

ORIGINAL RESEARCH–ALLERGY

Nonasthmatic nasal polyposis patients with allergy exhibit greater epithelial MMP positivity

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ABSTRACT

OBJECTIVE: To determine the influence of allergic rhinitis (AR) on the expression of matrix metalloproteinase (MMP)-9, MMP-2, and tissue inhibitor-1 of metalloproteinase (TIMP-1) in nasal polyposis.

STUDY DESIGN: A case-control study.

SETTING: A tertiary referral center.

SUBJECTS AND METHODS: The expression of MMP-9, MMP-2, and TIMP-1 was investigated in the nasal polyp tissue (NP) and maxillary sinus mucosa (MM) samples from 20 AR patients and 20 nonallergic patients undergoing endoscopic sinus surgery. Trichrome, periodic acid–Schiff, and hematoxylin–eosin staining were also performed and those expression levels were compared.

RESULTS: Infiltration of eosinophils was shown more intensely in NP rather than in MM, especially in the presence of AR. In the NP of AR patients, increased expression of MMP-9, MMP-2, and TIMP-1 was observed more prominently than in that of the control group. In case of MM, however, there was no significant difference between AR patients and the control group.

CONCLUSION: The presence of AR may enhance the expression of MMP-9, MMP-2, and TIMP-1 associated with airway remodeling in nasal polyposis.

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Nasal polyposis remains one of the most difficult challenges in rhinology. Its etiology and pathophysiology are still controversial, and surgical treatments are often inevitable due to frequent recurrences. Sinus mucosa in nasal polyposis are characterized by massive stromal edema, infiltration with inflammatory cells like eosinophils, lymphocytes, and plasma cells, alterations of the overlying epithelium, and, in some cases, hyperplasia of submucosal seromucous glands.^{1,2}

The fact that nasal polyposis and asthma share characteristic inflammatory features and histopathologic findings

of airway remodeling motivated many researchers to investigate the possibility that the airway remodeling occurring with nasal polyps is similar to that with asthma. Most of these studies have mentioned the change of the expression of matrix metalloproteinases (MMPs) or their tissue inhibitors (TIMPs).^{1–4} However, these findings were inconsistent. For example, Sun et al¹ reported that expression of MMP-9 and interleukin-5 mRNA are associated with nasal polyp, but MMP-2 is not. On the contrary, Bhandari et al³ found that MMP-2 may play a role in the airway remodeling of nasal polyps.

Allergic rhinitis (AR) and allergic asthma also show common pathophysiologic features, such as the infiltration of inflammatory cells, including eosinophils and lymphocytes, degranulation of mast cells, and expression of various cytokines in response to allergens. Additionally, the comorbidity of AR and asthma has been well known.^{5,6} With this background, many authors have tried to ascertain the characteristic nasal airway remodeling in AR. Nevertheless, association studies have had inconsistent and conflicting results.^{7,8} In addition, several researchers demonstrated the airway remodeling of nasal mucosa in AR using ovalbumin-sensitized murine models.^{9,10} However, those models still have limitations as a complete AR model because they used ovalbumin. Moreover, few reports have noted the differences of airway remodeling in nasal polyps in the presence of AR.

The aim of the present study is to investigate the expression of MMP-9, MMP-2, and TIMP-1 in the presence of AR and to speculate the influence of AR on airway remodeling of nasal mucosa in nasal polyposis.

METHODS

Patients

The study group consisted of 40 patients with nasal polyps (M:F = 1:1, median age 46 yrs, range 13–68 yrs) who underwent endoscopic sinus surgery at our hospital, be-

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tween August 2006 and September 2008. Twenty (50%) patients, comprising the experimental group (M:F = 1:1, median age 45 yrs, range 13–64 yrs), had a history of perennial AR (>2 years) that was confirmed by a positive skin prick test. The skin prick test was performed using 55 common aeroallergens (Allergopharma, Reinbek, Germany), including house dust mites (*Dermatophagoides pteronyssinus*, *Dermatophagoides farinae*), animal danders (cat epithelia, dog epithelia), molds (*Aspergillus fumigatus*, *Alternaria alternata*), pollens (mugwort, ragweed, alder, hazel, birch, oak), and cockroaches (*Blattella germanica*). Histamine and isotonic saline were used as positive and negative controls. A mean wheal diameter greater or equal to that of histamine in the absence of any reaction to the saline was considered a positive response. The allergen for house dust mites (85%) was most common, and the seasonal allergens including trees and grass (30%) followed. Allergy was defined as a positive response to one or more allergens on SPT. The remaining 20 patients, comprising the control group (M:F = 1:1, median age 47 yrs, range 14–68 yrs), had no clinical evidence of AR and had a negative skin prick test.

All patients had clinical symptoms and signs of chronic rhinosinusitis, including facial pain, congestion, and/or rhinorrhea, for more than 12 weeks, which had not improved after at least two courses of medical treatment that included antibiotics and topical steroids for at least 14 days. In all participants, nasal polyps were confirmed by nasal endoscopy and signs of nasal polyps in computed tomographic scans. Antibiotics and topical steroids were discontinued two weeks prior to surgery. Oral steroids were ceased one month before surgery. Acute infections were excluded by physical and laboratory examination. We also excluded the patients with asthma or an intolerance for nonsteroidal anti-inflammatory drugs. Five patients who had been diagnosed with asthma by a physician were excluded from our study. The Institutional Review Board of Seoul National University Hospital approved this case-control study. All partici-

pants provided written informed consent before involvement.

Specimen Collection and Handling

During endoscopic sinus surgery, nasal polyp tissue (NP) and maxillary sinus mucosa (MM) were obtained from each patient. The Lund scores for the maxillary sinuses from where the specimens were biopsied in AR and control groups were 0.95 ± 0.83 and 0.85 ± 0.59 , respectively ($P = 0.758$). NP and MM specimens were fixed in buffered formalin and embedded in paraffin for conventional histopathological examination and immunohistochemistry. Hematoxylin-eosin (H&E), periodic acid-Schiff (PAS), trichrome staining, and immunohistochemical staining for MMP-9, MMP-2, and TIMP-1 were performed for NP and MM.

H&E/PAS/Trichrome Staining

H&E staining was performed to measure the infiltration of eosinophils. Under light microscopic vision (original magnification, $\times 400$; BX51, Olympus, Tokyo, Japan), eosinophils were counted in four randomly selected fields of the submucosal area. The percentage of eosinophils among all infiltrating cells in the submucosa of NP or MM was compared. PAS staining was performed to observe the development of goblet cell hyperplasia. The result was presented as the number of goblet cells expressed per unit length (100 μm). The degree of subepithelial fibrosis was examined by measuring the trichrome-stained area per unit length. A single observer, not knowing the experimental conditions, counted all of the slides.

Immunohistochemical Staining for MMP-9, MMP-2, and TIMP-1

Immunohistochemical analysis was performed on 4- μm thick paraffin sections and air-dried for 24 hours at room temperature. Paraffin sections of all samples were rehy-

Table 1
Summary of findings

	AR (+)			AR (–)			<i>P</i> * value	
	MM	NP	<i>P</i> † value	MM	NP	<i>P</i> † value	MM	NP
Eosinophil (%)	5.8 ± 2.7	12.2 ± 3.8	0.001	2.4 ± 2.6	5.2 ± 1.9	0.001	<0.001	<0.001
Goblet cell count (/10 ² μm)	17.4 ± 17.2	15.8 ± 8.5	0.203	11.6 ± 11.0	12.2 ± 7.8	0.710	0.221	0.081
Subepithelial fibrosis	22.6 ± 17.6	20.9 ± 21.3	0.433	14.0 ± 6.9	13.3 ± 9.6	0.588	0.383	0.968
MMP-9 SA	26.3 ± 14.7	41.9 ± 25.1	0.001	17.6 ± 12.0	21.2 ± 10.4	0.232	0.068	0.003
MMP-2 SA	0.30 ± 0.24	0.48 ± 0.36	0.012	0.16 ± 0.21	0.19 ± 0.30	0.940	0.076	<0.001
TIMP-1 SA	2.3 ± 3.2	1.2 ± 1.5	0.003	1.11 ± 0.87	0.51 ± 0.43	0.001	0.355	0.035

Values given as mean $\pm$ SD. Bold *P* values are significant.

AR, allergic rhinitis; MM, maxillary sinus mucosa; NP, nasal polyp tissue; SA, stained area ($\mu\text{m}^2/\mu\text{m}$); TIMP-1, tissue inhibitor-1 of metalloproteinase.

**P* value for the differences between groups with and without AR. Mann-Whitney *U* test used.

†*P* value for the differences between MM and NP. Wilcoxon signed rank test used.

drated and subjected to antigen retrieval by microwave treatment over three cycles of 500 W for 10 minutes in citrate buffer solution (pH 6.0). Endogenous peroxidase activity was blocked with three percent hydrogen peroxide

in phosphate buffer solution (pH 7.6) containing 0.025% Tween 20 for 20 minutes at room temperature. Sections were subsequently incubated in 10 percent normal fetal bovine serum for 2 hours at 37°C, followed by monoclonal anti-human antibodies against MMP-2, MMP-9, or TIMP-1 (Santa Cruz, San Diego, CA) at a dilution of 1:50 overnight at 4°C, and detected with the peroxidase LSAB kit (DAKO, LSAB kit, Hamburg, Germany). Breast cancer (invasive ductal carcinoma) samples known to produce MMP-2, MMP-9, and TIMP-1 were stained and observed as positive controls in the same way as performed in previous studies.^{11,12} Negative controls were performed by omitting the primary antibody and using an irrelevant antibody of similar isotype.

Measurement of Stained Area

All slides were read by a single observer blinded to the experimental conditions. For a quantitative measurement, the areas of interest were measured by using an image analysis system (ImageJ version 1.40g; National Institutes of Health, Bethesda, MD) with an attached light microscope (BX51; Olympus, Tokyo, Japan). Ten fields (final magnification $\times 400$) along the epithelium were selected randomly, and the average of positively stained area in each field was calculated in μm^2 per μm of length of the epithelial lining.

Statistical Methods

Statistical analysis was performed using the statistical software package SigmaStat for Windows SPSS 12.0 (SPSS, Chicago, IL). Values were described as mean $\pm$ SD. When comparisons were made between groups, significance in between-group variability was established by using the Mann–Whitney *U* test. The Wilcoxon signed rank test was used to analyze the difference between MM and NP within each group. A statistical difference of $P < 0.05$ was considered significant. The statistical power analysis was performed in collaboration with Medical Research Collaboration Center in our institution.

RESULTS

Eosinophil Infiltration

The patients with AR had more submucosal eosinophil infiltration in both their NP and MM specimens than the

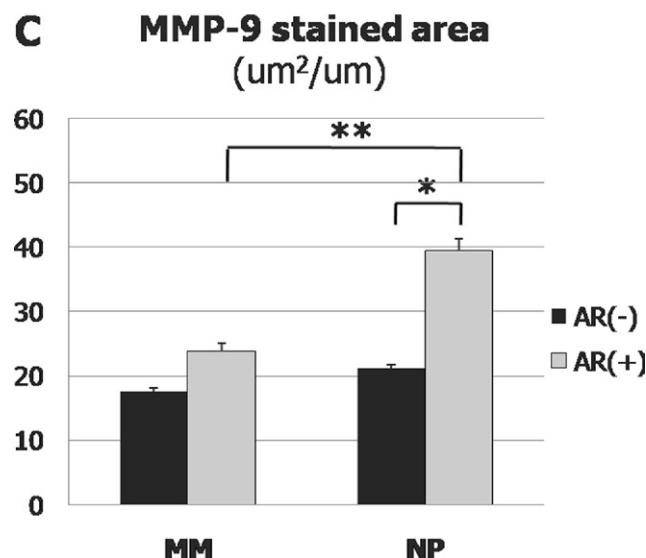
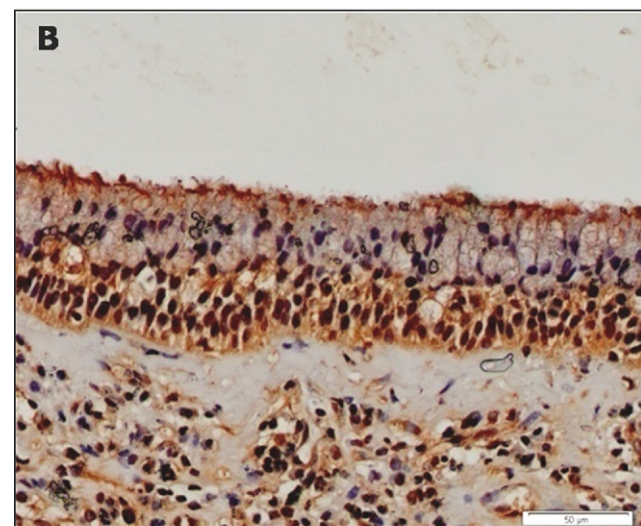
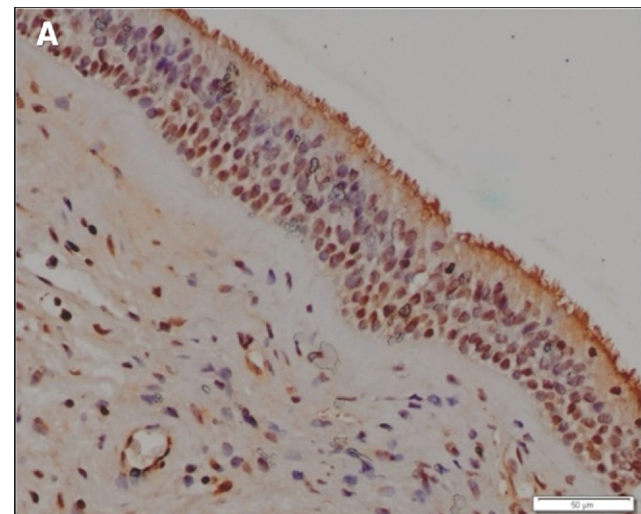
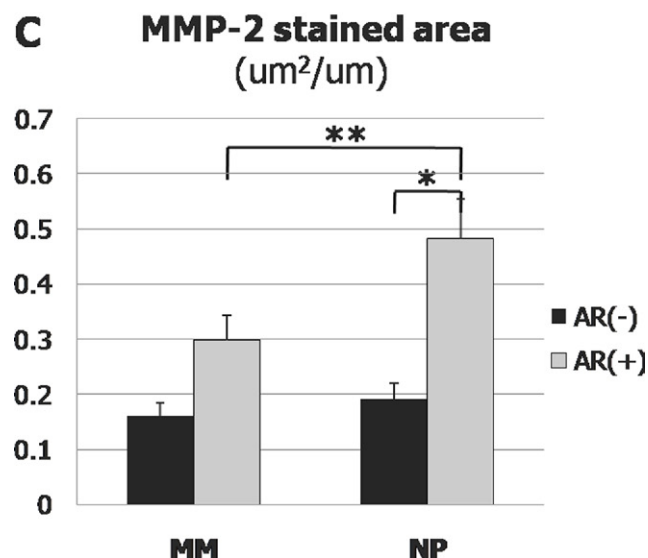
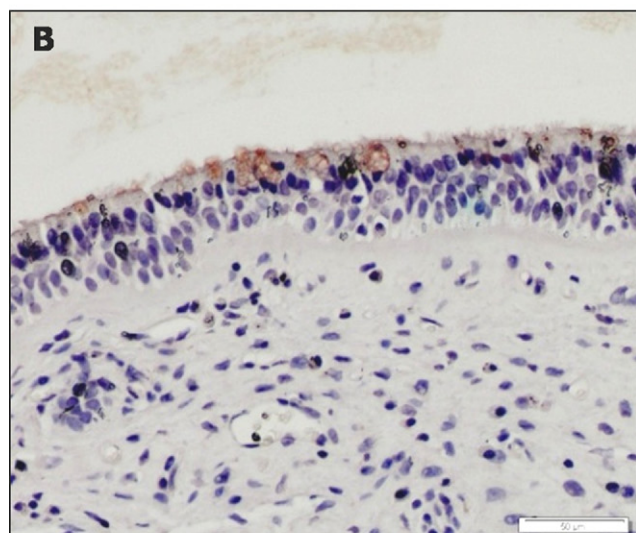
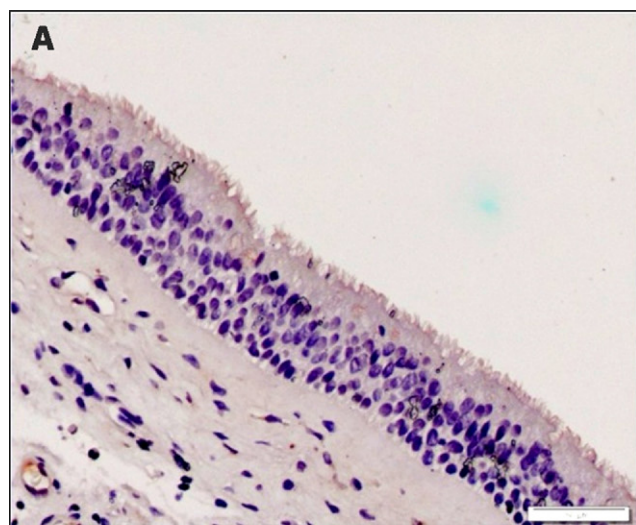


Figure 1 (A) Representative MMP-9 immunohistochemical staining of surface epithelium of NP in the control group (original magnification, $\times 400$). (B) Representative MMP-9 immunohistochemical staining of surface epithelium of NP in the AR group (original magnification, $\times 400$). (C) Differences in the expression of MMP-9 according to the presence of AR in nasal polyposis (* $P < 0.05$, Mann–Whitney *U* test, ** $P < 0.05$, Wilcoxon signed rank test). AR, allergic rhinitis; MM, maxillary sinus mucosa; NP, nasal polyp tissue.

control group ($P < 0.001$) (Table 1). Regardless of the presence of AR, eosinophils were found more frequently in NP than in MM ($P = 0.001$).



Goblet Cell Hyperplasia

Goblet cell counts in nasal mucosa were not significantly different between groups in or between MM and NP. On average, 10 to 20 goblet cells were found per 100 μm .

Subepithelial Fibrosis

The patients with AR showed more prominent subepithelial fibrosis in both MM and NP specimens than the control group. However, the differences were not statistically significant ($P = 0.383$ in MM, $P = 0.968$ in NP).

Expression of MMP-9, MMP-2, and TIMP-1

MMP-9 was diffusely distributed in surface epithelium (whole layer), extracellular matrix, vascular basement membrane, endovascular and perivascular cells, and polymorphonuclear cells (Figs 1A and 1B). MMP-2 or TIMP-1 expression was localized in the epithelial cells (mainly apical layer), whereas secretory glands, basal membrane, extracellular matrix, and endothelial cells were not stained (Figs 2A and B, 3A and B). In addition, MMP-2 and TIMP-1 were stained more weakly than MMP-9, and their intensity was more heterogeneous in both MM and NP.

In regards to the comparison of the stained area between the AR and control groups, MMP-9, MMP-2, and TIMP-1 were expressed more intensely in the AR group (Figs 1C, 2C, and 3C). These differences were statistically significant in NP, but not in MM (Table 1).

In comparing MM and NP tissues, MMP-9 and MMP-2 were stained in larger areas in NP than in MM. This finding was significant in the AR group, but not in the control group (Figs 1C and 2C). In contrast, TIMP-1 positivity was found to be lower in NP than in MM, and it was significant in both the AR and control groups (Fig 3C).

Table 1 summarizes these results for conventional histopathological examination and immunohistochemistry.

DISCUSSION

In this study, we investigated the expression of MMP-9, MMP-2, and TIMP-1 in two different sites of nasal tissues from patients with nasal polyposis, and speculated the influence of AR on airway remodeling of nasal mucosa in nasal polyposis.

MMPs are a group of Zn^{2+} -dependent endopeptidases, with more than 20 known members, which are able to

Figure 2 (A) Representative MMP-2 immunohistochemical staining of surface epithelium of NP in the control group (original magnification, $\times 400$). (B) Representative MMP-2 immunohistochemical staining of surface epithelium of NP in the AR group (original magnification, $\times 400$). (C) Differences in the expression of MMP-2 according to the presence of AR in nasal polyposis (* $P < 0.05$, Mann-Whitney U test, ** $P < 0.05$, Wilcoxon signed rank test). AR, allergic rhinitis; MM, maxillary sinus mucosa; NP, nasal polyp tissue.

degrade every component of the extracellular matrix. The extracellular activity of MMPs is regulated by TIMPs. The production and action of the MMPs are subject to strict control, ensuring limited proteolysis of the extracellular

matrix during cell migration, tissue remodeling, and repair.¹³

Based on common pathophysiologic findings of AR and asthma, several studies concerned with the airway remodeling of nasal mucosa in AR have been reported. Shaida et al⁸ examined MMPs and their inhibitors in the nasal mucosa of patients with perennial AR and found that mRNAs for MMP-2 and MMP-9 were not detected in large quantities in most samples. They concluded therefore that MMPs are not upregulated in the nasal mucosa of patients with perennial AR. However, van Toorenenbergen et al⁷ showed that eosinophil cationic protein and MMP-9 increased after the exposure of allergic antigens to the nasal cavity. In addition, it was found that the thickness of the basement membrane, which corresponds to regions positive for types I and III collagen antibodies, was statistically significantly greater in allergic patients than in nonallergic patients.¹⁴ Thus, it is known that airway remodeling exists in the nasal mucosa of patients with AR,^{14,15} although it appears that airway remodeling in the nose is less extensive than in the lungs.¹⁶ Nevertheless, the details of the nasal airway remodeling in AR are unclear and inconsistent compared with that in asthma.

Recently, it was reported that plasma MMP-9 levels were higher in allergic nasal polyp patients than in nonallergic nasal polyp patients and controls.¹⁷ In the group with allergic nasal polyps, plasma MMP-9 levels showed a positive correlation with eosinophil counts. These findings suggest that repetitive allergic attack could aggravate the airway remodeling in nasal polyposis. In our study, MMP-9, MMP-2, and TIMP-1 were expressed more intensely in the allergic group. Especially in NP of AR patients, increased expression of MMP-9, MMP-2, and TIMP-1 were observed more prominently than in that of the control group. However, we could not find any statistically significant differences between the allergic group and the control group in MM, except for the degree of eosinophilic infiltration. When we considered the limited number ($n = 20$ in each group) of samples involved in our study, the statistical power with nonparametric correction according to the expression of MMP-9, MMP-2, and TIMP-1 was calculated as 0.52, 0.48, and 0.35, respectively. Accordingly, a large-population study will be mandatory to determine whether the expression levels in MM are significantly different. If no significant differences are found, the possibility exists that differences in airway remodeling depending on the nasal mucosa site can exist in one individual.

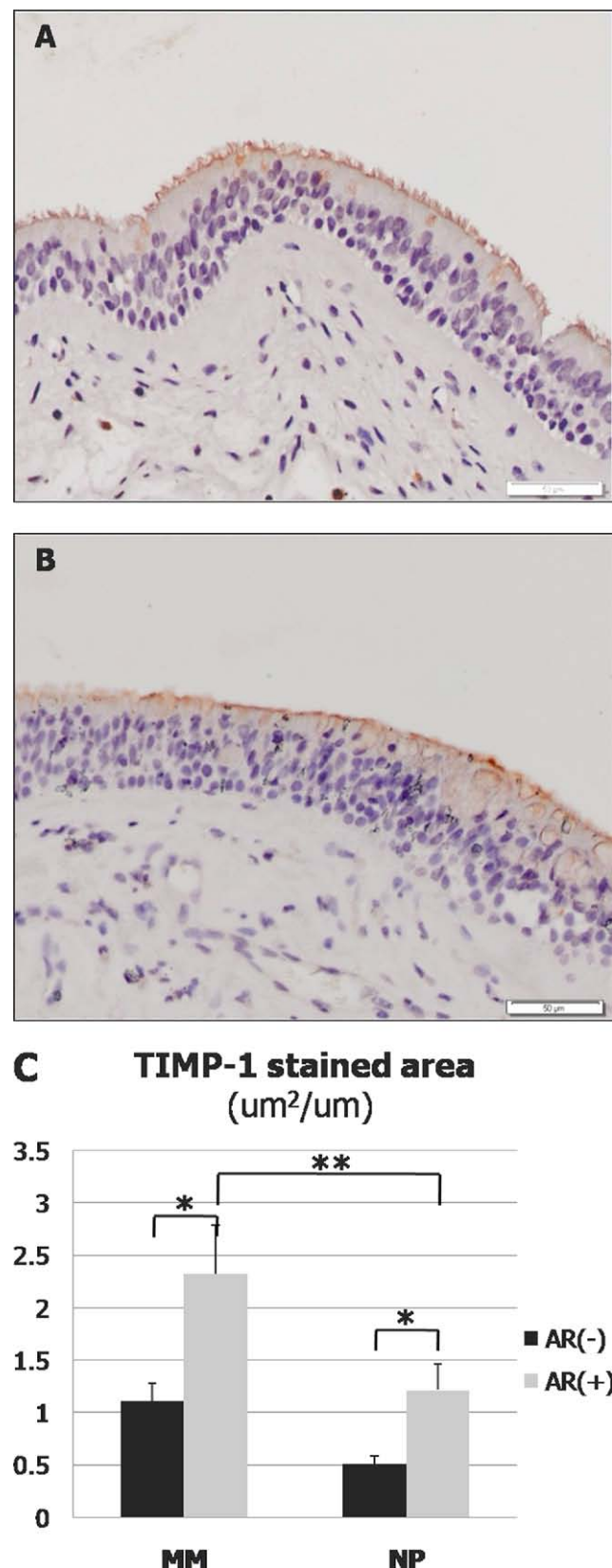


Figure 3 (A) Representative TIMP-1 immunohistochemical staining of surface epithelium of NP in the control group (original magnification, $\times 400$). (B) Representative TIMP-1 immunohistochemical staining of surface epithelium of NP in the AR group (original magnification, $\times 400$). (C) Differences in the expression of TIMP-1 according to the presence of AR in nasal polyposis (* $P < 0.05$, Mann-Whitney U test, ** $P < 0.05$, Wilcoxon signed rank test). AR, allergic rhinitis; MM, maxillary sinus mucosa; NP, nasal polyp tissue; TIMP-1, tissue inhibitor-1 of metalloproteinase.

We chose the MM as opposed to NP because, in most cases, maxillary sinuses are easily explored and biopsied. Moreover, polypoid changes of the MM are comparatively less frequent than those of the middle turbinate or the bulla ethmoidalis mucosa. However, the MM cannot represent the status of the whole nasal mucosa. Hence, a study including specimens from more diverse sites of nasal mucosa could provide more exact information in relation to the extent of airway remodeling in nasal tissues other than NP. In addition, we excluded the patients with a history of asthma, because their intake of anti-inflammatory medication, including corticosteroids, could have interfered with the results in this study. Thus, the findings are only reflective for nasal polyposis patients with presumably milder disease expression, without asthma.

In this study, MMP-2 was stained more weakly than MMP-9, and its intensity was more heterogenous in both MM and NP. In addition, TIMP-1 positivity was found to be lower in NP than in MM, and it was significant in both the allergic and nonallergic patients. Previously, several other reports mentioned that the expression level of TIMP-1 was significantly lower in nasal polyp patients compared to the chronic rhinosinusitis group.^{4,11} However, the study that compared the expression of TIMP-1 in NP and normal inferior turbinate mucosa showed no significant difference between those locations.⁸ Therefore, the difference of airway remodeling among the sites of nasal mucosa also should be further investigated in the future.

CONCLUSION

The presence of AR may enhance the expression of MMP-9, MMP-2, and TIMP-1 associated with airway remodeling in nasal polyposis. Additional studies are necessary to elucidate the role of MMPs and TIMPs in differentiating between allergic reaction and airway remodeling.

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AUTHOR CONTRIBUTIONS

Hyun-Woo Shin, data acquisition, data analysis and interpretation, initial writing and revision; **Doo Hee Han**, data analysis and interpretation; **Yune**

Sung Lim, data analysis and interpretation; **Hye Jung Kim**, data acquisition; **Dong-Young Kim**, manuscript revision; **Chul Hee Lee**, manuscript revision; **Yang-Gi Min**, manuscript revision; **Chae-Seo Rhee**, study concept and design, initial writing and revision, final manuscript approval.

DISCLOSURES

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