ARQUIVOS DE ASMA, ALERGIA E IMUNOLOGIA

Original Article

Evaluation of philagrin expression in esophageal biopsies of patients with eosinophilic esophagitis

Avaliação da expressão da filagrina em biópsias esofágicas de pacientes com esofagite eosinofílica

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ABSTRACT

Introduction: Filaggrin gene mutations have been classically associated with changes in the epithelial barrier in allergic diseases involving the skin and mucosal surfaces. Particularly in atopic dermatitis, the relationship between filaggrin, pathophysiological mechanism and clinical evolution hás been demonstrated. Recently, changes in the epithelial barrier with reduced expression of filaggrin have also been associated with immunological mechanisms involved in the pathogenesis of eosinophilic esophagitis. Due to dysfunction in the epithelial barrier, microorganisms and allergens are able to penetrate the epithelium of the esophageal mucosa, as well as in atopic dermatitis. **Objective:** To evaluated the possible correlation of filaggrin expression with histopathological findings in esophageal biopsies of patients with eosinophilic esophagitis. **Methods:** Filaggrin expression was investigated in situ by immunohistochemistry in esophageal biopsies in the following groups: Group I, control (n = 8), samples from healthy patients; Group II (n = 27), samples from patients with eosinophilic esophagitis. **Results:** The results demonstrated a decrease in the expression of filaggrin in the esophageal mucosa of patients with eosinophilic esophagitis. Additionally, the intensity of the immunohistochemical labeling was lower in the esophageal mucosa with greater infiltration of eosinophils. **Conclusion:** The reduction of filaggrin expression may be a pathophysiological phenomenon associated with an increase in the quantity of eosinophils in the esophageal mucosa, which may impact on the clinical evolution of eosinophilic esophagitis.

Keywords: Eosinophilic esophagitis, immunohistochemistry, esophageal mucosa, atopic dermatitis.

RESUMO

Introdução: Mutações do gene da filagrina vêm sendo associadas, classicamente, a alterações da barreira epitelial em doenças alérgicas com comprometimento da pele e das superfícies mucosas. Particularmente na dermatite atópica, a relação entre filagrina, mecanismo fisiopatológico e evolução clínica tem sido demonstrada. Recentemente, alterações da barreira epitelial com redução da expressão da filagrina, também têm sido associadas a mecanismos imunológicos envolvidos na patogênese da esofagite eosinofílica. Devido a disfunções na barreira epitelial, microrganismos e alérgenos são capazes de penetrarem no epitélio da mucosa esofágica, assim como na dermatite atópica. **Objetivo:** Avaliar a possível correlação da expressão da filagrina com os achados histopatológicos em biópsias esofágicas de pacientes com esofagite eosinofílica. **Métodos:** A expressão da filagrina foi investigada *in situ*, por imuno-histoquímica, em biópsias esofágicas nos seguintes grupos: Grupo I, controle (n=8), amostras provenientes de pacientes saudáveis; Grupo II (n=27), amostras provenientes de pacientes com esofagite eosinofílica. **Resultados:** Os resultados demonstraram uma diminuição da expressão da filagrina na mucosa do esôfago de portadores de esofagite eosinofílica. Adicionalmente, a intensidade da marcação imuno-histoquímica foi menor na mucosa esofágica com maior infiltração de eosinófilos. **Conclusão:** A diminuição da expressão de filagrina pode ser um fenomeno fisiopatológico associado ao aumento da quantidade de eosinófilos na mucosa esofágica, podendo impactar na evolução clínica da esofagite eosinofílica.

Descritores: Esofagite eosinofílica, imuno-histoquímica, mucosa esofágica, dermatite atópica.

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Introduction

Eosinophilic esophagitis (EoE) has been studied since the late 1970s and was best described in 1993 by Attwood et al. It is a chronic inflammatory, immune-antigen-mediated disease characterized by eosinophilic inflammation located in the esophagus, without involvement of other parts of the gastrointestinal tract (GIT), associated with symptoms of esophageal dysfunction, such as dysphagia, nausea, vomiting and impaction feed.[1,2] EoE is more frequent in patients with a personal and/or family history of atopy, such as food allergy, asthma and allergic rhinitis. Regarding prevalence, epidemiological studies indicate that EoE is as prevalent as idiopathic inflammatory bowel diseases, affecting 40 to 55 individuals/100,000 inhabitants.[3] However, as EoE was only recognized and characterized as an isolated entity in recent years, it is likely that many patients have already been wrongly diagnosed as having gastroesophageal reflux disease (GERD).[2-4]

The histopathological diagnosis obtained from esophageal biopsies material is defined by the presence of 15 or more eosinophils (Eo) per higher power field (400X). For diagnostic definition, the analysis of 2 to 4 samples from two different segments of the esophagus is recommended, regardless of the normality in the upper digestive endoscopy (UGE).[2,4] The diagnostic sensitivity of the histopathological exam can reach 100%, when at least five samples of esophageal biopsies are collected.[3] In peripheral blood circulation, eosinophilia is found, on average, in 50% of patients with EoE5.

Filagrin gene (FLG) mutations have been associated with changes in the epithelial barrier in allergic diseases that affect skin and mucosal surfaces, such as atopic dermatitis and asthma.[6-11] The dysfunction of the epithelial barrier represented mainly by the reduction of FLG expression interacts closely with the immunological mechanisms involved in the pathophysiology of these atopic diseases. Likewise, in EoE, dysfunction of the epithelial barrier in the compromised esophageal mucosa also appears to be a key factor in the pathophysiology. More recently, the abnormal expression of filaggrin has been suggested as a possible pathway of action in the pathophysiological mechanism of EoE.[12-14]

Due to an altered epithelial barrier, microorganisms and allergens are able to penetrate the esophageal mucosal epithelium. These antigens are recognized by epithelial cells through pattern recognition receptors (PRRs), which then release cytokines, such as thymic stromal lymphopoietin (TSLP), initiating a Th2 immune response. As a result of the Th2 response, eosinophils are recruited from the circulation, mainly by the local esophageal production of IL-5 and eotaxin.[9-13]

In the present study, the in situ expression of FLG was investigated in esophageal biopsies from patients with EoE. The results suggested that the expression of FLG in the esophageal mucosal epithelium is inversely proportional to the amount of eosinophils observed in the esophageal mucosa.

Material and methods

Patients

The initial study sample comprised 50 adult patients (N = 50), of both sexes, aged over 18 years, with clinical suspicion of eosinophilic esophagitis. The definitive diagnosis was made based on clinical and endoscopic features suggestive of EoE, associated with the presence of at least 15 eosinophils (EO) per microscopic field of higher magnification (400X) in the esophageal mucosa; the diagnosis wasconfirmed by histopathological analysis in 27 patients (n = 27), with at least six biopsies performed in different regions of the esophagus. Esophageal biopsies were performed for diagnostic purposes before starting the treatment of patients.

In order to control the study, esophageal samples from healthy patients were analyzed, composing the control group (Group I, n = 8). Esophageal biopsy samples from patients with EoE comprised Group II (n = 27). All samples from groups I and II were submitted to histopathological and immunohistochemical analysis.

This study was conducted in accordance with the World Medical Association Helsinki Declaration of Ethical Principles for Medical Research Involving Human Subjects, and approved by the Human Research Ethics Committee in accordance with the National Commission on Research Ethics – CONEP (Opinion 2.314.988 / CAAE 28260814.9.0000.5103 SUPREMA - University Society for Medical Education).

Histopathological processing

Esophageal biopsies were obtained from proximal, medial and distal portions in all patients included in the study. The samples were fixed in 10% formalin, embedded in a paraffin block and submitted to

microtomy with histological sections 5 to 6 µm thick. Histological sections were stained with hematoxylin and eosin for routine examination. The samples were examined with a Zeiss optical microscope (Hallbergmoos, Germany) by two independent observers trained in histopathology. After observation, representative areas of the esophageal mucosa were selected for descriptive histopathological analysis in a microscopic field of higher magnification (400x magnification), and three representative areas were chosen for digital photographic capture.

Detection in situ of FLG by immunohistochemistry

The immunohistochemical method for detecting FLG expression involved the following steps: deparaffinization for 20 min (60 °C) and imbibition in 3 baths of xylene for 3 min each; hydration in 100%, 95% and 70% alcohol for 3 min each; rinse in distilled water; blockade of endogenous peroxidase (H_2O_2 – 0.4% for 30 min /100 µL per cut); antigen recovery in a water bath at 95 °C for 40 min, in PBS; cool for 20 min at room temperature and rinse with PBS for 1 min; addition of 4 drops of BackgroundSniper on the section and incubation for 15 min (room temperature); rinse in PBS buffer (1 min). The prepared sections were incubated with Anti-Filaggrin primary antibody (Santa Cruz, Inc.) (1:100 µL Dilution) for 1 h. Afterwards, double rinse in PBS (2 min); dripping the Link Universal Trekkie secondary antibody onto the section and incubating for 20 min (room temperature); double rinse in PBS (2 min); incubation in a humid chamber for 10 min with TrekAvidin-HRP(Label): streptavidin (room temperature); double rinse in PBS (2 min each); incubation with chromogen Betazoid DAB Chromogen (DAB) homogenized in 1 ml PBS (5 min); double rinse in distilled water and in PBS; counterstaining with hematoxylin (1 min); double rinse in distilled water and then in PBS (1 min each); dehydration in three baths of 100% alcohol (1 min each) and followed by three baths in xylene (1 min each); blade assembly. The negative control was performed by omitting the primary antibody in a selected section. Positivity was determined by observing, under light microscopy, intracytoplasmic brown staining.

Positive immunohistochemical staining for FLG in the esophageal mucosal epithelium was evaluated according to immunoreactivity and intensity, and classified according to the scoring criteria.[14] The results were expressed as the mean scoreper study group. The criteria defining expression intensity and immunoreactivity are specified in Table 1. All analyzes were performed by two different examiners.

Statistical analysis

To compare the variables of the two groups, the Student's *t* test was used. SPSS 22 software was used. The significance level was considered for $p < 0.05$.

Results

Immunohistochemical analysis revealed a reduction ($p < 0.05$) in the number of cells positively labeled for

Table 1
Immunohistochemical reaction evaluation criteria for filaggrin[14]

Expression intensity	
0	No positive marking
1	Mild
2	Moderate
3	Intense
Immunoreactivity	
0	Absence of positivity
1	Positivity in more than 10% of cells (microscopic field 400x)
2	Positivity in 10 to 50% of cells (microscopic field 400x)
3	Positivity in more than 50% of the cells (microscopic field 400x)

filaggrin when comparing samples from the control group (score 3) and samples from patients with EoE (score 1). Table 2 demonstrates the immunoreactivity in the control group and in the EoE group.

Regarding the intensity of immunohistochemical staining, samples from group I (control) were strongly stained (score 3). A decrease in labeling intensity was observed in samples from group II (EoE). However, the results showed that the higher the number of eosinophils per microscopic field (400x magnification), the lower the intensity of immunohistochemical staining for filaggrin.

Thus, for comparative purposes, we divided the esophageal samples from patients with EoE into 2 subgroups with the cut-off point of 25 eosinophils per microscopic field because there is a correlation between this amount of eosinophils and a significant decrease in the intensity of filaggrin tissue labeling ($p < 0.05$).

Figure 1 presents the results of the marking intensity score respectively in each group.

Discussion

FLG is a structural protein of the skin and the loss of its function is associated with skin permeability and susceptibility to the development of atopic dermatitis and, in patients with EoE, it influences the permeability of the esophageal epithelial barrier. An important aspect to be considered is that interleukin 13 (IL-13) counter-regulates the expression of FLG in epithelial cells, promoting a mechanism by which food antigens activate the adaptive immunity of Th2 profile, which can alter the epithelial barrier function of the epithelial cells. esophageal mucosa, perhaps propagating the local inflammatory process and increased antigen uptake by epithelial and antigen-presenting cells. In this way, FLG influences the immunological tolerance mechanism that maintains the esophageal barrier intact, preventing the passage of protein particles, that could cause an allergic sensitization process. As in atopic dermatitis, mutations in the FLG gene promote a change in tissue hydration and consequent disruption of the epithelial barrier, facilitating sensitization by food and/or aeroallergens.[12-16]

Histologically, EOE is characterized by a predominantly chronic inflammatory infiltrate, which includes eosinophils, mast cells, basophils and Th2 cells. Similar to other atopic diseases, EOE is triggered by allergenic foods and aeroallergens, culminating in esophageal fibrosis or tissue remodeling. Like the skin in patients with atopic dermatitis, the mucosal epithelium of the esophagus has an altered barrier function in patients with OAE.[12-16] In the present study, using immunohistochemistry, we demonstrated a marked reduction in FLG expression in the esophageal mucosal epithelium of patients with EoE.

Table 2
Immunohistochemical staining evaluation score: immunoreactivity and intensity

Immunoreactivity		
A (Group 1 - control)	3	Positivity in more than 50% of the cells (microscopic field 400x)
B (Group II - EoE)	1	Positivity in more than 10% of the cells (microscopic field 400x)
Expression intensity		
A (Control group)	3	Intense
B (Group II - EoE) Subgroups > 25/E the field	1	Weak
B (Group II - EoE) Subgroups > 25/E the field	2	Moderate

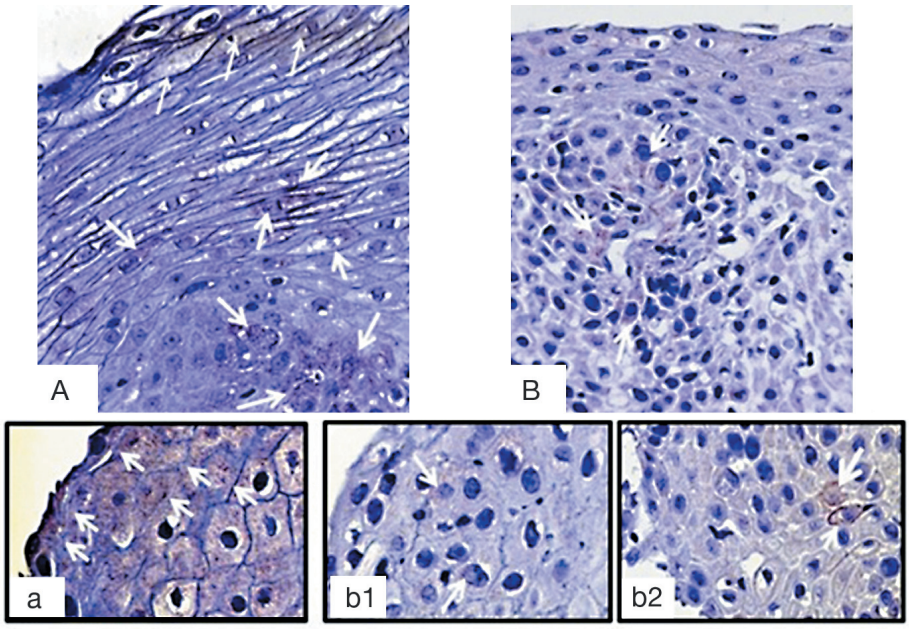

Figure 1
Immunohistochemical evaluation of filaggrin expression in esophageal samples. (**A**) presence of numerous positive cells (arrows), stained with anti-filaggrin antibody, in a sample from the control group. (**B**) Presence of some cells (arrows) with positive staining for filaggrin in a sample of Group EoE. (**b1**) Lower staining intensity for filaggrin in a sample with a number ≤ 25 eosinophils/400x field. (**b2**) Lower intensity of staining for filaggrin in a sample with a number ≥ 25 eosinophils/field 400x

The genetic predisposition to the development of EoE is well documented. This genetic susceptibility is due to the single nucleotide polymorphism that encodes the eotaxin-3 gene and also to mutations in the FLG[14] gene. The eotaxin-3 gene is considered the main promoter of eosinophil recruitment in the esophagus, therefore, the increased expression of this gene is related to an EoE phenotype characterized by an intense eosinophil infiltrate.

Recently, in epithelial cell culture, increased IL-13 synthesis was associated with changes in the epithelial barrier via decreased filaggrin expression.[15] Previously, Politi et al. (2017) investigated, also using immunohistochemistry, the in situ expression of FLG and the periostin molecule (PET) in esophageal biopsies of pediatric patients diagnosed with EoE. The authors found a downregulation of FLG and upregulation of PET in the esophageal mucosa of children with eosinophilic esophagitis, compared to control biopsies obtained from healthy subjects. Our results were similar to those described above,[14] the second study that demonstrated in situ, in human biopsies, the association between EoE and decreased FLG expression, and the first in samples from adult patients.

When we analyzed the score of filaggrin immunoreactivity, we observed that tissue samples from patients in the EoE group had a significantly lower positive labeling score for FLG ($p < 0.05$) than that observed in samples from the control group. This finding strengthens the hypothesis that changes in the epithelial barrier in EoE are important in the pathophysiology of this disease, similar to what is observed, for example, in atopic dermatitis.

The results, regarding the intensity of labeling for filaggrin, allow us to hypothesize that the number of eosinophils may be influencing the severity of epithelial dysfunction via loss of filaggrin expression. However, further studies are needed to better elucidate the role of filaggrin in the pathogenesis of EoE and the

significance of the intensity of tissue eosinophilic infiltration in terms of the clinicopathological evolution of this disease.

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