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Original Article

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Detection of Eosinophilic Cell-free Granules Based on Expression of CCR3 and MBP Markers in Esophageal Biopsy Specimens of Patients with Suspected and Confirmed Eosinophilic Esophagitis

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ABSTRACT

Purpose: Eosinophilic esophagitis (EoE) is the most well-known eosinophilic gastrointestinal disorder (EGID) characterized by the presence of a high number eosinophils within the esophageal epithelium and the clinical signs. Biopsies of patients with suspected EoE may not show a high number of eosinophils, however the presence of granules may help with the diagnosis. This study aims to evaluate the presence of cell-free eosinophil granules in the esophageal tissue of patients with suspected and confirmed EoE to accelerate the diagnosis and treatment of patients with low eosinophil count.
Methods: Fifteen patients with confirmed EoE and 15 suspected of EoE were included in this study. Patients' esophageal tissue biopsies were stained using immunohistochemistry (IHC) to identify eosinophils and their cell-free granules. For testing, anti-major basic protein (MBP) and anti-chemokine receptor type 3 (CCR3) were used as primary antibodies and a double-staining kit containing secondary antibodies conjugated to the enzyme and related chromogens were used.
Results: Cell-free granules with different degrees were observed in 53.3% and 93.3% of suspected and confirmed EoE samples, respectively. Furthermore, in esophageal biopsy of 73.3% of patients with suspected and 93.3% of patients with a definitive diagnosis of EoE, basal layer hyperplasia (BLH) was recognized.

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Conflict of Interest
The authors have no financial conflicts of interest.

Conclusion: The results of the present study showed that IHC can be applied to detect cell-free eosinophil granules in esophageal tissue. Observation of granules and basal cell hyperplasia in biopsies of suspected EoE patients whose eosinophil count is below the threshold can be valuable findings to make a definitive diagnosis for these patients.

Keywords: Esophagus; Immunohistochemistry; Degranulation

INTRODUCTION

Eosinophilic gastrointestinal disorders (EGIDs) are chronic inflammatory diseases characterized by persistent gastrointestinal symptoms and the infiltration of eosinophils into the gastrointestinal tract without a specific cause for eosinophilia, such as a parasitic infection or malignancy [1]. Eosinophilic esophagitis (EoE) is the most well-known type of EGIDs, characterized by a group of clinical, endoscopic and histopathological features. EoE was recognized as a distinct clinical condition in the early 1990s [2], and its incidence and prevalence have increased significantly since then and have become a major cause of dysphagia and food impaction [3]. This disease has a negative impact on the lives of patients and their families. In adolescents and adults, symptoms usually include dysphagia and food impaction in the esophagus. But, non-specific symptoms are seen in children, including growth retardation, vomiting, heartburn, and abdominal pain. Also, endoscopic examination of the esophagus in many but not all EoE patients shows the presence of rings, linear furrows, white exudate, and narrowing of the lumen [4]. Almost all guidelines refer to the observation of ≥15 eosinophils per high power field (Eos/HPF) in a biopsy specimen taken from the esophagus to diagnose the disease [5]. However, there are patients in whom, despite having symptoms associated with this disease, the number of eosinophils in their biopsy is less than the threshold defined for a definitive diagnosis. Eosinophil granules contain several compounds, such as major basic protein (MBP), which are involved in tissue and organ damage due to dysfunction or over activity of eosinophils [6]. In addition, these granules express various receptors such as chemokine receptor type 3 (CCR3) and cysteinyl leukotriene receptor (cysLTR) on their surface, which bind to their ligands and have a secretory mechanism that allows the granules to selectively release their specific protein components and act as extracellular secretory organs after eosinophil cytolysis [7]. Eosinophils cytolysis, which leads to cell destruction and the release of cell-free granules, has been observed in inflammatory conditions [8] and it is a common degranulation mechanism in many eosinophil-related diseases [9]. Therefore, the observation of cell-free granules in the tissue samples of patients can indicate the presence of eosinophils that are ruptured and cannot be identified directly. The objective of this study is to evaluate the presence of cell-free eosinophil granules in esophageal biopsy specimens of pediatric patients who exhibit EoE-related symptoms but have fewer than 15 Eos/HPF, likely attributed to eosinophil rupture and the absence of intact cells.

MATERIALS AND METHODS

Patients

In this case-control study 15 patients with suspected EoE and 15 with confirmed EoE were included as case and control groups respectively. The groups were age and sex matched. Patients were selected based on medical records referred to Roshan Azma Pathobiology Laboratory, Tehran, Iran (a referral lab for pediatric gastrointestinal pathology) and

Children's Medical Center, Tehran, Iran from 2017 to 2021, who underwent endoscopy and biopsy of esophageal tissue. Biopsy specimens were taken from the distal esophagus. Based on the available diagnostic criteria, biopsy specimens of patients with EoE related symptoms but fewer than 15 Eos/HPF in their biopsies, were selected as the suspected EoE (case) group and patients with a definite diagnosis (≥15 Eos/HPF and EoE related symptoms) were selected as confirmed EoE group (positive control) [10]. The number of eosinophils has been confirmed by a pathologist by examining slides stained with hematoxylin and eosin (H&E). Exclusion criteria were a history of underlying diseases such as malignancy and autoimmune diseases. Furthermore, patients with less than 15 Eos/HPF in their biopsies, who responded well to treatment with proton pump inhibitors (PPIs) were considered as patients with gastroesophageal reflux disease (GERD) and were not included in this study.

This study protocol was reviewed and approved by ethics committee of Children's Health Research Institute, Shahid Beheshti University of Medical Sciences with approval number IR.SBMU.RICH.REC.1399.005 and informed consent was obtained.

Clinical data

Demographics, history of allergies, and histopathological findings were recorded by reviewing patients' medical reports and filling out questionnaires.

Immunohistochemistry

Formalin-fixed paraffin-embedded tissue blocks were sectioned (5 microns thick) and immunohistochemistry (IHC) was performed according to the following protocol. The slides were first deparaffinized and rehydrated. Antigen retrieval was performed using pepsin enzyme (pepsin solution; Abcam). After blocking, the slides were incubated for one hour with mouse anti-human eosinophil major basic protein antibody (clone BMK-13, 1:25, Bio-rad) and then for one hour with rabbit anti-human CCR3 polyclonal antibody (1:50; MyBioSource) at room temperature. To identify the studied markers, a double stain IHC kit: M&R on human tissue (Abcam) was used. This kit contains Horseradish peroxidase (HRP)-polymer anti-mouse IgG, Alkaline phosphatase (AP)-polymer anti-rabbit IgG, and two distinct chromogens, permanent red (red) and emerald (green). Slides were incubated with a 1:1 mixture of anti-mouse IgG labeled with HRP and anti-rabbit IgG labeled with AP. The addition of HRP substrate (permanent red), counterstaining with hematoxylin, and addition of AP substrate (emerald) were performed respectively. Finally, the slides were dehydrated and cover slipped. Healthy esophageal tissue was used as negative control.

The degree of cell-free eosinophil granules in confirmed and suspected tissue samples were determined by visual observation of 10 microscopic fields of slides related to each sample with a magnification of 1,000 by two researchers independently. A semi-quantitative classification (0=none, +1=very low, +2=low and +3=moderate) was designed and the average of cell-free granules was reported. The findings of the immunohistochemical study were compared with the reported results of H&E staining.

Examination of basal layer hyperplasia

The severity of basal layer hyperplasia (BLH) was recorded based on the pathologist's diagnosis based on the examination of biopsy samples stained by H&E method. Epithelial thickness of up to 30% were considered as mild, 30–60% as moderate, and above 60% as severe BLH. Patients, who had different degrees of BLH in different parts of their biopsy slides were classified as low/moderate and moderate/severe.

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Statistical analysis

Data were statistically analyzed using SPSS 16.0 (SPSS Inc.). The age comparison of the cases was done using Mann-Whitney U-test. The gender and history of allergic diseases was investigated using Chi-Square test. Also, receiver operating characteristic (ROC) curve analysis was applied to investigate cell-free granules and BLH as predictors of the disease.

RESULTS

Patient characteristics

A total of 30 tissue samples from 30 patients were included in this study, of which 15 samples were definitively diagnosed with EoE and 15 were suspected of EoE. The confirmed EoE group had a mean age of 8.3 years with 40% of the cases being male. In the EoE suspected group, the mean age was 7.07 years with 46.7% of the cases being male. There was no significant difference in age and sex between two groups (p>0.05). 66.6% of cases with confirmed EoE and 41.7% of cases with suspected EoE had a history of food allergy. Sixty percent of cases with confirmed EoE and 33.3% of cases with suspected EoE had a history of other allergic diseases. Moreover, 73.3% and 58.3% of confirmed and suspected EoE patients had a history of allergic disease in their family members, respectively. There was no significant difference (p>0.05) between the two groups in terms of history of allergies (**Table 1**).

Significant number of cell-free granules in patients with confirmed EoE

Examination of IHC stained slides using two markers MBP and CCR3 clearly showed the presence of cell-free granules in esophageal biopsy of 53.3% patients in suspected group and 93.3% of patients with confirmed EoE (**Figs. 1 and 2**). While the cell-free granules in H&E stained slides were not clearly visible. There was a significant difference between the two groups in the amount of cell-free granules. In the suspected group, there were 7 (46.6%) biopsy samples with 0, 6 (40%) with +1, 2 (13.3%) with +2, and no patient had 3+ cell-free granules. In the confirmed EoE group, 1 (6.6%) biopsy sample with 0, 5 (33.3%) with +1, 4 (26.6%) with +2 and 5 (33.3%) with +3 cell-free granules were observed (**Fig. 3**).

Basal layer hyperplasia in EoE patients

Based on the pathologist's reports, in biopsy specimens of 93.3% of confirmed patients and 73.3% of patients with suspected EoE different degrees of BLH was found. Although BLH was observed more frequently in EoE patients compared to suspected cases, there was no significant difference between the two groups (**Fig. 4**).

Table 1. Patients' demographic information

Demographics	Suspected EoE (n=15)	Confirmed EoE (n=15)	p-value
Sex			
Female/male	53.3/46.7	60/40	0.7
Age	7.07±3.2	8.3±4.66	0.4
Positive (%)			0.13
History of food allergy	41.7	73.3	
History of other allergies	33.3	60	0.17
History of allergy in family	58.3	73.3	0.45

Values are presented as number only or mean±standard deviation.
EoE: eosinophilic esophagitis.

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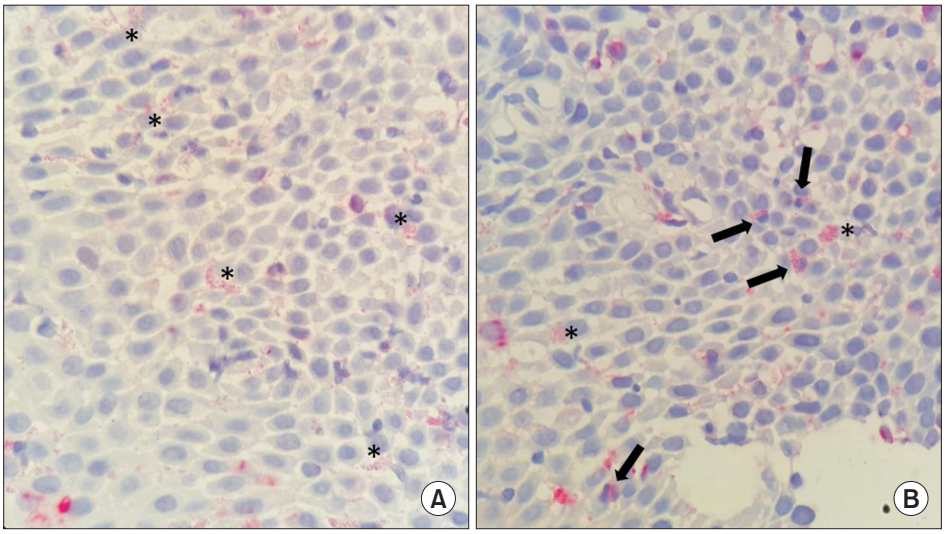

Fig. 1. Extracellular eosinophil granules in biopsies of patient with suspected and confirmed EoE using double-stain IHC technique. (A) Cell-free granules (stars) in esophagus biopsy of a patient with suspected EoE, magnification×1,000. (B) Cell-free granules (stars) and intact eosinophils (arrows) in esophagus biopsy of a confirmed patient, magnification×1,000.
EoE: eosinophilic esophagitis.

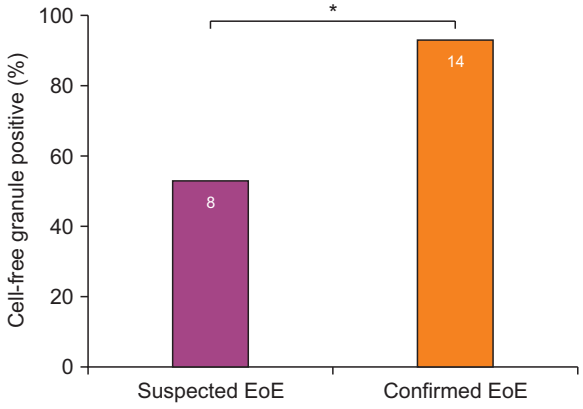

Fig. 2. Percentage of positive subjects regarding the presence of cell-free eosinophil granules in biopsy samples of suspected and confirmed EoE groups (*p<0.05).
EoE: eosinophilic esophagitis.

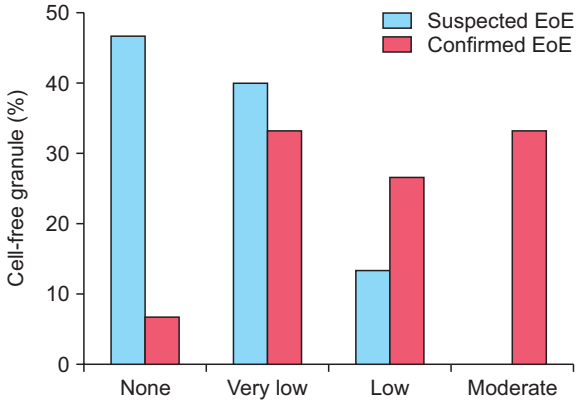

Fig. 3. Percentage of patients with different degree of cell-free granule levels in the confirmed and suspected EoE groups.
EoE: eosinophilic esophagitis.

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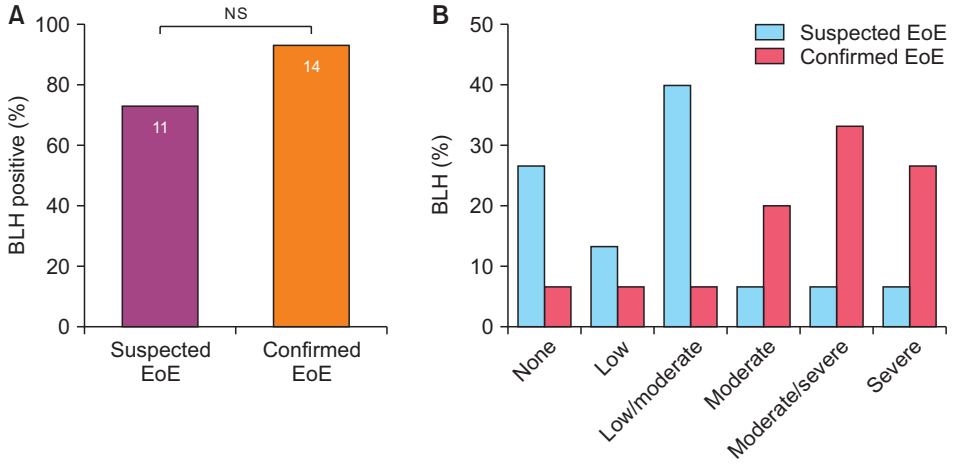

Fig. 4. Suspected and confirmed EoE groups in terms of BLH. (A) Percentage of BLH-positive cases in the two study groups (p>0.05). (B) Percentage of patients with different degrees of BLH in two study groups.
EoE: eosinophilic esophagitis, BLH: basal layer hyperplasia, NS: not significant.

Cell-free granules and BLH as EoE predictors

Statistical analysis showed that the presence of cell-free granules and BLH in biopsy specimens could act as predictors of EoE regardless of the number of eosinophils in the tissue. In ROC curve analysis, the area under the curve for cell-free granules was 0.82 (0.67–0.97) and the p-value was 0.003. These values for BLH were 0.78 (0.61–0.96) and 0.008, respectively.

DISCUSSION

Examination of IHC-stained slides using MBP and CCR3 markers showed the presence of cell-free granules in esophageal biopsy of 53.3% of suspected and 93.3% of confirmed EoE pediatric patients. Conversely cell-free granules were not clearly visible in H&E stained slides. This result indicates that in suspected patients with low eosinophils, the number of eosinophils are likely to disappear due to degranulation by cytolysis. In addition, the observation of cell-free granules in the samples of confirmed patients with high eosinophil count shows that the number of eosinophils counted by H&E staining is lower than the actual number probably because of abnormal morphology and degranulation of a number of these cells. These results corroborate findings of Protheroe et al.'s study [11] of EoE patients biopsies with IHC and the use of anti-eosinophil peroxidase (EPX) antibody, which clearly showed more intact cells and cell-free granules compared to the results of H&E staining. In addition, Peterson et al. [12] have also compared the presence of extracellular proteins of eosinophils in esophageal biopsies of EoE patients with clinical and endoscopic symptoms but eosinophils fewer than 15/HPF and those with >15 Eos/HPF using indirect immunofluorescence. Similar to our study, they found extracellular MBP1 in samples from both groups [12]. Therefore, the observation of cell-free granules in suspicious patients with clinical symptoms and endoscopic findings, despite eosinophil counts below the threshold, could be beneficial for the diagnosis of EoE.

Furthermore, examination of BLH in our study showed that 73.3% of suspected and 93.3% of confirmed EoE patients have different degrees of hyperplasia in their esophageal tissue samples. Whelan et al. [13] investigated the prevalence of epithelial changes, particularly BLH, in patients with symptomatic EoE but with Eos/HPF<15 as well. They found that

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BLH in these patients was associated with symptoms and endoscopic findings and the observation of BLH regardless of the number of eosinophils indicates an active progressive disease [13]. In addition, examining the status of BLH in the samples of EoE patients with Eos/HPF<15 by Godwin et al. [14] showed that BLH has a high prevalence in this group of patients. ROC curve analysis of BLH in present study confirmed its suitability as a marker in diagnosis of EoE irrespective of the eosinophil count. Cell free granules also demonstrated a good predictive value for diagnosing EoE, with an area under the curve indicating strong diagnostic accuracy.

In contrary to most studies showing that EoE has a significant prevalence in men [3,15] in our study, in both suspected and confirmed EoE groups, the number of females were higher than males although in general, no significant sex difference was observed in any of the two studied groups. Furthermore, in both groups of our study, a significant number of cases had a history of allergies. Several previous studies have also shown co-occurrence of allergic diseases or a history of these diseases in a remarkable percentage of patients with EoE [16-18]. These reports show a significant relationship between EoE and allergic diseases. Therefore, taking into account the allergy history in suspected EoE patients should be considered in favor of this diagnosis.

Since it is impossible to count cell-free eosinophil granules and there was no agreed method to measure them, the most important limitation in our study was that we had to determine the amount of cell-free granules semi-quantitatively. To moderate the effect of this limitation and get more accurate results, after designing the semi-quantitative classification, the slides were examined by two researchers independently and the average score of cell-free granules amount was reported.

Our investigations illustrated that despite the percentage difference, both suspected and confirmed EoE pediatric patients were statistically similar in terms of observation of BLH in biopsy samples, demographics and history of allergies. Cell-free granules were spotted in a considerable percentage of the cases of both groups. Given that cell-free granules and BLH serve as reliable predictors for this disease, these parameters can be employed for a definitive diagnosis of EoE and initiating treatment for suspected EoE patients.

In conclusion, EoE is a disease with variable amounts of eosinophil infiltration and activation. Our findings revealed that markers of eosinophil degranulation such as MBP and CCR3 can be valuable in recognizing pediatric patients with symptoms independent of the number of intact eosinophils.

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