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Original Article

Tobacco Smoking Could Accentuate Epithelial-Mesenchymal Transition and Th2-Type Response in Patients With Chronic Rhinosinusitis With Nasal Polyps

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ABSTRACT

Tobacco smoking (TS) has been known as one of the most potent risk factors for airway inflammatory diseases. However, there has been a paucity of information regarding the immunologic alteration mediated by TS in patients with chronic rhinosinusitis with nasal polyps (CRSwNP). To identify the effect of TS, we harvested human tissue samples (never smoker: n=41, current smoker: n=22, quitter: n=23) and analyzed the expression of epithelial-derived cytokines (EDCs) such as IL-25, IL-33, and thymic stromal lymphopoietin. The expressions of Th2 cytokines and total serum IgE showed a type-2 inflammatory alteration by TS. In addition, the epithelial marker E-cadherin and epithelial-mesenchymal transition (EMT)-associated markers (N-cadherin, α-SMA, and vimentin) were evaluated. Histological analysis showed that EDC expressions were upregulated in the current smoker group and downregulated in the quitter group. These expression patterns were consistent with mRNA and protein expression levels. We also found that the local Th2 cytokine expression and IgE class switching, as well as serum IgE levels, were elevated in the current smoker group and showed normal levels in the quitter group. Furthermore, the expressions of E-cadherin decreased while those of N-cadherin, α-SMA, and vimentin increased in the current smoker group compared those in the never smoker group. Taken together, these results indicate that TS contributes to the deterioration of pathogenesis by releasing local EDCs and Th2 cytokines, resulting in EMT in patients with CRSwNP. We verified that alterations of immunological response by TS in sinonasal epithelium can play a vital role in leading to CRSwNP.

Keywords: Tobacco smoking; Chronic rhinosinusitis with nasal polyps; Epithelial-derived cytokines; IgE; Epithelial-mesenchymal transition

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Conflict of Interest
The authors declare no potential conflicts of interest.

Abbreviations
CRS, chronic rhinosinusitis; CRSwNP, chronic rhinosinusitis with nasal polyps; CT, computed tomography; DM, diabetes mellitus; EDC, epithelial-derived cytokine; EMT, epithelial-mesenchymal transition; ESR, erythrocyte sedimentation rate; GLT, germline transcripts; HTN, hypertension; JESREC, Japanese Epidemiological Survey of Refractory Eosinophilic Chronic Rhinosinusitis; PST, post-switch transcripts; RT, room temperature; TS, tobacco smoking; TSLP, thymic stromal lymphopoietin; WBC, white blood cell.

Author Contributions
Conceptualization: Lee KI, Park SR; Data curation: Lee KI, Han Y, Ryu JS, In SM, Kim JY, Park JS, Kim J, Youn J; Formal analysis: Lee KI, Han Y, Ryu JS, Park SR; Funding acquisition: Lee KI, Park SR; Investigation: Lee KI, Han Y, Ryu JS, Park SR; Methodology: Lee KI, Han Y, Ryu JS, Park SR; Project administration: Lee KI, Park SR; Resources: Lee KI, In SM, Kim JY; Supervision: Park SR; Validation: Lee KI, Kim JY, Kim JS, Park SR; Visualization: Lee KI, Han Y, Ryu JS; Writing - original draft: Lee KI; Writing - review & editing: Lee KI, Park SR.

INTRODUCTION

Chronic rhinosinusitis (CRS) is one of the most prevalent upper airway diseases for which patients visit an otorhinolaryngology clinic (1). Phenotypically, CRS with nasal polyps (CRSwNP) is commonly associated with a negative impact on the quality of life, unresponsiveness to medical therapy, and a poorer prognosis after endoscopic sinus surgery compared to that of CRS without nasal polyps (CRSsNP) (2).

Tobacco smoking (TS) is known as one of the most potent risk factors of airway inflammation among environmental stimulants such as allergens, yellow dust, and particulate matter (3). The upper airway protects the lower airway by filtering and humidifying air prior to its arrival to the lungs (4). However, despite the concept of one airway between the upper and lower airway, relatively little information is available in the literature regarding the immunological effects of TS in patients with CRSwNP.

Epithelial-derived cytokines (EDCs) such as thymic stromal lymphopoietin (TSLP), IL-25, and IL-33 may have a crucial role in nasal polypogenesis and Th2-type immune responses (5,6). These EDCs can affect both innate and adaptive type 2 inflammatory responses and cause tissue remodeling, suggesting an important role in the pathomechanism of CRS (7). These innate cytokines stimulate type-2 innate lymphoid cells to secrete major cytokines, such as IL-4, IL-5, and IL-13 (8,9). A previous study reported that IL-4 and IL-5, major drivers of type-2 inflammation, are upregulated by TS in asthma (10,11). Likewise, we can predict the EDCs upregulated by TS in the sinonasal epithelium.

CRSwNP is typically characterized by a local Th2-type inflammatory response. Several studies have demonstrated an increase in local Th2 cytokine (such as IL-4, IL-5, and IL-13) production, local IgE class switching, and local IgE antibody production in patients with CRSwNP (12-14). Ig class switching occurs in B cells through Ig class switch recombination (CSR) to differentiate into IgG- or IgE- or IgA-producing B cells (15). Ig CSR requires the activation of B cells through stimuli such as the CD40 ligand and cytokines. Cytokine-induced germline transcripts (GLT) are a prerequisite for Ig CSR. IL-4 and IL-13 are the representative cytokines inducing GLTε transcription in human B cells (16,17). After Ig CSR, the GLTμ promoter becomes associated with each constant region gene and continues to be active, generating transcripts termed post-switch transcripts (PST) (18). Thus, GLTε and PSTε are indicators of active IgE class switching.

Epithelial-mesenchymal transition (EMT) is an immunological pathway whereby epithelial cells acquire mesenchymal features (19). Injury due to trauma, chemical, or pathogen disrupts the epithelial barrier integrity in the sino-nasal epithelium, leading to EMT (20). EMT is thought to be a key mechanism of CRS and nasal polypogenesis (7,21). Shin et al. (22) reported that hypoxic-inducible factor-1α (HIF-1α) mediates the development of nasal polyps by inducing EMT. The basal epithelial markers include E-cadherin, claudin, occludin, ZO-1, and MUC1, while EMT-associated markers include periostin, vimentin, N-cadherin, α-smooth muscle actin (α-SMA), snail, and slug (20). The epithelium of CRSwNP, is characterized by an attenuated expression of junctional proteins associated with tight junctions and a thickening of the basement membrane (21). In contrast, EMT-associated markers (N-cadherin, α-SMA, and vimentin) are intracellular filamentous genes expressed by mesenchymal cells, thus is thought to be another mesenchymal marker and also the hallmark on airway remodeling, and tissue fibrosis associated with EMT (21,23).

There is a scarcity of clarification regarding the immunological response in CRSwNP after TS exposure. We previously demonstrated that TS promoted type-2 inflammation to a greater extent than it did type-1 inflammation in a murine CRSwNP model (24). In the present study, we aimed to identify immunological alterations in the sinonasal epithelium by analyzing EDCs, type 2 cytokines, and EMT according to the TS status in patients with CRSwNP.

MATERIALS AND METHODS

Patients and surgical procedure

A total of 86 patients over 20 years old who underwent endoscopic sinus surgery at the department of otorhinolaryngology in our hospital from January 2019 and December 2020 were enrolled, and a medical chart review was performed retrospectively. Subjects were divided into three groups: the never smoker (n=41), current smoker (n=22), and quitter (n=23) groups. Additionally, the subgroup analysis was conducted according to the approximate mean value of smoking amount (20 pack-years) and the quitting duration (15 years) in each group (current smoker and quitter groups, respectively). The present study was approved by the committee of the Institutional Review Board of our hospital (2019-09-014, 2019-10-013). Written informed consent was obtained from all participants.

The diagnosis of CRSwNP is based on the criteria mentioned in the European Position Paper on Rhiniosinusitis and Nasal Polyps guidelines (25). The exclusion criteria used were as follows: fungal sinusitis, neoplasm, trauma, revision surgery, pregnancy, and having received immune-modulating drugs such as corticosteroids and antileukotriens in the 1-month period before surgery.

The skin prick test was performed to confirm the allergy status. The multiple-antigen simultaneous test was performed in patients in whom the skin prick test could not be performed. No subjects had a current upper respiratory infection. The computed tomography score was obtained using the Lund-mackay scoring system for assessing the extent of the disease. Additionally, complete blood count including inflammatory parameters such as white blood cells, the erythrocyte sedimentation rate, eosinophil count, and absolute neutrophil count were also evaluated. Detailed demographic characteristics of the patients are listed in **Supplementary Table 1**.

Endoscopic sinus surgery was performed by two experienced surgeons (K-IL and SMI) under general anesthesia as the standard fashion (26). We harvested the nasal polyp tissue from the most inflamed area. In contrast, healthy control patients could not be included in this study due to ethical reasons. Absorbable packing was applied in the sinonasal area for hemostasis. Patients were discharged in postoperative days 3–5 and visited at the out-patient clinic for 3–6 months. No recurrence or relapse was observed during the follow-up period in the included patients.

Immunofluorescence

To identify alterations in EDCs and EMT protein levels in nasal polyp tissues, the tissues were taken from a −70°C deep freezer, put in an icebox, and cut into 10 μm sections using a freezing microtome. Frozen sections were fixed with 4% paraformaldehyde for 10 min at room temperature (RT), permeabilized in 0.1% Triton X-100 (Sigma) for 30 min at RT and blocked with 4% BSA for 2 h at RT. Next, the samples were stained with the respective for E-cadherin (1:400, clone: 24E10; Cell Signal Technology, Danvers, MA, USA), N-cadherin

(1:125, clone: D4R1H; Cell Signal Technology), α-SMA (1:200, clone: 1A4; Sigma-Aldrich, St. Louis, MO, USA), and vimentin (1:200, clone: D21H3; Cell Signal Technology), as well as for IL-25 (1:100, clone: 68C1039.2; Abcam, Cambridge, UK), IL-33 (1:50, PA5-20397, Thermo Fisher Scientific, Waltham, MA, USA), and TSLP (1:500, ab47943; Abcam) as primary antibodies at 4°C overnight and were washed with 0.05% Tween-20 (Sigma) in PBS (PBST). The samples were incubated with anti-mouse-IgG-Alexa 488, (1:200, ab150013) or anti-rabbit-IgG-Alexa 647 (1:200, ab150079) secondary antibodies (both from Abcam) for 40 min at RT. Each section was examined under a high-power field (10×40) by an independent researcher blinded to the experiments. Positive staining was analyzed as positive signals at the nasal polyp. The quantification of immunofluorescence staining in each section was measured by using the Image J program (U.S. National Institutes of Health, Bethesda, MD, USA).

Western blot

Nasal polyp tissues were lysed with RIPA buffer (ThermoFisher Scientific) containing a protease inhibitor cocktail (Cell Signaling Technology) on sonication for 1 min 3times and centrifuged at 20,000 ×g for 30 minutes at 4°C. Protein concentrations were determined using a bicinchoninic acid protein assay kit (ThermoFisher Scientific). Total protein (30 μg) was separated via BoltTM 4%–12% Bis-Tris Mini Protein Gel (ThermoFisher Scientific) and electrotransferred to polyvinylidene difluoride membranes (ThermoFisher Scientific) using an iBlot2 Transfer Device (ThermoFisher Scientific). The membranes were blocked in 5% skim milk (BD Biosciences, San Jose, CA, USA) at RT for 2 h and then incubated with a specific primary antibody for E-cadherin (1:1,000), N-cadherin (1:1,000), α-SMA (1:200), vimentin (1:1,000), IL-25 (1:500), IL-33 (1:500), and TSLP (1:1,000) overnight at 4°C. The membranes were washed with tris-buffered saline containing 0.1% Tween-20 and then probed with HRP-conjugated secondary antibodies (Goat anti-Mouse IgG [H+L] Secondary Antibody, HRP, 1:5000, 31430, Thermo Fisher Scientific or Goat anti-Rabbit IgG (H+L) Secondary Antibody, HRP, 1:500, 65-6120, Thermo Fisher Scientific). The bands were detected using SuperSignal West Femto Chemiluminescent Substrate (Thermo Fisher Scientific) and LuminoGraph2 image system (ATTO). Samples from the three groups (the never smoker, the current smoker, and the quitter groups) were analyzed with the regard to IL-25, IL-33, TSLP, E-cadherin, N-cadherin, α-SMA, and vimentin.

RNA isolation and RT-PCR

RNA isolation and RT-PCR were performed as previously described (17). The primer sequences are listed in **Supplementary Table 2**. PCR was performed using Prime Taq DNA polymerase (Biofactories, Daejeon, Korea). PCR for β-actin was performed in parallel to normalize cDNA concentrations within each set of samples. The PCR products were detected through electrophoresis on a 2% agarose gel with 0.5× Tris-borate-EDTA buffer. A densitometric analysis was conducted using Image J software.

Isotype-specific ELISA

The concentrations of immunoglobulins (IgE, IgM, IgA, and IgG) in the serum of patients were measured as the previous method (9). A 96-well U bottom microplate was coated with 0.1-M sodium bicarbonate buffer (pH 9.3) containing specific antibodies overnight at 4°C. After washing with PBST, plates were blocked for 1 h with a minimum of 0.25% BSA solution. After washing with PBST again, 100 ml serum diluted in BSA solution and standard proteins were added to each well and then incubated for 1 h at 37°C. After washing, anti-isotype-specific antibodies conjugated with HRP were added to each well and then incubated for 1 h at 37°C. After incubating and washing, 3, 3', 5, 5'- tetramethylbenzidine liquid substrate was

added. To stop the reactions, sulfuric acid (0.05 M) was added to each well. Colorimetric samples were analyzed using an absorbance microplate reader at 450 nm.

Statistical analysis

Data on tissue histopathology, cytokines, immunoglobulins, epithelial markers, and EMT-associated markers are expressed as mean ± SEM. Cross-group comparisons were performed using the Mann–Whitney *U*-test. Comparisons among groups used the Kruskal–Wallis test with a Bonferroni's multiple comparisons test. The Mann–Whitney *U*-test and the Kruskal–Wallis test were conducted by GraphPad Prism (Ver.5.0, GraphPad Software, San Diego, CA, USA). A value of $p<0.05$ was considered statistically significant.

RESULTS

The current smoker group reveals clinical features of eosinophilic nasal polyps relatively

We analyzed the clinical characteristics of subjects in each CRSwNP subgroup according to the TS status. The serum eosinophil count tended to increase in the current smoker group compared with the never smoker group without statistical significance. Additionally, we calculated the Japanese Epidemiological Survey of Refractory Eosinophilic Chronic Rhinosinusitis (JESREC) score criteria to predict the endotype in each group according to the TS status. The JESREC score showed a similar outcome, although those differences were not statistically significant. Therefore, it can be assumed that the current smoker group tended to reveal features of eosinophilic nasal polyps. The detailed demographic data are presented in **Supplementary Table 1**.

EDCs are upregulated in current smokers and not in quitters

To determine the effect of TS in a nasal epithelial level, we compared EDCs (IL-25, IL-33, and TSLP) expression in patients with CRSwNP across groups (the never smoker, the current smoker, and the quitter groups). The expression of EDCs in sinonasal mucosa was significantly elevated in the current smoker group compared to that in the never smoker group (**Fig. 1**). Meanwhile, the expression of IL-25, and TSLP was reduced significantly in the quitter group compared to that in the current smoker group, although a reduced tendency without statistical significance was shown in the expression of IL-33 in the quitter group. Likewise, EDC protein levels showed a significant increase in the current smoker group compared to those in the never smoker group (**Fig. 2A**). A lack of upregulation was significantly detected for all EDCs in the quitter group compared to their levels in the current smoker group. Similar patterns were shown in the mRNA expression of EDCs (**Fig. 2B**). An increased mRNA expression of all EDCs was significantly revealed in the current smoker group compared to that in the never smoker group. On the other hand, a decreased tendency without statistical significance was revealed in the mRNA expression of all EDCs in the quitter group. In detail, an increased mRNA expression of IL-25 was significantly shown in patients with ≥20 pack-years smoking amount compared to those with <20 pack-years smoking amount. Alternatively, a decreased mRNA expression of IL-25 was significantly revealed in the patients with ≥15 pack-years of quitting duration compared to those with <15 pack-years of quitting duration. Similarly, an increased tendency of IL-33 and TSLP was shown without statistical significance in patients with ≥20 pack-years smoking amount (**Supplementary Fig. 1**). Based on the data, we presumed that TS can be associated with EDC expressions.

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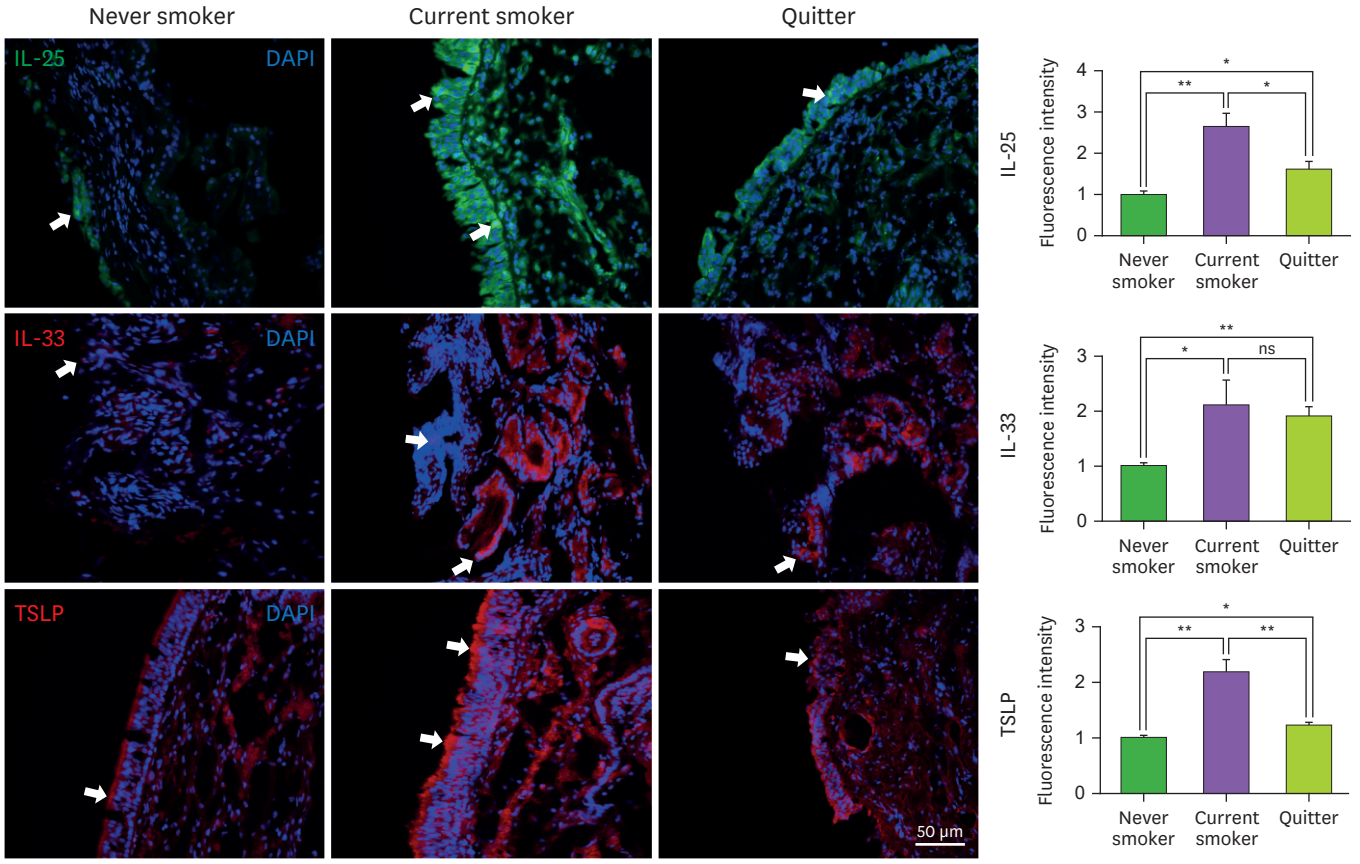

Figure 1. Histopathologic analysis of the expression of EDCs measured by immunofluorescence in patients with each chronic rhinosinusitis with nasal polyp subgroup according to the tobacco smoking status. The arrows indicate IL-25, IL-33, and TSLP positive cells in epithelial lining. The fluorescence intensity analysis of EDCs expression is shown in the graphs (Never smoker n=4, Current smoker n=4, Quitter n=4). The statistical analysis for numerical quantitative data was performed using the Kruskal-Wallis test with a Bonferroni's multiple comparisons test.
*p<0.05; **p<0.01, ***p<0.001, ns: not significant.

TS enhances local Th2 cytokine expression and IgE class switching

We performed RT-PCR with mRNAs from the nasal polyps of each group of CRSwNP patients to determine whether TS affects local Th2 cytokine expression and IgE class switching. The expression of the typical Th2 cytokines IL-4, IL-5, and IL-13 were enhanced in the current smoker group compared to those in the never smoker group, and these enhancements were decreased in the quitter group. Furthermore, the expressions of GLTε and PSTε were significantly enhanced in the current smoker group compared with those in the never smoker group. Additionally, the expressions enhanced by TS were diminished in the quitter group compared with those in the current smoker group (**Fig. 3**). On the other hand, the expression of IL-17 and IL-6 was enhanced in the current smoker group compared with those in the never smoker group, and there was no significant difference between the current smoker and quitter groups. Next, to determine the effect of TS on serum IgE levels in the patients with CRSwNP, we performed an isotype-specific ELISA. As shown in **Fig. 4**, serum IgE levels were substantially increased in the current smoker group compared with those in the never smoker group, while IgM, IgA, IgG levels were significantly decreased. Interestingly, the enhanced serum IgE levels in the current smoker group were diminished in the quitter group. Additionally, an increased tendency of serum IgE was shown without statistical significance in patients with ≥20 pack-years of the smoking amount. Meanwhile, we also found the decreased tendency of IgM in patients with ≥15 pack-years of the quitting duration

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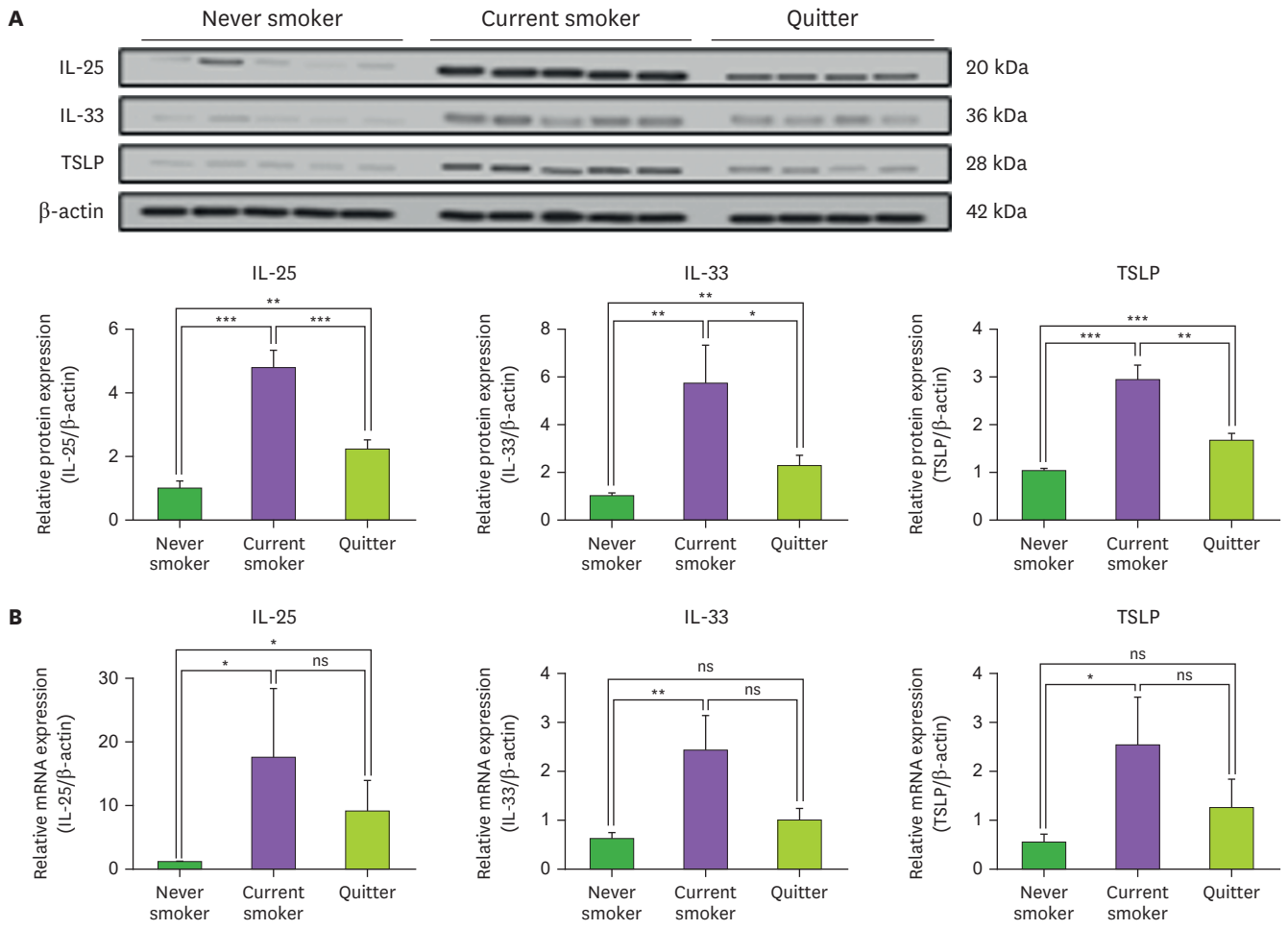

Figure 2. Effect of tobacco smoking on the protein and mRNA expression of EDCs in patients with chronic rhinosinusitis with nasal polyps. (A) Protein levels of EDCs measured by Western blotting (Never smoker: n=10, Current smoker: n=10, Quitter: n=9). (B) mRNA expression of epithelial-derived cytokines measured by RT-PCR (Never smoker: n=22, Current smoker: n=16, Quitter: n=14). The statistical analysis for numerical quantitative data was performed using the Kruskal-Wallis test with a Bonferroni's multiple comparisons test.
*p<0.05; **p<0.01, ***p<0.001, ns: not significant.

(**Supplementary Fig. 2**). According to these results, we posit that TS can also be associated with local IgE class switching by inducing IL-4 and IL-13 expression in patients with CRSwNP.

E-cadherin expression levels are decreased, while those of N-cadherin, α-SMA, and vimentin are increased in current smokers compared to their levels in never smokers

EMT is the process of immunological change whereby the mucosal epithelium losses its epithelial specialty and further acquires mesenchymal characteristics. Following EMT, E-cadherin is downregulated, while N-cadherin, vimentin, and α-SMA are upregulated. Histopathologically, a significant difference between the never smoker and current smoker groups was observed in E-cadherin, while the expression of E-cadherin was significantly ameliorated in the quitter group (**Fig. 5**). In contrast, a significant elevation in the fluorescence intensity of EMT-associated markers (N-cadherin, α-SMA, and vimentin) was observed in the current smoker group compared to that in the never smoker group. Interestingly, the immunofluorescence stainings of N-cadherin, α-SMA, and vimentin were significantly restored in the quitter group, nearly reaching the levels found in the never

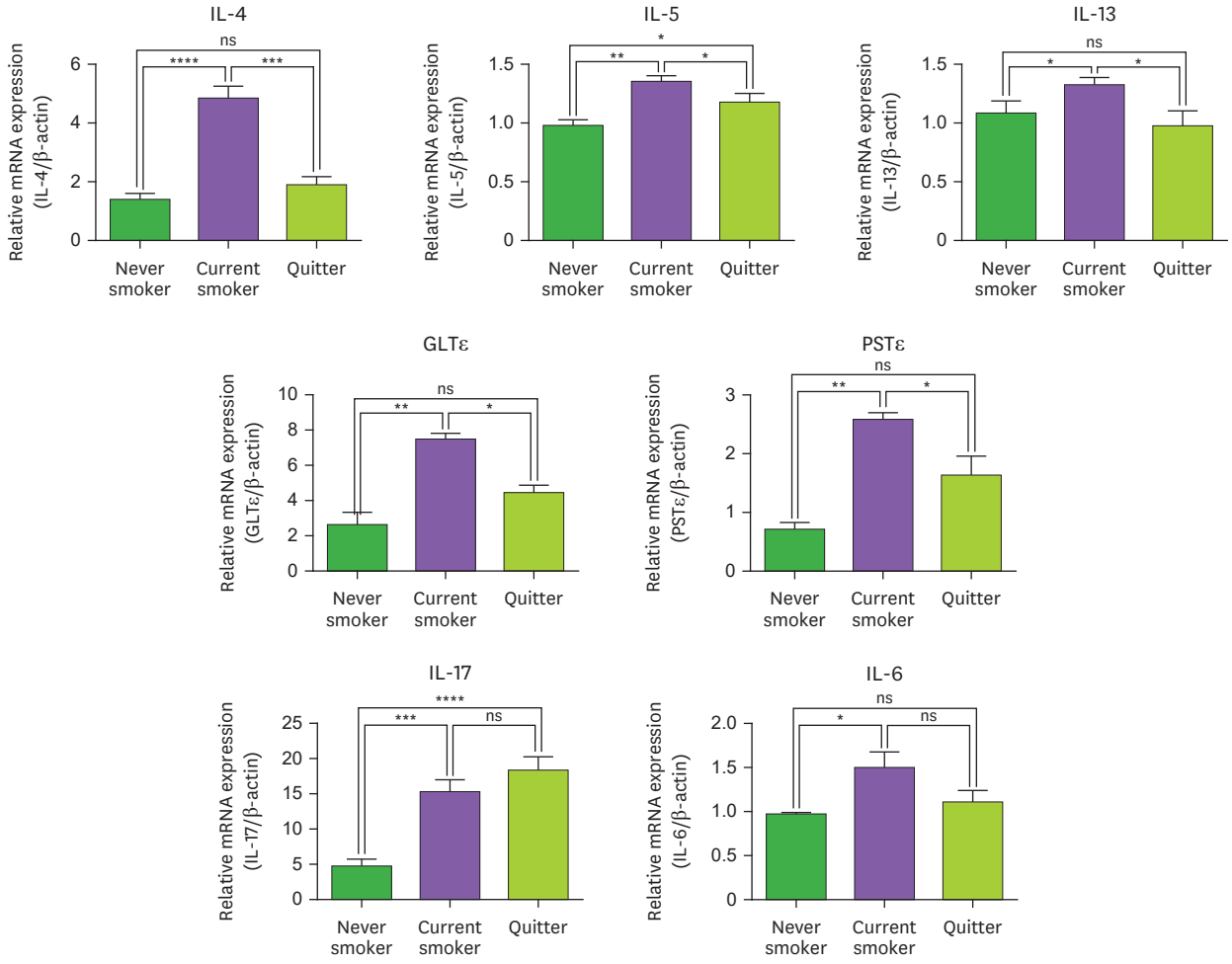

Figure 3. Expression of Th2 cytokines (IL-4, IL-5, and IL-13), GLTε, PSTε, IL-17, and IL-6 in patients with each chronic rhinosinusitis with nasal polyp subgroup according to the tobacco smoking status. The mRNA level of Th2 cytokines, GLTε, PSTε, IL-17, and IL-6 was measured by RT-PCR (Never smoker: n=6, Current smoker: n=6, Quitter: n=6). The statistical analysis for numerical quantitative data was performed using the Kruskal-Wallis test with a Bonferroni's multiple comparisons test.
*p<0.05; **p<0.01, ***p<0.001, ns: not significant.

smoker group. Next, we carried out Western blotting to confirm the histopathological results at the protein level. The protein levels of E-cadherin were significantly decreased in the current smoker group, while those in the quitter group were normalized to those in the never smoker group (**Fig. 6A**). On the other hand, a significantly increased protein expression of EMT-associated markers was shown in the current smoker group compared with those in the never smoker group. Meanwhile, the protein expression of EMT-associated markers in the quitter group tended to be reduced compared to that in the current smoker group (**Fig. 6A**). Then, a reduced tendency without statistical significance was observed in the mRNA expression of E-cadherin in the current smoker group compared to that in the never smoker group, while an enhanced mRNA expression of EMT-associated markers was detected in the current smoker group. In contrast, an increased tendency without statistical significance was shown in the mRNA expression of E-cadherin in the quitter group, while a reduced tendency in the expression of EMT-associated markers was revealed in the quitter group compared to that in the current smoker group (**Fig. 6B**). Additionally, an increased tendency of N-cadherin, and α-SMA was shown without statistical significance in patients with ≥20 pack-years smoking amount compared to that in patients with <20 pack-years smoking amount. Meanwhile, we

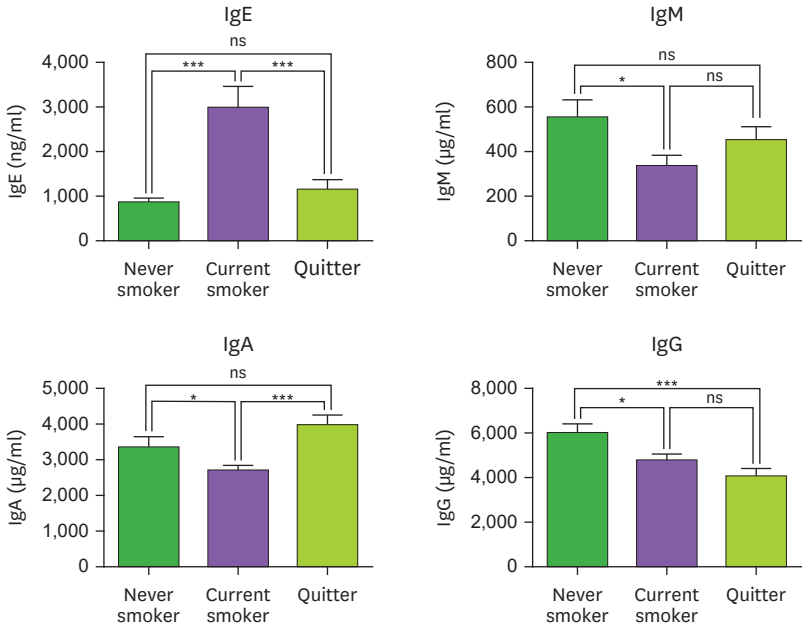

Figure 4. Serum Ig levels in patients with each chronic rhinosinusitis with nasal polyp subgroup according to the tobacco smoking status. The level of serum Ig was measured by isotype-specific ELISA (Never smoker: n=20, Current smoker: n=17, Quitter n=16). The statistical analysis for numerical quantitative data was performed using the Kruskal-Wallis test with a Bonferroni's multiple comparisons test.
*p<0.05; **p<0.01, ***p<0.001, ns: not significant.

found the increased tendency of E-cadherin and the decreased tendency of N-cadherin, and vimentin in patients with ≥15 pack-years of the quitting duration (**Supplementary Fig. 3**). These results suggest that TS promotes EMT, while quitting TS alleviates it.

DISCUSSION

Stimuli by innate cytokines induce the Th2 immune pathway, resulting in nasal polypogenesis, airway remodeling, and hypersecretion in the periphery (27). In our study, innate cytokines derived from the nasal epithelium, such as IL-25, IL-33, TSLP were elevated in the current smoker group. Interestingly, the elevated levels of these innate cytokines were decreased in the quitter group to the levels of the never smoker group. Thus, EDCs stimulate innate lymphoid cell 2 and Th2 cytokines, leading to a Th2-biased immunologic response (28,29). Our results revealed that local IgE expression was elevated in the current smoker group and reversed to the level of the never smoker group in the quitter group. In contrast, serum levels of IgM, IgA, and IgG were lower in the current smoker group compared to those of the never smoker group. These results imply that TS exposure is more associated with allergic inflammation and aggravated Th2 type inflammation rather than Th1 type. This is supported by previous researches using allergic or nasal polyp mice models, showing the additive effect of TS exposure on Th2-driven airway inflammation (24,30).

The exact underlying mechanism of the EMT process in CRSwNP is not known yet. It is widely assumed that TS accelerates airway epithelial barrier dysfunction (31). In a review paper, Schleimer (20) reported that the dysregulation of the injury-repair cycle on epithelial barrier function is paramount in the pathogenesis of CRS. The respiratory epithelium can

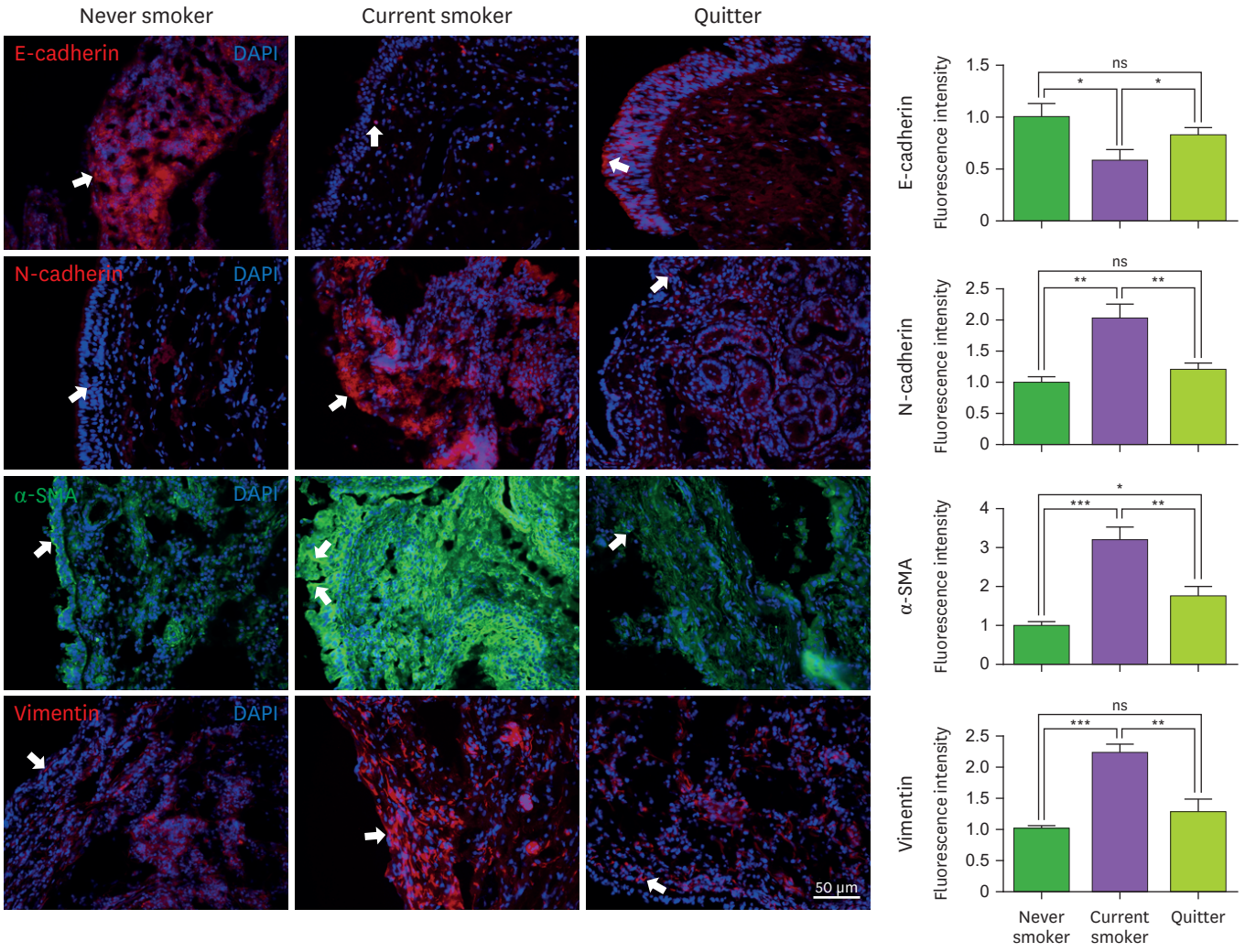

Figure 5. Histopathologic analysis of the expression of epithelial- and EMT-associated markers in patients with each chronic rhinosinusitis with nasal polyp subgroup according to the tobacco smoking status. The arrows indicate E-cadherin, N-cadherin, α-SMA, and vimentin positive cells in epithelial lining. The fluorescence intensity analysis of epithelial- and EMT-associated marker expressions is shown in the graphs (Never smoker: n=4, Current smoker: n=4, Quitter: n=4). The statistical analysis for numerical quantitative data was performed using the Kruskal-Wallis test with a Bonferroni's multiple comparisons test. $^{*}p<0.05$; $^{**}p<0.01$, $^{***}p<0.001$, and ns: not significant.

act as a critical barrier with innate immune and repair functions, and basal cell dysplasia is a hallmark of airway remodeling in CRSwNP (32). Alterations in epithelial composition might segregate with CRS phenotype under the influence of type 2 cytokines. Soyka et al. (33) reported that type 2 cytokines such as IL-4 and IL-13 may act as inducers of the EMT in CRS. In addition, another type 2 cytokine, IL-5, which is associated with eosinophils, is also essential in the barrier dysfunction, damage, and EMT induction in the airways (34,35). Based on the results from our present ex vivo study, we have demonstrated that TS may accentuate EDCs expression, the Th2-type response, leading to the EMT in patients with CRSwNP (**Fig. 7**). TS exposure has been shown to be associated with CRS and tissue remodeling in upper airways (36). We suggest that these sequential responses after the TS can disrupt epithelial barrier function and promote the development of the critical pathomechanism in CRSwNP tissues. Furthermore, increased levels of EDCs, IgE, and EMT markers were shown in the heavy smoker group. We think that TS has a cumulative effect also on this immunologic process in CRSwNP.

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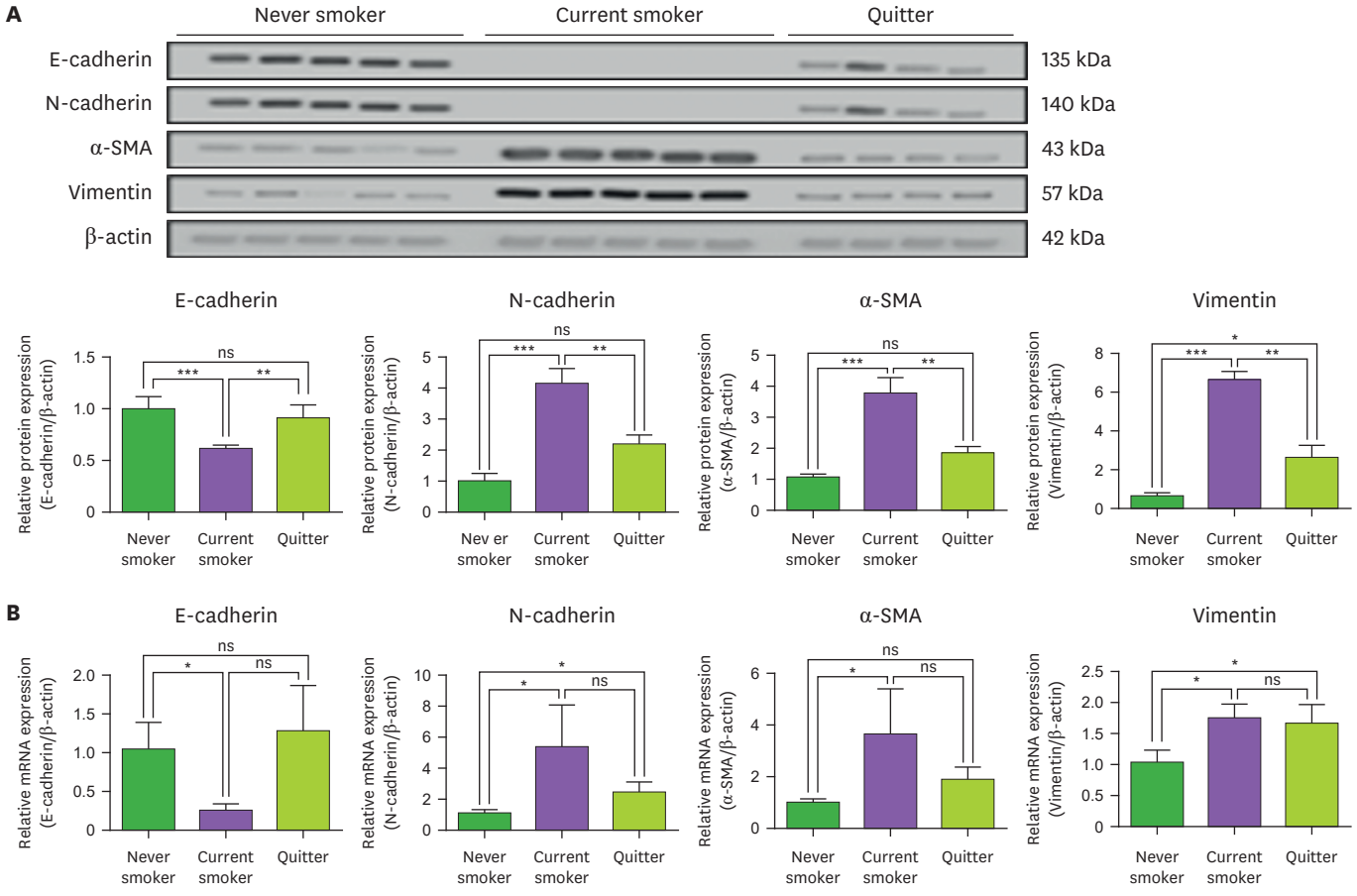

Figure 6. Protein and mRNA expression of epithelial- and EMT-associated markers in patients with each chronic rhinosinusitis with nasal polyp subgroup according to the tobacco smoking status. (A) Protein levels of epithelial- and EMT-associated markers were measured by Western blotting (Never smoker: n=10, Current smoker: n=10, Quitter: n=9). (B) The mRNA expression of epithelial- and EMT-associated markers was measured by RT-PCR (Never smoker: n=22, Current smoker: n=16, Quitter: n=14). The statistical analysis for numerical quantitative data was performed the Kruskal-Wallis test with a Bonferroni's multiple comparisons test. $^{*}p<0.05$; $^{**}p<0.01$, $^{***}p<0.001$, ns: not significant.

Meanwhile, Bequignon et al. (37) reported, by analyzing epithelial wound closure and ciliary beat frequency, that IL-6 could be a major cytokine in nasal polyp pathophysiology. Xia et al. (38) demonstrated that TS induces a release of IL-6 in the bronchial epithelium and promotes EMT via STAT3 activation, which causes pulmonary dysfunction. Likewise, we identified that the mRNA expression of IL-6 was increased in the current smoker group compared to the never smoker and quitter groups. Our results are in accordance with Xia's concept of IL-6's effect on EMT and indicate that TS stimulates the EMT process by inducing IL-6 expression. In this study, the mRNA expression of IL-6 and IL-17 was also revealed higher in the current smoker group compared to the never smoker group, whereas there was no significant difference between the current smoker and the quitter groups. These results were similar to that of Th2 inflammation. We derived that the TS can also affect the aggravation and restoration of neutrophilic or Th17 inflammation by analyzing IL-6 and IL-17.

TS provokes airway wall thickening and reduces mucociliary clearance and the infiltration of inflammatory cells in the airway mucosa in patients with chronic obstructive pulmonary disease and asthma (39,40). Furthermore, it was suggested that TS can induce the EMT and increase the metastatic ability of cancer cells in a previous *in vitro* and *in vivo* experiment (41). Tobacco contains a variety of active compounds that trigger EMT via different signaling

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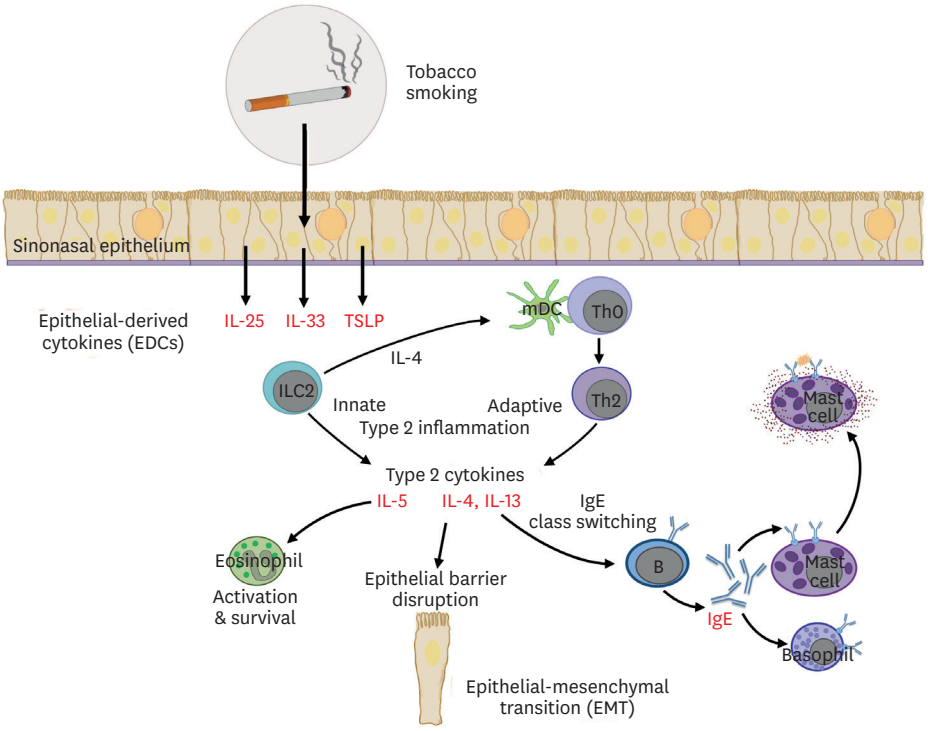

Figure 7. Proposed mechanism of tobacco smoking-induced EMT. EDCs such as IL-25, IL-33, and TSLP, which could be accentuated by tobacco smoking, and associated with the type-2 immune response.

pathways, and TS-induced EMT is mediated by reactive oxygen species (42). Meanwhile, Sun *et al.* (43) reported that 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone is a potent carcinogen in TS, and peroxiredoxin 1, which is modulated by 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone, is overexpressed in lung cancer cells. In addition, the immunomodulatory compounds in tobacco induce the release of EDCs from epithelial and airway smooth muscle cells, with the subsequent activation of dendritic cells and Th2 polarization, resulting in chronic inflammation (44). Thus, based on previous studies, it can be assumed that the aforementioned components of tobacco play a role as an aggravating factor in CRSwNP.

Nevertheless, this study still has some limitations. First, the smoking and quitting duration was not determined in detail according to the time period, although the subgroup analyses according to the approximate duration (20 pack-years of smoking and 15 years quitting duration) were performed. Second, *in vitro* and *in vivo* experiments were not performed. These experimental confirmations would be needed to confirm our results. Third, we did not consider the subclassification of CRS according to a phenotype (nasal polyp) or an endotype (eosinophil). In the future, large-scale *in vitro* and *in vivo* experimentation is needed to overcome these limitations and validate our findings.

In conclusion, histopathologically and immunologically, we verified that TS has an additive effect on the pathogenesis of CRSwNP. TS stimulates EDCs expression, activating the type-2 inflammation and EMT, and promoting epithelial barrier disruption in the sinonasal area. From a clinical viewpoint, this immunological understanding can help for diagnostic and therapeutic merits in patients with CRSwNP according to the smoking status. In addition, our findings may imply that TS-enhanced local Th2 cytokines and IgE expression contribute to the pathogenesis of CRSwNP. Our results are in accordance with the fact that anti-IgE monoclonal antibody

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treatment has already been clinically used in patients with CRSwNP (45,46). Likewise, we suggest that the prevention of epithelial barrier disruption and the avoidance of EMT, resulting in inhibition of the type-2 inflammation, could be another therapeutic target for CRSwNP.

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SUPPLEMENTARY MATERIALS

Supplementary Table 1
Clinical characteristics of patients

Click here to view

Supplementary Table 2
Primers used for RT-PCR

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Supplementary Figure 1
The mRNA expression of epithelial-derived cytokines in patients with chronic rhinosinusitis with nasal polyps according to the tobacco smoking amount or quitting duration. mRNA expression of epithelial-derived cytokines was measured by RT-PCR. Never smoker (n=22), smoker <20 pack-years (n=8), smoker ≥20 pack-years (n=8), Quitter ≥15 pack-years (n=8), Quitter <15 pack-years (n=6).

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Supplementary Figure 2
The serum Ig levels in patients with chronic rhinosinusitis with nasal polyps according to the tobacco smoking amount or quitting duration. The level of serum Ig was measured by isotype-specific ELISA. Never smoker (n=20), smoker <20 pack-years (n=10), smoker ≥20 pack-years (n=7), Quitter ≥15 pack-years (n=10), Quitter <15 pack-years (n=6).

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Supplementary Figure 3
The mRNA expression of epithelial- and EMT-associated markers in patients with chronic rhinosinusitis with nasal polyps according to the tobacco smoking amount or quitting duration. mRNA expression of epithelial- and EMT-associated markers was measured by qRT-PCR. Never smoker (n=22), smoker <20 pack-years (n=8), smoker ≥20 pack-years (n=8), Quitter ≥15 pack-years (n=8), Quitter <15 pack-years (n=6).

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