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Carine Blanchard, … , Bruce J. Aronow, Marc E. Rothenberg


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Eotaxin-3 and a uniquely conserved gene-expression profile in eosinophilic esophagitis

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Eosinophilic esophagitis (EE) is an emerging disorder with a poorly understood pathogenesis. In order to define disease mechanisms, we took an empirical approach analyzing esophageal tissue by a genome-wide microarray expression analysis. EE patients had a striking transcript signature involving 1% of the human genome that was remarkably conserved across sex, age, and allergic status and was distinct from that associated with non-EE chronic eosinophilic esophagitis. Notably, the gene encoding the eosinophil-specific chemoattractant eotaxin-3 (also known as CCL26) was the most highly induced gene in EE patients compared with its expression level in healthy individuals. Esophageal eotaxin-3 mRNA and protein levels strongly correlated with tissue eosinophilia and mastocytosis. Furthermore, a single-nucleotide polymorphism in the human eotaxin-3 gene was associated with disease susceptibility. Finally, mice deficient in the eotaxin receptor (also known as CCR3) were protected from experimental EE. These results implicate eotaxin-3 as a critical effector molecule for EE and provide insight into disease pathogenesis.

Introduction

Eosinophilic esophagitis (EE) is an emerging worldwide disease, as documented by recent case series from Switzerland, Australia, Canada, Japan, England, and the US (1–4). Of concern, EE appears to be a growing health problem with an annual incidence of at least 1 in 10,000 children (5). The primary symptoms of EE (chest and abdominal pain, dysphagia, heartburn, vomiting, and food impaction) are also observed in patients with chronic esophagitis (CE) including gastroesophageal reflux disease (GERD) (6–8). However, in contrast to GERD, EE occurs more frequently in males (80%), appears to have a common familial form, has a high rate of associated atopic disease (70%), and is typically associated with a normal pH probe recording of the esophagus (5, 9, 10). Distinguishing EE from GERD is important since EE patients do not respond to anti-GERD therapy but may respond to anti-inflammatory therapy and/or allergen elimination (6, 11–13). Whereas both GERD and EE are associated with esophageal eosinophils, the level of eosinophils in EE is much higher, greater than 24 eosinophils per high-power field (hpf) (×400) (11); the normal esophagus is devoid of eosinophils (14, 15). However, whether GERD and EE represent a continuum, with EE being a more severe manifestation, has not been adequately addressed. A clearer differentiation between these various eosinophilic states is clearly needed.

Dissection of experimental EE models in mice has revealed that EE can be triggered by both food and aeroallergens (16, 17). However, nearly 25% of people with EE are nonatopic individuals with no identifiable allergic sensitization (2, 5, 9, 18). It is critically important to understand the relationship between the allergic and nonallergic variants of EE; whether allergic and nonallergic eosinophilic esophagitis involves similar effector pathways has significant implications for therapeutic strategies. Murine modeling has established that EE is a Th2-associated disease (17, 19). IL-5 is required for disease pathogenesis in an experimental model (16); indeed, humanized anti–IL-5 appears to be effective in an early clinical study (20). Human EE is associated with overproduction of the Th2 cytokines IL-4 and IL-13 (18, 21). Although these Th2 cytokines have been implicated, the mechanism by which they lead to esophageal eosinophilia is unclear. While IL-4 and IL-13 are known to induce the eosinophil-specific eotaxin chemokines (e.g., eotaxin-1, eotaxin-2, and eotaxin-3) (22–25), their role has remained elusive, since they have not yet been demonstrated to be overproduced in EE, and eotaxin-1–deficient mice only develop a modest attenuation of experimental EE (16, 19).

In an effort to provide unbiased insight into disease pathogenesis, we took an empirical approach involving expression profiling of esophageal biopsy tissue from patients with EE and comparison of this tissue with tissue from patients with CE, as well as healthy controls (referred to herein as NL [normal]). Whole-genome-wide expression analysis uncovered a striking EE transcript signature that was similar across patients’ sex and age, but distinct from CE. Notably, allergic and nonallergic variants of EE were found to have a conserved esophageal transcriptome indicating overlapping effector pathways in the diseased tissue. Furthermore, the most highly induced transcript in EE was eotaxin-3; notably, levels of eotaxin-3 strongly correlated with disease severity, and a single-nucleotide polymorphism (SNP) in the eotaxin-3 gene was associated with disease susceptibility. Lastly, the importance of this

Nonstandard abbreviations used: CCR, CC chemokine receptor; CE, chronic esophagitis; CLC, Charcot-Leyden crystal; EE, eosinophilic esophagitis; FDR, false discovery rate; GERD, gastroesophageal reflux disease; hpf, high-power field; MBP, major basic protein; NL, normal; SNP, single-nucleotide polymorphism.

Conflict of interest: The authors have declared that no conflict of interest exists.

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The pathway was demonstrated by the protection from experimental EE observed in mice harboring a genetic deletion in the eotaxin receptor (CC chemokine receptor 3 [CCR3]).

Results

EE transcript signature. Esophageal biopsy samples derived from individual patients (see Supplemental Table 1; supplemental material available online with this article; doi:10.1172/JCI26679DS1) were subjected to whole-genome-wide transcript expression profile analysis using oligonucleotide-based DNA microarray chips. Of the 54,681 transcripts represented on these microarrays, 574 transcripts (Supplemental Table 2) were differently expressed ($P < 0.01$) in the EE group compared with normal healthy patients; thus, approximately 1% of the whole human genome transcripts define the transcript signature of EE. Hierarchical clustering of the signal intensities of the individual transcripts in each group showed a high similarity of transcript expression patterns among EE patients (Figure 1A). Of these, 344 transcripts were expressed more abundantly and 230 were expressed less abundantly in the EE patients compared with the NL group (Figure 1A). Gene ontology analysis of the EE transcript signature (Supplemental Table 3) revealed that the overexpressed genes were frequently involved in cell communication (26%), signal transduction (22%), response to external stimulus (20%), immune response (16%), and response to stress (11%). In contrast, the downregulated genes were composed of a distinct family of functional groups (Supplemental Table 4). The complete annotation of all the transcripts expressed differently in EE patients compared with the NL group is presented in Supplemental Table 5.

While there are numerous related families of dysregulated genes, it is notable that 5 mast cell genes were highly induced (including carboxypeptidase A3, 13-fold; high-affinity IgE receptor [FcεRI], 4-fold; and mast cell tryptase-α, 6-fold). Interestingly, the maximum mast cell count per hpf was significantly increased ($13.8 ± 1.8$ cells per hpf, mean ± SD, $n = 13, P < 0.0005$) in biopsies from EE patients compared with NL ($4.6 ± 0.3, n = 6$) and CE patients ($5.8 ± 1.1, n = 5$), as shown in Supplemental Table 1 and Figure 2A. Whereas evidence of extracellular eosinophil granule constituents (indicators of eosinophil activation) was appreciated in most EE tissue sections, there was no dramatic evidence of mast cell degranulation based on the absence of extracellular mast cell constituents (data not shown). Further assessment revealed that mast cells and
The identification of an EE transcript signature provided a valuable opportunity to uncover critical aspects of disease pathogenesis. First, we were interested in determining whether the allergic and nonallergic variants of EE had different transcript profiles. When we compared the full EE transcript profile between allergen-sensitized and non–allergen-sensitized EE patients, there was nearly complete overlap in the transcripts (Figure 1B) of the EE signature genome defined in Supplemental Table 5. Only 10 genes were differentially expressed, and only 1 was changed at least 2-fold (lymphocyte antigen 75 [LY75]; 2-fold increase in allergic). The human LY75 molecule has previously been shown to affect IL-4 signaling (26). When EE patients were divided into allergic and nonallergic groups based on aeroallergen or food allergen sensitization, no significant differences (false discovery rate [FDR] < 0.05) were observed between the positive and negative groups. As shown in Supplemental Table 1, a dramatic overlap exists between patients sensitive to food and aeroallergens and patients with a history of allergic disease (food anaphylaxis, allergic rhinitis, asthma, or atopic dermatitis). Second, we aimed to determine whether EE patients had variable expression of genes dependent on their age. Of the 574 dysregulated transcripts, no gene correlated with patient age within the EE transcript profile. Third, we were interested in analyzing the EE transcriptome as a function of disease severity. We hypothesized that a disease severity index (sum of the maximum eosinophil and lymphocyte counts) at the time of endoscopy, as indicated in Supplemental Table 1. However, no significant differences in the identified transcripts were found (FDR < 0.05), as shown in Supplemental Table 2. Taken together, these results demonstrate that the EE transcript profile is remarkably conserved between patients despite differences in sex, age, and allergic status.

Differentiation of EE and CE. We compared the transcript expression profile in patients who presented with symptoms of EE but were found to have CE. Cluster analysis was performed to stratify dynamic genes into related subgroups (Figure 1D). In Figure 1D, the EE transcripts are seen in clusters 4 and 5; the CE transcripts are seen in clusters 1, 2, and 3. CE patient biopsies showed an expression profile close to that of the NL patient biopsies. The CE transcript esophageal samples contained only 228 dynamic transcripts (Supplemental Table 2), approximately 0.4% of the tested genome, compared with NL samples (P < 0.01). Indeed, there were significant changes in tissue pathology including lymphocyte levels and the degree of epithelial cell hyperplasia in CE versus NL samples (Supplemental Table 1 and Figure 2B). These 228 transcripts (114 overexpressed, shown in combined clusters 1 and 2, and 114 downregulated in cluster 3) were rich in genes involved in intracellular cascades (10%) and biosynthesis (10%) (both in cluster 1) and cell growth and maintenance (23%) (in cluster 2) (Supplemental Table 6). Notably, no transcript was modified 5-fold or more in CE compared with NL samples (Figure 3). In contrast, 124 genes were modified 5-fold or more in EE compared with NL samples, including 42 transcripts that were modified 10-fold or more in EE; these dysregulated genes are shown in Figure 3. To define genes that could distinguish EE from CE, we directly compared the EE transcripts are seen in clusters 4 and 5; the CE transcripts were found to have CE. Cluster analysis was performed to stratify dynamic genes into related subgroups (Figure 1D). In Figure 1D, the EE transcripts are seen in clusters 4 and 5; the CE transcripts are seen in clusters 1, 2, and 3. CE patient biopsies showed an expression profile close to that of the NL patient biopsies. The CE transcript esophageal samples contained only 228 dynamic transcripts (Supplemental Table 2), approximately 0.4% of the tested genome, compared with NL samples (P < 0.01). Indeed, there were significant changes in tissue pathology including lymphocyte levels and the degree of epithelial cell hyperplasia in CE versus NL samples (Supplemental Table 1 and Figure 2B). These 228 transcripts (114 overexpressed, shown in combined clusters 1 and 2, and 114 downregulated in cluster 3) were rich in genes involved in intracellular cascades (10%) and biosynthesis (10%) (both in cluster 1) and cell growth and maintenance (23%) (in cluster 2) (Supplemental Table 6). Notably, no transcript was modified 5-fold or more in CE compared with NL samples (Figure 3). In contrast, 124 genes were modified 5-fold or more in EE compared with NL samples, including 42 transcripts that were modified 10-fold or more in EE; these dysregulated genes are shown in Figure 3. To define genes that could distinguish EE from CE, we directly compared the EE and CE transcriptomes. There was an overlap of only 40 genes between EE and CE (mainly in cluster 2) (Figure 1D and Supplemental Table 7), and only 5 of these overlapped with the EE transcript signature. Taken together, these results suggest that EE and CE are unlikely to share the same disease process. Furthermore, the identification of strongly induced genes that distinguish EE from CE (Figure 3) defines potential diagnostic criteria that are likely to distinguish these forms of esophagitis.

Disease severity index. We were interested in analyzing the EE transcriptome as a function of disease severity. We hypothesized that

Figure 2
Mast cell and lymphocyte counts in NL, CE, and EE patients. (A) The maximum mast cell count per hpf was assessed in patients 1–24 using immunohistochemistry. Biopsies were stained using monoclonal anti–human tryptase. (B) The maximum lymphocyte count per hpf was assessed in patients 1–24 on H&E staining. P values were calculated using the Welch t test (A and B). (C) The correlations between basal layer cell thickness and both maximum eosinophil level (r² = 0.47, P < 0.0005) and mast cell level (r² = 0.51, P < 0.0001) are shown. (D) The maximum eosinophil levels are presented as a function of maximum mast cell levels (r² = 0.18, P < 0.05). P values were based on Pearson correlation (C and D).
the number and magnitude of modified genes might be directly related to histological severity. We thus aimed to determine whether eosinophil levels would correlate with the number of altered genes. The population of patients with EE had peak eosinophil levels that varied between 24 and 218 eosinophils per hpf (Figure 1D). The number of dysregulated genes increased between eosinophil levels of 0 and 83 (Figure 4A). Similarly, the magnitude of gene changes directly correlated with eosinophil levels (Figure 1D). As such, 321 genes (55%) of the EE transcript signature are among the 1,943 genes that most correlated (P < 0.005) with eosinophil levels (Figure 4B). Interestingly, the mast cell gene signature was located in the 321 genes that correlated with eosinophil numbers.

**Eotaxin-3 expression in the esophagus.** Within the EE transcript signature, the gene with the greatest change was eotaxin-3, which was induced 53-fold (Figure 3). Other relevant eosinophil chemokines, such as eotaxin-1 and eotaxin-2, were induced less than 2-fold in EE samples. Given that our analysis was a whole-genome-wide approach and that the gene with the largest expression change was a relevant, specific, and potentially important eosinophil chemoattractant (24), we prioritized investigation of this finding. Using real-time PCR analysis (LightCycler), a mean 53-fold increase in eotaxin-3 mRNA compared with that in NL samples was observed (Figure 5A). Modest changes in eotaxin-1 (Figure 5B) and eotaxin-2 (Figure 5C) were observed in EE patients, although there was some variability among patients.

It was next important to determine whether the level of eotaxin-3 correlated with eosinophil levels in esophageal samples. As shown in Figure 6A, 2 methods (LightCycler quantification and microarray analysis) revealed a strong correlation between eotaxin-3 mRNA and peak eosinophil counts (P < 0.005). In addition, eotaxin-3 mRNA correlated with mast cell levels based on microarray analysis (P < 0.005) and on LightCycler quantification (P < 0.05) (Figure 6B).
In order to localize the eotaxin-3 production in the esophagus, in situ hybridization was performed. As shown in Figure 7, the eotaxin-3 antisense probe yielded a strong signal in the epithelial cell layer of EE patients only. Bright-field microscopy revealed that the eotaxin-3 signal was confined to a population of mononuclear epithelioid cells within the mucosa. The localization of the eotaxin-3 was always in epithelioid cells, whose locations were usually close to the proliferative region (basal layer) of the esophagus. Infiltrative eosinophils were eotaxin-3 negative, as were subepithelial structures such as esophageal papillae and underlying stroma. No signal was found in the biopsies of NL patients (Figure 7, A and B) or CE patients (data not shown). Hybridization of a control eotaxin-3 sense probe to biopsies of EE, CE, and NL patients revealed no significant background signal (Figure 7, G and H, and data not shown).

Eotaxin-3 protein level in the esophagus and in the blood. Eotaxin-3 protein levels were quantified in NL, CE, and EE patients in esophageal biopsies. Eotaxin-3 protein level in the esophagus of EE patients was significantly increased compared with that in NL and CE patients (Figure 8). In EE patients, the eotaxin-3 protein level was $580 \pm 316$ pg/mg protein (mean $\pm$ SD). Indeed, the eotaxin-3 protein level correlated with esophageal level of eosinophils ($r^2 = 0.74$, $P < 0.05$) (data not shown). The esophageal eotaxin-3 protein levels in NL and CE patient biopsies were below the detection limit of the ELISA (6 pg/mg protein). Eotaxin-3 protein level was also quantified in the plasma. A 2-fold increase in eotaxin-3 protein levels was observed between NL and EE plasma samples. Eotaxin-3 protein levels were $64 \pm 36$, $21 \pm 9$, and $25 \pm 21$ pg/ml in EE, CE, and NL plasma samples, respectively (mean $\pm$ SD, $P < 0.005$). Interestingly, while detectable in NL patient biopsies, eotaxin-1 and eotaxin-2 protein levels ($13.6 \pm 11.5$ and $18.4 \pm 12.7$ pg/mg protein, respectively, mean $\pm$ SD, $n = 4$) were not significantly increased in CE ($8.1 \pm 16.3$ and $23.3 \pm 18.8$ pg/mg protein, $n = 6$) and EE samples ($33.2 \pm 30.4$ and $27.6 \pm 21.8$ pg/mg protein).

Eotaxin-3 SNP frequency associates with EE. We hypothesized that polymorphism(s) in the eotaxin-3 gene might be associated with disease susceptibility. We checked the position and the frequency of known SNPs in the eotaxin-3 gene (promoter, exons, and untranslated regions) that were between 5% and 20% frequency in the white population using the public SNP databanks (http://www.hapmap.org; and PubMed SNP databanks, http://www.ncbi.nlm.nih.gov). In the promoter, we found 3 SNPs, but they did not modify or create the consensus sequence of a responsive element. No SNP had a frequency between 5% and 20% in the coding region of exons. In the 3’ untranslated region, no SNP matched our criteria. In the 3’ untranslated region, one SNP (rs2302009, 2,496 $T \rightarrow G$) was present in 20% of the white population. Therefore, we genotyped this SNP in patients with EE and control individuals without EE (Table 1). Genotypes of the SNP 2,496 $T \rightarrow G$ were in Hardy-Weinberg equilibrium in both EE patients and unrelated controls. Notably, the allele G was overrepresented in patients with EE compared with race/ethnicity–matched controls (32.1% versus 22.4%, $P = 0.0069$). Additionally, the frequency for genotype GG was significantly higher in patients with EE (odds ratio 4.55, 95% confidence interval 1.71–12.39, $P = 0.001$). The GG genotype was not predominant in atopic EE patients; of the 16 GG individuals identified, there were 7 and 9 allergen-sensitized and nonsens-
Considering this SNP with EE is not dependent on atopic status. In order to validate the case-control results that could generate false positives due to population stratification, a family-based transmission disequilibrium test was conducted (27). From heterozygous parents, the allele was preferentially transmitted to affected individuals compared with the alternative allele \( T \) (39 versus 18, \( P = 0.0054 \)). The odds ratio was 2.13 (95% confidence interval 1.57–2.69). Taken together, the results obtained from both case-control and family-based association analyses suggest that the eotaxin-3 gene may be associated with susceptibility to EE.

**Discussion**

EE is an emerging worldwide disease, yet there is little information concerning its underlying pathogenesis. As such, EE poses considerable diagnostic and therapeutic challenges, especially because esophageal eosinophilia has been associated with several other medical conditions, including GERD, parasitic infection, and hypereosinophilic syndromes (8, 28). In this study, several principles have emerged. Notably, we have identified a striking EE transcript signature involving approximately 1% of the human genome. This transcriptome is remarkably conserved between patients despite their age, sex, and allergic status. The apparent homogeneity is even more striking considering that EE has been described as having a non-uniform (i.e., patchy) distribution. Despite the presence of apparent atopic and nonatopic variants of EE, our results indicate that the downstream effector phase of the disease is conserved between these disease variants. This was a surprising finding, because, from the outset, we were concerned that there would be large variability in gene transcript levels among patients because of their divergent clinical presentations (including age and sex). Thus, despite millions of SNPs in the human genome, our results suggest that this complex disorder may have largely conserved disease mechanisms. This finding provides encouraging insight that relatively uniform successful pharmacological therapy may be achieved for EE. Our results are consistent with prior analysis of atopic and nonatopic variants of eosinophilic lung disease (asthma) (29); atopic and nonatopic patients have been shown to have the same cytokine mRNA expression in lung tissue. In our study, based on analysis of hundreds of genes, we present strong evidence that allergic and nonallergic variants of eosinophilic disorders have a common underlying pathogenesis. To our knowledge, this is the first time this type of analysis has been used to examine the etiology of allergic and nonallergic disease variants.
Importantly, our study identified eotaxin-3 as the gene most highly induced in EE. Given the role of this chemokine in regulating CCR3-expressing cell responses in vitro (e.g., eosinophil and mast cell) (30), we focused our attention on this gene product. Indeed, levels of eotaxin-3 expression in the esophagus strongly correlated with disease severity based on basal layer expansion and levels of eosinophils and mast cells. In order to further prove the importance of the identified pathway in vivo, mice with genetic deletion of the eotaxin receptor (CCR3) were shown to be protected from the development of experimental EE. While the mouse data are not directly comparable to the human data because of the different eotaxin genes in mice and humans, this experiment emphasizes the crucial role of CCR3 ligands in experimental EE. This result also indicates that other chemotaxatrant substances such as leukotrienes are not likely to have a dominant role in EE. The specific overexpression of eotaxin-3 (and not eotaxin-1 or eotaxin-2) is consistent with prior studies showing the absence of eotaxin-1 overexpression in EE patients (18). The reason that eotaxin-3 is specifically overexpressed compared with eotaxin-1 and eotaxin-2 deserves further study. It is interesting to note that a recent study has shown eotaxin-3 production by Th2 cytokine–stimulated skin keratinocytes, cells that share properties with esophageal epithelial cells (31). Indeed, other Th2-induced genes were increased in EE (e.g., SOCS, cytokine-inducible SH2 domain–containing protein-1, and IL-8). Although IL-13 has been shown to be overexpressed in EE patients (18), Th2 cytokine mRNA (e.g., IL-4 or IL-13) was not upregulated in EE. Perhaps these cytokine mRNAs might be produced by cells before they infiltrate the tissue or are present in such a low quantity that they are not detectable based on microarray profiling of whole-tissue RNA. Notably, Supplemental Table 3 shows that 54 transcripts are associated with external stress, suggesting that an external stimulus or injury may also induce eotaxin-3. Indeed, eotaxin-3 has been shown to be induced by TNF-α (31). It is thus interesting to speculate that eotaxin-3 may be induced in response to innate signaling, perhaps triggered by ingested stimuli.

We demonstrated that a specific genetic variation in the eotaxin-3 gene is likely associated with EE. This SNP (+2,496 T→G, rs2302009) locates at the 3’ untranslated region of the eotaxin-3 gene. Modification of mRNA stability may be the mechanism by which eotaxin-3 (+2,496 T→G) contributes to EE. Notably, the induction of inflammatory cytokines is often controlled at the level of mRNA stability (32); this appears to be important in glucocorticoid-induced eotaxin-1 downregulation (33), raising the possibility that responsiveness to glucocorticoids in EE could be influenced by eotaxin-3 (+2,496 T→G). Indeed, a preliminary report has implicated eotaxin-3 mRNA stability in regulating the level of this gene product in epithelial cells (34). At present, of the 19 EE patients who were analyzed for eotaxin-3 production (Supplemental Table 1), we would not expect a phenotypic difference between TG and TT; indeed, no phenotypic difference was observed (Supplemental Table 1 and data not shown). This shows that the genetic regulation of eotaxin-3 by this SNP does not universally occur in EE. As in other polygenic complex genetic disorders, such as asthma, the individual contributions of a myriad of genes are likely to be involved in EE (35). This SNP is in complete linkage disequilibrium with another SNP (rs7787623) that is approximately 3 kb upstream of the eotaxin-3 gene (www.hapmap.org). This indicates that any one of the SNPs tracks with the other and that this polymorphism at the promoter or control region of the eotaxin-3 gene, either alone or in combination with other markers, may be functionally important in EE. Recently, the SNP +2,496 has been reported to be associated with atopy in the Korean population (36, 37). However, the frequency for allele G in our white normal control population is considerably higher than in normal Koreans (21% versus 5%) (www.hapmap.org). This large allele frequency difference across human subpopulations is another indication that this SNP may be functionally important (38). Hopefully, this genetic finding may be used in combination with eotaxin-3 (+2,496 T→G) contributions to EE. Notably, the induction of inflammatory cytokines is often controlled at the level of mRNA stability (32); this appears to be important in glucocorticoid-induced eotaxin-1 downregulation (33), raising the possibility that responsiveness to glucocorticoids in EE could be influenced by eotaxin-3 (+2,496 T→G). Indeed, a preliminary report has implicated eotaxin-3 mRNA stability in regulating the level of this gene product in epithelial cells (34). At present, of the 19 EE patients who were analyzed for eotaxin-3 production (Supplemental Table 1), we would not expect a phenotypic difference between TG and TT; indeed, no phenotypic difference was observed (Supplemental Table 1 and data not shown). This shows that the genetic regulation of eotaxin-3 by this SNP does not universally occur in EE. As in other polygenic complex genetic disorders, such as asthma, the individual contributions of a myriad of genes are likely to be involved in EE (35). This SNP is in complete linkage disequilibrium with another SNP (rs7787623) that is approximately 3 kb upstream of the eotaxin-3 gene (www.hapmap.org). This indicates that any one of the SNPs tracks with the other and that this polymorphism at the promoter or control region of the eotaxin-3 gene, either alone or in combination with other markers, may be functionally important in EE. Recently, the SNP +2,496 has been reported to be associated with atopy in the Korean population (36, 37). However, the frequency for allele G in our white normal control population is considerably higher than in normal Koreans (21% versus 5%) (www.hapmap.org). This large allele frequency difference across human subpopulations is another indication that this SNP may be functionally important (38). Hopefully, this genetic finding may be used in combination

### Table 1

<table>
<thead>
<tr>
<th>SNP +2,496</th>
<th>Patients with EE&lt;sup&gt;a&lt;/sup&gt; (n = 117)</th>
<th>Unrelated individuals (without known EE) (n = 225)</th>
<th>Odds ratio (95% confidence interval)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Genotype frequency</td>
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<td></td>
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<tr>
<td>TT</td>
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<td>132 (58.7%)</td>
<td>1.00</td>
<td>NS</td>
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<tr>
<td>TG</td>
<td>43 (36.8%)</td>
<td>85 (37.8%)</td>
<td>1.15 (0.69–1.91)</td>
<td>NS</td>
</tr>
<tr>
<td>GG</td>
<td>16 (13.7%)</td>
<td>8 (3.6%)</td>
<td>4.55 (1.71–12.39)</td>
<td>0.0010&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>TG+GG</td>
<td>59 (50.4%)</td>
<td>93 (41.3%)</td>
<td>1.44 (0.90–2.32)</td>
<td>NS</td>
</tr>
</tbody>
</table>

<sup>a</sup>There is an overlap of 13% between the EE patients in this table and the EE patients in Supplemental Table 1. <sup>b</sup>Statistical significance was determined by exact test using shuffling approach; the P value was generated by 10<sup>6</sup> random permutations of the data.

Figure 9

Role of CCR3 in allergen-induced eosinophil recruitment to the esophagus of wild-type and CCR3-deficient (KO) mice. Mice were challenged with saline or allergen intranasally 3 times a week for 3 weeks. The esophagus was harvested 24 hours after the last intranasal treatment, and esophageal sections were stained with anti-MBP. Results represent the number of eosinophils (mean ± SD, n = 3) present in the esophagus per square millimeter. §P < 0.05 versus saline group, ¶P < 0.05 versus wild-type group.
Figure 10

Cellular and molecular mediators in EE. Microscopic assessment (lower panel; magnification, ×100) using a tryptase-specific antibody demonstrates scattered mast cells (bright-red-fluorescent cells marked by white arrows) among cytokeratin-positive epithelial cells (green-fluorescent cells, which are appropriately absent from the fibrovascular stroma within a papilla, marked “P”). Two eosinophils are designated by dashed circles. Eosinophils are identified by their characteristic red autofluorescence and nuclear morphology under higher magnification (e.g., lower left cell in top left panel; magnification, ×1000; green channel omitted). Nuclei are fluorescently counterstained (blue) with DAPI. We propose a model of EE pathogenesis involving eotaxin-3 expression by epithelial cells. Eotaxin-3 overexpression promotes chemoattraction of CCR3-positive eosinophils and expression of the CLC protein. An SNP in the eotaxin-3 gene is associated with EE. Mast cells (white arrows) accumulate in the esophagus, and mast cell genes (tryptase-α and carboxypeptidase A3) are overrepresented in the EE transcript signature (Supplemental Table 5). Eotaxin-3 drives eosinophil activation that leads to tissue damage. CRISP-3, cysteine-rich secretory protein-3.

Our data indicate a dysregulation of transcripts that primarily reflects the epithelial tissue (likely epithelial cells). Our primary interest was not to identify eosinophil transcripts, but rather diseased tissue transcripts that may explain pathogenesis, at least in part. Our analysis provides a new view on EE, since we propose that the disease involves a problem extrinsic to eosinophils involving overproduction of eotaxin-3 (and other gene products) by resident cells within the epithelium. Few eosinophil-derived gene products were present in the EE transcript signature; major basic protein (MBP) (1.5-fold change), eosinophil-derived neurotoxin (0.85-fold change), eosinophil peroxidase (0.94-fold change), and CCR3 (1.4-fold change) were absent in the signature. This may be due to the dilution of eosinophil transcripts with transcripts from relatively RNA-rich cells such as epithelial cells, fibroblasts, and mast cells. An absence of eosinophil transcript signature has been previously observed in murine models of asthma (43) and also in human atopic dermatitis lesions (44), even though these tissues also have abundant eosinophils. However, the Charcot-Leyden crystal (CLC) mRNA, an eosinophil-specific transcript, was dramatically overexpressed in EE. CLCs, along with other eosinophil products, likely promote proinflammatory changes including epithelial hyperplasia (16, 45–47). It is tempting to speculate about the significance of numerous other genes in the EE transcript signature (Figure 10). For example, peristin, a gene that is strongly overexpressed (47-fold) in EE patients, has been associated with epithelial cell growth, angiogenesis, and cellular adhesion (48, 49). Also of interest, cadherin-26 (overexpressed 23-fold in EE patients) is a member of the cadherin family of molecules that has been associated with a variety of inflammatory and epithelial
proliferation diseases. The most downregulated gene, CRISP-3 (cysteine-rich secretory protein-3), is an androgen-dependent transcript (50), perhaps linking the male gender predominance in EE. In EE patient biopsies, there was a profound dysregulation in genes involved in arachidonic acid metabolism (e.g., upregulation of 15-lipoxygenase and downregulation of 12-lipoxygenase). Interestingly, products of arachidonic acid metabolism have been shown to affect Th2 cytokine production or epithelial cell growth (51, 52). While a limited number of other chemokines, such as CXCL1, CXCL6, and CXCL8, were induced in EE, these chemokine receptors were not associated with their characteristic neutrophil accumulation in EE (data not shown). Perhaps molecules such as TNFAIP6 (53–55), induced 23-fold in EE, block neutrophil infiltration. It is interesting to note that eotaxin-3 is an antagonist of CCR1, CCR2, and CCR5 (56, 57), and this may inhibit the action of these chemokine receptors and other cell types. Other CCR3-positive cells may include dendritic cells and mast cells (58, 59), and these cells have indeed been shown to be increased in our study (Figure 2) and in EE in the literature (13, 18). Interestingly, Ig isotypes were found to be dramatically increased (more than 10-fold), emphasizing the potential of in situ B cell development and Ig secretion in the inflamed tissue of EE patients. The EE transcripts do not appear to represent an alteration in cell signature alone. For example, some mast cell genes are increased 2-fold (chymase), whereas others are increased 6-fold (tryptase) or 20-fold (carboxypeptidase A3), showing a dissociation from the 3-fold change in mast cell levels. Similarly, CLC protein is increased 20-fold, yet other eosinophil-specific genes are not increased. Epithelial cell hyperplasia may explain some of the increased gene levels; however, these genes are not present in CE patients even though CE also demonstrates epithelial hyperplasia. Despite the prominent epithelial hyperplasia observed in EE, numerous epithelial cell-specific genes (such as esphagin, esphagous cancer–related gene-2, and filaggrin) are decreased, suggesting a modification of the nature of the epithelium. Collectively, these findings support the view that the EE transcript profile reflects transcriptional dysregulation rather than simply cell signature changes. Taken together, our results draw attention to a variety of pathways that deserve further attention for etiopathogenesis and treatment.

These results provide unprecedented insight into the molecular aspects of EE, providing new targets for EE treatment strategies. In particular, we propose that EE is an eotaxin-3–associated disease and involves a markedly conserved genetic transcript signature (Figure 10). The magnitude of gene changes in EE compared with CE supports the notion that EE is a primary esophageal disease. Based on these results, we are hopeful that eotaxin-3 and/or CCR3 blockers may be beneficial for the treatment of EE. These findings are likely to contribute to prediction of the general outcome of EE and to the building of a molecular classification for diagnosis and therapy of esophagitis.

Methods

Esophageal samples. The patient characteristics are provided in Supplemental Table 1. Our population (patients 1–37) was selected without any regard to age, atopic status, or sex; samples from all the patients are included in Figure 5. Of these 37 patients, 24 were selected for microarray analysis, based on their diverse clinical features. None of the patients was taking glucocorticoids (topical or oral) at the time of the endoscopy or was on diet treatment; glucocorticoids and diet modification were considered exclusion criteria in this study. Patient biopsies, collected from the distal esophagus less than 5 cm from the lower esophageal sphincter, were submerged in formalin for routine pathological analysis with H&E staining. Diagnosis was established based on the maximum eosinophil count per hpf (>400), and basal layer expansion was established according to established criteria (6, 9, 11). Normal (NL) patients were defined as having 0 eosinophils per hpf and no basal layer expansion. The NL biopsies were obtained from patients who presented with symptoms typical of GERD and EE but were found to have completely normal endoscopic appearance and microscopic analysis. Typically, these patients are labeled as having functional abdominal pain. While these patients may not be completely normal, since they had gastrointestinal symptoms, they serve as a relevant control group for comparison with EE. Patients with CE were defined as having mild expansion of the basal layer (less than approximately one-third of epithelium) and/or no more than 23 eosinophils per hpf. EE patients were defined by at least 24 eosinophils per hpf and extensive basal layer hyperplasia (expansion to more than approximately one-third of epithelium). The maximum eosinophil and lymphocyte counts and thickness of the basal layer were assessed after H&E staining; using well-oriented transverse sections, the thickness of the basal layer was assessed by the number of cells containing a high-density nucleus. This study was approved by the Institutional Review Board of the Cincinnati Children’s Hospital Medical Center.

Assessment of allergen sensitization. Skin-prick testing was performed for a panel of 11 aeroallergens and 63 food antigens and assessed based on a 0–4 scale by comparison with the histamine control response. A score greater than or equal to 2 was considered positive. The number of positive skin-prick tests is provided in Supplemental Table 1. Patients with at least 1 positive skin-prick test were considered to be allergen sensitive. History of past or present atopic dermatitis, allergic rhinitis, eczema, or asthma is shown in Supplemental Table 1. The Pharmacia Immuno CAP System (Pharmacia Diagnostics) was used to quantify levels of food allergen–specific IgE levels (referred to as RAST, or RadioAllergoSorbent test); values greater than 0.35 kU/l were considered positive.

DNA microarray analysis. For each patient, 1 distal esophageal mucosal biopsy sample was immersed in RNAlater RNA stabilization reagent (QIAGEN) and stored at 4°C for less than 15 days. Total RNA was extracted using RNeasy Mini Kit (QIAGEN) according to the manufacturer’s recommendations. Hybridization to DNA microarray was performed by the Microarray Core at Cincinnati Children’s Hospital Medical Center, as previously reported (43). The genome-wide human Affymetrix U133 Plus 2.0 GeneChip was used, and gene transcript levels were determined using algorithms in the Microarray Analysis Suite and GeneSpring software (Silicon Genetics).

Ontology assessment. We subjected the list of differentially expressed transcripts to gene ontology analysis using DAVID (Database for Annotation, Visualization and Integrated Discovery) and EASE (Expression Analysis Systematic Explorer), Web-based applications (http://david.niaid.nih.gov/david/upload.asp) that allow access to a relational database of functional annotations (60, 61).

In situ hybridization. Esophageal biopsy samples were fixed in 4% paraformaldehyde/PBS, stored overnight at 4°C, and subsequently submerged in 30% sucrose (43). In brief, eotaxin-3 cDNA was generated using the primers ACCTGAGAAGGGCCTGATTT and GTAACTCTGGGAG-GAAACACCTCTCC and cloned into PCR2.2 vector (Invitrogen Corp.). The resulting plasmid was linearized by BglII, and probes were labeled with 35S-UTP. Hybridized, slides were washed under stringent conditions, and autoradiography was performed for 2–4 weeks at 4°C. The specificity of the hybridization was established using the eotaxin-3 sense riboprobe. Sections from NL, EE, and CE patients were hybridized and underwent autoradiography under identical conditions.
Esophageal sections were prepared from the BALB/c mice (National Cancer Institute) and CCR3-deficient mice (BALB/c background; a kind gift of A. Humble and C. Gerard, Harvard Medical School, Boston, Massachusetts, USA) were housed under specific pathogen-free conditions. Experimental EE was induced by exposure of mice to Aspergillus fumigatus antigen intranasally 3 times a week for 3 weeks as previously described (16). Mice were sacrificed 48 hours after the last challenge, and the esophagus was harvested and fixed in formalin. Eosinophil levels were determined by immunostaining for mouse eosinophil major basic protein (anti-MBP; a kind gift of J. Lee, Mayo Clinic, Scottsdale, Arizona, USA), as previously reported (16).

**Statistics.** Gene lists on microarray were obtained by study of differences in gene-expression levels between groups using the Welch T Test and 2-tailed Student’s t test (with or without Benjamini and Hochberg false discovery rate [FDR] correction). The EE transcript was obtained using the Welch T Test with FDR (P ≤ 0.01). The CE transcript signature was composed of the addition of the gene lists from the Welch T Test without FDR and genes from the Student’s t test without FDR (P ≤ 0.01). Genes differently expressed between allergic and nonallergic EE were composed of the addition of the gene lists from the Welch and Student's t tests without FDR (P ≤ 0.05). Ordered tree clustering was performed using standard correlation or distance. Correlation of gene expression with numeric clinical parameters or eosinophil levels was assessed using the Pearson correlation test with P value. Tests used to generate the gene lists and the number of genes in these lists are shown in Supplemental Table 2. These lists were filtered based on P value and/or fold changes. Statistical significance between groups of data was determined using an unpaired 2-tail Welch T test or Mann-Whitney U test with Bonferroni correction, and correlations of data with the number of eosinophils in the biopsies were determined using the Pearson correlation test with P value. P values less than or equal to 0.05 were considered statistically significant.

A case-control comparison was conducted at both genotype and allele frequency levels (rs2302009, SNP 2,497T→G), where the cases were from the probands of each family and a set of race/ethnicity-matched unrelated healthy individuals was collected as controls. The statistical significance was evaluated by exact test using a shuffling method, generated by 10^5 random permutations of the data. The Hardy-Weinberg equilibrium test, which compares the observed genotype with the expected genotype, was also conducted in cases and controls, respectively, using the χ^2 test (63). Next, the association between the SNP 2,497T→G and EE susceptibility was examined by the family-based transmission disequilibrium test to determine whether the affected child received the disease-associated allele more frequently than the alternative allele. The software TDT/S-TDT, version 1.1 (64), was used for analysis (65).

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