Immunohistochemical expression of filaggrin is decreased in proton pump inhibitor non-responders compared with proton pump inhibitor responders of eosinophilic esophagitis

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Abstract

Background Eosinophilic esophagitis (EoE) is an allergic gastrointestinal disease that features eosinophilic infiltration of esophageal mucosa, but the role of barrier dysfunction of the epithelium in its pathogenesis remains to be elucidated. Clinically, EoE is divided into proton pump inhibitor-non-responders (PPI-NR) and PPI-responders (PPI-R). Our main aims were to investigate the differences of expression of epidermal differential complex (EDC) proteins and desmoglein that are considered to play important roles in formation of the epidermal skin barrier between these two conditions and to seek the usefulness of the differences in pathological diagnosis. Conventional histopathological findings and allergic background were also compared.

Methods Twenty-nine PPI-NR and 44 PPI-R were recruited, and 35 reflux esophagitis (RE) patients were also enrolled. After clinical information and histopathological findings were reviewed, immunohistochemical expression of EDC proteins (filaggrin, loricrin, and involucrin) and desmoglein in all three groups were examined and semi-quantitatively scored.

Results Regarding allergic conditions, the prevalence of asthma was significantly higher in PPI-NR than in PPI-R. Other allergic conditions showed no differences. Histopathological findings did not exhibit statistical difference between PPI-NR and PPI-R. However, immunostaining score of filaggrin in PPI-NR was significantly lower than in PPI-R, although the expressions of involucrin, loricrin and desmoglein demonstrated no differences.

Conclusions The results suggest a role of reduced filaggrin expression in the difference of effectiveness of PPI treatment between PPI-NR and PPI-R. Moreover, immunohistochemical determination of filaggrin expression in EoE patients could be informative in clinical decision of how to treat the patients.

Keywords: Eosinophilic esophagitis · Epidermal differentiation complex · Filaggrin · Proton pump inhibitor-non responder · Proton pump inhibitor-responder

Introduction

Eosinophilic esophagitis (EoE) is a chronic inflammatory disease in which many eosinophils infiltrate into the epithelial layer of esophagus with an increasing number of patients all over the world. EoE is generally considered to be a Th2-dominant immune response to allergens, and this allergic reaction is now classified as non-immediate type that does not involve IgE. However, the mechanisms of barrier deficiencies of the squamous epithelium leading to immune responses in EoE are still incompletely understood [1,2].

The genes encoding protein molecules such as filaggrin, loricrin and involucrin that are expressed in the squamous epithelium are clustered in the chromosome 1q21. These proteins are essential for epidermal differentiation and constitute epidermal differentiation complex (EDC). Many allergic skin diseases e.g. atopic dermatitis are known to be related to decreased expression of filaggrin [3][4]. Recently, it has been reported that downregulation of EDC genes also contributes to the mechanism of development of EoE [5]. The expression of another barrier protein, desmoglein was also reported to be reduced in EoE [6]. Reduced expressions of EDC proteins and/or desmoglein have been considered to play a role in the pathogenesis of EoE by increasing the permeability of the epithelium, followed by entrance of allergens into the mucosa.

In clinical practice, the symptoms of about half of the patients with suspected EoE improve by taking proton pump inhibitor (PPI) [7, 8], so that these patients had been separated from EoE and classified as PPI-responsive esophageal eosinophilia (PPI-REE) until recently [9]. However, since there are many

reports that EoE and PPI-REE show no endoscopic, histopathological and molecular biological differences [10-12], the guidelines were developed in 2017 to remove PPI trials from diagnostic criteria of EoE [13], and PPI-REE is no longer distinguished from EoE. In recent years EoE has been divided into two groups: patients who respond to PPI treatment are called PPI responders (PPI-R), and patients who don't are called PPI non-responders (PPI-NR). To the best of our knowledge, there have been no reports on the differences of clinicopathological factors including expression of EDC proteins between PPI-NR and PPI-R. In this article we investigated to clarify whether reduced expressions of EDC proteins and/or desmoglein are correlated with the clinical differences between PPI-NR and PPI-R. In addition, we believe distinguishing the two conditions by pathological diagnosis with biopsy specimens will be useful for subsequent treatment strategies. We also compared the immunohistochemical findings of PPI-NR and PPI-R with those of RE patients, for which PPI is the standard therapy. Furthermore, the prevalence of allergic conditions was compared among the three groups. In this article, EoE includes PPI-NR and PPI-R unless otherwise stated.

Materials and methods

Patients

The database of Shimane University Hospital between 2005 and 2018 was searched to identify suitable patients. The final diagnoses rendered were based on clinical, endoscopic and histopathological findings

according the updated guidelines described above [13]. The patients were defined as PPI-R when their intraepithelial eosinophilic infiltration decreased to less than 15/HPF and the symptoms were completely relieved after 2 months of PPI therapy. If the symptoms were not relieved by the same therapy, they were defined as PPI-NR. Patients diagnosed as RE were also enrolled. A total of 108 patients (29 PPI-NR, 44 PPI-R and 35 RE patients) were recruited. Along with pathological examination, clinical information including the patients' age, gender, allergic background (bronchial asthma, food allergy, atopic dermatitis, rhinitis and hay fever) was collected. These pathological and clinical factors were compared between the three groups. The study protocol has been approved by the ethics committee of Shimane University School of Medicine (approval number: 20190715-1).

Histopathological and immunohistochemical findings

Endoscopic biopsy specimens taken when the patients were first diagnosed as either EoE or RE were reviewed by two of the surgical pathologists (N.N. and R.M.) in a blinded manner. The number of the specimens taken varied from 1 to 4 depending on the patients. The median values were 2 in all three groups. The biopsy samples were examined for the maximum number of eosinophils, lymphocytes, neutrophils and mast cells per high power field (HPF), and the presence or absence of basal hyperplasia and spongiosis in the mucosal epithelial layer. Immunohistochemical examination was performed on the specimens showing

the largest number of eosinophils in each subject. The formalin-fixed paraffin embedded tissues were sliced to a thickness of 4 μm, and immunostaining was performed using an automatic immunostainer (Bond Polymer System, Leica Byosystems, Germany) according to the manufacturer's instructions. The antibodies used were a mouse monoclonal anti-human filaggrin antibody (ab17808, Abcam, Cambridge, MA, 1:50), a mouse monoclonal anti-human loricrin antibody (ab24722, Abcam, Cambridge, MA, diluted at a 1:500), a mouse monoclonal anti-human involucrin antibody (ab53112, Abcam, Cambridge, MA, 1:100) and a mouse monoclonal anti-human desmoglein 1 antibody (sc-20114, Santa Cruz Biotechnology, Santa Cruz, CA, 1:50). Antigen retrieval was performed using BOND Epitope Retrieval Solution 2 (Leica) at 100 °C for 20 minutes (filaggrin, loricrin, involucrin) or BOND Epitope Retrieval Solution 1 (Leica) at 100 °C for 20 minutes (desmoglein 1).

Immunohistochemical staining was evaluated semi-quantitatively, combining scores of proportions of positive cells and staining intensity: the proportion score was determined in each of the four categories as 0 (no staining), 1 (up to 33%), 2 (34 to 66%) and 3 (67% or more) and the staining intensity was also scored as 0 (no staining), 1 (weak positivity) and 2 (strong positivity). The combined staining scores (0, 2, 3, 4 and 5) were calculated and employed for statistical analysis. The scores are briefly summarized in Table 3.

Statistical analysis

We compared the staining scores of four proteins, clinical factors and the counts of inflammatory cells on HE specimens between PPI-NR, PPI-R and RE. As we sought to determine the differences between the mean values of the three groups, we first employed one-way analysis of variance (ANOVA). Then Bonferroni's multiple comparison test was performed, based on the data obtained by ANOVA to determine which of the three between-group differences were statistically significant. Statistical significance was defined as $p<0.05$. All the analyses were carried out with SPSS (version 23.0, IBM SPSS Inc., Chicago, IL).

Results

Patient's background and histopathological characteristics

Age, gender and allergic background of the patients are shown in Table 1. There was a significant difference in age between EoE and RE, but no difference was observed between PPI-NR and PPI-R ($p = 0.174$). EoE was significantly more prevalent in males than RE. These results were similar to the previously published report [14]. The prevalence of total allergic diseases was highest in PPI-NR, followed in descending order by PPI-R and RE, but it was not significantly different between PPI-NR and PPI-R ($p=0.201$). Regarding

specific allergic diseases, asthma had a stronger association with PPI-NR than with PPI-R. Atopic dermatitis and allergic rhinitis were significantly more prevalent in PPI-R than in RE, but no differences were observed between PPI-NR and PPI-R ($p=0.223$ and $p=0.98$, respectively).

All kinds of inflammatory cells showed significant differences in number between EoE and RE. However, no statistical differences were demonstrated between PPI-NR and PPI-R in these cells including eosinophils ($p=0.234$). Histopathological findings including basal hyperplasia and spongiosis showed similar statistical tendency as inflammatory cells (Table 2).

Immunohistochemistry

Representative immunohistochemical images taken using a x40 objective of the four proteins of each disease and statistical analysis of the staining scores described above are shown in Fig. 1 through 4. Filaggrin exhibited a coarse granular pattern in the cytoplasm, whereas loricrin and involucrin showed a diffuse or fine granular staining pattern in the cytoplasm as well as on the cell membrane. Desmoglein 1 was expressed on the cell membrane. These staining patterns were exactly the same as those of the previous report [14]. The results of statistical analysis were shown with box-and-whisker plot. In Fig.1 through 4, crosses depicted mean values. Median values were identical to either first quartile or third quartile, as described in Table 3. The staining scores of all four molecules in EoE were statistically lower than in RE.

When we focused on the differences between PPI-NR and PPI-R, the expression of filaggrin in PPI-NR was significantly lower than in PPI-R (Fig. 1), while expression of loricrin, involucrin, and desmoglein 1 showed no differences between them (Fig.2-4). Utilizing the expression of filaggrin, we set the staining score cut-off to 2. Sensitivity, specificity, positive predictive value and negative predictive value of the score less than two, which means 0, for PPI-NR were 79%, 68%, 62%, and 83%, respectively. Since PPI-NR had a stronger association with asthma than PPI-R, positive predictive value increased to 80% when asthmatic background of EoE was taken into account (PPI-NR with asthma: 15 cases, PPI-R with asthma: 8 cases), though negative predictive value decreased to 63%. Sensitivity and specificity remained unchanged.

Discussion

EoE is more frequent in Western countries than in Eastern countries. However, the number of patients has been increasing even in Eastern countries like Japan. A great deal of research has made considerable progress in elucidating molecular pathogenesis of EoE, but the role of reduced expression of epithelial barrier proteins in the pathogenesis still remains to be clarified [1,2]. Clinically, the patients of EoE are often treated with PPI, but it is now well known that the drug is ineffective for about half of them.

It has been reported with molecular biological approach and immunohistochemistry that the expression of filaggrin, is reduced in EoE. Blachard et al. and Matoso et al. stated that there was a mutation

in the FLG gene in the region of EDC at 1q21 on chromosome 1 [5, 15]. Wu et al. also reported that filaggrin accumulation was reduced in EoE using Western blot technique [16]. Oshima et al. and Politi et al. tried to confirm it with immunohistochemistry [14, 17]. According to the former report, there was a tendency for decrease in the expression of filaggrin in EoE patients, although no statistical difference was demonstrated as compared with normal subjects. The latter report showed striking difference between EoE patients and normal subjects. However, comparison of expression of EDC proteins between PPI-NR and PPI-R has not yet been reported. In the present study, we have demonstrated for the first time that the immunohistochemical expression of filaggrin in PPI-NR is significantly lower than in PPI-R. We think this result should shed additional light on the mechanisms underlying ineffectiveness of PPI in PPI-NR. Also, it could occupy an important place in clinical practice, especially when making a treatment decision as described below.

In the initial stage of EoE development, allergens enter the esophageal mucosa, stimulate dendritic cells and induce Th2 cells. Epithelial damage of the esophagus is very likely to increase esophageal permeability and make it easier for allergens to pass through [2, 18]. It is quite conceivable that this phenomenon can be exacerbated in the presence of pre-existing barrier disorders. Consequently, cytokines such as IL-4, Il-5, and IL-13 produced and released by Th2 cells enhance the expression of eotaxin 3 in the epithelial cells and fibroblasts. Eotaxin 3 promotes infiltration of eosinophils into the epithelium. It is also known that IL-13 suppresses filaggrin production [5, 16]. When these processes are

repeated, fibrosis of the subepithelial layer progresses gradually to irreversible state [19, 20]. The mechanisms through which PPI works for a considerable number of EoE patients have not been clearly understood, but PPI has been known to inhibit secretion of eotaxin 3 and block binding of STAT6 to the eotaxin 3 promoter, resulting in suppression of inflammatory processes [21-23]. However, in addition to these possible anti-inflammatory effects of PPI, its suppressive effect on gastric acid secretion leading to repairment of the damaged mucosa has recently been reappraised [2, 18]. The reason why PPI does not work for PPI-NR has not yet been clarified, either. However, according to our results, it is speculated that an influx of larger number of allergens into the epithelium in PPI-NR due to decreased expression of filaggrin than in PPI-R could overcome either anti-inflammatory or acid blocking effect of PPI treatment. In contrast, since PPI-R retain more filaggrin than PPI-NR, PPI administration can improve the barrier function of the epithelium more readily, resulting in remission of EoE. Furthermore, since PPI-NR apparently have a stronger predisposition to asthma than PPI-R, it is also speculated that Th2-mediated immune response is more easily induced in PPI-NR after influx of allergens, which may also be related to ineffectiveness of PPI treatment. Tanaka et al. reported that obesity may be one of the nonallergic risk factors for EoE in Japanese adults [24]. However, it is unlikely that dosage based on weight is associated with difference of effectiveness of the PPI treatment between PPI-NR and PPI-R, because body mass index (BMI) of PPI-NR was statistically lower than PPI-R ($p=0.015$, Student t test, data not shown). As for the protein molecules other than filaggrin, no significant differences were observed between PPI-NR and PPI-

R, which might indicate expression of these molecules does not affect PPI treatment.

There have been many solid evidences that EoE is associated with other allergic diseases, especially with asthma [25, 26], but only a few reports have compared PPI-NR and PPI-R in reference to association with other allergic diseases. The present study showed the prevalence of asthma was significantly higher in PPI-NR than in PPI-R. This finding is generally in accordance with the previous study reporting that EoE (consistent with PPI-NR) was asthmatic as compared with PPI-REE and RE [12]. As for atopic dermatitis and allergic rhinitis, Doucet-Ladevèze R et al. reported that there was an overlap in the gene expression pattern between EoE and atopic dermatitis as well as between EoE and asthma [26]. Allergic rhinosinusitis was also reported to be associated with EoE [28]. In the present study, the prevalence of atopic dermatitis and that of allergic rhinitis were higher in PPI-NR than in RE, but no significant difference was observed between PPI-NR and PPI-R.

The present study showed that inflammatory cells infiltrated more heavily in EoE than in RE, but no significant difference in the numbers of inflammatory cells between PPI-NR and PPI-R was observed. This is also in line with the previous report [12]. Although there was no difference in the count of mast cells between the two conditions, it is of interest that Iwakura et al. reported that basophil not mast cell infiltration in the esophageal epithelium of the patients with EoE (compatible with PPI-NR) was higher than that in patients with PPI-REE employing immunohistochemistry [29].

The difference of filaggrin expression between PPI-NR and PPI-R may have an impact on

making clinical decisions. More specifically, if pathologists are able to differentiate PPI-NR from PPI-R based on filaggrin expression, their diagnosis can help clinicians to decide when to start topical steroid treatment or vonoprazan, a novel potassium-competitive acid blocker, before irreversible changes occur in the subepithelial tissue. Ishimura and Kinoshita reported that more than half of EoE patients who showed resistance to PPI therapy responded to vonoprazan that has been shown to provide more potent and sustained suppression of gastric acid secretion than PPIs [30]. It is important to note that the difference was demonstrated by immunohistochemistry, which is easy to perform even in laboratory settings where genetic tests cannot be carried out. When the cutoff of 2 was chosen for immunostaining score of filaggrin, sensitivity, specificity, positive predictive value and negative predictive value for PPI-NR were relatively high, though these values were not yet sufficient enough to separate the two conditions accurately. The values could become more informative in clinical decision when the patient has an asthmatic background according to our results.

There are some limitations to this retrospective study. To clearly elucidate how decreased filaggrin expression contributes to PPI refractoriness, immunohistochemistry alone is not sufficient. Comparison of protein levels with Western blot analysis and mRNA expression levels are also required. Moreover, in light of the relatively small number of the patients in this retrospective study, we need to enroll more patients. Furthermore, it is necessary to perform prospective study after making up the number of specimens from patients to clarify the usefulness of filaggrin expression test in treatment decision more

accurately.

Despite those limitations, we still think the results of the present study are worth reporting, because there have been no reports showing the difference of filaggrin expression between PPI-NR and PPI-R. We are now planning prospective study on EDC protein expression. Also, an investigation of whether or not there are any differences in the expressions of various kinds of proteins that are considered to be involved in the mechanisms of EoE between the two groups is now underway.

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Compliance with ethical standards

Ethical Statement The present study was performed at the Shimane University Hospital in accordance with the Declaration of Helsinki, and the protocol was approved by the ethics committee of Shimane University School of Medicine. Written informed consent was obtained from all subjects.

Conflict of interest Yoshikazu Kinoshita has received research funding from EA Pharma Co., Ltd.,

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Figure Legends

Fig. 1 Statistical analysis of immunostaining score of filaggrin and representative images of staining pattern obtained from PPI-NR, PPI-R and RE patients. Scale bars in the images are 50μm. *p<0.05

Box: interquartile range Dots: scores shown on the ordinate X: mean value

Fig. 2 Statistical analysis of immunostaining score of loricrin and representative images of staining pattern obtained from PPI-NR, PPI-R and RE patients. Scale bars in the images are 50μm. *p<0.05

Box: interquartile range Dots: scores shown on the ordinate X: mean value

Fig. 3 Statistical analysis of immunostaining score of involucrin and representative images of staining pattern obtained from PPI-NR, PPI-R and RE patients. Scale bars in the images are 50μm. *p<0.05

Box: interquartile range Dots: scores shown on the ordinate X: mean value

Fig. 4 Statistical analysis of immunostaining score of desmoglein and representative images of staining pattern obtained from PPI-NR, PPI-R and RE patients. Scale bars in the images are 50μm. *p<0.05

Box: interquartile range Dots: scores shown on the ordinate X: mean value