All patients had negative results for serum house dust mite (HDM) antibody, according to the AlaSTAT 3 g method (third generation; detection limit: 0.1 UA/mL; Siemens Healthineers, Tokyo, Japan) (**Table 1**).

Group Assignment

The participants were assigned to groups on the basis of JRQLQ scores obtained through inquiries before the start of treatment and during the first season (January, March, and June 2016) after the start of SLIT. For the JRQLQ symptom score, the total score for a group of 6 items—runny nose, sneezing, stuffy nose, itchy nose, itchy eyes, and watery eyes—was used (**Table 2**). Markedly severe symptoms were assigned 4 points, while the absence of a symptom was assigned 0 points; the maximum score was 24 points (4 points × 6 kinds of symptoms). Three scores were recorded, as follows.

Score 1: JRQLQ symptom score at the "time of symptom onset reaching a peak during the past 3 years", as described at the initial visit to our outpatient clinic.

Score 2: JRQLQ symptom score at the "time of symptom onset reaching a peak within 1 to 2 weeks", as described at the visits in January, March, and June.

Score 3: JRQLQ symptom score at the "time of symptom onset reaching a peak during this season", as described in June.

We assumed that the greater the difference between score 1 ("time of symptom onset reaching a peak during the past 3 years") and score 3 ("time of symptom onset reaching a peak during this season"), the greater the effectiveness of the treatment. We used the t-test, which is effective when the number of samples is small, to analyze variations in score differences. Furthermore, on the basis of the results, the patients were divided into 3 groups, and the upper and lower groups were compared. The 6 patients with the largest differences were assigned to the high-response group (HRG), the 5 patients with the smallest differences were assigned to the poor response group (PRG), and the remaining 6 patients were assigned to the intermediate group (**Fig. 1**). The 6 pa-

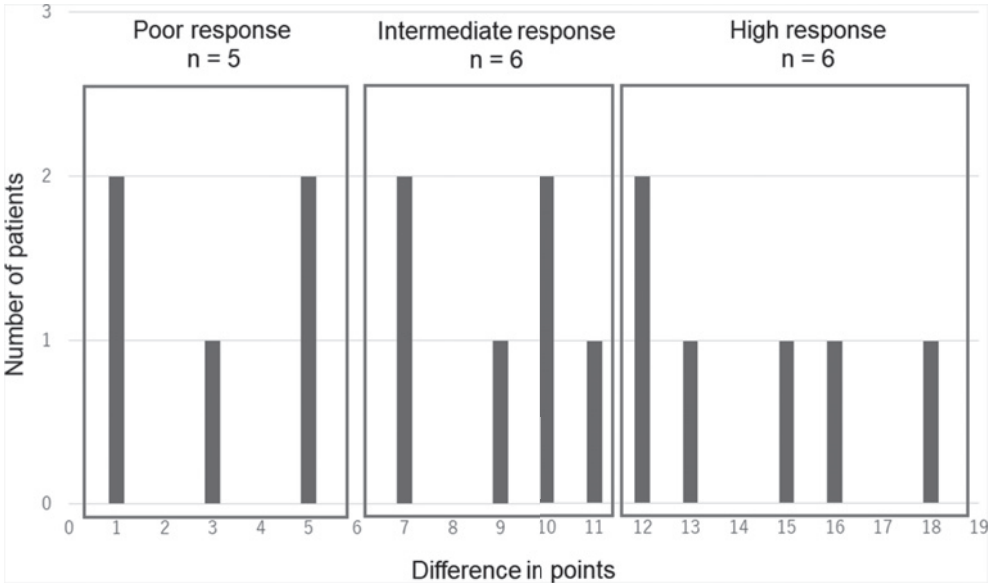

Fig. 1 Selection of patients for the high response and poor response groups. The t-test was used to analyze differences between the Japan Rhino-conjunctivitis Quality of Life Questionnaire (JRQLQ) symptom score at the "time of symptom onset reaching a peak during this season" (Score 3) and at the "time of symptom onset reaching a peak during the past 3 years" (Score 1) for 17 patients. The 6 patients with the largest differences were assigned to the high response group, and the 5 patients with the smallest differences were assigned to the poor response group.

tients in the HRG comprised 2 males and 4 females (mean age, 52.5 years; (range, 40-72 years), and the 5 patients in the PRG comprised 2 males and 3 females (mean age, 40.6 years; range, 13-50 years) (**Table 1**).

Cedar Pollen Dispersion During the Test Season

The amounts of cedar pollen dispersed in Ohta-ku, Tokyo (which is located close to our hospital), according to the nonprofit organization Pollen Information Association, Tokyo Metropolitan Institute of Public Health, were adopted as the references for total pollen dispersion in 2013 to 2016.

Blood Sampling

After blood samples were collected in January, March, and June, sera were promptly centrifuged (3,500 rpm, 5 minutes) and stored at −80°C. Cedar pollen allergen-specific immunoglobulin (Ig)G4/IgE antibody titers were measured by AlaSTAT 3 g (third generation; detection limit: 0.1 UA/mL; Siemens Healthineers). Levels of the cytokines interleukin (IL)-2, IL-5, IL-10, IL-12, IL-17, granulocyte macrophage colony-stimulating factor, interferon (IFN)-γ, vascular endothelial growth factor (VEGF), and transforming growth factor-β1 (TGF-β1) were measured by using the Beads Suspension Array Technique (BIO Plex 200, multiplex immunoassay with fluorescent microbeads; Bio-Rad Laboratories, Inc., Tokyo, Japan).

Ethical Considerations

Before the start of the study, the study protocol was approved by the Ethics Review Board of Nippon Medical School Musashi Kosugi Hospital (Approval No. 273-27-01). This study was carried out in accordance with the principles embodied in the Declaration of Helsinki of 1965 (as revised in Brazil 2013). All participants provided written informed consent, and patient anonymity was preserved by using methods approved by the Ethics Review Board.

Statistical Analysis

For quantitative comparisons of IgG4, IgE, and cytokine levels between the HRG and PRG, we used the 2-sample t-test with unequal variances. In the analysis of statistical differences, $p \geq 0.1$ indicated no difference, $0.1 > p \geq 0.05$ indicated a slight difference or a trend, and $p < 0.05$ indicated a significant difference.

Results

We compared the level of cedar pollen dispersion in 2016 (5,368 pollen grains/cm^2) to values for the 3 previous years (4,445 pollen grains/cm^2 in 2013, 5,479 pollen grains/cm^2 in 2014, and 4,310 pollen grains/cm^2 in 2015) and found no statistical difference between the values for the 4 years. The scores for HRG and PRG patients during the pollen season of 2016 were compared. There were no

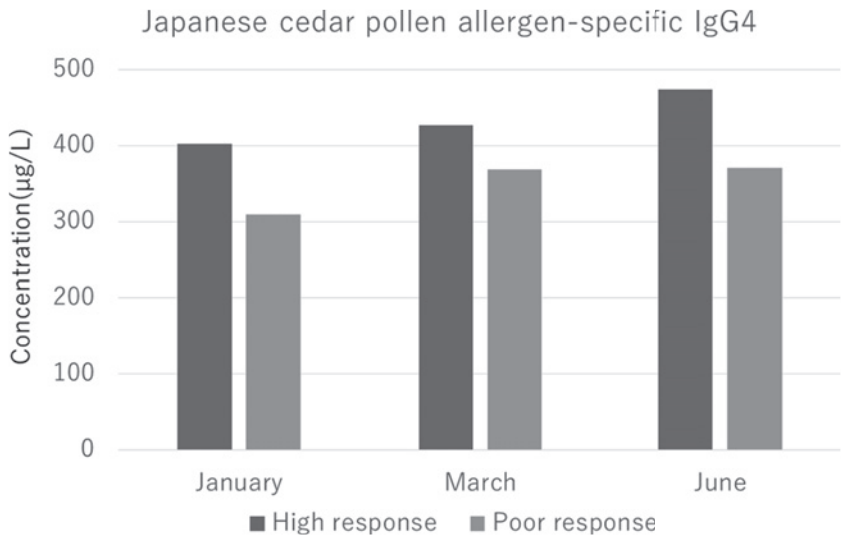

Fig. 2 Cedar pollen allergen–specific immunoglobulin (Ig)G4 levels (μg/L) in blood. There was no change in IgG4 level from January to June in either group (1-sided nonparametric t-test).

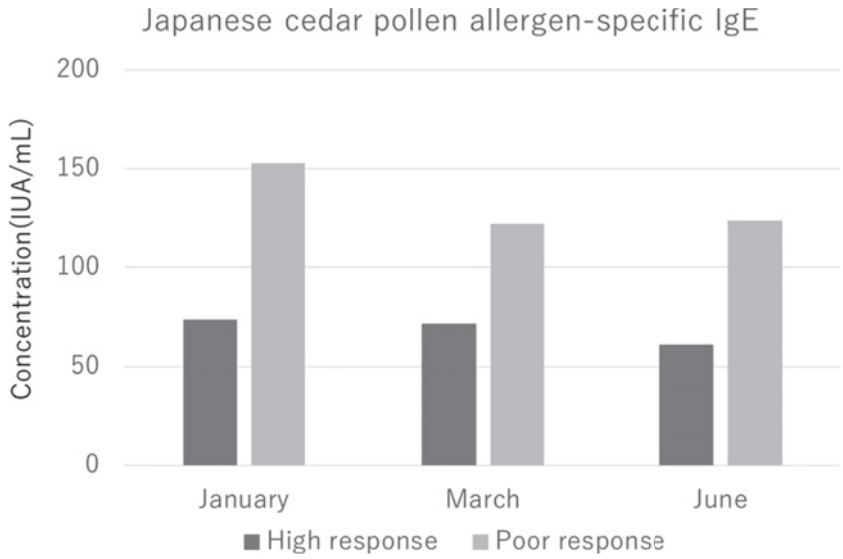

Fig. 3 Cedar pollen allergen–specific immunoglobulin (Ig)E levels (IUA/mL) in blood. There was no change in IgE level from January to June in either group (1-sided nonparametric t-test).

significant differences in symptom scores for the first season (Score 2) in January, March, or June between the 2 groups. However, there were significant differences in Scores 1 and 3 between the 2 groups ($p = 6.80 \times 10^{-6}$).

We also compared changes in serum IgG4 and IgE levels during the pollen season. Cedar pollen-specific IgG4 and IgE before treatment were not significantly different between the 2 groups. There was no change in IgG4 level from January to June in either group (**Fig. 2**) and no change in IgE level in either group (**Fig. 3**).

During the pollen season, levels of IL-2 and granulocyte macrophage colony-stimulating factor remained below the detection limits. IL-12p70 levels in January, March, and June were slightly higher in the HRG than in the PRG (January: $p = 0.07$; March and June: $p = 0.06$). IL-17 levels in June were significantly higher in the HRG than in the PRG ($p = 0.04$; **Fig. 4**); however, this tendency was not seen for IL-5, IL-10, or IFN-γ levels during the season (**Fig. 5**). VEGF levels in January, March, and June were slightly higher in the HRG than in the PRG (January: $p = 0.09$; March: $p = 0.06$; June: $p = 0.05$). This tendency was not seen for TGF-β1 level during the season (**Fig. 6**).

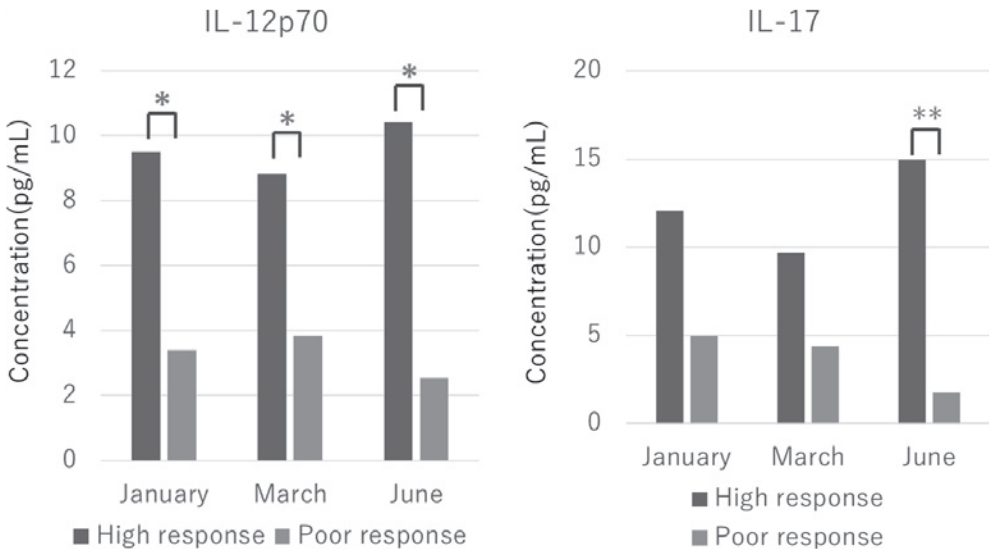

Fig. 4 Comparison of cytokine levels (interleukin [IL]-12p70 and IL-17) measured in January, March, and June of the first season. IL-12p70 levels in January, March, and June were slightly higher in the high-response group than in the poor-response group (January: p = 0.07; March and June: p = 0.06; 1-sided nonparametric t-test). IL-17 levels in June were significantly higher in the high-response group than in the poor-response group (p = 0.04; 1-sided nonparametric t-test) (*0.05 ≤ p<0.1; **p<0.05).

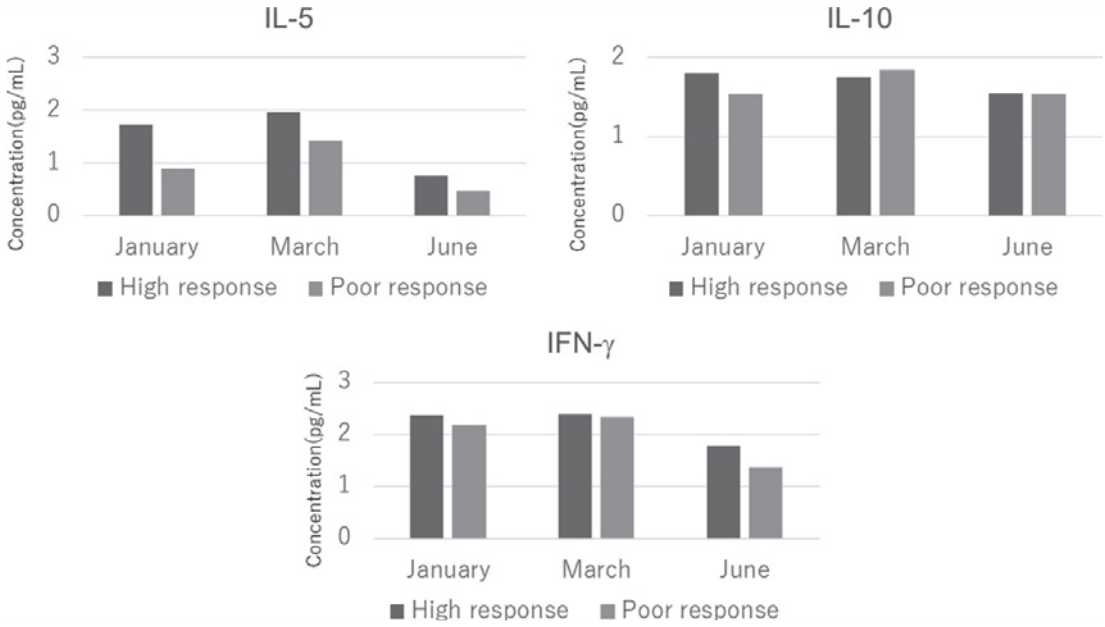

Fig. 5 Comparison of cytokine levels (interleukin [IL]-5, IL-10, and interferon [IFN]-γ) measured in January, March, and June of the first season. No trend was observed for any of the cytokines during the season (1-sided nonparametric t-test).

Discussion

Because the level of cedar pollen dispersion can markedly influence JCP symptoms and treatment responses, the level of dispersion must be confirmed every year. However, no significant yearly difference was observed in our data on cedar pollen dispersion for Ohta-ku, Tokyo, so yearly differences in dispersion levels were unlikely to have affected SLIT outcomes.

In this study, we used the JRQLQ to evaluate response to treatment. The JRQLQ is frequently used in Japan to evaluate subjective symptoms of allergic rhinitis. It comprises 3 evaluations: subjective symptoms, QOL score, and face scale, each of which is commonly used. In this study, we focused on symptom relief and thus used only

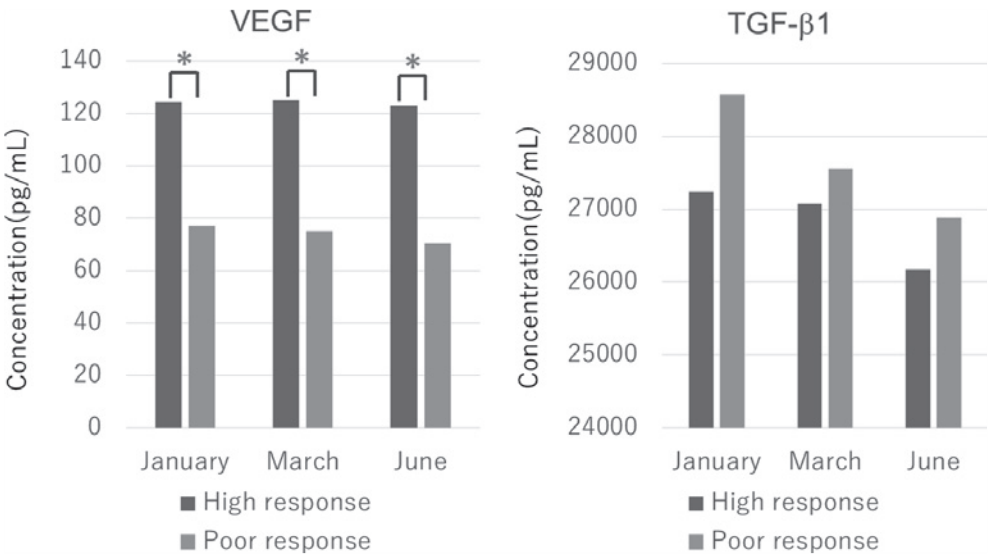

Fig. 6 Comparison of cytokine levels (vascular endothelial growth factor [VEGF] and transforming growth factor-β1 [TGF-β1]) measured in January, March, and June of the first season. VEGF levels in January, March, and June were slightly higher in the high-response group than in the poor-response group (January: $p = 0.09$; March: $p = 0.06$; June: $p = 0.05$; 1-sided nonparametric t-test). There was no such tendency in TGF-β1 level during the season (1-sided nonparametric t-test) (*$0.05 \leq p<0.1$; **$p<0.05$).

subjective symptoms. There are several other criteria that can be adopted to evaluate therapeutic response, e.g., a ≥ 30% reduction from the baseline visual analogue scale score[4], or a posttreatment improvement rating based on 5-grade assessments before treatment[5]. In this study, therapeutic response was evaluated not by objective physical findings but by symptoms in relation to patient satisfaction with the effects of SLIT. Restricting the evaluation to symptom score limits the objectivity of the estimates. However, the present evaluation was based on the degree of improvement in subjective symptoms, including satisfaction.

Although we expected a significant decrease in symptom scores in the HRG at the March inquiry, no such decrease was observed, perhaps because the difference in scores was assessed in this study, and because the effects of SLIT were more obvious in the second year than in the first year of treatment[6]. That is, in patients with severe symptoms and a high score before treatment, the score decreased along with relief of symptoms, which resulted in a marked difference in scores before and after treatment. However, in patients with mild symptoms and a low score before treatment, the scores could not decrease to negative values even when symptoms resolved; therefore, their smaller differences in scores may have been regarded as a limited response or no response. A patient in the PRG reported 22, 21, and 21 points for Scores 1, 2, and 3, respectively, whereas another such patient reported 5, 1, and 2 points, respectively. Thus, the scores varied greatly, and the number of patients was small, which may have led to the absence of significant differences between groups. In the future, only patients with severe symptoms before SLIT should be enrolled in this type of study.

IgG is an antibody that can bind numerous antigens. It is known to block the response mediated by IgE antibody against allergens, but its clinical significance remains to be clarified[7]. Regarding changes in cedar allergen-specific IgG4 level during SLIT, a comparative study of a drug and placebo found that IgG4 level in the drug-treated group increased after the start of treatment, transiently decreased after the pollen dispersion period, and then later increased again. In the placebo group, the changes were slight[8]. Another study reported that the association between change in IgG4 antibody titer and clinical symptoms was unclear[9]. Thus, no consensus has been reached. In the present study, we found no significant difference in IgG4 level. In the HRG, the increase in IgG4 level in June was greater than that in January in some, but not all, patients. Currently, it appears that IgG4 level cannot be used to predict the effect of treatment before the start of treatment or to assess treatment efficacy after the start of treatment. However, these findings require confirmation in a study with a longer follow-up period and a larger number of patients.

With respect to IgE, a previous study reported that

SLIT was more effective for patients with a higher cedar-specific/total IgE antibody ratio[10]. However, similar results were also obtained in the placebo group, and symptoms were milder in patients with a higher cedar-specific/total IgE antibody ratio. Thus, this variable does not appear to be useful for evaluating the effects of SLIT. In the present study, we did not compare cedar-specific IgE level with total IgE level, but there was no significant difference or an indication of tendency for a difference between the HRG and PRG; therefore, this variable appears to be unsuitable for predicting the effects or evaluating the efficacy of SLIT.

IL-12 induces differentiation of undifferentiated T cells to Th1 cells. A previous study suggested that IL-12-mediated Th1/Th2 balance control is a therapeutic strategy for allergic diseases[11]. Another study reported that increased IL-12p70 expression corrected the Th1/Th2 imbalance and inhibited allergic diseases[12]. In the present study, IL-12p70 level was always higher in the HRG than in the PRG. Another study similarly noted that pretreatment IL-12p70 level was high in patients with a strong response[13]. Thus, IL-12p70 may be a biomarker for predicting the therapeutic effects of SLIT and for evaluating its efficacy.

IL-17 is a cytokine with several functions, including neutrophil activation and fibrosis induction. Many studies have confirmed its involvement in autoimmunity and chronic inflammation. Li et al. reported that there were significant decreases in levels of plasma cytokines (IL-4 and IL-17) and complement components (C3a and C5a) 3 years after the start of SLIT for HDM allergy and that IL-17 was particularly useful for evaluating treatment response[14]. Sakashita et al. concluded that IL-17, C3a, and C5a levels after SLIT for JCP were lower than those at baseline in patients with a low symptom/medication score[15]. Makihara found a positive correlation between the number of IL-17-expressing cells in the nasal mucosa and symptom score, which suggests that IL-17 is involved in the pathogenesis of allergic rhinitis, especially in relation to symptom severity and eosinophilic inflammation[16]. Thus, many studies have investigated the usefulness of IL-17 as a biomarker for evaluating the therapeutic effects of SLIT. According to our study, serum IL-17 level was significantly higher in the HRG than in the PRG in June ($p = 0.04$). The physiological/pathological functions of IL-17, such as in neutrophil activation, suggest that its level would increase rather than decrease during SLIT. Although the significance of increases or decreases in IL-17 is unclear, it is considered to have at least some usefulness in the evaluation of treatment response after the start of treatment. In any case, IL-17 is believed to be a promising key cytokine marker for SLIT. Future analyses of much larger datasets from multicenter studies are necessary.

VEGF is a cytokine that functions in neovascularization and induction of vascular permeability by acting directly on vascular endothelial cells. VEGF levels were elevated in biological samples from the lower airway of asthma patients[17,18]. Regarding the upper airway, several studies have reported that VEGF levels in nasal discharge were significantly higher in patients with allergic rhinitis than in those with nonallergic rhinosinusitis[19,20]. In the present study, VEGF levels in January, March, and June tended to be higher in the HRG than in the PRG (January: $p = 0.09$; March: $p = 0.06$; June: $p = 0.05$). As VEGF level markedly increases during type I allergic responses, pure allergic inflammation, rather than neutrophilic responses, such as in bacterial infectious events, may have been more prevalent in the HRG than in the PRG, and patients with nonallergic inflammation, including infection, may have been present in the PRG. VEGF level, which tended to be higher before treatment in the HRG, may be appropriate for predicting the effects of SLIT and for evaluating its efficacy. In the future, a cut-off value for VEGF level should be investigated in a large study.

Shimada et al. measured cytokines in responders and nonresponders to sublingual immunotherapy[21]. Our results are mostly consistent with theirs, and any discrepancies are likely attributable to the different study designs.

In conclusion, the findings of this preliminary study, performed at Nippon Medical School Musashi Kosugi Hospital, indicate that IL-12p70 and/or VEGF might be useful for predicting the effects of SLIT. In the future, the usefulness and cut-off values of biomarkers, including IL-17, should be examined in a large-scale multicenter trial.

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Conflict of Interest: The authors declare no conflicts of interest.

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