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ORIGINAL ARTICLE

Upregulation of *IL17RB* during Natural Allergen Exposure in Patients with Seasonal Allergic Rhinitis

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ABSTRACT

Background: Seasonal allergic rhinitis (SAR) to Japanese cedar (*Cryptomeria japonica*; JC) is an IgE-mediated type I allergy affecting the nasal mucosa. However, the molecular mechanisms that underlie SAR are only partially understood. The aim of the study was to identify novel genes related to SAR during natural exposure to pollens, by using microarray analysis.

Methods: Subjects were 32 SAR patients and 25 controls. Total RNA was extracted from $CD4^+$ T cells isolated from peripheral blood mononuclear cells and subjected to microarray analysis with Illumina Human Ref8 BeadChip arrays. The Mann-Whitney test was performed to identify genes whose expression was altered during allergen exposure. Quantitative real-time reverse transcription-polymerase chain reaction (RT-PCR) was performed on samples collected from SAR patients and controls to verify the microarray results.

Results: Microarray analysis showed that the expression of 3 genes was significantly altered during allergen exposure. Among these 3 genes, the expression of interleukin 17 receptor beta (IL17RB) was confirmed to be upregulated in SAR patients compared to that of the *IL17RB* gene in healthy, non-allergic controls. The average fold change of *IL17RB* expression in the real-time RT-PCR experiment was 3.9 ($P = 0.003$).

Conclusions: The present study identified upregulation of *IL17RB* during natural allergen exposure in patients with SAR, which may further elucidate the molecular mechanisms underlying SAR.

KEY WORDS

allergen exposure, microarray, nasal mucosa, quantitative real-time RT-PCR, seasonal allergic rhinitis

INTRODUCTION

Allergic diseases such as asthma and allergic rhinitis are major causes of morbidity in developed countries, and their incidence is increasing. Seasonal allergic rhinitis (SAR) to Japanese cedar (*Cryptomeria japonica*) is an IgE-mediated type I allergy affecting the nasal mucosa. It is one of the most common allergic diseases in Japan, affecting 19.4% of the Japanese population,[1] and thus is a major public health issue. According to a national survey, the prevalence of rhinitis in Japan was 0.16 in 1992 and 0.21 in 2002.[2] We recently reported the prevalence of allergic rhinitis in an adult population of the Fukui area of Japan was 44.2% (681 of 1,540 subjects aged between 20 and 49 years), and the most common allergen in allergic rhinitis was Japanese cedar pollen (89.6%, 610 of 681 subjects with SAR).[3] SAR therefore contributes to the undermining of quality of life and decline in labor productivity.[4]

SAR is a chronic, inflammatory disease of the nasal mucosa caused by the infiltration of lymphocytes, mast cells, and eosinophils into the nasal mucosa. T-helper type 2 (Th-2) cytokines play a crucial role in orchestrating inflammatory responses. However, the molecular mechanisms that underlie SAR development are only partially understood. To understand the molecular basis of SAR, it would be helpful to examine the expression of genes in subjects with SAR during allergen exposure. Microarray techniques permit simultaneous analysis of the expression of many

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Table 1 Characteristics of the study population

	SAR (n = 32)	Control (n = 25)	P value
Age (year ± SD)	36.8±8.8 (25-50)	32.8±7.1 (18-47)	0.052
No. of Male/Female	16/16	12/13	0.334
Whole blood			
Neutrophil (%)	55.2 (range 39.5 to 76.0)	53.7 (range 36.3 to 76.0)	0.596
Lymphocytes (%)	33.7 (range 12 to 48.5)	37.4 (range 16.0 to 57.0)	0.154
Monotytes (%)	5.4 (range 3.3 to 8.0)	5.5 (range 3.6 to 7.3)	0.71
Eosinophils (%)	5.2 (range 0 to 18.0)	2.8 (range 0.3 to 7.3)	0.008
Basophils (%)	0.6 (range 0 to 1.5)	0.6 (range 0 to 1.8)	0.92
Total serum IgE (IU/mL)	50.1 (range 7 to 880)	28.4 (range 5 to 160)	0.049
JC-specific IgE (U_A/mL)	8.08 (range 0.79 to 86.1)	0.11 (range <0.34 to 0.56)	<0.00001

genes. Therefore, large-scale gene expression analysis by microarray may clarify which novel molecules are related to SAR.

Two types of tissues have been used for human microarray studies of allergic rhinitis. One is tissue from the nose, such as nasal polyps and nasal mucosa. Zhang *et al*.[5] performed microarray studies with nasal mucosa obtained from subjects with and without allergic rhinitis and found that several chemokine genes such as CC chemokine receptor (CCR) 2, CCR3, CCR5, CCR8, and CX3 chemokine receptor 1 were highly expressed in the nasal mucosa of subjects with allergic rhinitis compared to the expression of these genes in the mucosa of subjects without allergic rhinitis.

The other type of tissue is peripheral blood obtained from patients and controls. Larsson *et al*.[6] examined the transcriptional profiles of dendritic cells (DCs) after stimulation with grass pollen allergens and co-culture with autologous $CD4^+$ memory T cells. This study found a distinct T-cell-induced DC profile in atopic individuals, suggesting that T cells have a key instructive role in educating DCs in Th2-type responses. Benson *et al*.[7] performed microarray analysis using allergen-challenged $CD4^+$ T cells from patients with SAR and compared the expression level of $CD4^+$ T cells challenged with diluents only. This study found that tumor necrosis factor receptor superfamily member 4 (TNFRSF4), which is related to apoptosis, is significantly upregulated in allergen-challenged $CD4^+$ T cells. Allergic diseases are thought to be involved in the dysregulation of T cells, including $CD4^+$ lymphocytes. Therefore, examining changes in gene expression levels in $CD4^+$ T cells from SAR patients and healthy, non-allergic controls may improve our understanding of the molecular mechanism underlying SAR.

In the present study, we performed microarray analysis to identify changes in gene expression that reflect the status of SAR during natural allergen exposure and found that interleukin 17 receptor B (*IL17RB*) is upregulated during natural allergen exposure in SAR patients.

METHODS

SUBJECTS

Between 2003 and 2007, 1575 hospital workers and university students were invited to participate in an epidemiological survey of allergic rhinitis. All participants were of Japanese origin and were residents of Fukui Prefecture, Japan. The characteristics of the study population have been described in detail previously.[3] Total and specific IgE (produced in response to Japanese cedar, Dermatophagoides, *Dactylis glomerata*, *Ambrosia artemisiifolia*, *Candida albicans*, and *Aspergillus*) were measured using the CAP-RAST method (Pharmacia Diagnostics AB, Uppsala, Sweden).

We invited 56 of the 1575 survey subjects to participate in a gene expression analysis study and collected a 150-ml blood sample from each subject between February and April 2009, the time during which subjects were naturally exposed to Japanese cedar pollens. We also collected blood samples from the same individuals between November and December 2008, when they were not exposed to Japanese cedar pollens. Cases of SAR due to Japanese cedar polinosis (SAR group) were diagnosed on the basis of a positive history of rhinitis between February and April and high levels of Japanese cedar-specific IgE antibodies in the serum (RAST score $\geq$ class 2). We included only those SAR patients who were sensitized to Japanese cedar (i.e., no detectable allergen-specific IgE against dust mites, *D. glomerata*, *A. artemisiifolia*, *C. albicans* or *Aspergillus*; RAST score $\leq$ class 1). Subjects without allergies (control group) had to satisfy the following criteria: (1) no symptoms or history of allergic diseases, (2) no detectable, specific IgE antibodies against 6 common inhalant allergens (RAST score $\leq$ class 1), and (3) total serum IgE levels below the general population mean. The characteristics of subjects are listed in Table 1 and Table 2. The sample from one SAR patient (No. 19) was used only for realtime RT-PCR analysis because cRNA amplification for microarray experiment was not successful.

All participants provided written informed consent

Table 2 Treatment of SAR patients during natural pollen exposure

Sample No.	Age	Sex	Treatment
2	45	Female	Pranlukast Bepotastine besilate
3	41	Female	Epinastine hydrochloride
4	47	Female	Epinastine hydrochloride
11	27	Female	Olopatadine hydrochloride
12	30	Male	No treatment
14	25	Female	No treatment
15	28	Female	Fexofenadine hydrochloride
19	30	Male	Epinastine hydrochloride
23	30	Female	No treatment
24	47	Male	No treatment
25	27	Male	No treatment
30	50	Male	Olopatadine hydrochloride
32	49	Female	Epinastine hydrochloride
34	31	Male	No treatment
36	27	Male	No treatment
39	43	Male	Cetirizine hydrochloride
40	49	Male	No treatment
41	44	Female	No treatment
45	45	Male	Fexofenadine hydrochloride
53	45	Female	Epinastine hydrochloride
55	26	Female	Epinastine hydrochloride
56	28	Female	Epinastine hydrochloride
57	44	Male	Cetirizine hydrochloride
58	37	Male	Bepotastine besilate
59	25	Female	No treatment
60	25	Female	No treatment
65	44	Male	Epinastine hydrochloride
68	32	Male	Olopatadine hydrochloride
69	45	Female	Cetirizine hydrochloride
73	42	Female	No treatment
75	38	Male	Cetirizine hydrochloride
81	30	Male	Bepotastine besilate Olopatadine hydrochloride

to participate in the study. The study was approved by the ethical committees of the University of Tsukuba and the University of Fukui, Japan.

RNA EXTRACTION

Peripheral blood (150 ml) was taken from each subject. Peripheral blood mononuclear cells (PBMCs) were purified with Ficoll-Paque™ gradient (GE Healthcare, Piscataway, NJ, USA). $CD4^+$ T cells were isolated from PBMCs with a human CD4 Isolation kit (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany) according to the manufacturer's instructions. RNA was extracted from PBMCs with an RNeasy Mini Kit (Qiagen K.K., Tokyo, Japan) according to the manufacturer's instructions.

We used the Illumina BeadArray with single-color array (Illumina, San Diego, CA, USA) as a microarray platform. For the Illumina BeadArray assay, cRNA was synthesized with an Illumina® RNA Amplification Kit (Ambion, Austin, TX, USA) according to the manufacturer's instructions. In brief, 500 ng of total RNA from $CD4^+$ T cells were reverse transcribed to synthesize first- and second-strand cDNA, purified with spin columns, and then *in vitro* transcribed to synthesize biotin-labeled cRNA. A total of 750 ng biotin-labeled cRNA was hybridized to each Illumina Human Ref8 BeadChip array (Illumina) at 55℃ for 18 h. The hybridized BeadChip was washed and labeled with streptavidin-Cy3 (GE Healthcare) and then scanned with the Illumina BeadStation 500 System (Illumina). The scanned image was imported into BeadStudio software (Illumina) for analysis. Twenty-two thousand transcripts representing 8 whole-genome samples can be analyzed on a single Bead-

Table 3 Genes related to SAR identified by microarray

Gene Name	Description	Microarray		Real-time PCR		Accession †
		Fold change	*q*-value	Fold change	*P*-value	
ARID4B	AT rich interactive domain 4B	-1.6	0.049	1.1	0.204	NM_016374.5
SERPINE2	serpin peptidase inhibitor, member 2	-1.5	0.049	1	0.104	NM_006216.2
IL17RB	interleukin-17 receptor B	1.6	0.049	3.9	0.003	NM_018725.3

† GenBank accession numbers.

Chip. We included at least 1 technical replicate (i.e., the same cRNA sample) for each BeadChip. The correlation coefficients for identical RNAs were 0.995 to 0.996 (r^2) in the present study.

QUANTITATIVE REAL-TIME REVERSE TRANSCRIPTION-POLYMERASE CHAIN REACTION (RT-PCR)

$CD4^+$ T cells from the subjects in each group were purified by Ficoll-Paque™ gradient (GE Healthcare) and human CD4 T cell Isolation kit (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany). The purity of the isolated CD4 was over 98% in the present study. Total RNA was extracted from PBMCs with an RNeasy Kit (Qiagen). Quantitative real-time RT-PCR was performed with TaqMan Universal Master Mix and an Assay-on-Demand Gene Expression Kit (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's instructions. The endogenous control GAPDH was used to normalize the sample with the $\Delta\Delta C_T$ method for relative quantification with SDS software 2.2.0 (Applied Biosystems).

STATISTICAL METHODS

For the microarray analysis, background-corrected values for each probe on the BeadChip array were extracted using BeadStudio version 2.0 (Illumina). The detection limit corresponding to a 0.01 detection *p*-value was determined by using a normal model of intensities of 20 negative control probes that had no corresponding target in the sample.[8] The extracted values were exported to the software GeneSpring version 10 (Silicon Genetics, Redwood, CA, USA), and per chip and per gene normalizations were performed. The statistical significance of the microarray data was calculated using the Mann-Whitney test, and multiple tests were corrected by the Benjamini and Hochberg false discovery rate.[9]

Statistical significance of real-time RT-PCR was calculated by using the Wilcoxon signed rank-sum test (paired samples) and the Mann-Whitney U test (2 independent samples). In the paired samples, each sample from SAR patients and controls exposed to pollens was normalized to the sample from the same individual not exposed to pollens (sample-specific normalization). The correlation between normalized values of the microarray and those of the quantitative PCR experiments were performed with Spearman's rho test. Significance was defined as $P < 0.05$.

RESULTS

We first selected transcripts that were expressed by at least half (i.e., 28 samples) of the 56 samples on the Human Ref8 BeadChip arrays with detection *P* values < 0.01. Among the 10,477 expressed transcripts, those satisfying all the following criteria were selected as up- or downregulated transcripts in the microarray analysis: (1) more than 1.5-fold increase/decrease on average and (2) transcripts showing statistically significant differences between the SAR and control groups ($q < 0.05$).

A total of 4 transcripts were upregulated, and 15 transcripts were downregulated at the 1.5-fold level (19 transcripts total). The change in the expression of 3 (1/2 = up-/downregulated) transcripts was statistically significant ($q < 0.05$); 1 was up-regulated, and 2 were down-regulated. Genes that were up- and down-regulated in $CD4^+$ T cells between the SAR and control groups are listed in Table 3.

We then performed quantitative real-time RT-PCR to verify the results of the microarray analyses. Significant correlations between the microarray results and the results of the quantitative real-time RT-PCR were observed for *IL17RB* (Spearman's rho = 0.815, $P < 0.0001$) and *SERPINE2* (Spearman's rho = 0.877, $P < 0.0001$). However, no correlation was observed for *ARID4B* (Spearman's rho = -0.063, $P = 0.58$). The average fold change for *IL17RB* in the real-time RT-PCR experiment was 3.9 ($P = 0.003$, Table 3). Although good correlation was observed between the microarray results and the results of the quantitative real-time RT-PCR for *SERPINE2*, the average fold change for *SERPINE2* in the real-time PCR experiment was 1.0 ($P = 0.10$, Table 3), because of the existence of outliers.

In the quantitative real-time RT-PCR results of paired (exposed versus non-exposed) samples, *IL17RB* expression was elevated during natural pollen exposure in SAR patients but not in the controls (Fig. 1). *IL17RB* expression in SAR patients did not differ from that in controls when they were not exposed to cedar pollens ($P = 0.93$).

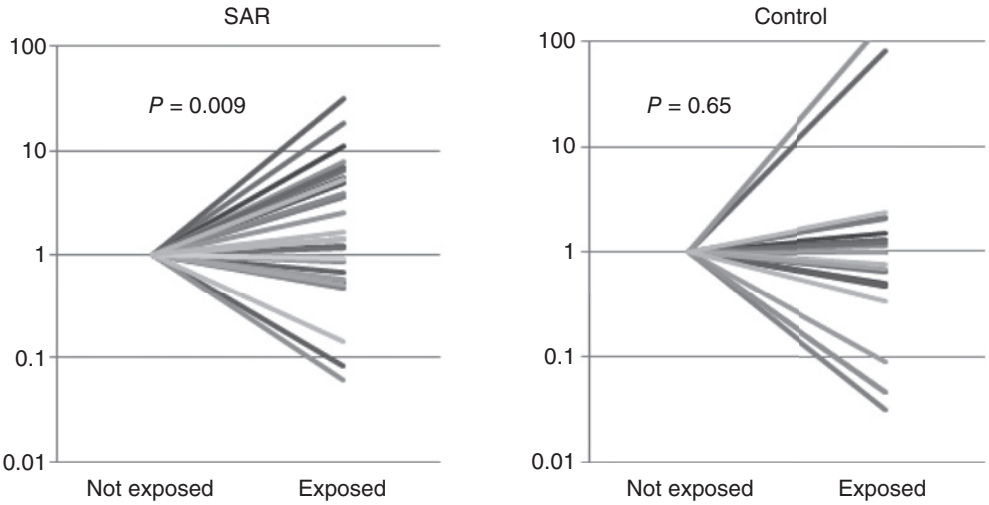

Fig. 1 Quantitative real-time RT-PCR validation of microarray data. Each sample from SAR patients and control individuals exposed to pollens was normalized to the sample from the same individual not exposed to pollens (sample-specific normalization). *P* values were calculated with Wilcoxon signed rank-sum test.

DISCUSSION

In the present study, we performed microarray analyses to identify genes related to SAR and identified 3 up- or downregulated genes related to SAR. Among these 3 genes, *IL17RB* was confirmed to be upregulated in real-time quantitative PCR analysis.

In our study, the number of genes that were significantly altered was small compared with those in other microarray studies, which used $CD4^+$ cells stimulated with allergens. One possibility for this difference is that gene expression change during natural allergen exposure in vivo is more subtle. Hansel *et al*.[10] performed microarray analyses using $CD4^+$ T cells from 84 subjects and did not find a dominant allergy-associated profile in $CD4^+$ T cells between allergic and non-allergic subjects. Because distinct $CD4^+$ T-cell-induced DC profiles were reported in atopic individuals,[6] changes in $CD4^+$ T cell profiles are likely to influence subsequent allergic responses, leading to the development of SAR.

Microarray experiments are now widely used to simultaneously analyze the expression of tens of thousands of genes. Quantitative real-time PCR is a commonly used method for validating microarray experiments. However, microarray and quantitative real-time PCR results sometimes disagree. In general, it has been reported that correlations increase with increasing degrees of change.[11] Dallas and colleagues reported poorer correlations between microarray expression scores for genes that exhibited fold-change differences of <1.5 compared with fold-change differences of >1.5.[12] We observed poor correlation for *ARID4B*. The poor correlations may be due, in part, to the existence of alternative, cross-hybridizing transcripts differentially recognized by the oligonucleotide probe sets and qRT-PCR probes, because several splice variants exist in *ARID4B*.

IL-17RB is the receptor for IL-17B and IL-17E (also known as IL-25).[13,14] IL-25 has been shown to induce Th-2 responses, and recent studies revealed that IL-17RB was highly expressed on a subset of naive and activated $CD4^+$ invariant natural killer (NK) T cells, but not on activated T cells and that IL-17RB$^+$ invariant NKT cells produced large amounts of Th-2 cytokines that were substantially increased by IL-25 stimulation.[15,16] It has also been reported that *IL17RB* knockout mice did not exhibit histological signs of lung inflammation, while marked infiltration of inflammatory cells were observed in wild-type mice.[17] Recently, Wang *et al*. performed microarray experiments using PBMCs stimulated with allergens or diluents in vitro, and reported that *IL17RB* was the most significantly upregulated gene on allergen stimulation in SAR patients when compared with that in controls. They also observed a significant increase in *IL17RB* gene expression from microarray data of allergen-challenged CD4 cells from SAR patients as compared to diluent-challenged cells. This is in agreement with our findings, which reveal upregulation of *IL17RB* in SAR patients naturally exposed to pollens, although the degree of changes in gene expression that we observed was lower than that in the study by Wang *et al*. Therefore, it is speculated that the differential gene expression pattern of *IL17RB* in SAR patients during allergen exposure may be related to the development of SAR.

In conclusion, the present study identified upregulation of *IL17RB* during natural allergen exposure in patients with SAR. Because the blockade of

IL-17RB has been reported to prevent IL-25-induced lung inflammation and Th-2 type cytokine secretion,[17] antagonists for IL-17RB could be a novel therapeutic target for allergic diseases.

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