

[ORIGINAL ARTICLE]

Serum Biomarkers for the Diagnosis of Eosinophilic Esophagitis and Eosinophilic Gastroenteritis

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Abstract:

Objective Clinically useful serum biomarkers for the diagnosis and monitoring of eosinophilic gastrointestinal diseases are not available. This study was conducted to examine the possible value of eosinophil-related proteins as serum biomarkers.

Methods The serum concentrations of 49 cytokines, chemokines, and other proteins were measured in 29 patients with eosinophilic gastrointestinal diseases and 80 controls.

Results The levels of interleukin (IL)-5, IL-33, eotaxin-3, and thymic stromal lymphopoietin (TSLP), previously reported as possible biomarkers of eosinophilic esophagitis, were not significantly elevated in the serum. In contrast, the B cell-attracting chemokine (BCA)-1/chemokine (C-X-C motif) ligand (CXCL) 13 and hemofiltrate C-C chemokine (HCC)-1/CC chemokine ligand (CCL) 14 α levels were significantly elevated, while the granulocyte chemotactic protein (GCP)-2/CXCL6 levels were suppressed in patients with eosinophilic esophagitis as well as in those with eosinophilic gastroenteritis. The cutaneous T cell-attracting chemokine (CTACK)/CCL27, stromal cell-derived factor (SDF)-1/CXCL12, macrophage inflammatory protein (MIP)-3 β /CCL19, and squamous cell carcinoma antigen (SCCA) 2 levels were elevated only in patients with eosinophilic esophagitis. However, there were large overlaps of data obtained from the patient and control groups, indicating that these serum biomarkers are not adequately sensitive for clinical use with presently available assay systems.

Conclusion Of the 49 investigated serum proteins, none were shown to be adequately sensitive for use as biomarkers for the diagnosis or monitoring of eosinophilic gastrointestinal diseases.

Key words: eosinophilic esophagitis, eosinophilic gastroenteritis, biomarker, cytokine

(Intern Med 56: 2819-2825, 2017)

(DOI: 10.2169/internalmedicine.8763-16)

Introduction

Eosinophilic gastrointestinal diseases are chronic allergic gastrointestinal conditions characterized by dense infiltration of eosinophils in the gastrointestinal tract (1). These diseases can be divided into eosinophilic esophagitis (EoE) and eosinophilic gastroenteritis (EGE), based on the involved portion of the gastrointestinal tract (2). In Japan, the preva-

lence of EGE has been reported to be higher than in other countries (3). In Western countries, the prevalence of EoE has rapidly increased in the past two decades and is now the second-most frequently encountered esophageal inflammatory disease (4-6). In Japan as well, since the first report of a typical adult case in 2006, the prevalence of EoE has been steadily increasing (7-9). EoE and EGE are now considered major gastrointestinal allergic diseases, along with many allergic diseases, such as bronchial asthma, allergic rhinitis,

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Received: December 26, 2016; Accepted: February 23, 2017; Advance Publication by J-STAGE: September 25, 2017

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and atopic dermatitis.

For the diagnosis and monitoring of the activities of EoE and EGE, an endoscopic investigation and histological assessment of biopsy specimens are considered to be required (1, 10, 11). However, endoscopic abnormalities are often non-specific and difficult to identify (12-14). Furthermore, histological findings for grading eosinophil infiltration are not always accurate because of the uneven patchy distribution of eosinophils in the esophago-gastro-intestinal mucosa (1, 10, 14). In addition, an endoscopic examination is invasive, and repeated examinations for activity evaluations are difficult to perform.

Non-invasive blood tests, if sensitive and specific enough, would be better diagnostic options for these purposes. We previously investigated whether or not peripheral blood leukocyte and eosinophil numbers, total IgE concentration, antigen-specific IgE titers, the anti-*Helicobacter pylori* antibody, or C-reactive protein could serve as biomarkers for the diagnosis and activity grading of EoE and EGE, although we obtained discouraging results (3, 15, 16). Nevertheless, a pilot study on the value of serum cytokine and chemokine levels as biomarkers suggested the rationale for further research with possible promising results (17). In that study, cytokines and chemokines were similarly elevated in the serum of EoE and EGE patients, which suggested a similar pathophysiology for these two types of eosinophilic gastrointestinal diseases, as was also suggested by the findings of mucosal mRNA measurement (18).

In the present study, to identify potential biomarkers for the diagnosis and monitoring of EoE and EGE, we measured the levels of 49 different cytokines, chemokines, and other proteins in serum obtained from patients with eosinophilic gastrointestinal diseases as well as normal healthy subjects.

Materials and Methods

Patients

Twenty-nine patients with symptomatic eosinophilic gastrointestinal disease (19 with EoE and 10 with EGE) were enrolled. The diagnosis of eosinophilic gastrointestinal disease was confirmed by the presence of gastrointestinal symptoms and histo-pathological identification of pathological eosinophil infiltration in examinations performed according to the recent guidelines for EoE and Talley's diagnostic criteria for EGE (1, 10, 19). The mean age of the patients with EoE was 52.9±2.9 years, including 14 (73.7%) men, while that of the patients with EGE was 42.8±7.1, including 5 (50%) men. Serum samples were collected and immediately stored at -30°C until use.

As controls, 80 healthy subjects (mean age 54.5±1.1 years, 55 men) were also enrolled, and their serum was collected without any drug administration. Twenty-three of the 80 normal controls reported a history of allergic disease, mainly seasonal allergic rhinitis, while no such history was

reported by the remaining 57.

This study was conducted under the approval of the Ethics Committee of Shimane University and in accordance with the Declaration of Helsinki.

Cytokine and chemokine measurements

We determined the levels of 15 cytokines and 31 chemokines using a multiplex assay kit (MILLIPLEX: Merck Millipore, Darmstadt, Germany), according to the manufacturer's instructions. The cytokines investigated were interleukin (IL)-1β, IL-3, IL-5, IL-6, IL-9, IL-10, IL-12, IL-13, IL-17, granulocyte-colony stimulating factor (G-CSF), granulocyte macrophage colony-stimulating factor (GM-CSF), interferon (IFN) γ, tumor necrosis factor (TNF) α, IL-33, and thymic stromal lymphopoietin (TSLP), and the chemokines included IL-8, eotaxin, fractalkine, growth related oncogene (GRO), interferon gamma-induced protein (IP)-10, monocyte chemoattractant protein (MCP)-1, MCP-3/CC chemokine ligand 7 (CCL7), macrophage-derived chemokine (MDC)/CCL22, macrophage inflammatory protein (MIP)-1α, MIP-1β, regulated upon activation, normal T cell expressed and secreted (RANTES), 6CKine/CCL21, B cell-attracting chemokine (BCA)-1/chemokine (C-X-C motif) ligand (CXCL) 13, cutaneous T cell-attracting chemokine (CTACK)/CCL27, epithelial cell-derived neutrophil-activating peptide (ENA)-78/CXCL5, eotaxin-2/CCL24/myeloid progenitor inhibitory factor (MPIF)-2, eotaxin-3/CCL26, I-309/CCL1, MCP-2, MCP-4, MIP-1δ/MIP-5/CCL15, stromal cell-derived factor (SDF)-1/CXCL12, thymus and activation regulated chemokine (TARC)/CCL17, granulocyte chemotactic protein (GCP)-2/CXCL6/ LPS-induced CXC chemokine (LIX), hemofiltrate C-C chemokine (HCC)-1/CCL14α, interferon-inducible T-cell alpha chemoattractant (I-TAC)/CXCL11, lymphotactin, monokine induced by gamma interferon (MIG)/CXCL9, MIP-3α/CCL20, MIP-3β/CCL19, and neutrophil-activating peptide (NAP)-2/CXCL7.

An enzyme-linked immunosorbent assay performed as previously reported (20, 21) was used to measure the levels of extracellular matrix protein, periostin, and squamous cell carcinoma antigen (SCCA) 1 and 2 in serum, since those have been shown to be associated with the pathogenesis of several allergic diseases.

Statistical analyses

Statistical comparisons between groups were performed using Mann-Whitney's U test. The data are presented as the medians (25th-75th percentile). p<0.05 was considered to indicate a statistically significant difference.

Results

Of the 49 serum proteins investigated, only 19 chemokines and 3 proteins were successfully measured in all 109 subjects. In contrast, the serum concentrations of the cytokines and remaining 12 chemokines could not be determined

Table. Serum Protein Concentration as Biomarkers in Cases with EoE, EGE, and Controls.

	EoE (n=19)		EGE (n=10)		Control		
	52.9	14/5	42.8	5/5	Total (n=80)	with allergy (n=23)	without allergy (n=57)
Age					54.5	50.6	56.0
M/F					55/25	14/9	41/16
Eotaxin (pg/mL)	105.0 (81.1-135.0)		96.0 (70.0-121.0)		110.5 (95.1-136.3)	118.0 (107.5-150.0)	106.0 (92.2-131.0)
GRO (pg/mL)	647.0 (476.5-737.5)		565.5 (306.5-890.8)		721.0 (596.8-875.5)	774.0 (669.5-885.0)	685.0 (590.0-865.0)
MDC (pg/mL)	1,068.0 (743.0-1,286.0)		732.0 (522.8-1,215.3)		1,028.5 (812.8-1,248.0)	1,131.0 (860.5-1,455.5)	1,013.0 (794.0-1,125.0)
IP-10 (pg/mL)	349.0 (253.0-389.5)		312.0 (180.5-423.3)		294.0 (243.8-353.3)	321.0 (227.5-353.5)	285.0 (245.0-339.0)
MCP-1 (pg/mL)	381.0 (328.5-503.0)		376.5 (234.5-524.3)		405.5 (337.8-519.0)	389.0 (292.0-500.5)	421.0 (342.0-523.0)
RANTES (pg/mL)	4,002.0 (2,787.0-5,272.0)		4,708.5 (3,586.3-5,292.5)		3,922.0 (3,410.8-4,364.0)	4,065.0 (3,656.0-4,682.0)	3,871.0 (3,371.0-4,200.0)
Eotaxin-2 (pg/mL)	588.0 (530.0-762.0)		592.5 (398.3-949.3)		471.5 (356.0-687.5)	386.0 (250.0-471.5)	512.0 (369.0-730.0)
BCA-1 (pg/mL)	26.6 (20.7-36.5)*		37.3 (34.9-64.2)*		19.3 (15.7-24.8)	20.0 (17.5-25.2)	18.6 (15.4-24.4)
TARC (pg/mL)	110.0 (67.0-133.5)		75.5 (53.8-185.3)		85.3 (58.9-120.0)	112.0 (68.4-139.5)	79.9 (57.3-109.0)
CTACK (pg/mL)	1,140.0 (942.0-1,327.0)*		730.5 (618.0-1,072.0)		912.5 (745.3-1,014.3)	803.0 (684.0-981.0)	930.0 (761.0-1,019.0)
SDF-1 (pg/mL)	3,343.0 (2,637.5-4,239.5)*		2,995.5 (2,671.8-3,604.8)		2,766.0 (2,042.8-3,126.0)	2,938.0 (2,584.5-3,247.0)	2,551.0 (1,950.0-3,034.0)
ENA-78 (pg/mL)	738.0 (592.5-1,026.5)		762.0 (481.8-864.3)		727.0 (546.5-975.8)	735.0 (586.0-947.5)	720.0 (542.0-975.0)
MIP-1 δ (pg/mL)	1,727.0 (1,304.0-2,328.5)		1,796.5 (1,538.5-2,103.5)		2,026.0 (1,392.8-3,186.3)	2,035.0 (1,383.0-3,076.5)	1,964.0 (1,393.0-3,144.0)
NAP-2 (pg/mL)	8,636.5 (5,769.0-10,032.5)		2,991.5 (1,592.5-8,494.3)		6,677.5 (5,061.0-8,401.0)	5,871.0 (3,777.5-8,123.5)	6,911.0 (5,269.0-8,453.0)
GCP-2 (pg/mL)	64.1 (54.0-92.8)*		62.7 (50.8-84.4)*		81.4 (68.1-99.9)	82.4 (70.4-102.5)	80.9 (68.0-99.8)
I-TAC (pg/mL)	61.3 (38.1-111.5)		68.3 (46.8-111.5)		61.2 (46.1-85.1)	49.2 (44.0-77.7)	63.2 (49.9-85.0)
HCC-1 (pg/mL)	3,968.0 (3,483.0-5,061.0)*		3,757.5 (3,188.0-5,250.8)*		2,814.0 (2,328.5-3,534.0)	2,504.0 (2,182.5-3,324.0)	2,995.0 (2,415.0-3,673.0)
MIP-3 β (pg/mL)	106.0 (95.0-175.0)*		128.0 (86.2-173.3)		93.1 (73.0-129.5)	97.4 (69.5-121.5)	91.7 (74.8-1,131.0)
MIG (pg/mL)	845.0 (470.0-1,463.5)		659.5 (460.5-1,246.5)		833.5 (609.5-1,220.8)	826.0 (586.5-1,235.0)	841.0 (617.0-1,209.0)
SCCA1 (ng/mL)	0.7 (0.6-0.9)		0.5 (0.2-0.5)		0.6 (0.4-0.8)	0.8 (0.4-1.0)	0.6 (0.4-0.8)
SCCA2 (ng/mL)	0.7 (0.4-1.0)*		0.3 (0.1-0.6)		0.4 (0.3-0.7)	0.4 (0.2-0.8)	0.4 (0.3-0.6)
Periostin (ng/mL)	84.0 (78.8-96.5)		108.8 (69.8-137.4)		90.0 (73.8-109.3)	94.0 (77.5-120.0)	90.0 (73.0-104.0)

*p<0.05. * p<0.01 significantly different from total control. Median (25-75th percentile)

EoE: eosinophilic esophagitis, EGE: eosinophilic gastroenteritis, M: male, F: female, GRO: growth related oncogene, MDC: macrophage-derived chemokine, IP: interferon gamma-induced protein, MCP: monocyte chemoattractant protein, RANTES: regulated upon activation, normal T cell expressed and secreted, BCA: B cell-attracting chemokine, TARC: thymus and activation regulated chemokine, CTACK: cutaneous T cell-attracting chemokine, SDF: stromal cell-derived factor, ENA: epithelial cell-derived neutrophil-activating peptide, MIP: macrophage inflammatory protein, NAP: neutrophil-activating peptide, GCP: granulocyte chemoattractant protein, I-TAC: interferon-inducible T-cell alpha chemoattractant, HCC: hemofiltrate C-C chemokine, MIG: monokine induced by gamma interferon, SCCA: squamous cell carcinoma antigen

in at least some of our subjects, because of the detection limits of the assay methods employed. The 19 chemokines and 3 serum proteins successfully measured in all obtained samples were eotaxin, GRO, IP-10, MCP-1, MDC, RANTES, BCA-1, CTACK, ENA-78, eotaxin-2, MIP-1 δ , SDF-1, TARC, GCP-2, HCC-1, I-TAC, MIG, MIP-3 β , NAP-2, SCCA1, SCCA2, and periostin (Table).

Serum concentrations of IL-5, IL-13, IL-33, eotaxin-3, and TSLP, whose production has been reported to be ele-

vated in patients with eosinophilic gastrointestinal disease, did not reach measurable levels in many of our patients or control subjects. Only 8, 3, 2, 3, and 1 of 29 serum samples obtained from EoE and EGE cases showed detectable levels of IL-5, IL-13, IL-33, eotaxin-3, and TSLP concentrations, respectively.

Effects of allergy history on biomarker concentrations in normal controls

When the serum concentrations of the successfully measured 19 chemokines and 3 proteins were compared between normal controls with and without an allergy history, there were no significant differences found (Table). Thus, the 80 control subjects were compared as a single group with the patients with EoE and EGE for the subsequent analyses.

Effects of disease activity and proton pump inhibitor responsiveness on biomarker concentrations

Serum samples were obtained from 11 patients with EoE and 8 with EGE during an active disease stage before the start of treatment, as well as from 8 patients with EoE and 2 with EGE during an inactive disease stage. None of the patients with EoE or EGE in an inactive disease stage were receiving corticosteroid therapy at the time of serum sample collection, although 8 patients with EoE were being administered a proton pump inhibitor (PPI) at that time. There were no significant differences found in the serum concentrations of 22 proteins between the active and inactive stages in these patients. Therefore, patients in both the active and inactive stages of EoE and EGE were analyzed as single groups. Nine patients with EoE favorably responded to treatment with a PPI and were classified as PPI-responsive esophageal eosinophilia (PPI-REE). When the concentrations of the 22 serum proteins were compared between PPI-REE and PPI non-responsive EoE patients, no significant differences were found. Therefore, the PPI-REE and non-responsive cases were analyzed as a single EoE group in this study.

Serum biomarker concentrations in EoE and EGE patients

When the EoE and EGE patients were compared with the control group, the concentrations of BCA-1 and HCC-1 were found to be significantly elevated in both patient groups (Table). In contrast, the serum concentration of GCP-2 was significantly decreased in both of those patient groups. The CTACK, SDF-1, MIP-3 β , and SCCA2 levels were significantly elevated only in patients with EoE. However, despite the significant differences regarding serum chemokines and proteins between the EoE/EGE and control groups, large overlaps in the findings among these groups were found (Figure).

Discussion

In the present study, we determined the levels of 49 cytokines, chemokines, and proteins in serum obtained from patients with EoE or EGE and control subjects. Of those, the serum concentrations of IL-5, IL-13, IL-33, eotaxin-3, and TSLP, which have been reported to have pathogenetic roles in eosinophilic gastrointestinal diseases, were not successfully measured in the majority of patients, likely be-

cause of the detection sensitivity of the assay system. BCA-1 and HCC-1 levels were significantly elevated in the present patients with EoE and EGE, while those of CTACK, SDF-1, MIP-3 β , and SCCA2 were elevated only in EoE patients. GCP-2 was significantly lower in patients with EoE and EGE than in healthy controls.

Despite the statistically significant differences found, these chemokines and serum proteins are not reliable for use as biomarkers for the diagnosis of EoE and EGE, because there were large overlaps between the patients with eosinophilic gastrointestinal diseases and normal controls. In addition, the lack of differences between the active and inactive disease stages show the limited value of these serum biomarkers for disease activity monitoring.

Gastrointestinal endoscopy and histopathological examinations of biopsy specimens are now considered to be absolutely necessary for obtaining an accurate diagnosis, as well as for monitoring the activity of eosinophilic gastrointestinal disease (1, 10). However, because endoscopy is burdensome for patients, repeated endoscopic examinations are not practical in many cases. Therefore, serum biomarkers would be very helpful if any useful ones existed. With regard to the pathogenetic mechanism of eosinophilic gastrointestinal diseases, Th2 type immune activation and chronic eosinophil-related allergic reactions are believed to be important (22). IL-5, IL-13, IL-33, eotaxin-3, and TSLP are known to have important pathogenetic roles, and their production in involved gastrointestinal mucosa has been reported to be elevated (23, 24). Therefore, these cytokines and chemokines have been proposed as candidate serum biomarkers for EoE and EGE (17).

In this study, we measured the serum concentrations of 49 cytokines, chemokines, and serum proteins. However, there were no increases detected in the levels of IL-5, IL-13, IL-33, eotaxin-3, or TSLP. The findings obtained in our previous study revealed that a considerable number of patients had undetectable levels of those cytokines, which was similar to the results of the present study obtained using the same assay kit (17). It is likely that the serum release of IL-5, IL-13, IL-33, eotaxin-3, and TSLP from the involved tissue is restricted because of the limited and patchy involvement of the gastrointestinal tract and limited activity of immune-mediated inflammation. Therefore, any increase in the serum concentration of these cytokines/chemokines would be difficult to detect using the assay system employed in the present study.

We previously speculated that IL-5 and IL-15 might be effective biomarkers of EoE and EGE, although their serum concentrations were difficult to detect in some cases because of the limited sensitivity of the assay system used (17). In the present study, the detection limit of the assay system was again a problem, indicating the need to develop assays with higher sensitivity.

A study of infants with EGE found that the concentrations of IL-33 and TSLP in serum were significantly elevated in association with increased mRNA expression in

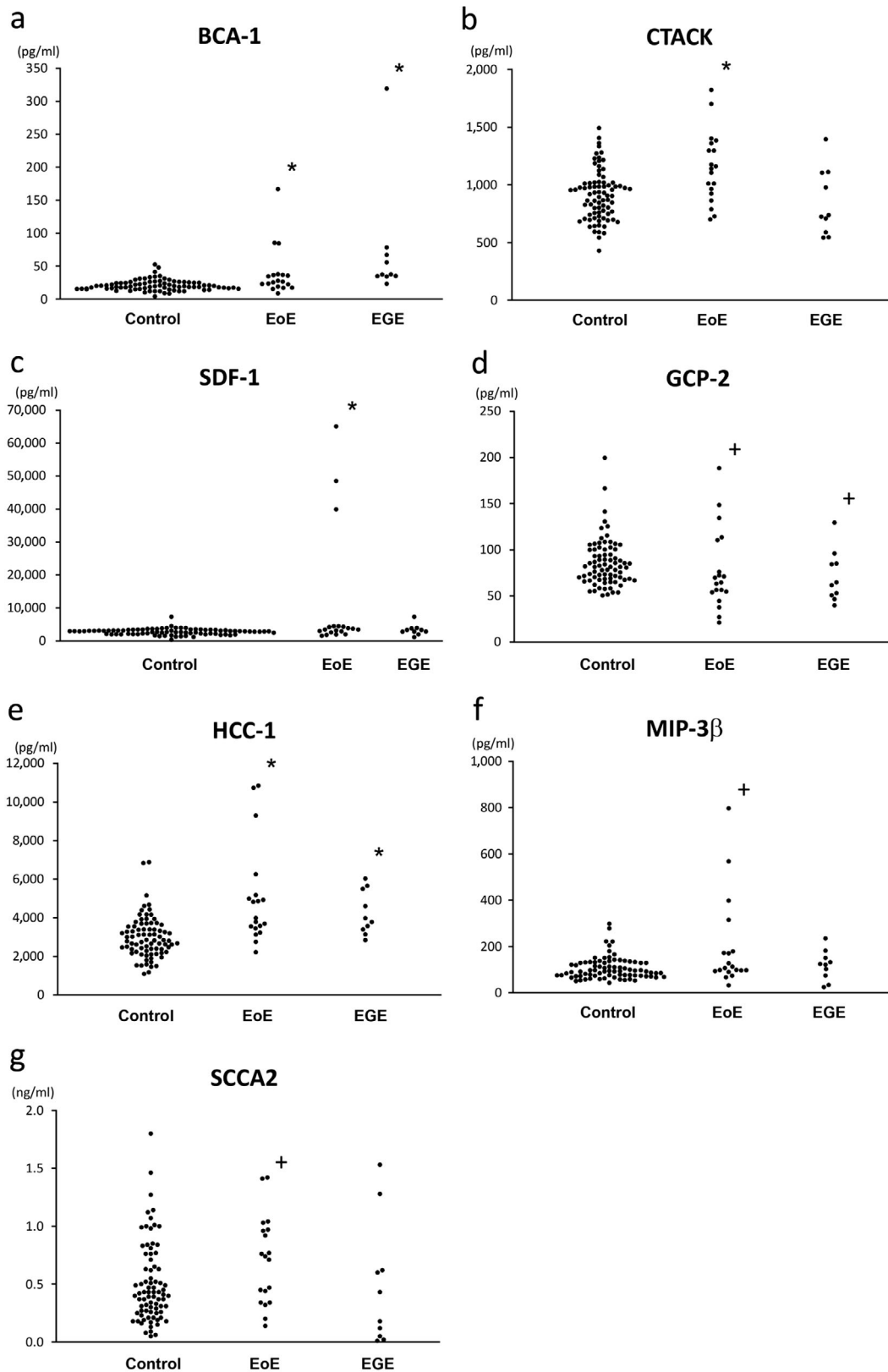


Figure. Serum concentrations of BCA-1/CXCL13 (a), CTACK/CCL27 (b), SDF-1/CXCL12 (c), GCP-2/CXCL6 (d), HCC-1/CCL14 α (e), MIP-3 β /CCL19 (f), and SCCA2 (g) in patients with eosinophilic esophagitis (EoE) and eosinophilic gastroenteritis (EGE), as well as in control subjects. Each dot indicates a single case. * $p < 0.01$, significantly different from control. + $p < 0.05$, significantly different from control.

colonic mucosa (25). In those cases, a higher disease activity and greater disease extent may have been responsible for

the elevated serum levels, although there was still some overlap between the patients and controls in that study.

Of the serum proteins measured in our study, BCA-1 and HCC-1 were significantly elevated in both EoE and EGE patients, while GCP-2 was significantly decreased in those same patients. In contrast, the CTACK, SDF-1, MIP-3 β , and SCCA2 levels were significantly elevated only in patients with EoE. BCA-1 is a chemotactic factor for B cells, and GCP-2 is a chemotactic factor for neutrophilic granulocytes. HCC-1 activates monocytes, and CTACK is associated with the homing of memory T lymphocytes and plays a role in T cell-mediated inflammation. SDF-1 and MIP-3 β have roles in the immunoregulatory and inflammatory responses, and SCCA2 may be a serum biomarker of Th2-type immune activation. Although the changes observed in our study were not marked or consistent, they might suggest important roles of Th2 and B lymphocyte-mediated immune response in eosinophilic gastrointestinal diseases.

The mechanisms by which these chemokines are elevated or depressed in EoE and EGE patients have yet to be clarified. However, those observations suggested that these chemokines can be used as biomarkers. When considering the clinical usefulness as serum biomarkers, both the sensitivity and specificity must be good. However, when we plotted individual case data obtained in the present study, it was difficult to determine the cut-off values for the levels of BCA-1, CTACK, SDF-1, GCP-2, HCC-1, MIP-3 β , and SCCA2 for identifying EoE and EGE, because of large overlaps between the patients and controls (Figure). Thus, these chemokines and serum proteins are considered not sensitive enough to be used as biomarkers. We did not include patients with other diseases, such as reflux esophagitis or inflammatory bowel disease, as a disease control group; therefore, the specificities of the present biomarkers were not investigated. The possible combination of these biomarkers for the diagnosis and activity monitoring of eosinophilic gastrointestinal disease should be investigated in a future study.

Recently, Dellon et al. measured 14 serum biomarkers (IL-4, IL-5, IL-6, IL-9, IL-13, TGF- α , TGF- β , TNF- α , eotaxin-1, eotaxin-2, eotaxin-3, TSLP, a major basic protein, and eosinophil-derived neurotoxin) in cases with EoE and controls and found no significant differences in the levels between the patient and control groups (26). In addition, they measured and compared these serum biomarkers before and after treatment and found no treatment-related changes (26). The mRNA expression of periostin, a pro-allergic mediator, was previously reported to be significantly elevated in cases of EoE (27). Dellon et al. also measured its expression in serum as a possible biomarker and found its level to be slightly increased in EoE patients. However, the level of periostin in the serum did not significantly change after topical steroid treatment, despite a significant change in its expression in the esophageal mucosa (28).

Our results obtained in Japanese patients with EoE coincide quite well with the findings noted above. Even though we increased the number of examined serum biomarkers to 49, it was difficult to obtain definitive results useful for the

diagnosis and activity monitoring of EoE.

The limitations of our study include the low number of EoE and EGE patients enrolled, as such patients are rare in Japan, as well as the restriction of our enrollment to adult subjects only. Non-endoscopic monitoring with serum biomarkers may be better for pediatric cases. In addition, we only measured the levels of cytokines and chemokines in serum once in the same patients. It may be interesting and important to evaluate whether or not those serum levels change with disease activity, as that information may be useful for the clinical assessment, including evaluating the therapeutic efficacy, in affected patients. Further studies are necessary to determine useful serum biomarkers or combinations of biomarkers not only in adults but also in pediatric patients.

In conclusion, none of the 49 serum biomarkers investigated were adequately sensitive for the diagnosis or activity monitoring of patients with eosinophilic gastrointestinal diseases.

The authors state that they have no Conflict of Interest (COI).

Financial Support

This work was funded by Health and Labor Sciences Research Grants, Research on Intractable Diseases, from the Ministry of Health, Labor and Welfare, Japan.

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