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Original Article

Significance of IgG4-positive cells in severe eosinophilic chronic rhinosinusitis

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Abbreviations:
CRS, chronic rhinosinusitis; CRSsNP, chronic rhinosinusitis without nasal polyps; CRSwNP, chronic rhinosinusitis with nasal polyps; ECRS, eosinophilic chronic rhinosinusitis; FEV_1/FVC, 1-s forced expiratory volume/forced vital capacity; HPF, high power field; IgG4-RD, IgG4-related disease; JESREC, Japanese Epidemiological Survey of Refractory Eosinophilic Chronic Rhinosinusitis; NP, nasal polyps; SEB, staphylococcal enterotoxin B; UT, uncinate tissues

ABSTRACT

Background: IgG4 production is regulated by type 2 (IL-4 and IL-13) and regulatory (IL-10) cytokines involved in the pathophysiology of chronic rhinosinusitis (CRS). We sought to determine the pathophysiological characteristics of IgG4-positive cells in sinonasal tissues in CRS, especially eosinophilic CRS (ECRS).

Methods: IgG4-positive cells in uncinate tissues (UT) and nasal polyps (NP) were examined by immunohistochemistry. Associations between the number of IgG4-positive cells and clinicopathological factors were analyzed. Receiver operating characteristics (ROC) analysis was performed to determine the cut-off value of IgG4-positive cells in tissue that can predict the post-operative course.

Results: IgG4 was mainly expressed in infiltrating plasma and plasmacytoid cells, and the number of IgG4-positive cells was significantly higher in NP, especially those from severe ECRS patients, than in UT. In CRS patients, the number of IgG4-positive cells significantly and positively correlated with blood and tissue eosinophilia, radiological severity, and serum level of total IgE. The number of infiltrating IgG4-positive cells was significantly higher in patients with a poor post-operative course (sustained sinus shadow 6 months after surgery) than in those with a good one. The number of IgG4-positive cells in NP could discriminate patients with a good or a poor post-operative course (area under the curve: 0.769). Also, 73.3% sensitivity and 82.5% specificity were achieved when the cut-off value was set at 17 cells/high-power field.

Conclusions: Our results suggest that the local expression of IgG4 on cells may be used as a biomarker that reflects the pathophysiology of CRS, including the post-operative course.

Introduction

Chronic rhinosinusitis (CRS) with nasal polyps (CRSwNP), especially eosinophilic chronic rhinosinusitis (ECRS), is one of the phenotypes of CRS known as an intractable upper airway

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inflammatory disease.[1] Although the precise etiology and pathophysiology underlying this disease remain poorly understood, altered humoral immunity, including IgG, IgA, and IgE, appears to be involved.[2–4] For example, nasal polyps (NP) contain significantly higher amounts of IgG and IgA, especially antinuclear antibodies, than control nasal tissues.[4]

IgG4 is the least abundant IgG subclass in human serum, comprising approximately 5% of the total IgG.[5] In general, IgG4 is regarded as an anti-inflammatory antibody because of the lack of complement activation, inability to undergo antigen cross-linking due to Fab-arm exchange, and high affinity for inhibitory FcγRIIb.[5,6] It also inhibits the activity of IgE.[6,7] Similar to IgE, which is known to be involved in the pathogenesis of ECRS, type 2 cytokines, including IL-4 and IL-13, and a co-stimulatory signal through CD40/CD40L are crucial for IgG4 production.[2,3,6] In addition, IL-10, which also plays a substantial role in the pathogenesis of ECRS, enhances the production of IgG4.[6,8,9] An important feature of IgG4 production is its association with chronic high-dose antigen exposure.[6] For example, chronic exposure to cat allergen induces IgG4 production, especially in patients with cat allergy.[10]

CRS is known to accompany IgG4-related disease (IgG4-RD) in 9.2%–51.9% of patients.[11–13] IgG4-RD is a recently established systemic disorder characterized by mass-forming lesions in mainly exocrine tissues, such as the pancreas, lacrimal glands, and submandibular glands, consisting of fibrosclerosis and lymphoplasmacytic infiltration, especially numerous IgG4-positive plasma cells.[14] Although the precise pathogenesis of IgG4-RD remains unclear, type 2 cytokines, including IL-4 and IL-13, and regulatory cytokines, including IL-10 and TGF-β produced by mast cells, are known to play a substantial role.[15,16] On the other hand, little is known on whether IgG4 or IgG4-positive cells play a role in the pathogenesis of CRS, including ECRS, regardless of IgG4-RD. NP contained significantly increased levels of IgG4 compared with uncinate process tissues (UT) from not only healthy control subjects but also patients with CRSsNP.[17] A recent report showed that the number of IgG4-positive plasma cells is increased in nasal mucosal specimens in CRS, and that hyperplasia of the nasal glands was observed significantly more frequently in patients with high IgG4 expression than in those with low IgG4 expression.[18]

In the present study, we sought to determine the pathophysiological characteristics of IgG4-positive cells in sinonasal tissues in CRS. We found that local IgG4-positive cell infiltration may be a novel biomarker that not only reflects the severity of CRS, but also predicts the post-operative course.

Methods

Patients

Seventy-one Japanese patients with CRS (47 males and 24 females; mean age, 55.8 years) were enrolled. None of all patients met the diagnostic criteria for IgG4-RD.[14] Among these, 57 patients exhibited NP (CRSwNP) and the remainder demonstrated no visible NP in the middle meatus (CRSsNP; n = 14). Patients with CRSwNP were divided into non-ECRS (n = 27) and ECRS (n = 30) groups based on the Japanese Epidemiological Survey of Refractory Eosinophilic Chronic Rhinosinusitis (JESREC) criteria. In brief, the JESREC scoring system assesses unilateral or bilateral disease, the presence of NP, degree of blood eosinophilia, and dominant shadow of ethmoid sinus in computed tomography (CT) scans. A case was diagnosed as ECRS if it showed a JESREC score of 11 or more and tissue eosinophilia of 70 or more per high-power field (HPF; × 400). The severity of ECRS was further determined by the JESREC algorithm using factor A (presence of both blood eosinophilia equal to or more than 5% and an ethmoid-dominant shadow on CT scan) and factor B (comorbid bronchial asthma or nonsteroidal anti-inflammatory drug intolerance) as follows: cases negative for both factors A and B, cases positive for either factor A or B, or cases positive for both factors A and B were grouped into mild, moderate, or severe ECRS groups, respectively.[1] Using this algorithm, 30 ECRS patients were divided into the mild (n = 8), moderate (n = 12), and severe (n = 10) ECRS subgroups. All CRSsNP patients were diagnosed as being non-ECRS using this criterion. To eliminate the effect of medications on the expression of IgG4, patients were excluded if they received pharmacotherapy for CRS, such as oral corticosteroids, within 8 weeks prior to surgery, or other medications, such as macrolide antibiotics and intranasal corticosteroids, within 3 weeks prior to surgery. During surgery, NP and UT were taken from patients with CRSwNP and CRSsNP, respectively. Serum samples were kept from 17 patients (non-ECRS: n = 6, mild ECRS: n = 4, moderate ECRS: n = 2, severe ECRS: n = 5). As the control, 13 non-CRS patients (e.g., patients with blowout fracture, posterior ethmoidal cyst, or sphenoidal cyst) with normal UT at inspection were enrolled (4 males and 9 females; mean age, 61.4 years). After surgery, CRS patients received medications including macrolides and mucolytic agents for 2 months, together with saline douching which was continued as long as they could. In addition, NP patients received systemic corticosteroids (prednisolone: started with 20 mg/day then gradually decreased for 1 month) followed by intranasal corticosteroids. Furthermore, ECRS patients basically receive oral antileukotrienes. Informed consent for participation in the study was obtained from each patient, and the study was approved by the Human Research Committee of the Okayama University Graduate School of Medicine and Dentistry (1505-030).

Patients' characteristics

The clinical characteristics of the patients are presented in Table 1. Twenty patients were asthmatic and all had NP. Half (n = 10) of the asthmatic patients were regarded as atopic showing elevated serum IgE concentration above 170 IU/ml (Pharmacia, Uppsala, Sweden). Among the asthmatic patients, 5 patients were

Table 1
Subjects characteristics.

Groups	Non-CRS (UT)	CRSsNP (UT)	Non-ECRS (NP)	Mild ECRS (NP)	Moderate ECRS (NP)	Severe ECRS (NP)
Number	13	14	27	8	12	10
Age (years old)	61.4 (41–92)	60.5 (35–75)	57.6 (36–84)	56.8 (34–70)	52.5 (32–78)	49.5 (33–74)
Sex (female/male)	9/4	6/8	8/19	2/6	5/7	3/7
Blood eosinophil rate (%)	2.1 (0–5.9)	2.5 (0.8–8.6)	3.2 (0.1–7.3)	3.8 (2.6–4.7)	5.6 (1.7–7.9)	11.4 (6.9–22.8)
Serum toal IgE (IU/mL)	34.5 (2–99)	141.7 (4–923)	164.6 (4-1322)	150.4 (10–452)	256.4 (34-1768)	476.2 (34-1899)
FEV1/FVC ratio (%)	83.8 (68.7–92.7)	77.3 (66.5–84.7)	78.3 (47.2–91.9)	76.3 (73.2–86.4)	76.0 (49.2–92.3)	72.8 (52.6–89.5)
CT grading score (Lund–Mackay)	1.9 (1–3)	6.5 (1–14)	13.4 (3–24)	14.3 (8–22)	13.4 (7–21)	16.6 (10–24)
Comorbidity of asthma (n)	0	0	5	0	5	10
Comorbidity of NSAIDs intorelance (n)	0	0	2	0	1	3

Data are shown as the mean with ranges in parentheses.

diagnosed with aspirin-exacerbated respiratory disease (AERD) based on their history of asthma attacks precipitated by non-steroidal anti-inflammatory drugs. Prior to surgery, the serum level of total IgE, blood eosinophil rate, and 1-s forced expiratory volume/forced vital capacity (FEV_1/FVC) ratio were determined for each CRS patient. A radiological assessment of the severity of rhinosinusitis in each patient was also performed using the Lund–Mackay system.[19] Sections from surgically excised UT and NP were stained with hematoxylin and eosin, and the average number of eosinophils per HPF was determined.

In CRSwNP patients complaining of a loss of smell, objective olfaction was evaluated using identification threshold in orthonasal T&T olfactometer testing (n = 51) and the latent time in retronasal intravenous olfactory testing (n = 49).[20,21] In order to examine the objective residual inflammation after surgery, CT examination was performed 6 months after surgery in 55 of the 57 patients who agreed to receive the examination. The post-operative course was defined as good when the post-operative CT score was less than half of the pre-operative score.

Histological examination and immunohistochemistry

Surgical specimens were fixed in 10% formaldehyde and embedded in paraffin. Serial sections (4 μm) were cut from each paraffin-embedded tissue block, and several sections were stained with hematoxylin and eosin, and IgG4 immunostaining.[15,16] Immunohistochemistry was performed on paraffin sections using an automated Bond Max stainer (Leica Biosystems, Melbourne, Australia) with anti-human IgG4 mAb (HP6025, 1:400; The Binding Site, Birmingham, UK) as the primary antibody. The number of IgG4-positive cells was estimated for areas with the highest density of positive cells. Three different HPFs (× 10 eyepiece and × 40 objective lenses) in each section were counted by a pathology reviewer (YG) blinded to the patients' characteristics such as JESREC criteria, and the average number of positive cells per HPF was determined; eosinophil numbers were counted as well. Double immunohistochemistry using anti-human IgG4 mAb and anti-human CD138 mAb (MI15; 1:200; Dako, Glostrup, Denmark) was performed to identify whether IgG4-positive cells were plasma cells.

Determination of serum IgG4

Levels of serum IgG4 in a limited number of patients (n = 17) were determined using IgG4 Human ELISA kit (Thermo Fisher Scientific, Waltham, MA) according to the manufacturer's instructions.

Statistical analysis

Values are given as the median. The nonparametric Mann–Whitney U test was used to compare data between groups, and Wilcoxon's signed rank test was used to analyze data within each group. A Kruskal–Wallis test followed by a Dunn test was used for multiple comparisons. Correlation analyses were performed using Spearman's rank correlation. Statistical analyses were performed with GraphPad Prism 6 software (GraphPad software, Inc., La Jolla, CA). Receiver operating characteristics (ROC) analyses were performed to determine the cut-off values for IgG4-positive cells. *P*-values for sensitivity and specificity were calculated using JMP Pro 13.2 (SAS Institute Inc., Cary, NC), and logistic regression analyses were conducted by STATA 12.1 (StataCorp, College Station, TX). *P*-values less than 0.05 (two-tailed) were considered to be statistically significant.

Results

Local expression of IgG4 in nasal mucosae

IgG4 was mainly expressed in infiltrating mononuclear cells both in UT and NP (Fig. 1A–D). The number of IgG4-positive cells in the UT and NP ranged from 0 to 7 (median: 0) and from 0 to 162 (median: 6), respectively, per HPF. Overall, the number of IgG4-positive cells was significantly higher in NP than in UT ($P < 0.001$; Fig. 1E). Double immunohistochemistry showed that infiltrating IgG4-positive cells (red: cytoplastic staining) were stanied by anti-CD138 mAb (brown: membrane staining) (Fig. 1F).

Comparison of IgG4-positive cells among the phenotypes of CRS

Samples were divided into six groups according to the phenotype of the CRS: UT from non-CRS (n = 13), UT from CRSsNP (n = 14), NP from non-ECRS (n = 27), NP from mild ECRS (n = 8), NP from moderate ECRS (n = 12), and NP from severe ECRS (n = 10) (Table 1). The Kruskal–Wallis test revealed a significant difference in the number of infiltrating IgG4-positive cells among these groups ($P < 0.001$; Fig. 2A). The Dunn test further showed that, although the number was not different between UT from non-CRS and UT from CRSsNP ($P = 0.725$), the number was significantly higher in each group of NP (non-ECRS: $P = 0.003$; mild ECRS: $P = 0.009$; moderate ECRS: $P = 0.038$; severe ECRS: $P < 0.001$) than in UT from non-CRS. The highest degree of infiltration with IgG4-positive cells was seen in NP from severe ECRS, and this phenotype showed significantly more infiltration than UT from CRSsNP ($P < 0.001$) and NP from non-ECRS ($P = 0.013$) and moderate ECRS ($P = 0.039$).

Among the 57 patients with NP, 20 were asthmatic. A significantly higher number of IgG4-positive cells was seen in asthmatic patients than in non-asthmatic patients ($P < 0.001$; Fig. 2B). The presence of atopic status (serum IgE > 170 IU/ml) did not affect the number of IgG4-positive cells in NP between non-atopic (n = 10) and atopic (n = 10) asthmatics (P = 0.630 by Mann–Whitney U test; Fig. 2C). In addition, 5 of the 57 NP patients had concomitant AERD. A significantly higher number of IgG4-positive cells was seen in AERD patients when compared to non-AERD patients ($P = 0.011$; Fig. 2D).

Pathophysiological significance of IgG4-positive cells in CRS

We next pathophysiologically characterized the degree of IgG4-positive cells in sinonasal tissues. A significant positive correlation was seen between the number of infiltrating IgG4-positive cells and eosinophils in sinonasal tissues (n = 84, r = 0.509, $P < 0.001$; Fig. 3A). The number was also significantly and positively correlated with peripheral blood eosinophilia (n = 84, r = 0.445, $P < 0.001$; Fig. 3B), the radiological severity of CRS (n = 77, r = 0.390, $P < 0.001$; Fig. 3C), and the serum total IgE level (n = 76, r = 0.238, $P = 0.038$; Fig. 3D). In contrast, no correlation was seen between the number of infiltrating IgG4-positive cells and the FEV_1/FVC ratio (n = 83, r = −0.036, $P = 0.743$; Fig. 3E). Olfactory testing was performed in CRSwNP patients complaining of a loss of smell. No significant correlation between the degree of infiltrating IgG4-positive cells and the results of identification threshold in orthonasal T&T olfactometer testing was seen (n = 51, r = 0.223, $P = 0.115$; Fig. 3F); however, there appeared to be a positive correlation between the cell number and the latent time in retronasal intravenous olfactory testing (n = 49, r = 0.238, $P = 0.097$; Fig. 3G). Our preliminary result using 17 serum samples showed that the levels of serum IgG4 significantly and positively correlated with the number of infiltrating IgG4-positive cells in tissue (r = 0.629,

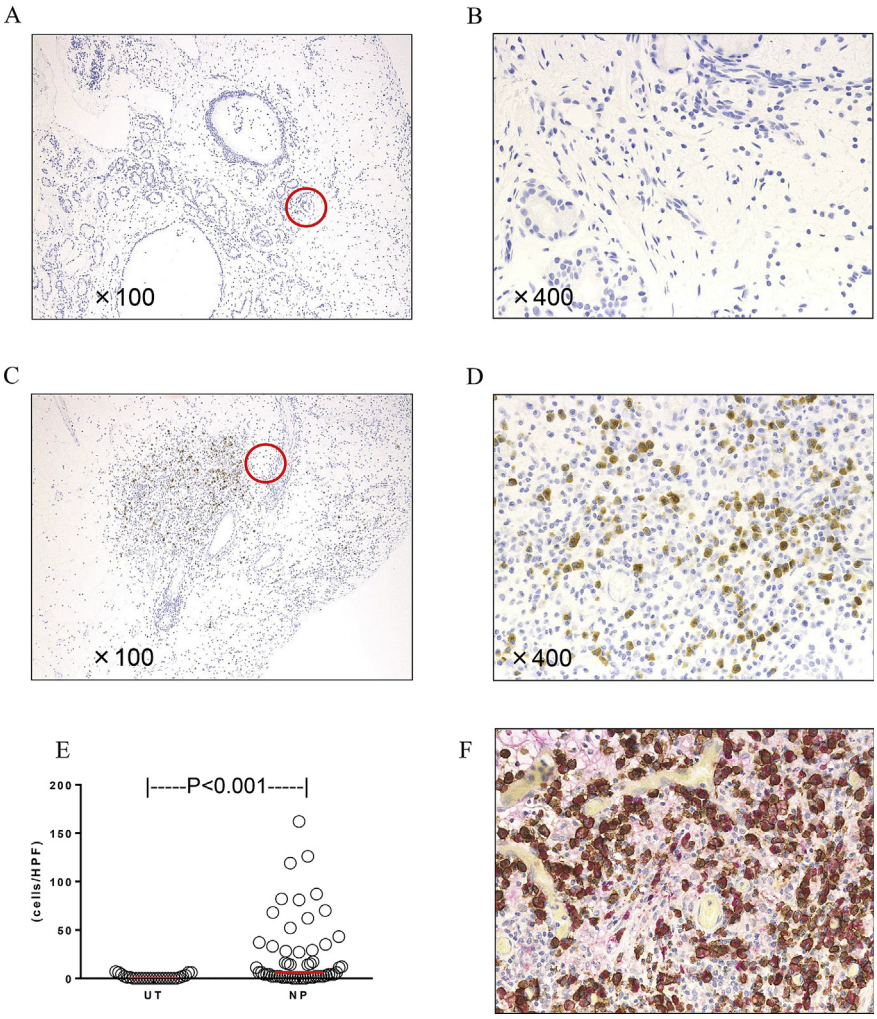


Fig. 1. Immunohistochemical staining of IgG4 in UT **(A, B)** and NP **(C, D)** with different magnifications (A and C: ×100, B and D: ×400). **E:** Comparison of numbers of IgG4-positive cells between UT and NP. P value was determined by Mann–Whitney U test. **F:** Double immunohistochemistry using anti-human IgG4 mAb (red) and anti-human CD138 mAb (brown).

$P = 0.008$). The Kruskal–Wallis test revealed a significant difference in serum IgG4 levels among patients with non-ECRS, mild/moderate ECRS and severe ECRS ($P = 0.006$). The Dunn test further showed a significant difference between mild/moderate ECRS and severe ECRS ($P = 0.009$).

Significance of infiltrating IgG4-positive cells on the post-operative course in CRSwNP

Of the 57 patients with CRSwNP, 55 received a CT examination 6 month after surgery. The number of infiltrating IgG4-positive cells was significantly and positively correlated with the post-operative CT score ($r = 0.459$, $P < 0.001$; Fig. 4A), showing a stronger correlation than the pre-operative CT score. The rate of the residual CT score (Post-operative CT score/Pre-operative CT score × 100) was also significantly and positively correlated with the number of infiltrating IgG4-positive cells in NP ($r = 0.340$, $P = 0.003$; Fig. 4B). In the evaluation of the post-operative course, a course was considered to be good if the CT score 6 month after endoscopic sinus surgery was less than half of the pre-operative score; of the 55 patients evaluated, 40 (72.7%) patients showed a good post-operative course, and 15 (27.3%) patients showed a poor post-operative course. The number of infiltrating IgG4-positive cells was significantly higher in the patients with a poor post-operative course than in those with a good one ($P = 0.002$; Fig. 4C).

Finally, we assessed the possibility of using the number of IgG4-positive cells in NP as a biomarker for predicting the post-operative course in patients with CRSwNP using ROC analysis. The number of IgG4-positive cells in NP appeared to be a better diagnostic test for predicting the post-operative course after endoscopic sinus surgery (area under the curve (AUC): 0.769; 95% confidence interval (CI): 0.612 – 0.926) than the number of eosinophils in tissue (AUC: 0.512; 95% CI: 0.332 – 0.692) or the number of eosinophils in peripheral blood (AUC: 0.658; 95% CI: 0.493 – 0.823) (Fig. 5A). This analysis showed that the best cut-off for IgG4-positive cells was 17 cells/HPF, and the corresponding sensitivity and specificity for predicting the post-operative course was 73.3% (95% CI: 44.9 – 92.1) and 82.5% (95% CI: 67.2 – 92.7), respectively. With this cut-off value, 11 of the 18 patients (61.1%) with more than or equal to the cut-off value exhibited a poor post-operative course, whereas 4 of the 37 patients (10.8%) with less than the cut-off value exhibited a poor post-operative course ($P < 0.001$ by the chi-square test; Fig. 5B). Logistic regression analysis showed that the odds ratio (OR) for a poor post-operative course was 12.96 (95% CI: 3.18 – 52.82), and the results essentially remained the same after we adjusted by age and sex (OR: 17.60; 95% CI: 3.46 – 89.54), the

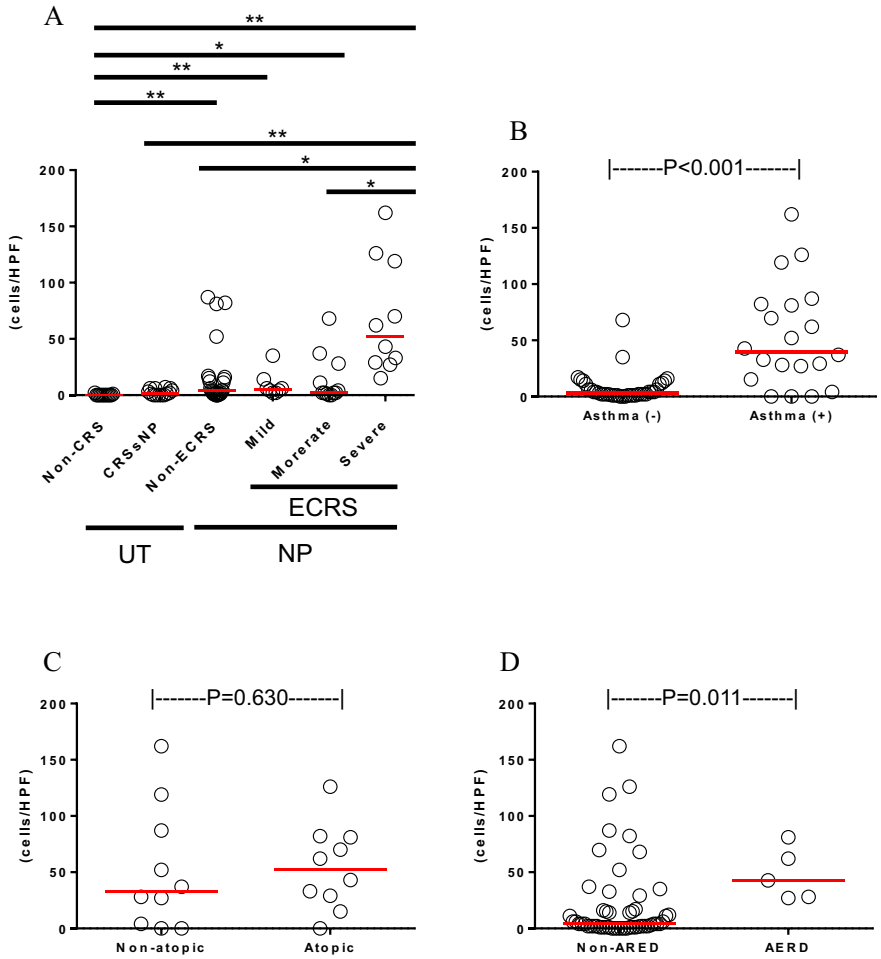


Fig. 2. Comparison of numbers of tissue IgG4-positive cells based on phenotypes. **A:** Comparison among six subgroups including UT from non-CRS patients, UT from CRSsNP patients, NP from non-ECRS patients, NP from mild, moderate and severe ECRS patients. **B:** Comparison between asthmatic and non-asthmatic patients. **C:** Comparison between non-atopic and atopic patients with asthma. **D:** Comparison between AERD and non-AERD patients. P value was determined by Dunn test (**A**) and Mann–Whitney U test (**B** and **C**). * $P < 0.05$. ** $P < 0.01$.

severity of ECRS (OR: 16.63; 95% CI: 3.14 – 88.21), and asthma comorbidity (OR: 27.50, 95% CI: 2.47–306.2) (Fig. 5C).

Discussion

In the present study, we characterized IgG4-positive cells in CRS, especially ECRS. We found that severe phenotypes of ECRS showed the highest infiltration of IgG4-positive cells. The number of infiltrating IgG4-positive cells was significantly and positively correlated with the degree of local and blood eosinophilia, radiological severity of CRS, and the level of serum total IgE. In addition, a high infiltration of IgG4-positive cells in NP was associated with poor control after surgery. Although we did not examine the role of IgG4 in the pathogenesis of ECRS, these results may provide a basis for the diagnostic use of local IgG4-positive cells as a biomarker for not only confirming the severity of ECRS, but also for predicting the outcome after surgery.

IgG4 was mainly expressed in infiltrating plasma and plasmacytoid cells in sinonasal tissues. It is well known that IgG4-positive cells mainly infiltrate the periductal and/or fibrosclerotic areas of IgG4-RD lesions.[14] Morphologically, the IgG4-positive cells in sinonasal tissues also seem to be plasma cells since the characteristic appearance of plasma cells, such as eccentric nucleus with cartwheel-like chromatin and enriched cytoplasm, was seen in the IgG4-positive cells, suggesting that local class switching toward IgG4 was induced in sinonasal tissues.[22] In addition, infiltrating IgG4-positive cells expressed CD138 in double immunohistochemistry, confirming that IgG4-positive cells were plasma cells.

The number of IgG4-positive cells was significantly higher in NP than in UT, especially those from non-CRS patients. Although several endotypes and regional diversity do exist, the general inflammatory profiles of CRS have been characterized. In general, CRSsNP show neutrophil infiltration with upregulation of non-type 2 cytokines, such as IL-6, IL-8, and IFN-γ, whereas CRSwNP show eosinophil infiltration with upregulation of type 2 cytokines, such as IL-4, IL-5, and IL-13.[23–25] Because IL-4 and IL-13 are crucial factors for inducing isotype switching to IgG4, the present findings suggest that a microenvironment favoring type 2 inflammation induces class switching toward IgG4 and/or the infiltration of IgG4-positive cells in NP.[6] This is supported by the result that no significant difference in the number of infiltrating IgG4-positive cells was found between UT from non-CRS and CRSsNP patients.

Severe phenotype of ECRS showed an augmented infiltration of IgG4-positive cells into NP than other phenotypes in CRSwNP. On the other hand, no significant difference in radiological severity determined by the Lund–Mackay system was seen among the phenotypes (Table 1: $P = 0.522$ by Kruskal–Wallis test), suggesting that IgG4-positive cells in NP are phenotype-specific augmentation but not comparative increase.

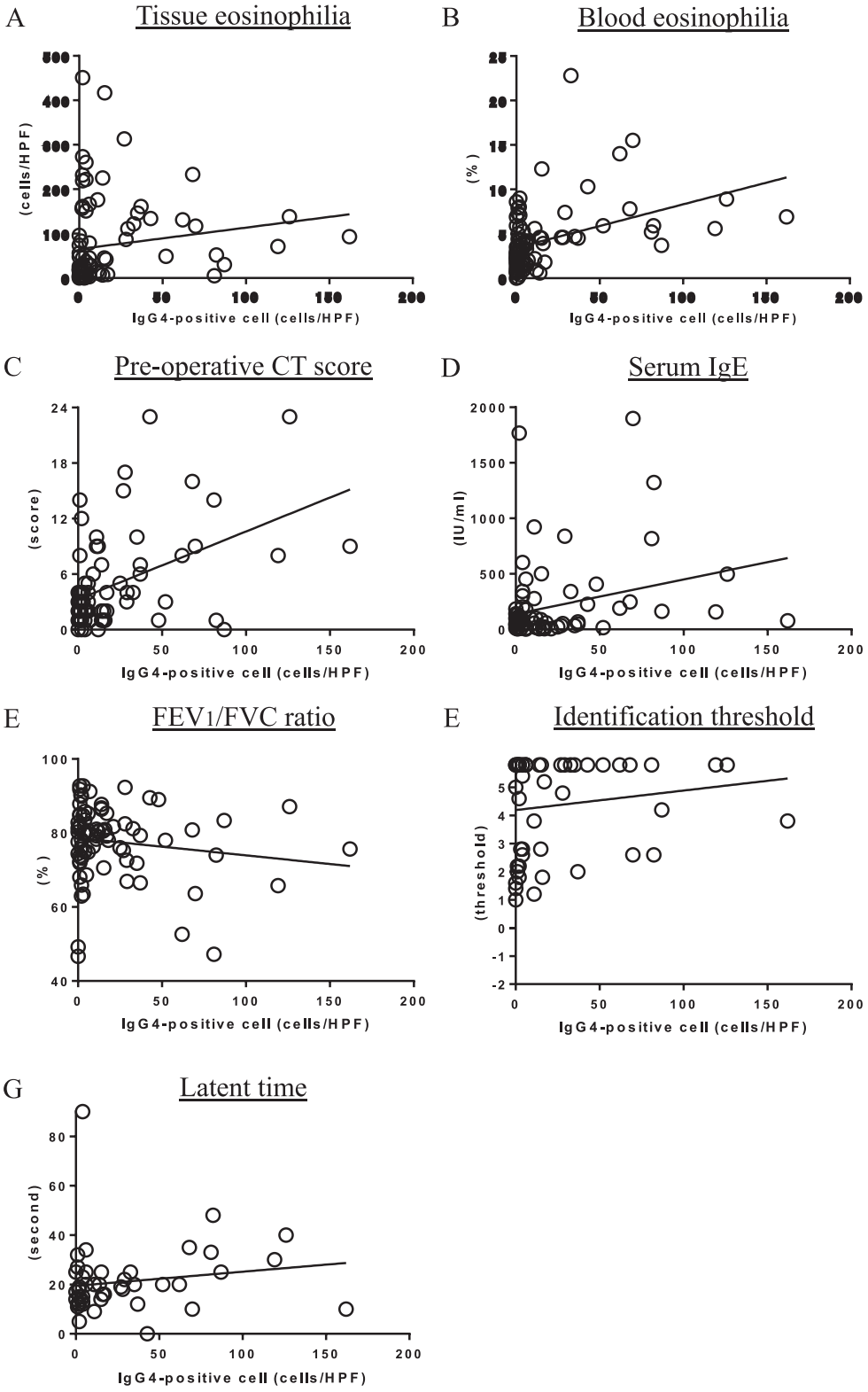


Fig. 3. Relationship between the numbers of tissue IgG4-positive cells and pathophysiological characterizations of CRS including tissue eosinophilia **(A)**, blood eosinophilia **(B)**, pre-operative CT score of CRS **(C)**, serum total IgE levels **(D)**, FEV_1/FVC ratio **(E)**, identification threshold in orthonasal T&T olfactometry testing **(F)**, and latent time in retronasal intravenous olfactory testing **(G)**.

Significant and positive correlations were seen between the number of infiltrating IgG4-positive cells in sinonasal tissues and the level of serum total IgE. In the past, we failed to find any association between the level of serum total IgE and the local levels of inflammatory molecules, including prostaglandin D_2 synthase, prostaglandin E_2 synthases, IL-17, IL-18, and IL-22.[26–29] The present study is the first to report a significant correlation, and this correlation may reflect a similarity to class switching toward IgE and

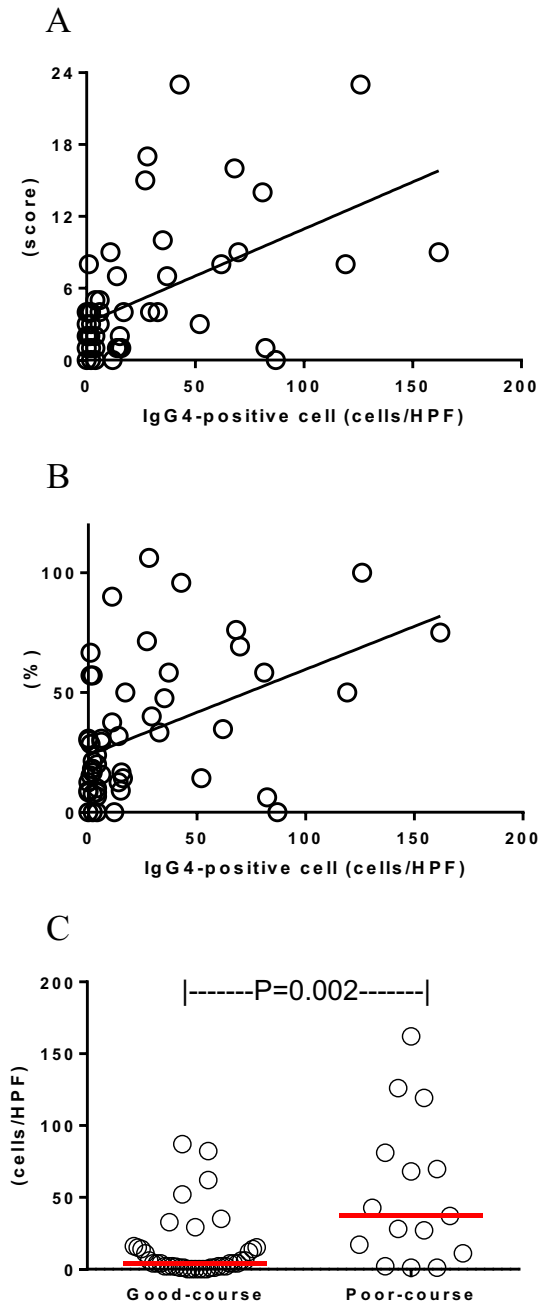


Fig. 4. Characterization of IgG4-positive cells in post-operative course after endoscopic sinus surgery. Relationship between the numbers of tissue IgG4-positive cells and CT score 6 months after surgery **(A)** and rate of residual CT score **(B)**. **C:** Comparison of numbers of IgG4-positive cells between well-controlled and poor controlled patients after surgery. P value was determined by Mann–Whitney U test.

IgG4.[6] IgG4 levels in sinus tissue may be associated with tissue IgE rather than serum IgE. Investigation to determine a correlation between local numbers of IgG4-positive cells and IgE-positive cells in currently under the way.

A positive correlation was also found between the number of infiltrating IgG4-positive cells and the degree of local and blood eosinophilia. However, this finding is not consistent with a previous report that found no significant difference in the degree of local eosinophilia between CRS patients with high IgG4 (more than 10 positive cells/HPF and a ratio of IgG4- to IgG-positive cells of >40%) and low IgG4 infiltration.[18] This discrepancy may be due to different subject populations and/or different evaluation criteria. In our case, non-CRS patients were included in order to investigate the correlation in sinonasal tissue. Indeed, when we excluded the cases with non-CRS, the correlation became weaker ($n = 57$, $r = 0.254$, $P = 0.056$ by Spearman rank correlation). However, when we divided CRS patients into high IgG4 (>10 positive cells/HPF) and low IgG4 (≤10 positive cells/HPF) groups based on the criterion set by Ohno *et al.*, the high IgG4 group showed significantly more severe eosinophilia both in tissue ($P = 0.014$ by the Mann–Whitney test) and peripheral blood ($P < 0.001$) than the low IgG4 group.[18]

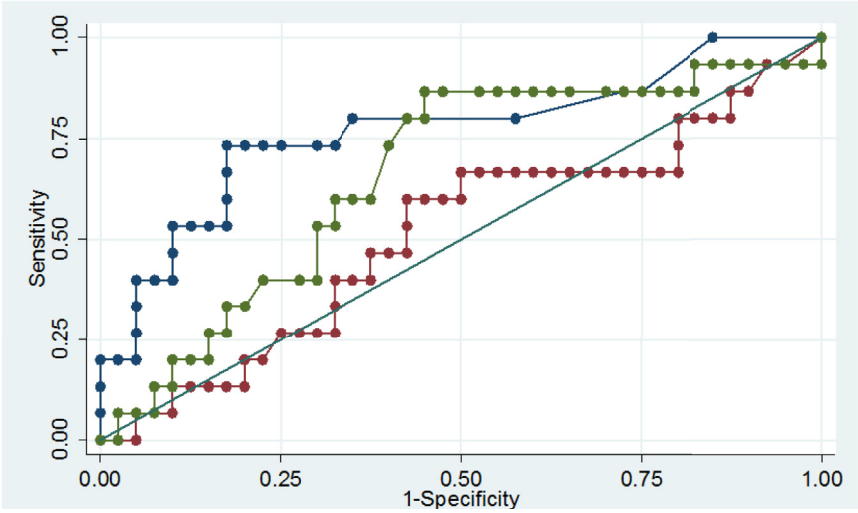
CRSwNP patients often have comorbid asthma and AERD.[19] Asthmatic patients show significantly higher infiltration of IgG4-positive cells than non-asthmatic patients. However, the role of IgG4 in the pathogenesis of asthma remains unclear. Allergen-specific immunotherapy for allergic asthma induces allergen-specific IgG4 production, and IgG4 may act as a blocking antibody that prevents the activation and degranulation of mast cells and basophils by competing with the allergen binding of sIgE bound to Fcε receptors on these effector cells.[30] In addition, AERD patients also show significantly higher infiltration of IgG4-positive cells than non-AERD patients. A recent report analyzing 12,323 patients who had undergone IgG subclass testing found isolated elevation of serum IgG4 in patients with AERD ($P = 0.017$), nasal polyposis ($P = 0.020$), eosinophilia ($P = 0.038$), and celiac disease ($P = 0.020$).[31] A higher, albeit not significant, expression of IL-13 was observed in NP from ARED patients as compared with non-AERD patients.[32] In addition, enhanced expression of IL-4 mRNA was seen in peripheral blood mononuclear cells in response to aspirin, especially in AERD patients.[33] Therefore, the higher expression of IgG4-positive cells in AERD patients may reflect the hypersensitivity to NSAIDs that induces type 2 cytokines, including IL-4 and IL-13.

The number of IgG4-positive cells was correlated with post-operative CT score which was stronger than pre-operative CT score. This suggests that IgG4-positive cells contribute to persistent sinus inflammation especially after surgery. When we set 17 cells/HPF as a cut-off level, poor post-operative control could be predicted at 73.3% sensitivity and 82.5% specificity. Biomarkers for predicting treatment outcomes in CRSwNP have been proposed, including eosinophilia in UT, blood basophil counts and rates, the levels of serum periostin, and the levels of staphylococcal enterotoxin-specific IgE in serum and tissues.[34–36] The present results suggest that tissue expression of IgG4 is an additional biomarker reflecting the severity and post-operative course of CRSwNP, especially ECRS. We previously reported that the numbers of eosinophilis in tissue was an important factor for predicting recurrence of NP after surgery.[1] In the present study, the number had little prognostic value (AUC: 0.512). We think that this inconsistency is due to different endpoint (CT findings versus recurrence of NP at inspection).

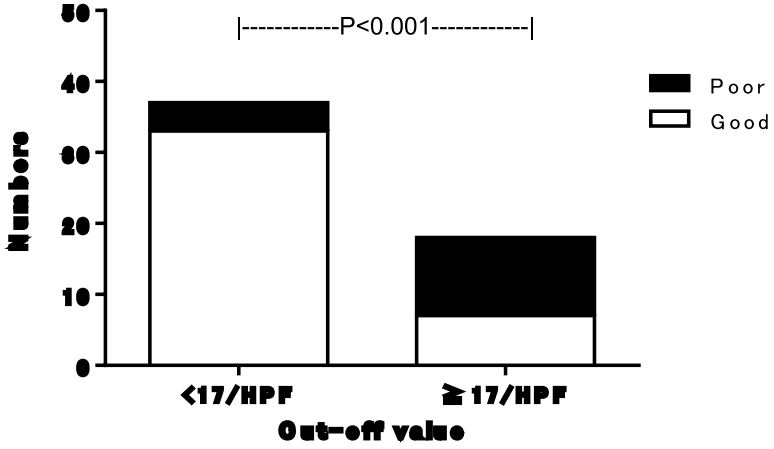
Interestingly, our preliminary result demonstrated a significant and positive correlation between the numbers of infiltrating IgG4-positive cells in tissues and serum levels of IgG4, suggesting that the serum IgG4 level is also associated with the severity in ECRS. Future study with large numbers of samples is required to confirm this phenomenon. In addition, it is important to determine whether IgG4 expressed in sinus tissue is antigen-specific or polyclonal in the future. A recent case study showed that IgG4 specific for desmoglein 3, an autoantigen of Pemphigus vulgaris, was detected in nasal and paranasal mucosa in a patient of refractory CRS with IgG4-RD.[37]

In conclusion, we showed evidence that the local expression of IgG4 is associated with disease severity and treatment outcome in CRS, especially ECRS. In particular, the degree of expression correlated with the degree of not only local eosinophilia, but also the serum total IgE level, suggesting that IgG4 expression reflects high

A



B



C

	Model 1	Model 2	Model 3	Model 4
IgG4	12.96 [3.18-52.85]	17.60 [3.46-89.54]	16.63 [3.14-88.21]	27.50 [2.47-306.2]
Age		1.00 [0.94-1.05]	1.00 [0.94-1.06]	1.00 [0.94-1.06]
Sex		0.42 [0.08-2.31]	0.42 [0.08-2.29]	0.44 [0.08-2.52]
Severity			1.25 [0.26-5.95]	1.38 [0.28-6.91]
Asthma				0.48 [0.05-5.03]

Fig. 5. ROC analysis to predict post-operative course in CRSwNP. **A:** ROC curve for the number of IgG4-positive cells in tissue (blue), blood the number of eosinophils in peripheral blood (green) and the number of eosinophils in tissue (red) for distinguishing subjects with well and poor course after surgery. **B:** Comparison of numbers of well and poor course patients based on the cut-off value (17 cells/HPF) of IgG4-positive cells. P value was determined by Chi-square test. **C:** Logistic regression results using the cut-off value of IgG4-positive cells (17 cells/HPF). Model 1: crude. Model 2: adjusted by age (continuous) and sex (female vs male; reference). Model 3: adjusted by age, sex, and severity of ECRS (non ECRS: 0, mild ECRS: 1, moderate ECRS: 2, severe ECRS: 3). Model 4: comorbid asthma. Odds ratios [95% confidential interval] for poor course patients after surgery were shown.

type 2 inflammation. In contrast, although the present study did not include patients with IgG4-RD, the induction of IgG4 class switching may have regional diversity because IgG4-RD, one of the best characterized diseases in which IgG4 is involved, is more frequently seen in Asia, including Japan, than in other regions.[38] Therefore, ECRS may share a similar immune reaction as IgG4-RD, and future study is required to determine whether the findings seen in this investigation are reproducible in other regions outside of Japan.[24]

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Conflict of interest

The authors have no conflict of interest to declare.

Authors' contributions:

TK, YS, KN and MO designed the study and wrote the manuscript. SK, THi, THa and KK contributed to collection of tissue samples and patients' information. TF, YG and YO contributed to immunohistochemistry. AM, ST and MT performed the statistical analysis and interpretation of the results.

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