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What is This?
Expression of Chitinases in Hypertrophied Adenoids of Children

Kyung Wook Heo, MD, PhD1*, Dae Young Hur, MD, PhD2*, Seong Kook Park, MD, PhD1, Young Il Yang, MD, PhD3, Hyun Ho Kwak, MD1, and Tae Yong Kim, MD1

Abstract

Objectives/Hypothesis. Chronic rhinosinusitis (CRS), otitis media with effusion (OME), and allergic rhinitis (AR) are common conditions that have been associated with hypertrophied adenoids in children, and adenoidectomy is clinically recommended. The investigators assayed the expression level and site of acidic mammalian chitinase (AMCase) and chitotriosidase (ChT) in hypertrophied adenoids of children to determine the expression levels of 2 chitinases in relation to CRS, OME, and AR.


Setting. A tertiary care facility.

Methods. Hypertrophied adenoids from 41 children were harvested during adenoidectomy. Medical records were reviewed and the subjects were grouped according to the presence of CRS, OME, and AR. Messenger RNA (mRNA) and protein expression of AMCase and ChT in adenoid tissues was assessed by reverse transcription polymerase chain reaction and Western blotting. Immunohistochemical staining revealed the sites of AMCase and ChT expression.

Results. mRNA and protein of AMCase and ChT were present in adenoids of all subjects. CRS was a significant variable in AMCase mRNA and protein expression. CRS, OME, and AR were significant variables in ChT mRNA and protein expression. Both AMCase and ChT were expressed in histiocytes and vascular endothelial cells of adenoid tissues.

Conclusions. The findings suggest that chitin-containing pathogens or dysregulated immune responses to them in the hypertrophied adenoids of children could be factors contributing to CRS, OME, and AR via AMCase or ChT overexpression.

Keywords

adenoids, chitinase, rhinosinusitis, otitis media with effusion, allergic rhinitis

Received February 7, 2011; revised April 12, 2011; accepted April 26, 2011.

A recurrent or chronic infection in the adenoids may manifest as chronic rhinosinusitis (CRS), recurrent acute otitis media, or persistent otitis media with effusion (OME), indicating that the adenoids are reservoirs of pathogenic organisms. Etiologically, OME has been associated with upper respiratory infection, Eustachian tube dysfunction, allergic rhinitis (AR), and immunological and environmental factors. Also, AR is one reason for adenoid hypertrophy (AH) in children, and in a majority of children with asthma, asthmatic symptoms improved after adenotonsillectomy.

Chitin, the second most abundant polysaccharide in nature, is found in the structural coatings of fungi, the exoskeletons of many arthropods, and parasitic nematodes. The chitin coat provides protection for pathogens from harsh conditions inside the host. Chitin is a recognition element for tissue infiltration by innate cells implicated in allergic and helminth immunity, and this process can be negatively regulated by a vertebrate chitinase. Human subjects have 2 chitinases encoded in their genome: acidic mammalian chitinase (AMCase) and chitotriosidase (ChT). The substrate for these chitinases is presumably environmental chitin because no mammal has a chitin synthase.
A role for AMCase in asthma pathophysiology was suggested by the demonstration that AMCase expression increased in the lungs of ovalbumin-sensitized mice that developed airway hyperresponsiveness compared with control animals. Elevated expression of AMCase was also observed in the lung tissue of patients with asthma compared with healthy subjects. ChT is not a housekeeping enzyme, and macrophages are able to produce large amounts of this enzyme under specific circumstances. Although the physiological functions of ChT are still unclear, evidence exists that ChT is a component of innate immunity and may be involved in defending against pathogens containing chitin.

Acidic mammalian chitinase (AMCase) messenger RNA (mRNA) has been detected in the sinus mucosa of CRS with nasal polyps, and it was reported that AMCase and ChT were elevated in nasal polyps. However, the expression of the 2 chitinases has not previously been characterized in the adenoids, and their impact on the pathophysiology of CRS, OME, and AR has not been studied. Furthermore, their site of expression has not been determined. We examined the levels and sites of AMCase and ChT expression in the adenoids in relation to CRS, OME, and AR.

Materials and Methods

Subjects

In total, 41 children who showed preoperative AH were included in the study. Some subjects underwent tonsillectomy with or without tympanostomy tube insertion.

Informed consent was obtained from each child’s parents. The study was approved by the institutional review board of Inje University Busan Paik Hospital.

We defined AH radiologically as an adenoidal–nasopharyngeal ratio greater than 0.6, which secondarily caused nasal obstruction, snoring, mouth breathing, and/or pausing of breathing during sleep. Subjects were classified into groups according to the presence of CRS, OME, and/or AR.

The diagnosis of CRS was based on the guideline of the American Academy of Pediatrics. Symptoms included cough, nasal obstruction, or rhinorrhea that lasted for more than 90 days and an abnormal Water view (such as mucosal thickening or total haziness of the maxillary sinus) with abnormal physical examination findings (such as mucopurulent rhinorrhea or postnasal drip). OME was diagnosed by compatible microscopic findings, B- or C-type tympanogram, and/or conductive hearing loss greater than 20 dB lasting at least 3 months despite appropriate medical treatment. The diagnosis of AR was based on clinical symptoms of sneezing, watery rhinorrhea, and nasal congestion with a documented positive skin prick test and/or positive multiple allergosorbent test (MAST; Hitachi Chemical Diagnostics, Mountain View, California). The result of the MAST was taken to be positive when the MAST value of 1 of the allergens was more than class 3. The skin prick test or MAST was performed using common perennial allergens in Korea, including Dermatophagoides pteronyssinus, Dermatophagoides farinae, cockroach, Aspergillus, Cladosporium, Alternaria, dog fur, and cat fur. Before surgery, each child underwent routine ear, nose, and throat examination as well as paranasal sinus and neck lateral x-rays. Subjects with nasal polyps were excluded, as were those who had received any steroids (systemic or topical), nonsteroidal anti-inflammatory drugs, antihistamines, leukotriene receptor antagonists, or macrolide antibiotics in the month prior to this study.

Of the 41 subjects, patients with simple AH were classified into the AH group, patients also with CRS were classified into the CRS group, patients with OME were classified into the OME group, patients having both CRS and OME were classified into the CRS + OME group, patients having AR were classified into the AR group, and patients with both CRS and AR were classified into the CRS + AR group.

Reverse Transcription–Polymerase Chain Reaction

After surgical removal of hypertrophied adenoids, total RNA was isolated using the RNAeasy mini-kit (Qiagen, Hilden, Germany). RNA was transcribed into complementary DNA using oligo(dt) primers (Bioneer, Daejeon, Korea) and reverse transcriptase. Polymerase chain reaction (PCR) amplification was performed using specific primer sets (Bioneer) for human AMCase (upstream primer 5’-CGC CTA CCT CTA CTC TGG TAC TGA TTG-3′, downstream primer 5’-ACC TGA AAT CTC TCG TAT CCA GCA GCA ACC AC-3′, 121-bp product) and ChT (gene name Chit1; upstream primer 5’-CCC TGG TAC AGG ACT TGG C-3′, downstream primer 5’-ACC TCG TAT CCA GCA GCA ACC AC-3′, 121-bp product). As a control, a specific primer set for β-actin (upstream primer 5’-AAG AGC TAT GAG ACT TGG C-3′, downstream primer 5’-CAG GAG GAG CAA TGA TCT TG-3′) was used, which yielded a 200-bp product. Polymerase chain reaction (25 cycles of 20 seconds at 94°C, 10 seconds at 50°C, 30 seconds at 72°C) was performed using Prime Taq premix (Genet Bio, Daejeon, Korea). The PCR products were analyzed by agarose gel electrophoresis and visualized with ethidium bromide under UV light using the Multiple Gel-DOC system (Fujifilm, Tokyo, Japan). Densitometry measurements were made using Multi Gauge (version 2.3; Fujifilm).

SDS-PAGE and Western Blot Analysis

Western blot analysis was performed for AMCase and ChT as follows. Extracted human adenoid tissues were lysed in RIPA buffer (EIPIS, Daejeon, Korea). The proteins (10 µg per sample) were immediately heated for 5 minutes at 100°C. Total tissue lysates were subjected to SDS-PAGE on gels containing 15% (wt/vol) acrylamide under reducing conditions. Separated proteins were transferred to nitrocellulose membranes using a semidy technique at 80 mA for 2 hours. Membranes were blocked by treatment with 5% skim milk in Tris-buffered saline (TBS), supplemented with 0.1% Tween 20 (TBST), for 1 hour and subsequently incubated with the primary monoclonal or polyclonal antibodies in a final concentration of 1 µg/mL or at a final dilution of 1:1000, respectively, overnight in TBS. After 3 washes in TBST, membranes were incubated with peroxidase-conjugated secondary antibodies (final dilution, 1:3000) in TBS for 1 hour and subsequently washed as above. Detection was performed using the chemiluminescent substrate ECL (GE Healthcare, UK).

Densitometry measurements were made using Multi Gauge (version 2.3; Fujifilm).
performed by chemiluminescence using the ECL kit (Enhanced Chemiluminescence; Amersham Life Science, Brunschweig, Germany) and, subsequently, the Multiple Gel-DOC system (Fujifilm). The primary antibodies anti-AMCase, anti-ChT, and anti-β-actin were used, as described below.

**Antibodies**

Primary antibodies for Western blotting were goat antihuman AMCase (clone Y-14; Santa Cruz Biotechnology, Santa Cruz, California), goat antihuman ChT (clone C-18; Santa Cruz Biotechnology), and rabbit antihuman β-actin (Cell Signaling Technology, Beverly, Massachusetts). Secondary antibodies for Western blotting were horseradish peroxidase (HRP)–conjugated rabbit polyclonal anti-goat immunoglobulin G (IgG) (H&L) antibody (KOMA Biotech Inc, Seoul, Korea), HRP-conjugated goat polyclonal anti-mouse IgG (H&L) antibody (Zymed Laboratories, San Francisco, California), and HRP-conjugated goat polyclonal anti-rabbit IgG (H&L) antibody (KOMA Biotech Inc).

**Immunohistochemical Staining**

Immunohistochemical staining was performed using the EnVision system (DAKO, Hamburg, Germany). Cryosections (5 µm) were fixed in a 1:1 mixture of acetone and methanol, incubated with polyclonal goat anti-AMCase (1:1000; clone Y-14; Santa Cruz Biotechnology) or polyclonal goat anti-ChT (1:1000; clone C-18; Santa Cruz Biotechnology), and then incubated with HRP-conjugated anti-goat IgG (DAKO). After incubation with diaminobenzidine as a substrate, sections were mounted using Aqua Tex (Merck, Darmstadt, Germany). Negative control incubations used normal mouse IgG as the primary antibody.

**Statistical Analyses**

Densities of reverse transcription (RT)-PCR and Western blotting were analyzed statistically. The statistical significance of differences according to 5 variables (gender, age, CRS, OME, AR) was assessed by analysis of variance (ANOVA) first and then by a post hoc test using the Waller-Duncan method and multiple regression method (SPSS, version 17K; SPSS Inc, Chicago, Illinois). Differences were deemed to be statistically significant when P values were less than .05.

**Results**

**Characteristics of the Study Groups**

The AH group consisted of 5 patients. Ten subjects were in the CRS group, 3 were in the OME group, 10 were in the CRS + OME group, 10 were in the AR group, and 3 were in the CRS + AR group. Detailed demographic data are shown in Table 1. All subjects with AR showed positive result to mites in allergy test, 2 subjects to cockroaches, and 1 subject to crab and shrimp. There was no subject with asthma. In AR group, 3 subjects had mild atopic dermatitis.

**Expression of AMCase and ChT mRNA**

Semiquantitative RT-PCR was used to determine AMCase and ChT mRNA expression relative to the β-actin control in adenoid tissues. AMCase and ChT mRNA were detected in all adenoid tissues (Figure 1).

The expression ratio of AMCase mRNA was the highest in the CRS group, followed by the CRS + OME, AR, CRS + AR, OME, and AH groups, in descending order. Significant differences were detected among the groups in AMCase mRNA expression (ANOVA; P < .001). In the post hoc test, AMCase mRNA expression in the AH group was significantly lower than in all other groups. In the multiple regression test, CRS was the only significant variable regarding AMCase mRNA expression (Table 2; P = .01).

| Table 1. Characteristics of Patients According to Groups |

<table>
<thead>
<tr>
<th>Group</th>
<th>No. of Patients</th>
<th>Age Distribution, y, Range (Mean)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AH</td>
<td>5 (3, 2)</td>
<td>5-15 (10)</td>
</tr>
<tr>
<td>CRS</td>
<td>10 (7, 3)</td>
<td>3-14 (6.9)</td>
</tr>
<tr>
<td>OME</td>
<td>3 (2, 1)</td>
<td>5-7 (5.7)</td>
</tr>
<tr>
<td>CRS + OME</td>
<td>10 (5, 5)</td>
<td>3-5 (3.9)</td>
</tr>
<tr>
<td>AR</td>
<td>10 (8, 2)</td>
<td>3-11 (7.1)</td>
</tr>
<tr>
<td>CRS + AR</td>
<td>3 (1, 2)</td>
<td>5-7 (5.7)</td>
</tr>
</tbody>
</table>

Abbreviations: AH group, patients with only adenoid hypertrophy; AR group, patients with allergic rhinitis additionally; CRS group, patients with chronic rhinosinusitis additionally; CRS + AR group, patients with both chronic rhinosinusitis and allergic rhinitis; CRS + OME group, patients with both chronic rhinosinusitis and otitis media; OME group, patients with otitis media with effusion additionally.

Figure 1. Reverse transcription-polymerase chain reaction analysis of acidic mammalian chitinase and chitotriosidase in 41 adenoid tissues. AH group, patients with only adenoid hypertrophy; CRS group, patients with chronic rhinosinusitis additionally; CRS + AR group, patients with both chronic rhinosinusitis and allergic rhinitis; CRS + OME group, patients with both chronic rhinosinusitis and otitis media; OME group, patients with otitis media with effusion additionally.
The expression ratio of ChT mRNA was the highest in the CRS + OME group, followed by the CRS + AR, CRS, AR, OME, and AH groups, in descending order. Significant differences were found among the groups in ChT mRNA (ANOVA; $P < .001$). In the post hoc test, ChT mRNA expression in the AH group was significantly lower than in all other groups. ChT mRNA expression was significantly higher in the CRS, CRS + OME, and CRS + AR groups than in the AR and OME groups.

In the multiple regression test, gender ($P = .021$), CRS ($P < .001$), OME ($P = .001$), and AR ($P < .001$) were significant variables with regard to ChT mRNA expression (Table 3).

**Expression of AMCase and ChT protein**

We detected AMCase and ChT protein in all adenoid tissues (Figure 2). The expression ratio of AMCase protein was highest in the CRS + AR group, followed by the CRS, CRS + OME, OME, AR, and AH groups, in descending order. Significant differences were observed among the groups in AMCase protein expression (ANOVA; $P < .001$). In the post hoc test, AMCase protein expression in the AH group was significantly lower than in all other groups. Also, the AR group showed significantly lower expression of AMCase protein than all other groups, except the AH group. In the multiple regression test, CRS ($P = .021$), OME ($P < .001$), and AR ($P < .001$) were significant variables regarding AMCase protein expression (Table 4).

The expression ratio of ChT protein was highest in the CRS + AR group, followed by the CRS + OME, OME, CRS, AR, and AH groups, in descending order. Significant differences were detected among all subjects and all groups in ChT protein (ANOVA; $P < .001$). In the post hoc test, ChT protein expression in the AH group was significantly lower than in all other groups. Also, the CRS + AR and CRS + OME groups showed the highest ChT protein expression. ChT protein expression in the CRS and OME groups was higher than in the AR group. In the multiple regression test, CRS ($P < .001$), OME ($P < .001$), and AR ($P < .001$) were significant variables regarding ChT protein expression (Table 5).

**Immunohistochemical Staining**

In adenoid tissues, AMCase-positive and ChT-positive cells were exclusively detected in the histiocytes of subepithelial stroma and vascular endothelial cells (Figure 3).

**Discussion**

The proliferating adenoid is involved in chronic, nonspecific inflammation, resulting in chronic adenoiditis or chronic

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**Table 2. Relationships between Acidic Mammalian Chitinase Messenger RNA Expression and Variables**

<table>
<thead>
<tr>
<th>Variable</th>
<th>$\beta$</th>
<th>Standard Error</th>
<th>$P$ Value</th>
<th>$R^2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td>-0.041</td>
<td>0.023</td>
<td>.818</td>
<td></td>
</tr>
<tr>
<td>Gender</td>
<td>-0.270</td>
<td>0.111</td>
<td>.067</td>
<td></td>
</tr>
<tr>
<td>CRS</td>
<td>0.457</td>
<td>0.128</td>
<td>.010</td>
<td>0.335</td>
</tr>
<tr>
<td>OME</td>
<td>0.309</td>
<td>0.148</td>
<td>.099</td>
<td></td>
</tr>
<tr>
<td>AR</td>
<td>0.326</td>
<td>0.146</td>
<td>.078</td>
<td></td>
</tr>
</tbody>
</table>

Abbreviations: AR, allergic rhinitis; CRS, chronic rhinosinusitis; OME, otitis media with effusion.

**Table 3. Relationships between Chitotriosidase Messenger RNA Expression and Variables**

<table>
<thead>
<tr>
<th>Variable</th>
<th>$\beta$</th>
<th>Standard Error</th>
<th>$P$ Value</th>
<th>$R^2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td>-0.001</td>
<td>0.018</td>
<td>.992</td>
<td></td>
</tr>
<tr>
<td>Gender</td>
<td>-0.253</td>
<td>0.089</td>
<td>.021</td>
<td></td>
</tr>
<tr>
<td>CRS</td>
<td>0.661</td>
<td>0.103</td>
<td>&lt;.001</td>
<td>0.643</td>
</tr>
<tr>
<td>OME</td>
<td>0.514</td>
<td>0.119</td>
<td>.001</td>
<td></td>
</tr>
<tr>
<td>AR</td>
<td>0.463</td>
<td>0.117</td>
<td>&lt;.001</td>
<td></td>
</tr>
</tbody>
</table>

Abbreviations: AR, allergic rhinitis; CRS, chronic rhinosinusitis; OME, otitis media with effusion.

**Table 4. Relationships between Acidic Mammalian Chitinase Protein Expression and Variables**

<table>
<thead>
<tr>
<th>Variable</th>
<th>$\beta$</th>
<th>Standard Error</th>
<th>$P$ Value</th>
<th>$R^2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td>0.090</td>
<td>0.014</td>
<td>.527</td>
<td></td>
</tr>
<tr>
<td>Gender</td>
<td>-0.033</td>
<td>0.066</td>
<td>.771</td>
<td></td>
</tr>
<tr>
<td>CRS</td>
<td>0.848</td>
<td>0.076</td>
<td>&lt;.001</td>
<td>0.581</td>
</tr>
<tr>
<td>OME</td>
<td>0.108</td>
<td>0.088</td>
<td>.128</td>
<td></td>
</tr>
<tr>
<td>AR</td>
<td>0.222</td>
<td>0.086</td>
<td>.462</td>
<td></td>
</tr>
</tbody>
</table>

Abbreviations: AR, allergic rhinitis; CRS, chronic rhinosinusitis; OME, otitis media with effusion.

**Figure 2.** Western blot analysis of acidic mammalian chitinase and chitotriosidase in 41 adenoid tissues. AH group, patients with only adenoid hypertrophy; CRS group, patients with chronic rhinosinusitis additionally; OME group, patients with otitis media with effusion additionally; CRS + OME group, patients with both chronic rhinosinusitis and otitis media with effusion; AR group, patients with allergic rhinitis additionally; CRS + AR group, patients with both chronic rhinosinusitis and allergic rhinitis.
Table 5. Relations between Chitotriosidase Protein Expression and Variables

<table>
<thead>
<tr>
<th>Variable</th>
<th>β</th>
<th>Standard Error</th>
<th>P Value</th>
<th>R²</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td>−0.080</td>
<td>0.013</td>
<td>.467</td>
<td></td>
</tr>
<tr>
<td>Gender</td>
<td>−0.124</td>
<td>0.062</td>
<td>.164</td>
<td></td>
</tr>
<tr>
<td>CRS</td>
<td>0.691</td>
<td>0.072</td>
<td>&lt;.001</td>
<td>0.754</td>
</tr>
<tr>
<td>OME</td>
<td>0.537</td>
<td>0.083</td>
<td>&lt;.001</td>
<td></td>
</tr>
<tr>
<td>AR</td>
<td>0.679</td>
<td>0.081</td>
<td>&lt;.001</td>
<td></td>
</tr>
</tbody>
</table>

Abbreviations: AR, allergic rhinitis; CRS, chronic rhinosinusitis; OME, otitis media with effusion.

One is tempted to suggest that when chitin-containing pathogens enter a host, the innate antipathogen response contains oxidants and chitinases that induce chitin fragmentation. The resulting intermediate-sized fragments serve as signals to induce and amplify local inflammation by activating pattern recognition receptors and pathways like nuclear factor-κB. This would continue until the invader has been successfully dealt with and smaller chitin fragments are generated. These small fragments would induce molecules like interleukin-10 that feedback to control the local inflammatory response.18

Acidic mammalian chitinase (AMCase) and ChT are members of the glycosyl hydrolase 18 family, and both have the ability to hydrolyze chitin.15 AMCase is a 50-kDa protein that contains a 30-kDa N-terminal catalytic domain. AMCase is produced by lung epithelial cells, macrophages, and eosinophils at sites of Th2 inflammation. AMCase was shown to be expressed in an exaggerated fashion in epithelial cells and inflammatory cells in tissues from patients with moderate to severe asthma under the influence of interleukin-13.7 Genetic studies in human populations have also linked polymorphisms in AMCase to asthma susceptibility in children, suggesting that inherent defects in this chitinase could underlie airway inflammation and allergic responses.19

A recent report11 showing that AMCase mRNA was expressed at significantly higher levels in the nasal mucosa of people with severe sinus inflammation than in control subjects supports the concept that severe and persistent CRS may be a consequence of a misplaced immune response against parasites that are not really present. In this study, CRS was a significant variable causing the elevation of AMCase mRNA and protein in adenoid tissues. Also, AMCase was primarily expressed in stromal histiocytes and vascular endothelial cells.

Chitotriosidase (ChT) was the first discovered human analog of chitinases. In human tissue, ChT is heterogeneous with respect to its isoelectric point and molecular mass.9 The production, storage, and secretion of ChT by macrophages and neutrophils point to a role in innate immunity.20 Recombinant ChT has been found to inhibit hyphal growth of chitin-containing fungi, suggesting a physiological role in the defense against chitin-containing pathogens.21 Recently, the spectrum of the antimicrobial action of ChT was suggested to extend to bacteria.10,20

Lung chitinase shows high activity at near-neutral pH values and no activity at low pH values,21 indicating that ChT is the primary active lung chitinase, rather than AMCase, the expression of which was strongly dependent on genetics and on smoking status.7 Reese et al6 reported a protective role of chitinase activity and chitin-binding proteins in asthma and allergies. In this study, CRS, OME, and AR were significant variables causing the elevation of ChT mRNA and protein in adenoid tissues. Also, ChT was primarily expressed in stromal histiocytes and vascular endothelial cells. These results suggest that overexpression of ChT in the adenoids could affect the development and maintenance of CRS, OME, and AR. Because subjects in our OME group underwent operations 3
months after diagnosis, the expression of chitinases in the OME and CRS + OME groups probably represented mainly the chronic, rather than acute, status of their adenoid tissues. If a certain chronic condition of the adenoid tissues raises the pH, AMCase may decrease and ChT might still be active and sustain the OME for more than 3 months, resulting in OME surgery. This hypothesis partially corresponds with a previous report stating that nasal secretions in acute otitis media are more acidic than in prolonged otitis media. However, the exact in vivo immune regulatory effects of ChT remain unclear and further experiments are required to determine whether ChT is a protagonist or a bystander in hypertrophied adenoids. If the exact roles of chitin and chitinases in hypertrophied adenoids can be determined, chitinases may be a target for new drug treatments under these conditions.

Conclusion
We found that AMCase was elevated in CRS and ChT was elevated in CRS, OME, and AR in hypertrophied adenoids from children. Also, AMCase and ChT were expressed in histiocytes and vascular endothelium. We suggest that chitin-containing pathogens or a dysregulated immune response to them in hypertrophied adenoids of children could contribute to CRS, OME, and AR via AMCase or ChT overexpression.

Author Contributions
Kyung Wook Heo, writer, data analysis and interpretation; Dae Young Hur, data collection and analysis; Seong Kook Park, study design, data interpretation; Young Il Yang, data interpretation, writer; Hyun Ho Kwak, data collection and analysis; Tae Yong Kim, data collection and analysis.

Disclosures
Competing interests: None.
Sponsorships: The National R&D Program for Cancer Control, Ministry for Health, Welfare and Family Affairs, Republic of Korea (grant 0920040): funding for supply of antibodies and kits for Western blotting and immunohistochemical staining.

Funding source: Grant from Inje University, 2010: funding for supply of antibodies and kits for PCR.

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