Subepithelial neutrophil infiltration as a predictor of the surgical outcome of chronic rhinosinusitis with nasal polyps*

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Abstract

Background: Neutrophils present as major inflammatory cells in refractory chronic rhinosinusitis with nasal polyps (CRSwNP), regardless of the endotype. However, their role in the pathophysiology of CRSwNP remains poorly understood. We investigated factors predicting the surgical outcomes of CRSwNP patients with focus on neutrophilic localization.

Methods: We employed machine-learning methods such as the decision tree and random forest models to predict the surgical outcomes of CRSwNP. Immunofluorescence analysis was conducted to detect human neutrophil elastase (HNE), Bcl-2, and Ki-67 in NP tissues. We counted the immunofluorescence-positive cells and divided them into three groups based on the infiltrated area, namely, epithelial, subepithelial, and perivascular groups.

Results: On machine learning, the decision tree algorithm demonstrated that the number of subepithelial HNE-positive cells, Lund-Mackay (LM) scores, and endotype (eosinophilic or non-eosinophilic) were the most important predictors of surgical outcomes in CRSwNP patients. Additionally, the random forest algorithm showed that, after ranking the mean decrease in the Gini index or the accuracy of each factor, the top three ranking factors associated with surgical outcomes were the LM score, age, and number of subepithelial HNE-positive cells. In terms of cellular proliferation, immunofluorescence analysis revealed that Ki-67/HNE-double positive and Bcl-2/HNE-double positive cells were significantly increased in the subepithelial area in refractory CRSwNP.

Conclusion: Our machine-learning approach and immunofluorescence analysis demonstrated that subepithelial neutrophils in NP tissues had a high expression of Ki-67 and could serve as a cellular biomarker for predicting surgical outcomes in CRSwNP patients.

Key words: neutrophil, neutrophil elastase, sinusitis, nasal polyp, machine learning

Introduction

Chronic rhinosinusitis (CRS) is one of the most prevalent chronic diseases characterized by inflammation of the sinonasal mucosa that lasts for more than 12 weeks. Currently, it is classified into CRS with nasal polyps (CRSwNP) and CRS without nasal polyps, based on nasal endoscopic findings of the presence of NP[(1)]. Among these, CRSwNP is considered a heterogeneous disease, and its inflammatory endotypes are influenced by both geographic and ethnic differences among affected individuals[(2-5)]. Additionally, two geographically different studies based on a cluster approach demonstrated that CRS has diverse inflammatory endotypes, which have distinct clinical features[(6,7)]. Severe

immunologic Type 2 CRS is characterized by neutrophilic and eosinophilic markers and is associated with nasal polyposis and a comorbidity such as asthma in European countries[6]. Interestingly, subsets of refractory CRS have also been represented as forms of nasal polyposis with no evidence of asthma and have shown moderate to severe neutrophilic counts and IL-8 expression in a Chinese CRS cohort[7].

Neutrophilic infiltration is attributed to the reaction of epithelial cells stimulated by Charcot-Leyden crystals, which are degradation byproducts of eosinophils[8]. IL-36+ neutrophils have been proposed as a cellular marker for refractory CRS in China and Korea. In our previous study, we hypothesized the role of imbalance between neutrophil elastase and alpha 1 anti-trypsin in refractory CRS[9,10]. Although there are geographical differences in CRS endotypes, neutrophils as well as eosinophils may contribute to the refractoriness of CRSwNP. However, it remains unclear whether neutrophils are a cellular biomarker for refractory CRS[11-13].

Recently, the machine-learning approach has enabled computational models composed of multiple processing layers to present the prediction of data with multiple levels of abstraction[14,15]. Thus, numerous researchers have begun to focus on machine learning as a promising technology to solve major problems in various clinical fields[15-17]. Therefore, in this study, we investigated factors affecting the surgical outcomes of CRSwNP patients using the machine-learning algorithm with a focus on neutrophilic localization. We speculated that applying a machine-learning algorithm might provide new insights, helping us to further understand the role of neutrophils in the pathogenesis of CRSwNP.

Materials and methods

Patients and tissue samples

The diagnosis of CRS was based on personal history, physical examination, nasal endoscopy, and sinus computed tomography (CT) findings, according to the 2012 European position paper on rhinosinusitis and NP (EPOS) guidelines[1]. All enrolled patients were classified into subgroups according to the algorithm of the Japanese Epidemiological Survey of Refractory Eosinophilic Chronic Rhinosinusitis study: control, non-eosinophilic CRS (non-ECRS), mild ECRS, moderate ECRS, and severe ECRS[18,19]. Screening items for ECRS were bilateral disease sites, NP, sinus CT findings, eosinophilia in peripheral blood, and comorbidities (bronchial asthma and aspirin-exacerbated respiratory disease/nonsteroidal anti-inflammatory drug-exacerbated respiratory disease). If total score was 11 or higher, the case was diagnosed as ECRS. Cases of lesser than 11 were classified into non-ECRS. ECRS group was classified into three subgroups: mild, moderate, and severe ECRS according to factors A and B. Factor A was determined by clinical parameters such as >5% blood eosinophils and ethmoid-dominant CT finding. Factor B was comorbidity including asthma and NSAIDs intolerance[18].

Samples of NP tissues were obtained from patients with CRSwNP during routine endoscopic sinus surgery. The endotype of CRSwNP was defined according to histopathological findings of haematoxylin and eosin-stained tissue samples (ECRSwNP: >10% eosinophils per high-power field, non-ECRSwNP: ≤10% eosinophils per high-power field)[20]. The atopic status of study participants was evaluated using the ImmunoCAP® assay (Thermo Scientific Inc., Waltham, MA, US) to detect IgE antibodies against six common aeroallergens (house dust mites; molds; trees; weeds; grass pollen; and animal dander). An asthmatic patient was defined as one who exhibited chronic airway symptoms (dyspnoea, cough, wheezing, and/or sputum), reversible airflow limitations, an increase in forced expiratory volume of ≥12% or 200 mL in 1 second after using a bronchodilator, or a methacholine provocation test result of PC20 ≤16 mg/mL. Disease severity was evaluated on CT images based on the Lund-Mackay (LM) CT scoring system. LM CT scores, global osteitis scores, and olfactory CT scores were calculated on preoperative CT scans[21,22]. Odor threshold was evaluated using the butanol threshold test. According to National Institute on Alcohol Abuse and Alcoholism standards, 14 g of alcohol is defined as one "standard drink." One-quarter bottle of soju (Korean distilled spirit), 350 mL beer, and one glass of wine contain the same amount of alcohol as one "standard drink." Based on these guidelines, alcohol consumption of patients was calculated in grams.

Patient characteristics are presented in Table 1. Briefly, the disease control status (DCS) of individual patients was evaluated 1 year after surgery and used to classify the patients into controlled and refractory (partly controlled plus uncontrolled) groups according to the EPOS guidelines, considering the presence and severity of four major sinonasal symptoms, namely sleep disturbance and/or fatigue, nasal endoscopic evaluation, and need for oral medication[1]. All patients provided written informed consent for study participation, and this study was approved by the Institutional Review Board of Seoul National University Hospital, Boramae Medical Center (No. 10-2018-80).

Immunohistochemical analysis

As previously described[23], immunohistochemical staining of the obtained NP samples was performed using the Polink-2 Plus Polymerized horseradish peroxidase (HRP) broad DAB-Detection System (Golden Bridge International Labs., WA). Briefly, tissue sections were incubated in 3% hydrogen peroxidase to inhibit endogenous peroxidases. Heat-induced epitope retrieval was then performed by microwaving the samples in 10 mmol/L citrate buffer (pH 6.0). Thereafter, the sections were incubated for 60 minutes at room temperature with primary antibodies consisting of mouse anti-human neutrophil elastase (HNE; 1:500; R&D Systems, Minneapolis, MN, USA). Furthermore, the sections were incubated in broad antibody enhancer and then in poly-

Table 1. Potential factors affecting the surgical outcomes of CRSwNP patients and indicators based on neutrophilic localization.

Features	CRSwNP patients (n=129)
Surgical outcomes	Good: 75/Poor: 54
Age, years	50.4 ± 13.7
Sex	Female: 31/Male: 98
Smoking	Non-smoker: 88 Ex or current smoker: 41
Heavy alcohol consumption	No: 112/Yes: 17
Body-mass index, kg/m2	24.3 ± 3.1
Asthma	No: 113/Yes: 16
Allergic rhinitis	No: 70/Yes: 59
Histologic endotype	Eosinophilic: 35 Non-eosinophilic: 94
JESREC score	Non-eosinophilic: 53 Mild eosinophilic: 35 Moderate eosinophilic: 32 Severe eosinophilic: 9
Lund-Mackay score	15.4 ± 5.2
Global osteitis score	14.2 ± 9.3
Olfactory CT score	5.3 ± 2.5
Sinus dominance on CT	Ethmoid sinus: 81 Maxillary sinus: 48
Tissue eosinophil count	42.1 ± 55.6
HNE epithelial count	13.9 ± 20.1
HNE subepithelial count	34.9 ± 35.9
HNE perivascular count	4.9 ± 6.1
Serum eosinophil, %	4.3 ± 3.0
Serum eosinophil count	299.3 ± 227.8
Serum neutrophil count	3925.3 ± 1410.8

Values are either expressed as n or mean ± standard deviation. CRSwNP: chronic rhinosinusitis with nasal polyps; JESREC: Japanese Epidemiological Survey of Refractory Eosinophilic Chronic Rhinosinusitis; CT: computed tomography; HNE: human neutrophil elastase.

mer HRP, followed by staining using the DAB-Detection System reagent. Finally, the slides were counterstained with haematoxylin. Positive cells were counted in the four densest visual fields (400×), and the average value obtained from two independent observers was considered. Additionally, we counted HNE-positive cells in different infiltrated areas (epithelial, subepithelial, and perivascular).

Immunofluorescence analysis

To determine the proliferation markers of neutrophils in NP tissues, immunofluorescence analysis was conducted using primary antibodies consisting of anti-Ki-67 (1:350; Abcam, Cambridge, MA, USA), anti-Bcl-2 (1:50; Abcam), and anti-HNE (1:200; Abcam). After 24 hours of incubation with primary antibodies at 4°C, the samples were incubated with secondary antibody Alexa Fluor 488-conjugated goat anti-mouse IgG (1:1,000; Abcam) or Cy3-conjugated goat anti-rabbit (1:500; Abcam) for 1 hour at room temperature. Thereafter, the nuclei were stained with 4-,6-diamidino-2-phenylindole (DAPI, 1:5,000; Sigma-Aldrich) for 2 minutes. The tissues were mounted using Fluoroshield Mounting Medium (ab104135; Abcam). Fluorescent images were obtained using the CELENA® S Digital Fluorescence Imaging System (Logos Biosystems, Annandale, VA, USA). The proportion of HNE-positive cells for each Ki-67- and Bcl-2-positive cells was calculated and analysed in three randomly selected fields.

Statistical analysis

Statistical analyses were performed using R version 3.4.2 software and GraphPad Prism software 7.0 (GraphPad Software Inc., La Jolla, CA, USA). In this study, to identify and rank the important factors influencing patients' surgical outcomes, we used the decision tree and random forest models of machine learning. The reasons for choosing these methods were ease of understanding because of their prominent visual intuition and their dramatic ability to identify unbiased variables that affect the mass of variables. The decision tree model is a classifier based on a tree-like representation of conditions. This algorithm is widely used for classification tasks; however, they have a disadvantage of overfitting if not finely tuned. In this study, we used a binary decision tree implemented in the R software 3.3.1 (R Foundation for Statistical Computing, Vienna, Austria) using the party package. It is useful in recursive partitioning for continuous, censored, ordered, nominal, and multivariate response variables in a conditional inference framework. Briefly, the procedural steps for this algorithm are as follows: 1) test the global null hypothesis of independence between any of the input variables and the response (which may be multivariate as well). Stop if this hypothesis cannot be rejected. Otherwise, select the input variable with the strongest association to the response. This association is measured by a P-value corresponding to a test for the partial null hypothesis of a single input variable and the response; 2) implement a binary split in the selected input variable; and 3) recursively repeat steps 1) and 2).

Meanwhile, the random forest model refers to an ensemble of regression trees where a set of T unpruned regression trees are generated based on bootstrap sampling from the original training data. In this study, we used the random forest model to predict surgical outcomes based on a suite of predictor variables. This algorithm was implemented in the R software using the randomForest package and was optimized through three parameters: 1) ntree- the number of trees grown from a bootstrapped sample; 2) mtry- the number of predictors randomly tested at each node; and 3) nodesize- the minimum size of the terminal node. For each node, the optimal node-splitting feature is selected from a set of m features that are picked randomly

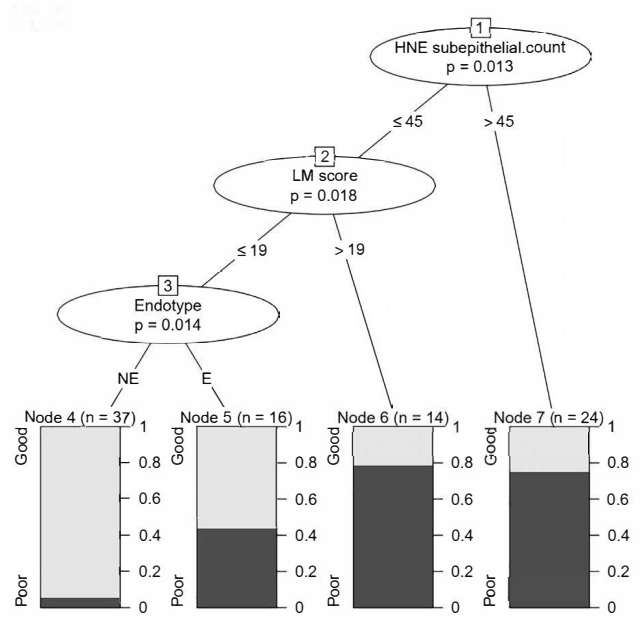

Figure 1. The decision tree for distinguishing chronic rhinosinusitis patients with nasal polyps based on the surgical outcomes. HNE: human neutrophil elastase; LM score: Lund-Mackay score; NE: non-eosinophilic; E: eosinophilic.

from the total M features. This calculated variable importance scores and then eliminated variables of small importance based on importance ranks (descending order). Variable importance was measured by mean squared error of a variable p, which is the average increase in prediction error among all regression trees when the out-of-bag data for variable p is randomly permutated.

Results

Prediction of the surgical outcomes for CRSwNP using the decision tree algorithm

To investigate factors influencing the surgical outcomes in CRSwNP patients, we employed machine-learning methods such as the decision tree algorithm. Figure 1 shows the decision tree generated from findings in CRSwNP patients. A decision tree algorithm consists of several nodes, which divide patients based on different variables. According to this machine-learning algorithm, the number of HNE-positive cells in the subepithelial area, LM score, and endotype were the most important factors, in that order, that could predict the surgical outcomes in patients. On this decision tree algorithm, Leaf no. 7 demonstrated that among patients with >45 HNE-positive cells in the subepithelial area, 75% would show poor surgical outcomes. Additionally, we found that among patients with ≤45 subepithelial HNE-positive cells and LM CT scores of >19, approximately 80% would show poor surgical outcomes (Leaf no. 6). The endotype of CRSwNP was also found to be effective in predicting the outcome in CRSwNP patients. The decision tree algorithm classified patients according to the endotype into ECRSwNP or non-ECRSwNP types. Thus, among non-ECRSwNP patients with ≤45 subepithelial HNE-positive cells and LM CT scores of ≤19, >90% would show a good outcome. The differences in the surgical

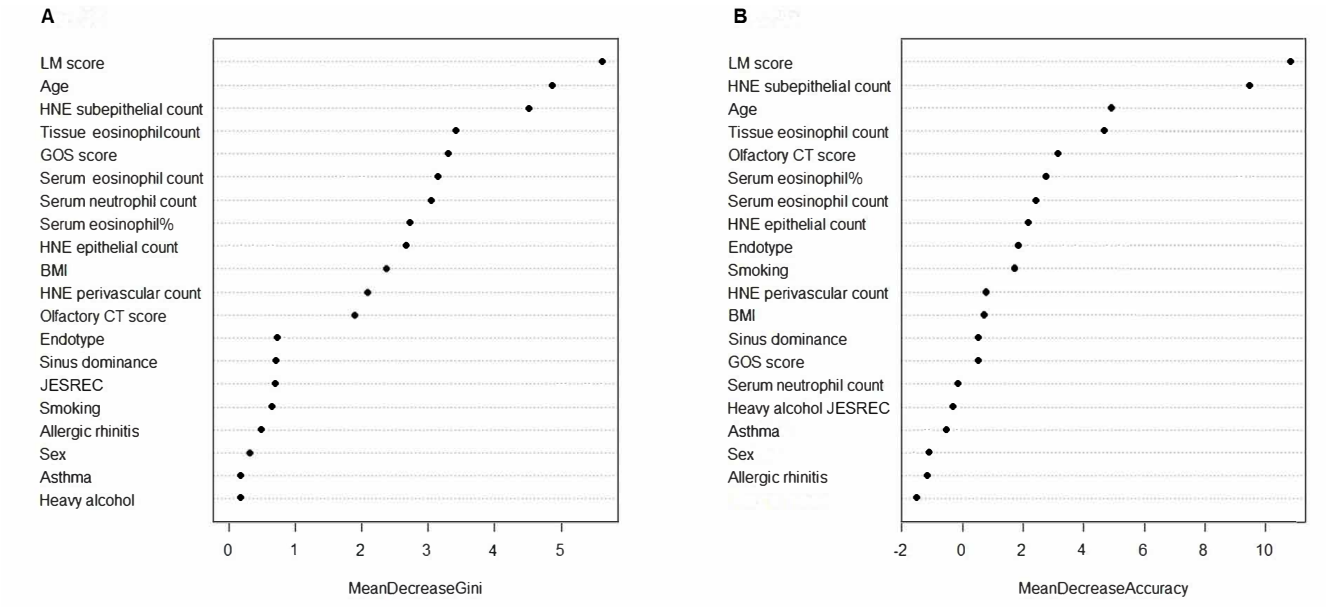

Figure 2. Result of the random forest model for the evaluation of surgical outcomes in chronic rhinosinusitis patients with nasal polyps according to (A) the mean decrease in the Gini index and (B) the mean decrease in accuracy. LM score: Lund-Mackay score; HNE: human neutrophil elastase; GOS: Global Osteitis Score; BMI: body-mass index; CT: computed tomography; JESREC: Japanese Epidemiological Survey of Refractory Eosinophilic Chronic Rhinosinusitis.

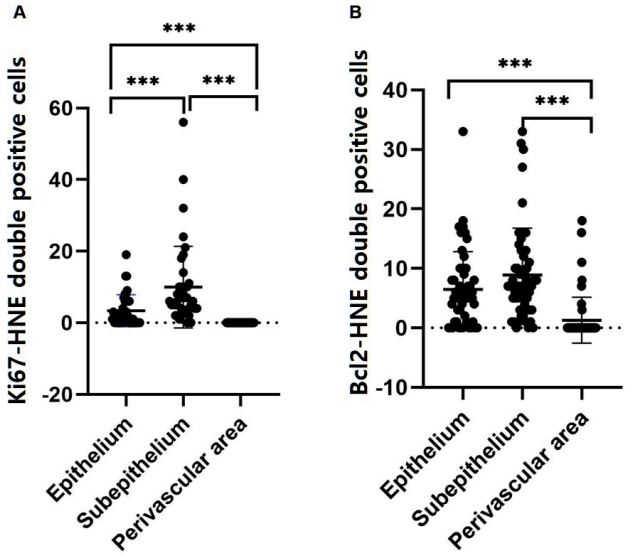

Figure 3. Comparison of proliferative markers associated with human neutrophil elastase (HNE)-positive cells according to the infiltrated area in the nasal polyp tissue based on immunofluorescence staining. (A) Ki-67 and HNE-double positive cells. (B) Bcl-2 and HNE-double positive cells.

outcomes of CRSwNP patients in different nodes demonstrated the ability of the tree to differentiate among patients based on the number of subepithelial HNE-positive cells, LM CT scores, and endotypes, which were confirmed using the chi-square test (P=0.013, P=0.018, and P=0.014, respectively).

Prediction of the surgical outcomes for CRSwNP using the random forest algorithm

To enhance the predictive value of the decision tree, we employed the random forest algorithm as an ensemble. In the random forest analysis, we found that the out-of-box error estimate was 15.96%. In other words, the mean accuracy in the correct prediction of good outcome after surgery in CRSwNP patients was 84.04%. The index that measures this model error is called the Gini index or Gini coefficient. Variables with the least values of the Gini index are more important in predicting the model. The random forest algorithm has two methods of determining the important variable among independent variables. When a variable is added to the model, according to the first method, the important variable is determined as the extent of decrease in the Gini coefficient, and according to the second method, the important variable is determined as the extent of decrease in accuracy after the removal of the variable from the model. Figure 2A shows the list of variables in decreasing importance of their values in reducing error and improving predictive accuracy when included in the random forest model. Regarding the mean decrease in the Gini index, the three highest-ranked variables were perceived LM CT scores, age, and number of subepithelial HNE-positive cells, in that order. Meanwhile, regarding the mean decrease in accuracy, LM CT scores, number of subepithelial HNE-positive cells, and age were the three most important factors, in that order, that could affect the accuracy of the random forest model (Figure 2B).

Proliferation of neutrophils in NP tissue

Next, to determine whether subepithelial neutrophils showed phenotypes different to those in epithelial and perivascular areas, immunofluorescence staining of NP tissues was performed (Figure 3). On immunofluorescence analysis, we found that the number of Ki-67/HNE-double positive cells was significantly greater in the subepithelial area than in the epithelial and perivascular areas. We also found that the number of Bcl-2/HNE-double positive cells in the epithelial and subepithelial areas was greater than that in the perivascular area. Moreover, the ratio of Ki-67/HNE-double positive cells to HNE-positive cells was higher in the subepithelial area than in the epithelial and perivascular areas (P<0.001). However, there was no significant difference in the ratio of Bcl-2/HNE-double positive cells to HNE-positive cells among the three areas (epithelial, subepithelial, and perivascular) (Figure 4), implying that HNE-positive cells in the subepithelial area have greater proliferative features than those in other areas.

Discussion

It is well known that type 2-skewed inflammation with increased eosinophilic infiltration is associated with refractoriness and comorbidities in CRSwNP. Recently, various studies have underlined the role of neutrophils and their related biomarkers in the development of CRSwNP[9-11,24,25]. Though increasing evidence has highlighted the importance of neutrophils in the pathogenesis of CRSwNP, several aspects of neutrophilic inflammation in CRSwNP remain unknown. In this study, based on the findings from the decision tree algorithm, we found that the number of subepithelial HNE-positive cells, LM score, and endotype were the most important factors that predicted the surgical outcomes of CRSwNP patients. This algorithm also demonstrated that >45 subepithelial HNE-positive cells and LM CT scores of >19 predicted poor surgical outcomes in patients, whereas ≤45 subepithelial HNE-positive cells, LM CT scores of ≤19, and non-ECRSwNP predicted good surgical outcomes. Additionally, our random forest algorithm showed 84.04% accuracy for the prediction of surgical outcomes in CRSwNP patients. Interestingly, the random forest model showed that the LM CT scores, age, and number of subepithelial HNE-positive cells were the three highest-ranked variables affecting the mean decrease in the Gini index and accuracy. Thus, these machine-learning approaches revealed that subepithelial HNE-positive cells in NP tissues were clinically more important than HNE-positive cells in other areas such as epithelial and perivascular. Based on these findings,

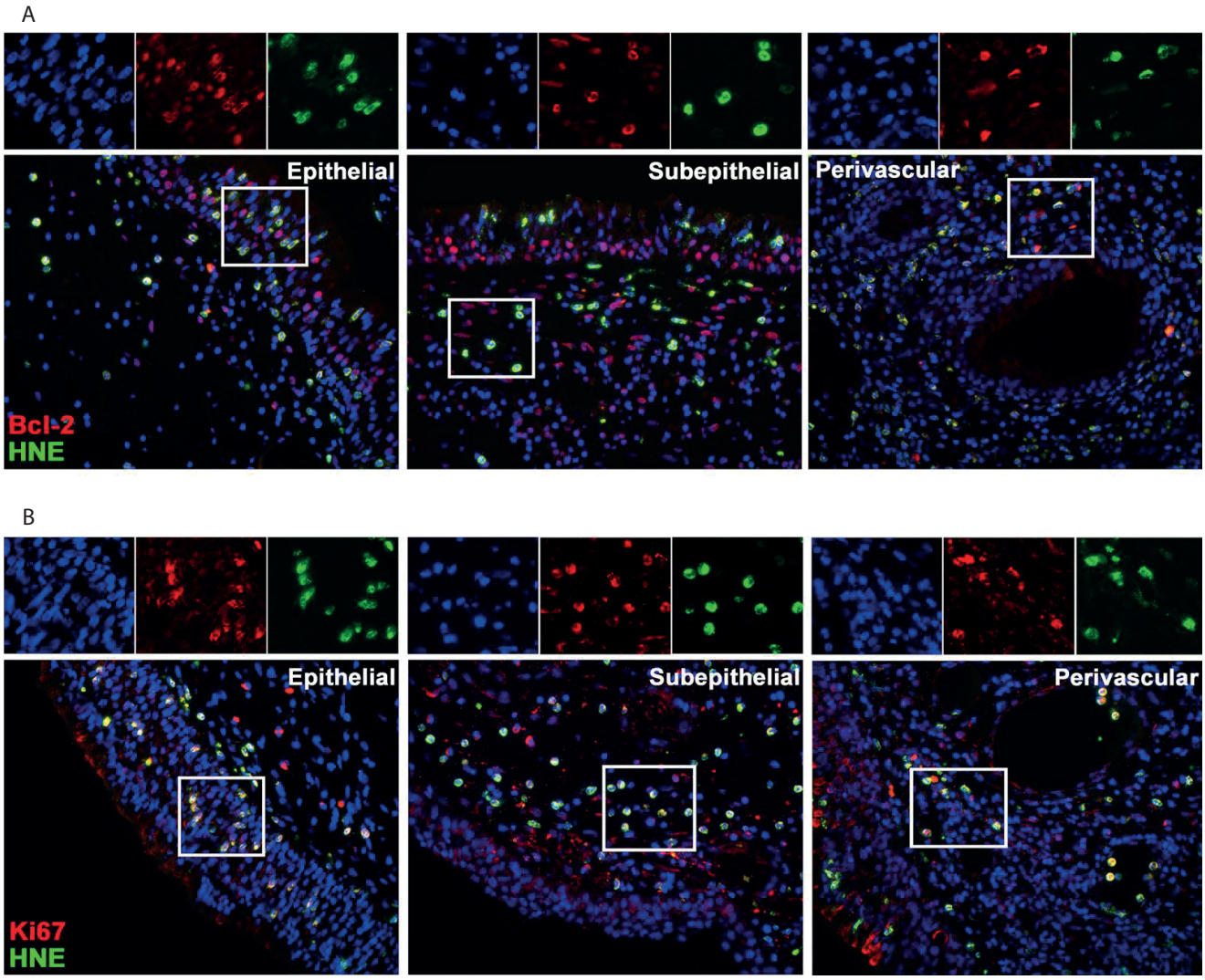

Figure 4. Representative images of immunofluorescence staining. (A) Ki-67 (1:350) and human neutrophil elastase (HNE, 1:200)-double positive cells. (B) Bcl-2 (1:50) and HNE (1:200)-double positive cells.

we further assessed the proliferation of neutrophils using Ki-67 and Bcl-2. The number of Ki-67/HNE-double positive cells and the ratio of Ki-67/HNE-double positive cells to HNE-positive cells were higher in the subepithelial area than in the epithelial and perivascular areas; thus, the proliferation of HNE-positive neutrophils was greater in the subepithelial area.

To date, it has been well known that an increased infiltration of neutrophils in the NP of CRSwNP patients is associated with poor corticosteroid response and disease prognosis[26]. However, studies regarding the role of neutrophils in the pathophysiology of CRSwNP are lacking. Neutrophils consist of granules, which comprise myeloperoxidase, elastase, cathepsin G, elastase, proteinase 3, and defensins[27]. These preformed mediators are released from the granules into the surrounding environment during degranulation. Among these mediators, elastase plays an important role in the coordination and activation of inflammatory reactions through processing of the extracellular matrix, cytokines, chemokines, and receptors[28]. A previous study showed colocalization of HNE-positive neutrophils in NP tissues with oncostatin M upon staining, which impaired barrier function[29]. Additionally, another study showed that HNE-positive neutrophils in NP tissues could cleave full-length IL-36γ into its activated form, which contributes to the inflammatory reaction in CRSwNP patients[10]. Moreover, a recent study reported that HNE-positive neutrophils and IL-36α were the strongest risk factors for refractory CRSwNP[9]. Our study also showed that HNE-positiveneutrophils might be more strongly associated with refractoriness than myeloperoxidase+ neutrophils. Interestingly, we used the decision tree and random forest models to explore factors affecting the surgical outcomes in CRSwNP patients. Similar to previous studies[9,10,29], we found that the number of subepithelial HNE-positive neutrophils in NP tissue may be an important predicting factor for the surgical outcomes in CRSwNP patients, but using machine learning. The

decision tree is an important machine-learning algorithm for predictive modelling and can be used to represent decisions visually and explicitly[30-32]. It is a graphical representation that uses branching methodology to exemplify all possible outcomes based on certain conditions. The internal node represents a test on the attribute, the branch depicts the outcome, and the leaf represents a decision made after computing the attributes. Meanwhile, the random forest model, as implied by its name, consists of large number of individual decision trees that operate as an ensemble based on bootstrapped samples of classification[33-35]. Each individual tree in the random forest is a class prediction, and the class with the most votes is the model's prediction. In the random forest, the mean decrease in accuracy is computed by permuting out-of-bag data; thus, the prediction error in the out-of-bag portion of the data is recorded by each tree. The same procedure is employed after permuting each predictor variable. The differences between the two are averaged over all trees and normalized by the standard deviation of the differences. In addition, the mean decrease in the Gini index is the total decrease in node impurities from splitting the variable, averaged over all trees. Thus, the node impurity is measured using the Gini index on the classification model. In this study, subepithelial HNE-positive neutrophils in NP tissue were the first internal node on the decision tree and the highest-ranked factor in the random forest. Thus, we further assessed the degree of neutrophil proliferation in the tissues using immunofluorescence analysis. We found that in NP tissues, HNE-positive neutrophils in the subepithelial area showed a higher rate of proliferation than did those in other areas.

However, we did not investigate the pathophysiological mechanism underlying the pathophysiologic mechanism of subepithelial neutrophils in the refractoriness of CRSwNP. Future studies should investigate this aspect.

Conclusion

The present study investigated factors predicting the surgical outcomes of CRSwNP patients using a machine-learning algorithm and immunofluorescence analysis, with focus on neutrophilic localization. We observed that subepithelial neutrophils play an important pathological role in CRSwNP patients. Specifically, a higher number of subepithelial HNE-positive neutrophils may be a cellular marker in refractory CRSwNP. These findings suggest that clinicians who treat patients with CRSwNP should pay careful attention to the number of subepithelial neutrophils.

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Authorship contribution

DWK had full access to all data obtained in the study and takes responsibility for the integrity of data and accuracy of data analysis; Conceptualization: DWK and DKK; methodology, formal analysis, resources, data curation: DKK, HSL, KME, YS, JKK, YSK, MKK, SJ, and SCH; writing-original draft preparation, writing-review and editing: DWK and DKK; supervision: DWK; project administration: HSL, KME and DKK; funding acquisition: DWK and DKK. All authors have read and agreed to the published version of the manuscript. This research was supported by Research Program 2020 funded by Seoul National University College of Medicine Research Foundation (to Dae Woo Kim)

Conflict of interest

The authors have no conflicts of interest to declare.

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