

Expression of Pendrin and Periostin in Allergic Rhinitis and Chronic Rhinosinusitis

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ABSTRACT

Background: Pendrin and periostin are newly identified mediators of the inflammatory process. The expression of these proteins in human sinonasal tissue and their roles in allergic rhinitis and chronic rhinosinusitis remain to be elucidated. This study investigated the expression of pendrin and periostin in sinonasal tissue of patients with allergic rhinitis, chronic rhinosinusitis, and aspirin-induced asthma. Prospective control study conducted at Yamagata University, Japan.

Methods: Surgical samples were investigated by means of real-time reverse transcription-polymerase chain reaction to evaluate the expression of pendrin and periostin mRNA. The presence and location of pendrin and periostin were determined by immunohistochemistry and Western blotting.

Results: Pendrin and periostin production was significantly higher in patients with nasal disorders than in controls. Further significant increases in periostin expression were noted in patients with chronic rhinosinusitis with nasal polyps and in those with aspirin-induced asthma. Immunohistochemistry revealed positive staining for pendrin in epithelial cells and submucosal glands and for periostin in the basement membrane in all three disorders, and additionally for periostin in nasal polyp tissue in chronic rhinosinusitis and aspirin-induced asthma.

Conclusions: Production of pendrin and periostin is upregulated in allergic rhinitis, chronic rhinosinusitis with nasal polyps, and aspirin-induced asthma. These findings suggest that pendrin can induce mucus production and that periostin can induce tissue fibrosis and remodeling in the nasal mucosa. Therefore, these mediators may be therapeutic target candidates for allergic rhinitis, chronic rhinosinusitis with nasal polyps, and aspirin-induced asthma.

KEY WORDS

allergic rhinitis, aspirin-induced asthma, chronic rhinosinusitis, nasal polyp, pendrin, periostin

INTRODUCTION

The presenting symptoms of nasal disorders such as allergic rhinitis, chronic rhinosinusitis with nasal polyps, and aspirin-induced asthma include nasal discharge and nasal congestion, which are responsible for not only decreasing quality of life, but also impeding the activities of daily living. These pathological conditions, which lead to mucus overproduction and edema in the nasal mucosa, might be precipitated by the actions of molecules such as pendrin and pe-

riostin. Periostin is an extracellular matrix protein originally isolated from an osteoblast cell line; its production is induced by IL-4 and IL-13 in airway epithelial cells.^{1,4} Periostin is also a regulator of fibrosis and collagen deposition, and although it has been recognized for its important role in myocardial repair and remodeling following myocardial infarction,² its overproduction in the nasal mucosa has been reported to contribute to polyp formation.⁵ Although we know that pendrin is genetically associated with Pendred syndrome, pendrin has recently been found to induce

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Table 1 Patient data and immunohistochemistry, qRT-PCR, and Western blotting parameters

Assay	No.	Sex (male/female)	Age	CT stage	No. of patients with asthma (%)
Immunohistochemistry					
AR	10	5/5	51.2 (24-72)	0	1 (10%)
CRS	10	5/5	49.3 (28-69)	3.4 ± 0.5	3 (30%)
AIA	10	4/6	50.5 (26-74)	3.7 ± 0.5	10 (100%)
Control	7	3/4	43.6 (31-68)	0	0 (0%)
Total	37	17/20			
qRT-PCR					
AR	10	5/5	51.2 (24-72)	0	1 (10%)
CRS	19	9/10	50.4 (28-74)	3.5 ± 0.6	6 (32%)
Control	7	3/4	43.6 (31-68)	0	0 (0%)
Total	36	17/19			
Western blotting					
AR	6	3/3	52.1 (24-72)	0	0 (0%)
CRS	14	7/7	51.1 (28-71)	3.3 ± 0.5	5 (36%)
Control	7	3/4	43.6 (31-68)	0	0 (0%)
Total	27	13/14			

AR, allergic rhinitis; CRS, chronic rhinosinusitis with nasal polyps; AIA, aspirin-induced asthma; qRT-PCR, quantitative reverse transcription polymerase chain reaction; CT, computed tomography.

increased mucus production in airway epithelial cells, suggesting its potential involvement in asthma-induced airway inflammation and occlusion.⁶⁻¹² However, current evidence for the relationship of pendlin and periostin with various nasal disorders is scarce. Therefore, we investigated the expression of pendlin and periostin in nasal tissue from patients with allergic rhinitis, chronic rhinosinusitis with nasal polyps, and aspirin-induced asthma.

METHODS

SUBJECTS

This study was approved by the ethics committee of the Yamagata University Faculty of Medicine and conducted with informed consent from the patients. Forty-six patients who underwent functional endoscopic sinus surgery, septal surgery, or inferior turbinate surgery were enrolled for assessments involving immunohistochemistry, quantitative real-time polymerase chain reaction (RT-PCR), and Western blotting (Table 1). Patients were allocated to one of four groups: allergic rhinitis, chronic rhinosinusitis with nasal polyps, aspirin-induced asthma, or control. The control group consisted of patients who were undergoing septoplasty because of anatomic variations and who had no sinus disease; inferior turbinate mucosal samples were taken from these patients during surgery. Chronic rhinosinusitis with nasal polyps was diagnosed according to the clinical criteria of Meltzer *et al.*⁹ on the basis of patient history, clinical examination, nasal endoscopy, and sinus computed tomography. Diseased sinus mucosal tissue and nasal polyp tissue were collected during surgery. Subjects were

excluded if they had received oral steroids or immunotherapy in the 3 months before surgery or if they had undergone previous sinus surgery.

IMMUNOHISTOCHEMISTRY FOR DETECTING PENDRIN AND PERIOSTIN

For immunohistochemical examination of periostin we used the labeled streptavidin-biotin complex method. Deparaffinized tissue sections were rehydrated in alcohol. The sections were then autoclaved for 10 min at 120°C in citrate phosphate buffer (pH 6.0) for antigen retrieval. Endogenous peroxidase activity was blocked with 0.3% H₂O₂ for 30 min. The sections were then incubated with normal skim milk in phosphate-buffered saline (PBS) for 10 min to block nonspecific background staining. Polyclonal anti-pendlin antibody and polyclonal anti-periostin antibody generated by immunizing rabbits with specific peptides were kindly provided by Dr. Izuhara.^{3,6} Polyclonal antibody against pendlin (diluted 1 : 100) and polyclonal antibody against periostin (diluted 1 : 500) were applied as the respective primary antibodies, and tissue sections were incubated overnight at 4°C. The sequences of these peptides are available on request. After the sections had been washed with PBS, biotinylated goat anti-rabbit IgG was applied, and the sections were incubated for 1 h at room temperature. Slides were developed with diaminobenzidine and counterstained with hematoxylin.

Positive staining for pendlin was quantitatively analyzed by design-based stereology.¹³ Measurements were recorded by a blinded investigator using a ×20 objective lens. The volume density of immunostain-

ing was calculated by quantification of the volume of stained mucosal epithelium referenced to the surface area of the surveyed basal lamina (cubic micrometers per square micrometer).

ASSESSMENT OF SLIDES

Immunostained sections were assessed under an Olympus microscope with a $\times 200$ eyepiece reticle. Cell counts were expressed as mean per high-power field (0.202 mm^2) values. At least two sections were immunostained, and more than five areas were evaluated via the graticule. Results are expressed as the number of positive cells per field.

QUANTITATIVE PCR ANALYSIS OF PENDRIN AND PERIOSTIN

Total RNA was isolated from nasal tissue using an RNeasy Mini Kit with RNase-Free DNase Set (Qiagen, Hilden, Germany). RNA was dissolved in RNase-free water and stored at -80°C according to the manufacturer's protocols. Preparations were quantified, and their purity was determined by standard spectrophotometric methods. cDNA was produced in a $20\text{-}\mu\text{L}$ reaction tube from $1 \mu\text{g}$ total RNA, 20 units of AMV reverse transcriptase (Roche Diagnostics, Mannheim, Germany), $1 \times$ AMV reaction buffer, 10 nM deoxynucleotide triphosphates, and $3.2 \mu\text{g}$ random hexamers. The reactions were incubated at 25°C for 10 min, 42°C for 1 h, and 94°C for 5 min. Primer sequence pairs were as follows: human pendrin, 5'-TTG ACG GTC CAT GAT GCT A-3' (forward) and 5'-TTC AGG ATG CAA GTG TAC G-3' (reverse) (product size, 187 bp); human periostin, 5'-TTT GCT GGC ACC TGT GAA TA-3' (forward) and 5'-TTT GCC TCC GAT GGT TTC-3' (reverse) (product size, 154 bp). β -actin was used as an internal control. Two β -actin primer pairs (LightCycler human β -actin primer set, Roche Diagnostics) were also used.

Quantitative PCR analysis was performed using the LightCycler instrument (Roche Diagnostics). Each reaction contained $5 \mu\text{L}$ cDNA, $0.5 \mu\text{M}$ of each primer, $1 \times$ Detection Mix, and $1 \times$ LightCycler Fast-Start DNA Master SYBERGreen I in a $20\text{-}\mu\text{L}$ volume. Each reaction capillary underwent 10 min incubation at 95°C before 45 cycles of 95°C for 10 s, 60°C for 10 s, and 72°C for 8 s. Melting curve analysis in SYBER Green I format after the end of the amplification cycles was performed at 95°C for 0 s, 70°C for 15 s, and 95°C for 0 s. PCR runs were concluded with incubation at 40°C for 30 s. This melting curve analysis was performed to verify the specificity of each primer. Agarose-gel observations revealed that no non-specific PCR products were amplified under these conditions. All mRNA levels are expressed as the number of copies per 1000 copies of β -actin.

WESTERN BLOTTING FOR PERIOSTIN

Antiperiostin serum was generated by purified His-

tagged or V5 epitope/His-tagged periostin possessing exons 17 to 21 in the C-terminal portion, together with complete Freund adjuvant (Sigma Aldrich, St. Louis, MO, USA) for immunization of New Zealand White rabbits. Nasal tissue was lysed in PBS containing 1% SDS, and the tissue lysates were boiled with SDS sample buffer containing 7 M urea. Samples were applied to SDS-PAGE and electrophoretically transferred to polyvinylidene difluoride membranes (GE Healthcare Bio-Sciences, Piscataway, NY, USA). The membranes were blotted with anti-periostin serum and periostin was visualized on Hyperfilm ECL (GE Healthcare Bio-Sciences).

STATISTICS

Control group means (arithmetic mean \pm SD) were compared with patient group means using the Mann-Whitney *U*-test at $P = 0.05$.

RESULTS

PRODUCTION OF PENDRIN IN NASAL MUCOSA

Pendrin-positive staining was demonstrated in apical membrane of epithelial cells by means of immunohistochemical staining (Fig. 1A-D). Production of pendrin protein was significantly higher in tissues from patients with allergic rhinitis (mean = 13.2), chronic rhinosinusitis with nasal polyps (mean = 12.8), or aspirin-induced asthma (mean = 12.8) than in control tissues (mean = 3.7) ($P < 0.05$) (Fig. 1E).

PRODUCTION OF PERIOSTIN IN SINONASAL TISSUES

Periostin-positive staining was observed in the basement membrane, extracellular matrix, nasal polyp tissue, and infiltrating cells by means of immunohistochemical staining (Fig. 2A-E). Production of periostin protein was significantly greater in tissues from patients with allergic rhinitis (mean = 14.8), chronic rhinosinusitis with nasal polyps (mean = 32.4), or aspirin-induced asthma patients (mean = 32.5) than in control tissues (mean = 5.1) ($P < 0.05$) (Fig. 2A-E). Patients with chronic rhinosinusitis or aspirin-induced asthma had significantly stronger protein production in their nasal polyps than did patients with allergic rhinitis (Fig. 2F).

EXPRESSION OF PENDRIN AND PERIOSTIN mRNAs IN SINONASAL TISSUES

Expression of the mRNAs of pendrin was significantly greater in the tissues of patients with allergic rhinitis (mean = 751) or chronic rhinosinusitis with nasal polyps (mean = 707) than in control tissues (mean = 53.6) ($P < 0.05$) (Fig. 3A). Expression of the mRNAs of periostin was significantly greater in the tissues of patients with allergic rhinitis (mean = 117) or chronic rhinosinusitis with nasal polyps (mean = 1500) than in control tissues (mean = 39.7) ($P < 0.05$) (Fig. 3B). Patients with chronic rhinosinusitis with

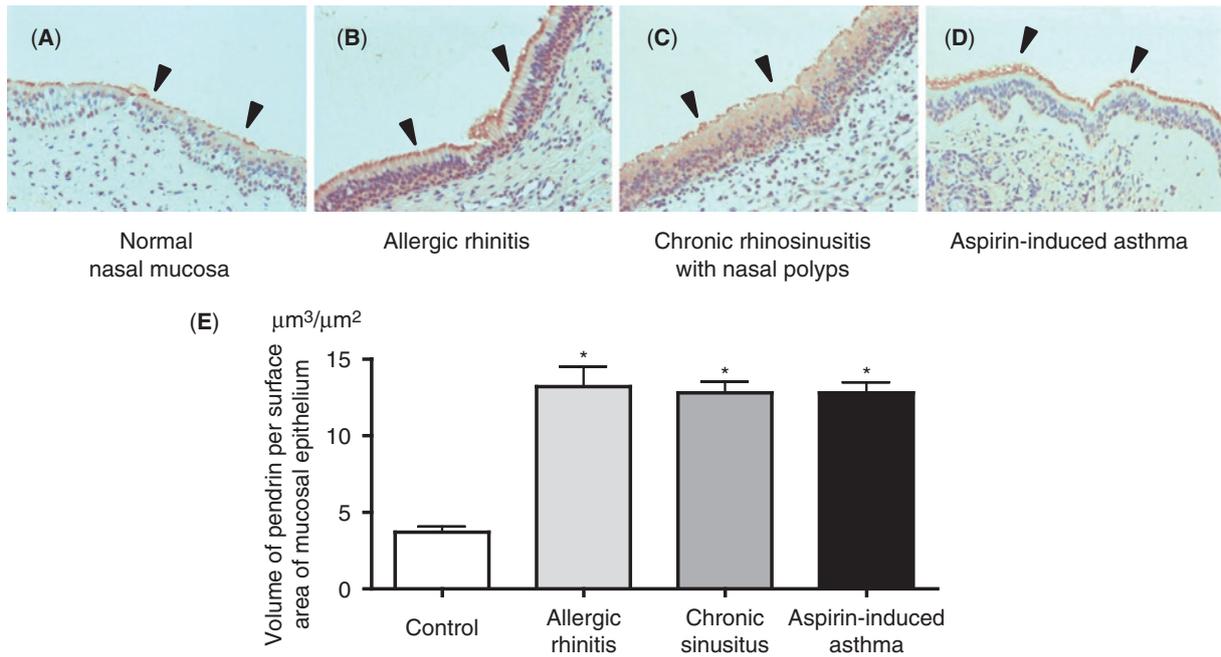


Fig. 1 Immunohistochemical staining of pendrin in the nasal mucosa (black; arrow) of controls (A) and patients with allergic rhinitis (B), chronic rhinosinusitis with nasal polyps (C), and aspirin-induced asthma (D) (original magnification: $\times 100$). Pendrin staining is more intense for allergic rhinitis, chronic rhinosinusitis with nasal polyps, and aspirin-induced asthma compared with controls. Results are expressed as mean \pm SD ($n = 10$ each group) (E). * $P < 0.05$ vs. control.

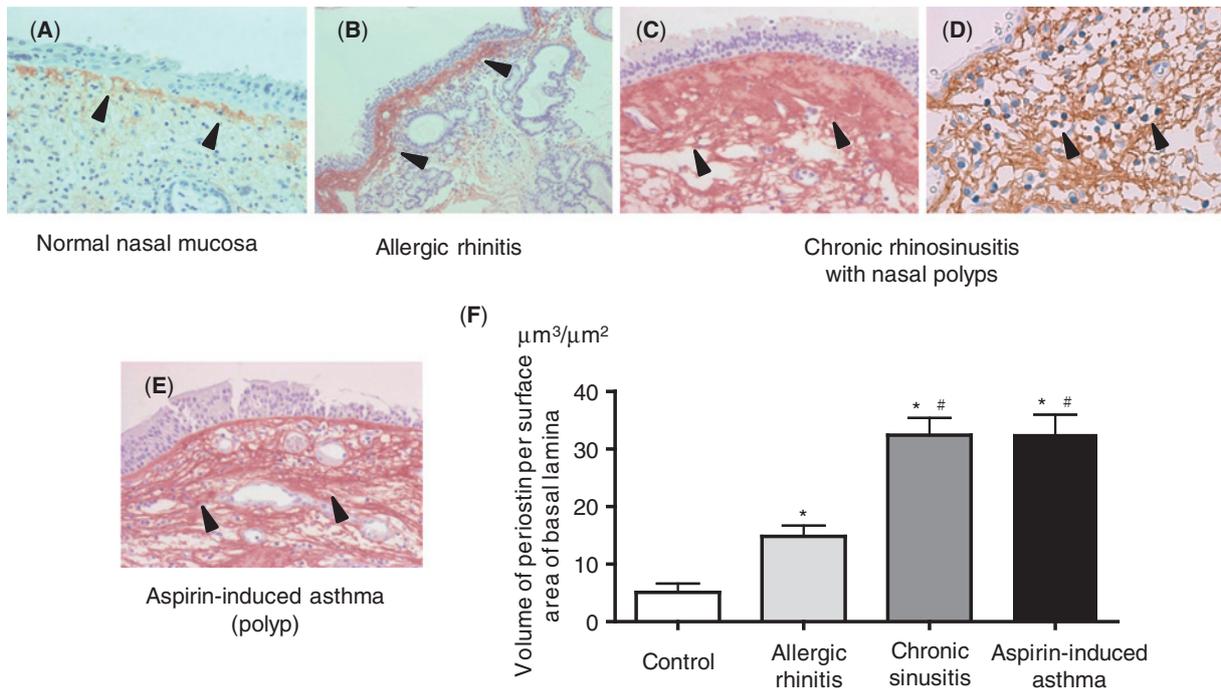


Fig. 2 Immunohistochemical staining of periostin in nasal mucosa (black; arrow) of control (A), allergic rhinitis (B), chronic rhinosinusitis with nasal polyps (C), high power field image of periostin positive infiltrating cells in chronic rhinosinusitis with nasal polyps (D) and aspirin-induced asthma (E) (original magnification $\times 100$, high power field $\times 400$). Periostin staining is more intense in allergic rhinitis, chronic rhinosinusitis with nasal polyps, and aspirin-induced asthma than in the control. Results are shown as means \pm SD ($n = 10$ each group) (F). * $P < 0.05$ vs. control; # $P < 0.05$ vs. allergic rhinitis.

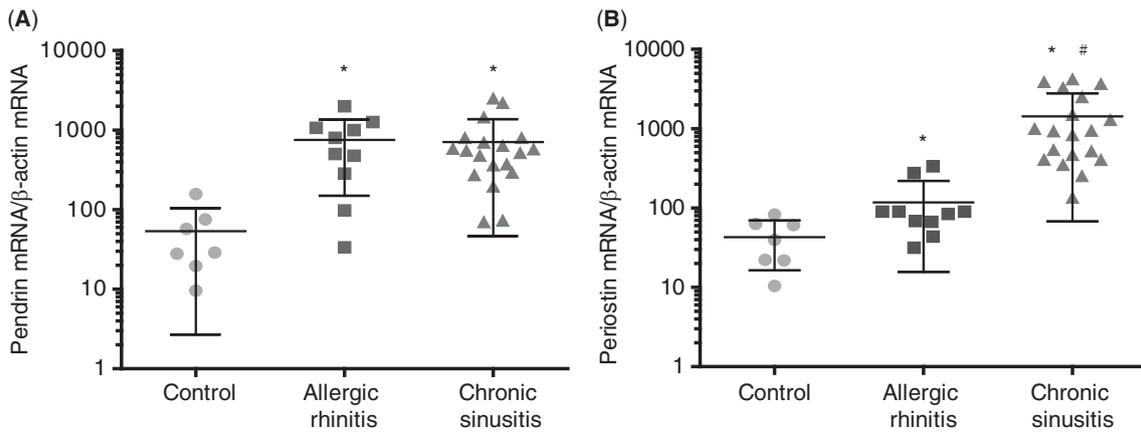


Fig. 3 Quantitative RT-PCR analysis of pendrin (A) and periostin (B) mRNA in sinonasal mucosal tissue from controls and patients with allergic rhinitis, chronic rhinosinusitis, or aspirin-induced asthma. All mRNA levels are expressed as copies per 1000 copies of *ACTB*, the β -actin housekeeping gene. Results are shown as mean \pm SD. * $P < 0.05$ vs. control; # $P < 0.05$ vs. allergic rhinitis.

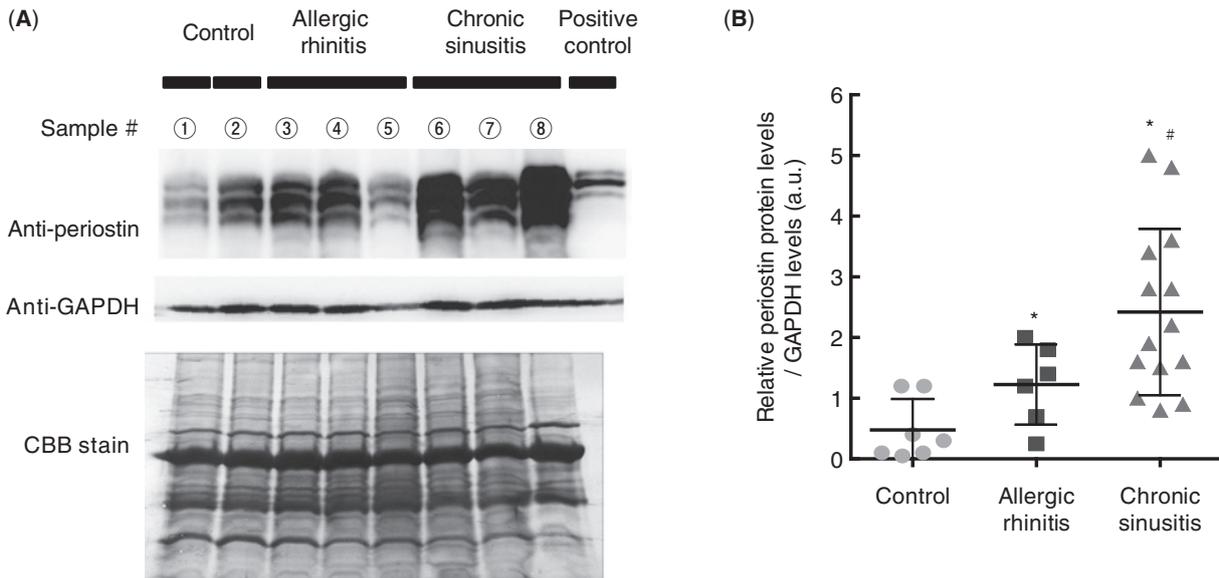


Fig. 4 Western blotting quantification of periostin in sinonasal mucosal tissue from controls and patients with allergic rhinitis, chronic rhinosinusitis, or aspirin-induced asthma. Supernatants from fibroblasts stimulated with IL-13 were used as positive controls. Results are expressed as mean \pm SD ($n = 10$ each group).

nasal polyps had significantly stronger periostin mRNA expression in their sinonasal tissues than did patients with allergic rhinitis ($P < 0.05$).

PRODUCTION OF PERIOSTIN PROTEIN IN SINONASAL TISSUES

Production of periostin protein was significantly greater in the tissues of patients with chronic rhinosinusitis with nasal polyps (mean = 2.42) than in the tissues of controls (mean = 0.40) or patients with allergic rhinitis (mean = 1.23) ($P < 0.05$) (Fig. 4).

DISCUSSION

Although pendrin and periostin have been implicated in various immunological events,¹⁻⁸ their physiologic functions and potential roles in pathologic conditions remain to be defined. Here we reveal for the first time that pendrin and periostin expression is increased in the sinonasal tissue of patients with allergic rhinitis, chronic rhinosinusitis with nasal polyps, and aspirin-induced asthma. Moreover, patients with chronic rhinosinusitis with nasal polyps had higher levels of pendrin and periostin production. In sinonasal tissue,

pendrin production was observed in epithelial cells and submucosal gland cells,^{4,6} while periostin production was observed in basement membrane cells and infiltrating cells. Periostin was also expressed in nasal polyps of patient with chronic rhinosinusitis and in those with aspirin-induced asthma.

Chronic rhinosinusitis is one of the most frequently encountered chronic nasal diseases, and the complexity of this condition in the presence of nasal polyps complicates the study of its etiological therapeutics.¹⁰⁻¹² Patients with chronic rhinosinusitis whose symptoms are refractory to treatment often develop nasal polyps. Growth of these polyps leads to obstruction of the sinonasal passages, and surgery may be necessary in advanced cases to remove the polyps to restore sinus ventilation. Proliferation and thickening of the mucosal epithelium, with focal squamous metaplasia, glandular hyperplasia, subepithelial fibrosis, and stromal edema with numerous blood vessels, have been histologically observed in nasal polyps.¹⁰ One of the most important characteristics of chronic rhinosinusitis is the prolonged and exaggerated inflammatory reaction in the paranasal mucous membranes.

Samter's triad is a condition diagnosed with aspirin-induced asthma in a subset of patients with chronic rhinosinusitis with nasal polyps, and it is characterized by nasal polyps, asthma, and aspirin intolerance.^{5,13-17} In individuals with aspirin-induced asthma, nonsteroidal anti-inflammatory agents, including aspirin, lead to immediate and severe bronchospasm which requires urgent emergency treatment.¹⁶ It is hypothesized that a common stimulus causes inflammation of both the sinonasal and the bronchopulmonary mucosa.

Allergic rhinitis is a Th2-mediated inflammatory disease characterized by mucosal eosinophilia, airway hyperresponsiveness, and mucus overproduction.^{10,12} With subsequent progression of allergic rhinitis, the airway undergoes structural and phenotypic changes resulting in airway remodeling, including damage to epithelial cells, goblet cell metaplasia, subepithelial fibrosis, and smooth muscle hyperplasia and hypertrophy.¹¹

Pendrin is an anion transporter that plays an important role in mucus production.⁵ Mucus overproduction is a prominent feature of both bronchial asthma and chronic obstructive pulmonary disease and is a major determinant of morbidity and mortality in these diseases.⁷ In both asthma and chronic obstructive pulmonary disease, pendrin is upregulated in airway epithelial cells in association with mucus overproduction.⁷ Our findings show that pendrin was also overexpressed in the sinonasal tissue, including epithelial cells and submucosal gland cells, of patients with allergic rhinitis, chronic rhinosinusitis with nasal polyps, and aspirin-induced asthma. The precise mechanism of mucus overproduction under these condi-

tions remains poorly understood, but it is reported that pendrin induces the production of several inflammatory mediators, including TGF- α , EGF, and TNF- α , which regulate transcription of the *MUC5AC* gene via the activation of transcription factors.^{6,7,14} Mucus production may be induced by not only the direct effect of pendrin on airway epithelial cells, but also the indirect effect of pendrin through recruitment of inflammatory cells, particularly neutrophils.⁶

Periostin is a 90-kDa member of the fasciclin-containing family; it functions as part of the extracellular matrix and its production by airway epithelial cells is induced by IL-4 and IL-13.³ Periostin is secreted by fibroblasts and has a cysteine-rich domain at its N-terminal end, four tandem fasciclin I domains, and an alternatively spliced domain at its C-terminal end.³ Periostin is upregulated in the airway epithelia of patients with bronchial asthma and is considered to contribute to remodeling under this condition.³ Periostin regulates goblet cell metaplasia and mucus production in airway inflammation, including in bronchial asthma.¹⁷ Our findings suggest that periostin plays an important remodeling role in allergic rhinitis and a polyp formation role in chronic sinusitis and aspirin-induced asthma.

The upregulation of pendrin and periostin in allergic rhinitis, chronic rhinosinusitis with nasal polyps, and aspirin-induced asthma suggests pathogenic mediation by these proteins. Consequently, pendrin and periostin may be therapeutic target candidates for these diseases.

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