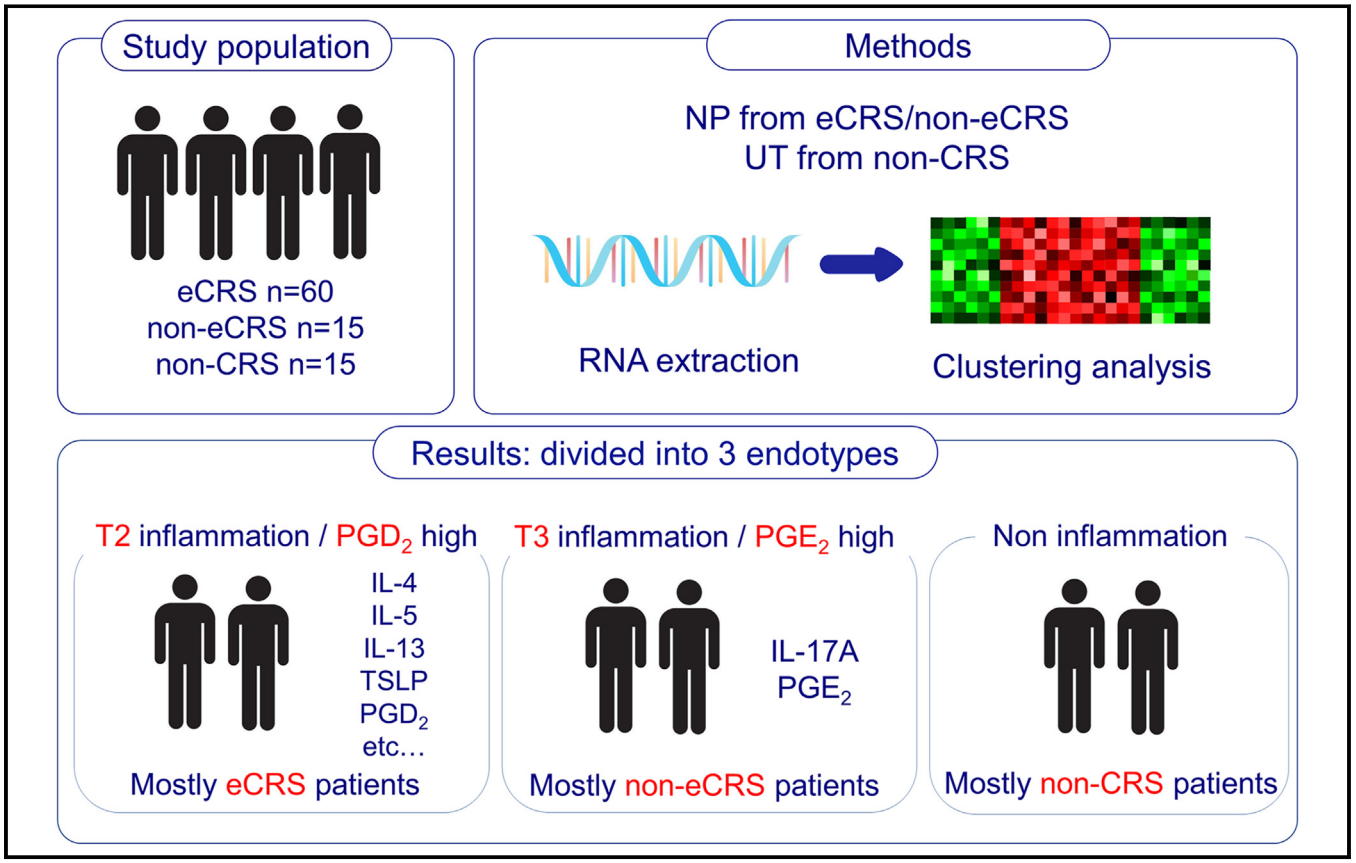
Macroarray expression analysis of cytokines and prostaglandin metabolism–related genes in chronic rhinosinusitis

Aiko Oka, MD,[a] Kengo Kanai, MD,[a] Takaya Higaki, MD,[c] Seiichiro Makihara, MD,[c] Yohei Noda, MD,[c] Shin Kariya, MD,[d] Mizuo Ando, MD,[c] Wataru Nishimura, MD,[b] and Mitsuhiro Okano, MD[a] *Narita and Okayama, Japan*

GRAPHICAL ABSTRACT



Background: Chronic rhinosinusitis (CRS) can be divided into endotypes by functional or pathophysiologic findings.
Objective: The aim of this study was to analyze the expression of cytokines, prostaglandin (PG) synthases, and their receptors related to the pathogenesis of CRS, especially those contributing to nasal polyp (NP) formation.
Methods: NPs and uncinate tissue (UT) samples were collected from 90 patients who underwent endoscopic sinus surgery. They included 75 patients with CRS (including 45 with eosinophilic CRS [eCRS] and 30 with non-eCRS) and 15 patients without CRS. A total of 30 genes were selected for our original DNA array plate to analyze the levels of expression of 10 cytokines (IFN-γ, IL-4, IL-5, IL-10, IL-13, IL-17A, IL-22, IL-25, IL-33, and TSLP), 4 prostaglandin synthases (prostaglandin D_2 [PGD_2] synthase, prostaglandin E_2 synthase, COX-1, and COX-2), and their 16 receptors. Clustering analysis was performed according to the expression results, and clinical findings of patients from each cluster were investigated.

From the [a]Department of Otorhinolaryngology and [b]the Department of Molecular Biology, International University of Health and Welfare School of Medicine, Narita; [c]the Department of Otolaryngology, Head and Neck Surgery, Okayama University Graduate School of Medicine, Dentistry and Pharmaceutical Sciences, Okayama; and [d]Department of Otolaryngology-Head and Neck Surgery, Kawasaki Medical School, Okayama.

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Corresponding author: Aiko Oka, MD, 852 Hatakeda, Narita, Chiba Prefecture 286-8520, Japan. E-mail: 19m3019@g.iuhw.ac.jp.

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Results: The samples could be divided into 3 clusters. Cluster 1 showed elevated levels of expression of *IL4, IL5, IL13, TSLP, IL1RL1* (ST2 [an IL-33 receptor]), *HPGDS*, and *GPR44* (CRTH2, a PGD_2 receptor); cluster 2 showed elevated levels of expression of *IL17A* and *PTGES*; and cluster 3 showed an elevated level of expression of *IL25*. Regarding clinical features, the main characteristics of each cluster were as follows: NPs from patients with eCRS for cluster 1, NPs and/or UT samples from patients with non-eCRS for cluster 2, and UTs from patients with non-CRS for cluster 3.
Conclusion: The results suggest that there are associations between type 2 inflammation/PGD_2 and eCRS and also between type 3 inflammation/prostaglandin E_2 and non-eCRS. (J Allergy Clin Immunol Global 2023;2:100123.)

Key words: *Chronic rhinosinusitis, endotyping, prostaglandin, cytokine, receptor, clustering analysis, gene expression, personalized medicine*

Abbreviations used

CRS: Chronic rhinosinusitis
CRSsNP: Chronic rhinosinusitis without nasal polyps
CRSwNP: Chronic rhinosinusitis with nasal polyps
CT: Computed tomography
eCRS: Eosinophilic chronic rhinosinusitis
h-PGDS: Hematopoietic prostoglandin D synthase
HR: Hazard ratio
ILC: Innate lymphoid cell
JESREC: Japanese Epidemiological Survey of Refractory Eosinophilic Chronic Rhinosinusitis
m-PGES-1: Microsomal prostaglandin E synthease-1
NP: Nasal polyp
PGD_2: Prostaglandin D_2
PGE_2: Prostaglandin E_2
UT: Uncinate tissue

Chronic rhinosinusitis (CRS) is a chronic inflammatory disease defined by the presence of nasal symptoms such as nasal discharge, nasal obstruction, loss of smell, and facial pain for at least 12 weeks. CRS can be divided into phenotypes by clinical findings and into endotypes by functional or pathophysiologic findings. Patients experiencing the same phenotype of CRS (eg, CRS with nasal polyps [CRSwNP]) often show different clinical courses and responses to treatments such as systemic corticosteroids, surgery, and biologic drugs, and these differences are likely caused by differences in endotype. Nasal polyp (NP) formation is affected by several mechanisms, including type 2 and/or epithelial-derived cytokines,[1] lipid mediators such as prostaglandins (PGs) and leukotrienes,[2,3] and dysregulation of the coagulation system.[4,5] Clustering analysis is currently used to classify CRS endotypes; for example, olfactory dysfunction in CRS is reported to be associated with inflammatory cytokines from the olfactory mucus, such as IL-5 and IL-13,[6] and NP formation and asthma as a comorbidity in CRS are reported to be associated with IL-5/*Staphylococcus aureus* enterotoxin B–specific IgE positivity in nasal tissues.[7] Although cytokines have been investigated in those reports, the roles of cytokine receptors, lipid mediators, and their receptors are not fully understood. Our previous studies suggest that prostaglandin D_2 (PGD_2) is positively associated with tissue remodeling and eosinophilic inflammation in CRS, whereas prostaglandin E_2 (PGE_2) is negatively associated with them.[8-10] Another report has shown that levels of cyclooxygenase-2 (COX-2) and PGE_2 are decreased in NP tissues.[3] In addition, the PGD_2/PGE_2 ratio has been reported to be high in patients with CRS with aspirin-intolerant asthma.[10,11]

In the present study, we investigated the levels of gene expression of cytokines and synthases for PGD_2/PGE_2 together with their receptors (especially those contributing to NP formation) in sinonasal tissues from patients with and without CRS and classified the samples using clustering analysis. As many genes are associated with NP formation, we prepared an original DNA array plate coated with 30 selected gene DNAs. The results provide insight for clear classification of CRS endotypes and also contribute to the clinical use of gene expression for CRS diagnosis.

METHODS

Study population

Patients with CRS having an indication for and undergoing endoscopic sinus surgery at Okayama University were enrolled. CRS was divided into CRSwNP and CRS without NPs (CRSsNP) according to the criteria established by the European Position Paper on Rhinosinusitis and Nasal Polyps 2020 (EPOS 2020).[12] CRSwNP was further divided into the categories noneosinophilic CRS (non-eCRS), mild eosinophilic CRS (eCRS), moderate eCRS, and severe eCRS based on the Japanese Epidemiological Survey of Refractory Eosinophilic Chronic Rhinosinusitis (JESREC) criteria.[13] In accordance with these criteria, we checked the eosinophil counts of NPs in 3 different hpfs) of the densest areas with cellular infiltrate beneath the epithelial surface; an average count higher than 70/hpf was defined as eCRS. Fifteen patients were enrolled for each group. All patients with CRSsNP were classified as belonging to the non-eCRS group according to the JESREC criteria. NP samples were collected from patients with CRSwNP, and uncinate tissue (UT) samples were collected from patients with CRSsNP, respectively. A total of 15 control (non-CRS) patients (n = 15) who underwent sinus surgery for paranasal sinus cyst, sinonasal tumors, and blowout fractures were enrolled, and samples of their UT were collected. Before surgery, the serum level of total IgE, blood eosinophil rate, and ratio of FEV_1 value to forced vital capacity were determined for each patient with CRS. Tissue samples were collected during the surgery and stored in RNAprotect Tissue Reagent (Qiagen, Hilden, Germany) at −80°C within 3 hours after the surgery until use. The characteristics of the subjects in each group are shown in Table I. Postoperative recurrence was defined as the occurrence of NPs for more than 28 days after the surgery according to nasal endoscopy performed by an otorhinolaryngologist. No patients had used systemic glucocorticoids for at least 1 month before surgery. All patients underwent computed tomography (CT) examination within 1 month before surgery, and the radiologic severity of their CRS was graded by using the Lund-Mackay CT scoring system.[14] Informed consent was obtained from each of the patients. The ethics committee of Okayama University Graduate School of Medicine approved this research and study protocol (approval no. 1505-030).

Measurement of gene expression for cytokines, PGs, and receptors

Total RNA was extracted from tissue samples (NP or UT) by using an RNeasy Mini Kit (Qiagen) and purified with DNase

TABLE I. Characteristics of the subjects in the non-eCRS, mild eCRS, moderate eCRS, severe eCRS, CRSsNP, and non-CRS groups

Group	Non-eCRS	Mild eCRS	Moderate eCRS	Severe eCRS	CRSsNP	Non-CRS	*P* value
Group size (no.)	15	15	15	15	15	15	
Age (y), median (IQR)	51.7 (22-75)	60.3 (37-74)	59.1 (28-74)	57.7 (43-73)	56.3 (15-75)	56.7 (34-74)	.823
Male sex/female sex (no.)	3/12	0/15	3/12	3/12	9/16	5/10	.006
Tissue eosinophil count (no.)	8.5	186.6	309.5	308.0	5.8	0.0	<.001
Blood eosinophil rate, median (IQR)	1.98 (0.7-5.4)	4.43 (3.0-7.4)	6.73 (5.0-9.9)	7.81 (2.6-12.3)	2.53 (0-10.8)	3.1 (0.6-5.5)	<.001
Serum total IgE level, median (IQR)	394.9 (9-2040)	317.2 (23-1274)	166.8 (2-456)	476.7 (35-2589)	148.3 (3-539)	220.5	.033
FEV_1/FVC ratio (%), median (IQR)	79.2 (59.4-99.2)	74.7 (51.8-83.6)	76.9 (62.8-95.3)	73.4 (53.9-95.0)	81.3 (67.7-95.4)	75.6 (61.6-85.5)	.26
Comorbidity of asthma (no.)	0	0	2	15	0	0	<.001
AERD (no.)	0	0	0	2	0	0	.069
Postoperative recurrence (no.)	0	0	3	6	0	0	.0003
CT score, median (IQR)	13 (10-16)	11 (10-14)	13 (11-17)	17 (12-22)	6 (5-10)	1.5 (1-2)	<.001

FVC, Forced vital capacity; *IQR*, interquartile range.
Differences between groups were assessed by using the Kruskal-Wallis test followed by the Dunn test for multiple comparisons or chi-square analysis.

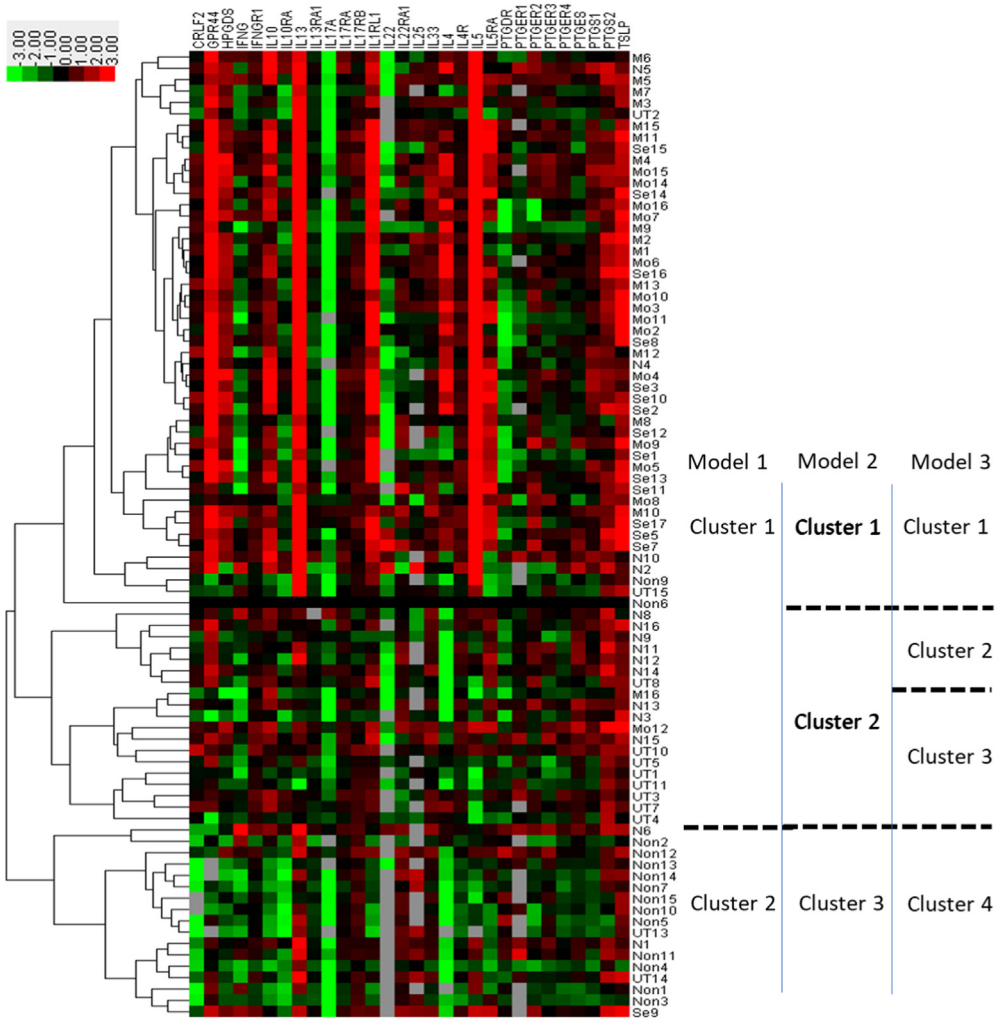

FIG 1. Heatmap of 30 expressed genes with a dendrogram. Hierarchic cluster analysis identified 2 to 4 potential clusters (models 1 to 3) based on gene expression signatures.

according to the manufacturer's protocol. cDNA was synthesized by using an iScript Advanced cDNA Synthesis kit for RT-qPCR (Bio-Rad, Hercules, Calif) as per the manufacturer's instructions. Quantitative PCR (qPCR) was performed using a Bio-Rad CFX96 real-time PCR detection system (Bio-Rad) for 40 cycles at 95°C for 5 seconds and 60°C for 30 seconds. SsoAdvanced Universal SYBR Green Supermix (Bio-Rad) was used for the PCR assays. The PrimePCR Realtime PCR Assay (Bio-Rad) was used as a primer for control assays. The comparative Ct ($\Delta\Delta$Ct) method was used for data normalization. Glyceraldehyde-3-

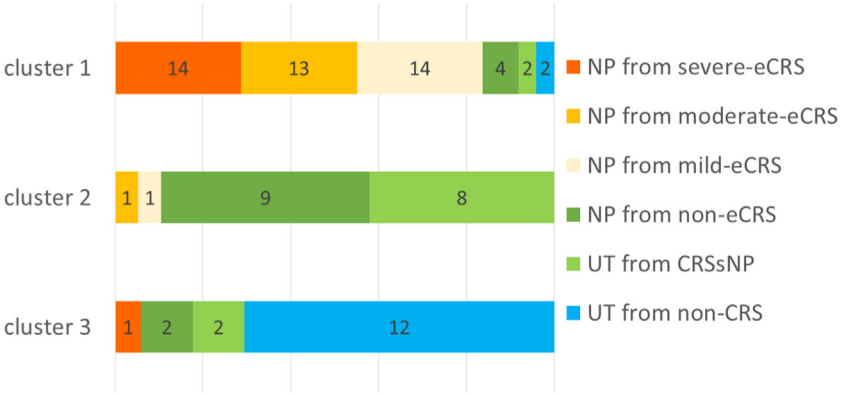

FIG 2. Proportion of sinonasal samples in cluster 1, cluster 2, and cluster 3 in model 2. Sinonasal samples include NPs from patients with severe eCRS, NPs from patients with moderate eCRS, NPs from patients with mild CRS, NPs from patients with non-eCRS, UT samples from patients with CRSsNP, and UT samples from patients with non-CRS.

phosphate dehydrogenase (GAPDH) was used as an internal control, and the control patient with the lowest contamination of genomic DNA was used to obtain the control sample. A total of 5 samples (1 from a patient with moderate eCRS, 3 from patients with CRSsNP, and 1 from the control patient) were removed after control assays for RNA quality.

In total, 30 genes were measured. The genes for cytokines were *IFNG, IL10, IL13, IL17A, IL22, IL25, IL33, IL4, IL5*, and *TSLP*. The genes for cytokine receptors were *CRLF2* (TSLP receptor), *IFNGR1, IL10RA, IL13RA1, IL17RA, IL17RB* (IL-25 receptor), *IL1RL1* (ST2 [IL-33 receptor]), *IL22RA1, IL4R*, and *IL5RA*. The genes for PGD_2 and PGE_2 synthases were *HPGDS* (hematopoietic prostaglandin D synthase [h-PGDS] and PGD_2 synthase), *PTGES* (microsomal prostaglandin E synthase-1 [m-PGES-1] and PGE_2 synthase), *PTGS1* (COX-1), and *PTGS2* (COX-2). The genes for PGD_2 and PGE_2 receptors were *GPR44* (CRTH2 [a PGD_2 receptor]), *PTGDR* (DP), *PTGER1* (EP1), *PTGER2* (EP2), *PTGER3* (EP3), and *PTGER4* (EP4). The mRNA data were analyzed for data summarization and normalization by using CFX Maestro Software 2.0 (Bio-Rad). The data were log_2-transformed and median-centered by genes using the Adjust Data function of CLUSTER 3.0 software and then further analyzed by using hierarchic clustering with average linkage. Finally, we performed tree visualization by using Java TreeView software.

Statistical analysis

Values are given as medians with interquartile ranges. The chi-square test was used for independence between the groups. For multiple comparisons, we used the Kruskal-Wallis test followed by the Dunn test. Statistical analyses were performed with GraphPad Prism 6 software (GraphPad Software, La Jolla, Calif). *P* values less than .05 (2 tailed) were considered statistically significant.

RESULTS

Gene expression in tissue samples divided by cluster analysis

We used hierarchic cluster analysis incorporating the 30 genes to identify potential subendotypes of CRS. The analysis identified 2 to 4 potential clusters (models 1 to 3) based entirely on gene expression signatures (Fig 1).

When the samples were divided into 2 clusters mechanically by computer-based cluster analysis, cluster 1 (n = 68) mostly contained samples of NPs and UT from patients with CRS (see Fig E1 in the Online Repository at www.jaci-global.org), and cluster 2 (n = 17) mostly contained samples of UT from patients with non-CRS (see Fig E1). Cluster 1 had higher levels expression of the genes *IL4, IL5, IL10, IL13, TSLP, IL4R, IL5RA, IL10RA, CRLF2, IL1RL1, HPGDS, GPR44*, and *PTGS1* than cluster 2 did. Cluster 2 had higher levels of expression of the genes *IL25* and *PTGDR* than in cluster 1 (Fig E2).

When the samples were divided into 3 clusters, the largest grouping (cluster 1 [n = 49]) mostly contained NPs from patients with eCRS, cluster 2 (n = 19) mostly contained NPs from patients with non-eCRS and UT samples from patients with CRSsNP, and cluster 3 (n = 17) mostly contained UT from patients with non-CRS (Fig 2). Cluster 1 carried a type 2–high signature, with elevated levels of expression of the genes *IL4* ($P < .0001$), *IL5* ($P < .0001$), *IL13* ($P < .0001$), *TSLP* ($P = .0002$), *IL1RL1* ($P < .0001$), *HPGDS* ($P < .0001$), and *GPR44* ($P < .0001$) compared with the levels in the other clusters. Cluster 2 had higher levels of expression of the genes *IL17A* ($P = .0008$) and *PTGES* ($P < .0001$) than in the other clusters. Cluster 3 had higher expression of the gene *IL25* ($P = .0022$) than in the other clusters. The levels of expression of the genes *IL4R* ($P < .0001$), *IL10RA* ($P < .0001$), *CRLF2* ($P < .0001$), and *PTGS1* ($P < .0001$) were higher in clusters 1 and 2 than in cluster 3. The levels of expression of the gene *IL10* were significantly different in each cluster, with the highest level in cluster 1 and the lowest in cluster 2 ($P < .0001$) (Fig 3 and Table II).

When the samples were divided into 4 clusters, the largest grouping (cluster 1 [n = 49]) mostly contained NPs from patients with eCRS, cluster 2 (n = 7) mostly contained NPs from patients with non-eCRS, cluster 3 (n = 12) mostly contained UT from patients with CRSsNP, and cluster 4 (n = 17) mostly contained UT from patients with non-CRS (see Figs E3 and E4 in the Online Repository at www.jaci-global.org). Cluster 1 had higher levels of expression of the genes *IL4, IL5, IL13, TSLP, IL5RA, IL1RL1, HPGDS*, and *GPR44* than in the other clusters. Cluster 2 had a higher level of expression of the gene *IL17A* than in the other

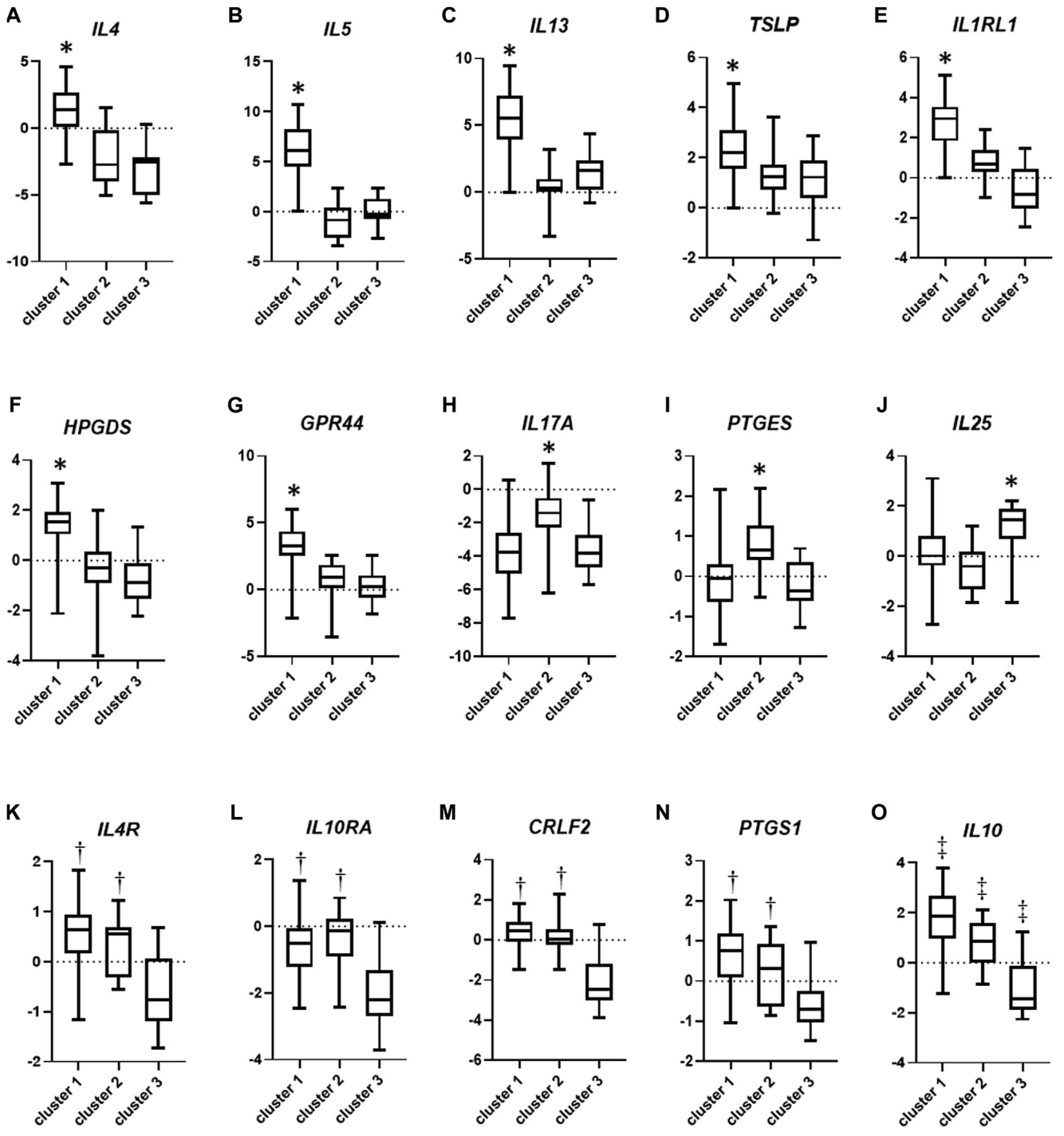

FIGURE 3. Genes showing a significant difference in the amount of expression between the 3 clusters. **A,** *IL4*. **B**, *IL5*. **C,** *IL13*. **D**, *TSLP*. **E**, *IL1RL1*. **F**, *HPDGS*. **G**, *GPR44*. **H,** *IL17A*. **I**, *PTGES*. **J,** *IL25*. **K,** *IL4R*. **L**, *IL10RA*. **M,** *CRLF2*. **N,** *PTGS1*. **O,** IL10. **P* < .01 versus the other clusters. †*P* < .01 versus cluster 3. ‡*P* < .01 versus each cluster.

clusters. Cluster 3 did not show any significant differences in levels of gene expression. Cluster 4 had higher expression of the gene *IL25* than in the other clusters. The levels of expression of the genes *IL4R, CRLF2, IL10, IL10RA*, and *PTGS1* were higher in clusters 1 to 3 than in cluster 4 (see Fig E4).

Clinical characteristics of each cluster

When the samples were divided into 3 clusters, each cluster generally contained samples from patients with eCRS, samples from patients with non-eCRS, and samples from patients without CRS, respectively (Fig 2), so we adopted these 3 clusters for

TABLE II. Characteristics of the subjects in each cluster

Characteristic	Cluster 1 (n = 49)	Cluster 2 (n = 19)	Cluster 3 (n = 17)	*P* value
Phenotype (no.)				
Severe-eCRS NP	14	0	1	
Moderate-eCRS NP	13	1	0	
Mild-eCRS NP	14	1	0	
Non-eCRS NP	4	9	2	
CRSsNP UT	2	8	2	
Non-CRS UT	2	0	12	
Tissue eosinophil count/hpf, median (IQR)	152 (92-225.5)	0.5 (0-5.8)	10 (0-31.9)	<.0001
Blood eosinophil rate, median (IQR)	5.9 (4.2-8.1)	2.0 (1.4-3.9)	1.8 (1.4-2.5)	<.0001
Serum total IgE level, median (IQR)	145 (71-366)	56 (20-525)	87 (22.5-375.5)	.0716
FEV1/FVC ratio (%),median (IQR)	75.1 (69.5-80.1)	77.8 (72.7-81.1)	83.6 (72.3-87.6)	.1109
Asthma as a comorbidity (no.)	18	0	1	.0009
AERD (no.)	2	0	0	.4712
Postoperative recurrence (no.)	9	0	0	.0248
CT score, median (IQR)	12 (10.5-17)	13 (6-15)	2 (1-6.75)	<.0001
log_2-transformed ΔΔCt value, cytokine-related gene, median (IQR)				
IL4	1.362 (0.1128-2.675)	–2.737 (–4.002 to –0.1154)	–2.548 (–4.982 to –2.225)	<.0001
IL5	6.119 (4.508-8.212)	–0.8528 (–2.630 to 0.4167)	–0.3113 (–0.7677 to 1.272)	<.0001
IL10	1.860 (0.9665-2.692)	0.8613 (0.0084-1.573)	-1.454 (–1.894 to –0.1322)	<.0001
IL13	5.564 (3.974-7.203)	0.3117 (0.0396-1.002)	1.641 (0.1836-2.367)	<.0001
IL17A	–3.777 (–5.058 to –2.593)	–1.407 (–2.277 to –0.5054)	-3.804 (–4.676 to –2.726)	.0008
IL22	–1.638 (–2.996 to –0.4776)	–2.304 (–3.705 to –1.299)	-4.148 (–4.148 to –4.148)	.178
IL25	0.0145 (–0.3748 to 0.7990)	-0.3943 (–1.317 to 0.1979)	1.467 (0.6952-1.889)	.0022
IL33	0.6529 (0.2015-1.181)	0.7604 (–0.0187 to 1.298)	0.4232 (–0.3780 to 0.8624)	.2869
TSLP	2.192 (1.559-3.118)	1.263 (0.7385-1.709)	1.222 (0.3926 to 1.882)	.0005
IFNG	–0.8498 (–1.544 to 0.3019)	–0.8522 (–2.101 to –0.2694)	–0.9049 (–2.087 to 0.6450)	.6053
Cytokine receptor–related gene, median (IQR)				
IL4R	0.6441 (0.1645-0.9456)	0.5570 (–0.3196 to 0.6917)	–0.7575 (–1.186 to 0.0652)	<.0001
IL5RA	1.872 (0.2806-2.486)	0.7447 (–0.3730 to 1.354)	-0.6351 (–1.514 to 0.5840)	.0002
IL10RA	-0.5213 (–1.219 to –0.0552)	-0.1492 (–0.9003 to 0.2282)	–2.203 (–2.695 to –1.313)	<.0001
IL13RA1	-0.4273 (–0.6077 to 0.0369)	-0.1488 (–0.4586 to 0.0621)	-0.4812 (–0.8991 to –0.1098)	.2143
IL17RA	0.2273 (–0.1072 to 0.6292)	0.3577 (–0.1492 to 0.6708)	0.1112 (–0.3598 to 0.4341)	.2946
IL17RB	0.4957 (0.2622-0.7498)	0.2632 (–0.4265-0.6990)	0.6588 (0.4474-0.8543)	.0146
IL22RA1	0.2565(0.4972-0.9809)	0.2322 (–0.6748 to 1.070)	0.9563 (0.2878-1.083)	.0975
IL1RL1	2.957 (1.837-3.543)	0.6586 (0.2929-1.371)	–0.8221 (–1.540 to 0.4663)	<.0001
CRLF2	0.4527 (–0.0883 to 0.8835)	0.0659 (–0.2582 to 0.5465)	–2.477 (–3.018 to –1.198)	<.0001
IFNGR1	0.3745 (0.0161-0.6859)	0.4030 (0.1694-0.9523)	0.0805 (–0.5682 to 0.6193)	.0572
Lipid mediator–related gene, median (IQR)				
HPGDS	1.559 (1.046-1.941)	-0.3021 (–0.8942 to 0.3348)	–0.8866 (–1.529 to –0.0954)	<.0001
PTGES	–0.0379 (–0.6252 to 0.2927)	0.6497 (0.4042-1.258)	-0.3738 (–0.6113 to 0.3525)	<.0001
PTGS1	0.7646 (0.0844-1.191)	0.3180 (–0.6372 to 0.9294)	-0.6950 (–1.016 to –0.2552)	<.0001
PTGS2	1.380 (0.8477-1.874)	1.685 (1.219-2.045)	0.9090 (–0.5817 to 1.703)	.0226
Lipid mediator receptor–related gene, median (IQR)				
GPR44	3.289 (2.533-4.372)	0.9085 (0.1333-1.837)	0.2225 (–0.5935 to 1.076)	<.0001
PTGDR	–1.185 (–2.320 to –0.0587)	–0.5921 (–1.719 to 0.0965)	0.2019 (–0.6567 to 0.7112	.0026
PTGER1	–0.2891 (–1.032 to 0.2233)	0.1516 (–0.2086 to 0.7137)	0.7514 (0.0646-1.421)	.0034
PTGER2	0.6890 (–0.2457 to 1.069)	0.1800 (–0.3067 to 0.6497)	–0.4887 (–1.399 to 0.1755)	.0037
PTGER3	0.0956 (–0.4363 to 0.8230)	–0.1537 (–0.9318 to 0.5111)	–0.0019 (–0.4315 to 0.5772)	.278
PTGER4	0.1071 (–0.4382 to 0.4488)	–0.3705 (–0.5950 to 0.2947)	–0.5735 (–0.9947 to –0.1413)	.0039

AERD, Aspirin-exacerbated respiratory disease; *FVC*, forced vital capacity *IQR*, interquartile range.
Differences between groups were assessed by using the Kruskal-Wallis test followed by the Dunn test for multiple comparisons or chi-square analysis.

further analysis (Table II). The blood eosinophil rate ($P < .0001$), tissue eosinophil count ($P < .0001$), and CT score ($P < .0001$) were significantly different between each cluster (Kruskal-Wallis test). The rates of asthma as a comorbidity ($P < .0009$) and postoperative recurrence ($P < .0248$) were also significantly different between each cluster (chi-square test). The hazard ratio (HR) of postoperative recurrence was 4.325 ($P = .0419$) in cluster 1 versus in cluster 2 and 3. The Dunn test further revealed that cluster 1 had a significantly higher blood eosinophil rate ($P < .0001$ for each) and tissue eosinophil count ($P < .0001$ and $P = .002$, respectively) than did clusters 2 and 3, and that clusters 1 and 2 had significantly higher CT scores than did cluster 3 ($P < .0001$ and $P = .0003$, respectively [Fig 4]). As for tissue neutrophil count, serum total IgE value, and ratio of FEV_1 value to forced vital capacity ratio in this study, there was no difference between clusters ($P = .152$; $P = .0716$; and $P = .1109$, respectively).

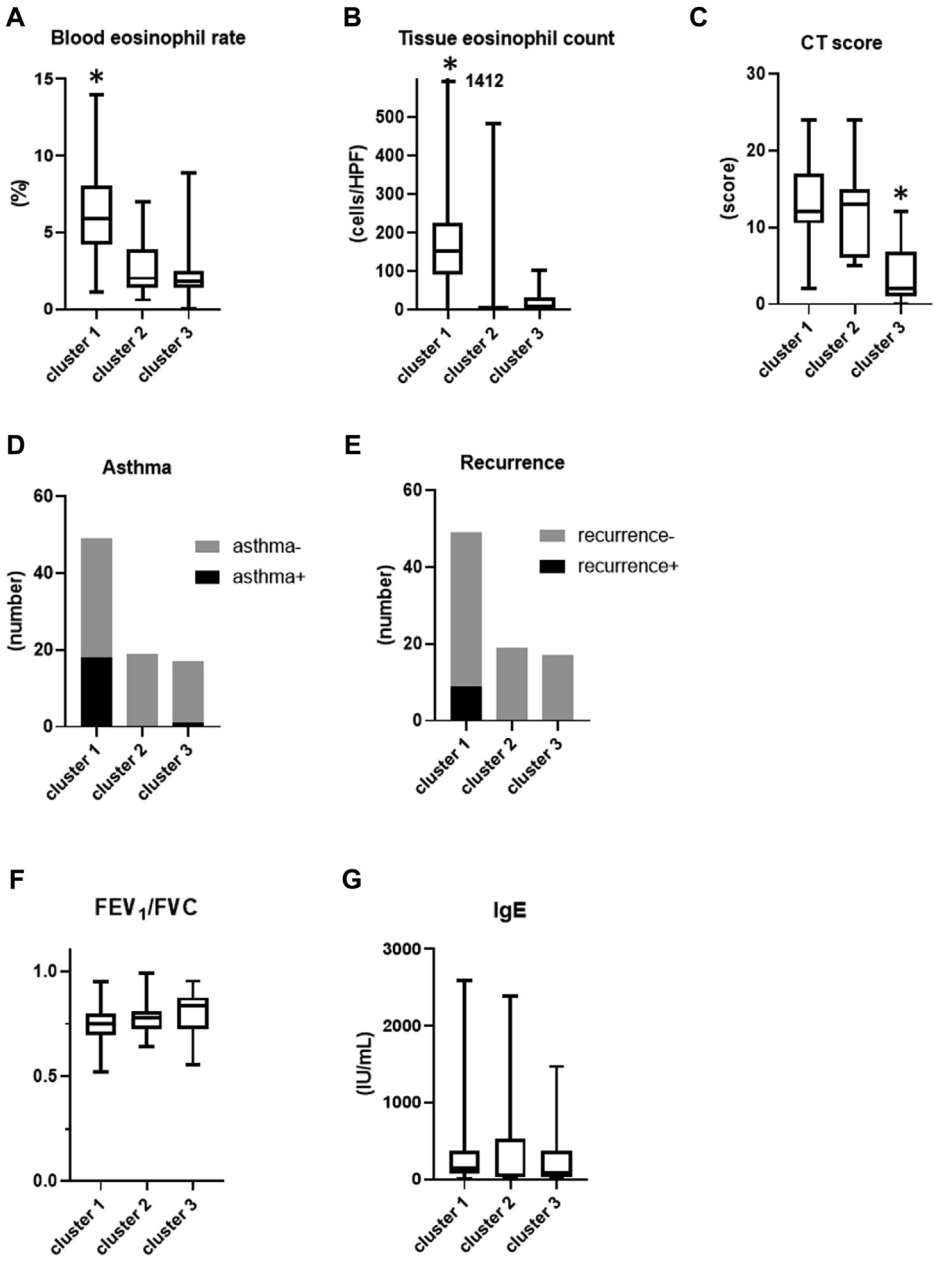

FIG 4. Clinical characteristics showing a significant difference between the 3 clusters. **A**, Blood eosinophil rate. **B**, Tissue eosinophil count. **C,** CT score. **D,** Number of persons with comorbid asthma. **E**, Number of persons with postoperative recurrence. **F**, Ratio of FEV_1 value to forced vital capacity (FVC). **G,** Total IgE level. $*P < .01$ versus the other clusters.

Minimizing genes for inflammatory endotyping

Finally, we chose the following 5 genes to minimize the clustering factors: *IL5, IL25, IL1RL1, PTGES*, and *GPR44*. *IL5, IL25*, and *PTGES* were selected because they had the highest (significantly so) gene expression in each cluster, whereas *IL1RL1* and *GPR44* were selected because they had the highest (significantly so) gene expression among cytokine receptors and PG receptors, respectively. With use of these 5 genes, the samples were divided into 3 clusters whose main components were almost the same as those of the 3 clusters divided by using 30 genes (Fig 5), as cluster 1 contained samples from patients with eCRS, cluster 2 contained samples from patients with non-eCRS, and cluster 3 contained samples from patients with non-CRS.

DISCUSSION

In the present study, we have examined the levels of gene expression of cytokines, enzymes for PGD_2 and/or PGE_2 synthesis, and their receptors in nasal tissues from patients with CRS and patients with non-CRS. We performed cluster analysis according to these levels of gene expression and found that a division into 3 clusters properly reflected the pathophysiologic significance of CRS. Regarding clinical features, the main components of each

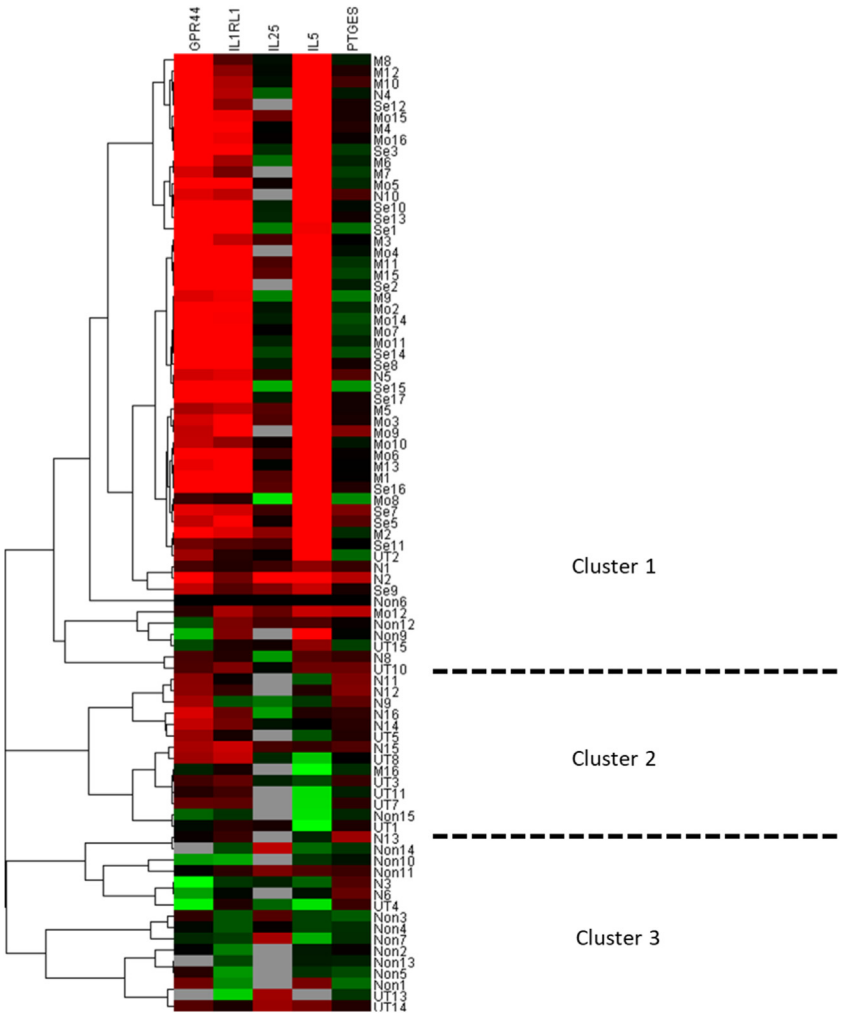

FIG 5. Heatmap of the levels of expression of 5 genes with a dendrogram.

cluster were as follows: NP tissue from patients with eCRS for cluster 1, NP tissue and UT from patients with non-eCRS for cluster 2, and UT samples from non-CRS patients for cluster 3.

Cluster 1, the largest cluster, showed elevated levels of expression of the genes *IL4, IL5, IL13, TSLP, IL1RL1, HPGDS*, and *GPR44*. IL-4, IL-5, and IL-13 are typical type 2 cytokines, suggesting that cluster 1 is characterized by a type 2–high signature. This is supported by the finding that cluster 1 had a significantly higher level of blood or tissue eosinophilia as well as significantly higher rates of asthma as a comorbidity and postoperative recurrence than the other clusters did. These results are compatible with those of a previous report showing that severe type 2 inflammation in CRS is associated with high rates of eosinophilia, asthma as a comorbidity, and postoperative recurrence.[15] There is some controversy regarding the cutoff number of tissue eosinophil counts reflecting type 2 CRS. Although several studies have defined eCRS by using criteria according to which the eosinophil count per hpf is higher than 10 eosinophils/hpf,[12] we chose the JESREC criteria, in which intractable eCRS was defined as an eosinophil count higher than 70 eosinophils/hpf in this study. Our results suggest that the JESREC criteria for tissue eosinophilia more clearly reflect CRS endotyping. In addition, the HR of recurrence (4.325) was higher than that for existing methods such as eosinophil counts in peripheral blood (HR = 1.52) or tissue (HR = 3.45) alone and higher than the 70 eosinophils/hpf mentioned in other reports.[13,16]

TSLP is produced from epithelial cells and induces type 2 cytokines from T_H2 cells, type 2 innate lymphoid cells (ILC2s), mast cells, eosinophils, and basophils. A strong correlation has been reported between the number of TSLP-expressing cells in NPs and tissue eosinophil counts or tissue IgE levels.[17] Genome-wide association studies have shown that a genetic variant near TSLP is associated with the number of eosinophils in mucosal tissues of patients with CRSwNP.[18] Our finding of elevated gene expression of *TSLP* in cluster 1, in which blood and/or tissue eosinophilia was seen, is consistent with the findings of these reports and suggests that TLSP is a key regulator in type 2 inflammation in CRSwNP.

IL1R1 (ST2) is the receptor for IL-33 and is expressed on the membranes of various immune cells such as T_H2 cells, regulatory T cells, ILC2s, macrophages, mast cells, eosinophils, basophils, and neutrophils. IL-33 is mainly released from epithelial cells by cell stress or damage, and immune cells are activated via ST2/IL-33 signaling, leading to a type 2 response through nuclear factor-κB activation.[19] In CRS, ILCs, eosinophils, and epithelial cells express ST2.[20-22] Shaw et al reported that ILCs from NPs

produce IL-13 in response to IL-33.[20] Baba et al showed that the level of expression of ST2 mRNA in NPs from patients with eCRS is significantly higher than the levels in samples from patients with non-eCRS.[21] The present result showing that expression of *IL1R1* was significantly increased in cluster 1 with high type 2 characterization is consistent with the results of these previous reports and confirms that IL-33/ST2 signaling is important in type 2 inflammation in CRS.

HPGDS is the gene encoding h-PGDS, which is the main terminal synthase for PGD_2 in the airway.[23] In general, h-PGDS is expressed on inflammatory cells, including mast cells, T_H2 cells, and ILC2s, which are associated with type 2 inflammation.[23] Higher expression of *HPGDS* in cluster 1 is compatible with the findings of our previous report showing that the levels of h-PGDS mRNA in sinonasal tissues are positively and significantly correlated with tissue eosinophilia and the radiologic severity of CRS.[10] Together with the finding of high expression of *GPR44* (the gene encoding CRTH2, which is the receptor for PGD_2 and is known to be expressed on cells associated with type 2 inflammation such as T_H2 cells and ILC2s), these results suggest upregulated PGD_2 metabolism in cluster 1.

In contrast, elevated levels of gene expression of *IL17A* and *PTGES* were seen in cluster 2, whose main constituents were samples from patients with non-eCRS and patients with noneosinophilic CRSsNP. IL-17A is produced in type 3 inflammatory diseases and induces neutrophil migration, especially on mucosa, via IL-8 and/or G-CSF production.[24-26] IL-17A has been reported to be associated with neutrophilic inflammation in both CRSwNP and CRSsNP.[27,28] As for tissue neutrophil infiltration in this study, there was no difference between clusters. This result suggests that the effect of IL-17A is not restricted to neutrophilic inflammation but can affect type 3 inflammation. *PTGES* is the gene encoding m-PGES-1, which is the main terminal synthase for PGE_2 in the airway. m-PGES-1 is an enzyme that is induced from stimulation by microbial components such as LPS.[29] PGE_2 enhances *Staphylococcus aureus* enterotoxin B–induced IL-17A production by NP cells.[30] In addition, PGE_2 is known to suppress the release of type 2 cytokines in NP tissue cells.[31] These results suggest that cluster 2 mainly contains non–type 2 CRS caused by IL-17A via the action of PGE_2, which suppresses type 2 inflammation.

Cluster 3, which mainly consisted of samples from patients with non-CRS, showed elevated gene expression of *IL25*. Tuft cells, especially solitary chemosensory cells, express taste receptors as a primary source of IL-25 in the human sinonasal epithelium.[32] Although IL-25 induces type 2 inflammation, it also acts on tissue homeostasis because of constitutive production in the airways, and it has a protective role against pathogens such as *Aspergillus* or viruses.[33] In addition, the levels of other type 2 cytokines in cluster 3were low, which suggests that this cluster is characterized by a noninflammatory signature.

In addition, the levels of expression of the genes *IL4R* (a receptor for both IL-4 and IL-13) and *CRLF2* (a TSLP receptor) were significantly higher in both clusters 1 and 2. According to the finding that clusters 1 and 2 mainly contained samples from patients with CRS whereas cluster 3 mainly contained samples from patients with non-CRS, these results suggest that signals through IL-4 and/or TSLP receptors induce chronic inflammation regardless of the inflammation type. This result is consistent with a previous report showing no significant difference in TSLP receptor expression of inflammatory infiltrates and epithelial cells between CRSwNP and CRSsNP[34] and that dupilumab, an anti–IL-4Rα antibody, suppresses inflammation without regard to eosinophilic status in CRSwNP.[35]

The gene expression of *IL10* was high in cluster 1 and low in cluster 3, and the expression of *IL10R* was high in clusters 1 and 2. Although IL-10 has been reported to be a major cytokine that is secreted in response to inflammation and counterregulates both innate and acquired immune responses,[36,37] the effect of IL-10 on NPs remains controversial. One report showed a positive correlation between IL-10 and Lund-Mackay CT score and concluded that the upregulation of IL-10 leads to decreased pathogen clearance and maintenance of inflammation in CRSwNP tissues.[38] However, another report showed that impaired IL-10 production may contribute to sustained inflammation in eosinophilic CRSwNP.[39] Furthermore, although baseline production of IL-10 is significantly higher in NP cells than in UT cells, impaired IL-10 production by NP cells in patients with severe CRSwNP in response to *S aureus* enterotoxin B was shown in our previous report.[40]

In this study, the level of expression of the gene *IFNG* did not differ between the clusters ($P = .605$). This result suggests that the role of type 1 inflammation in clustering or endotyping CRS is negligible. Tan et al reported that IFN-γ level is not significantly elevated in CRSsNP when compared with the levels in controls or patients with CRSwNP within the same tissue type,[41] which is compatible with our results.

According to the results of this study, the cluster 1 component, namely, eCRS, can be said to be PGD_2 type 2 inflammation, and the cluster 2 component, namely, non-eCRS/CRSsNP, can be said to be PGE_2 type 3 inflammation.

We chose 5 genes to minimize the clustering factors, namely, *IL5, IL25, IL1RL1, PTGES*, and *GPR44*. For custom commercial plates, the costs are estimated to be approximately $100 per person for 30 genes and approximately $10 per person for 5 genes. Thanks to our original gene plate, the examination of gene expression costs less than use of microarray analysis, in which the levels of expression of many genes are comprehensively checked. In addition, the total time from extracting RNA to finishing qPCR is about 7 hours. This study may contribute to a new low-cost and quick method for CRS classification, leading to personalized medicine.

This study has some limitations. First, our study was retrospective and its subjects were Japanese patients only. Prospective studies with subjects from other parts of the world are needed to confirm the results. Some non-eCRS samples were divided into cluster 1, although the levels of expression of 30 genes were used for clustering analysis. There may be some other genes that are more suitable for clustering. The 5 samples removed after the control assay for RNA quality may have also affected the results, and further studies with a greater number of samples are desirable. Although we used the best measure to store the samples at the current moment, better methods to collect and keep samples are also desired.

Conclusion

In conclusion, nasal tissue samples from patients who underwent endoscopic sinus surgery could be divided into 3 clusters according to their levels of gene expression. The results suggested that there were associations between type 2 inflammation/PGD_2 and eCRS, as well as between type 3

inflammation/PGE_2 and non-eCRS. With the use of our prepared original gene plate, the cost to analyze gene expression is lower if a smaller number of genes critical for NP formation are selected.

DISCLOSURE STATEMENT

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Key messages

- **Gene expression of NPs or nasal mucosa can be used to estimate endotypes of CRS.**
- **NPs from patients with eCRS have high levels of expression of type 2 cytokines and PGD_2 synthase.**
- **NPs and UT samples from patients with non-eCRS have high levels of expression of type 3 cytokines and PGE_2 synthase.**

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