

Increase of high mobility group box chromosomal protein 1 in eosinophilic chronic rhinosinusitis with nasal polyps

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Background: Chronic rhinosinusitis with nasal polyps (CRSwNP) is characterized by eosinophil-dominant infiltration in Europe and the United States. However, CRSwNP in Asia has shown different immunopathologic features. High-mobility group protein box 1 (HMGB1) is a DNA-binding protein that has been suggested to be involved in various chronic inflammatory diseases. The objective of this study is to investigate whether HMGB1 is augmented in the Chinese eosinophilic CRSwNP and if non-eosinophilic CRSwNP is associated with interleukin 5 (IL-5), IL-8, and tumor necrosis factor α (TNF- α).

Methods: Nasal polyps specimens were collected from 41 patients with CRSwNP (20 eosinophilic and 21 non-eosinophilic) undergoing functional endoscopic sinus surgery (FESS). Biopsies of uncinate process, and ethmoidal mucosa from 9 non-CRS patients were used as controls by means of immunohistochemistry (IHC) staining, Western blotting, and quantitative reverse-transcription polymerase chain reaction (qRT-PCR).

Results: HMGB1-positive expression was significantly increased in the epithelium and among the large inflammatory cells infiltration in Eos CRSwNP and non-Eos CRSwNP as compared with controls ($p < 0.001$). The HMGB1 pro-

tein and messenger RNA (mRNA) levels of HMGB1, IL-5, IL-8, and TNF- α were significantly higher in eosinophilic CRSwNP than those from controls and non-eosinophilic CRSwNP, but no significant differences in these markers were found between non-eosinophilic CRSwNP and controls. HMGB1 expression levels correlated significantly and positively with IL-5, IL-8, and TNF- α ($r_s = 0.665, 0.771$, and 0.724 , respectively; $p < 0.001$) and slightly with eosinophil infiltration ($r_s = 0.149$; $p = 0.012$) and the blood eosinophils count ($r_s = 0.225$; $p = 0.001$) in all samples.

Conclusion: Upregulation of HMGB1 could be a significant marker typically in eosinophilic CRSwNP and it may also contribute to the pathogenesis of CRSwNP along with IL-5, IL-8, and TNF- α . © 2014 ARS-AAOA, LLC.

Key Words:

HMGB1 protein; Chinese patients; chronic rhinosinusitis with nasal polyps; eosinophilic inflammation; cytokine

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Chronic rhinosinusitis (CRS) is characterized by inflammation of the nasal and paranasal sinuses mucosa. Although CRS is a heterogeneous disease, the underlying

pathophysiology remains unsolved. CRS with nasal polyps (CRSwNP) is considered a subgroup of CRS with high recurrence after surgery and distinctive eosinophil infiltrating in the nasal polyps in both Europe and the United States.¹⁻³ However, studies in Asians have shown different immunopathologic features of CRSwNP than those in whites.^{1,4-7} Zhang et al.⁵ found that Chinese CRSwNP was characterized by a TH1/TH17 cell pattern with a minor eosinophilic, a more neutrophilic, and interleukin 17 (IL-17)-driven type of inflammation. Based on previous studies in different subgroups of CRSwNP in China, it underlines the need to define novel targets involved in pathogenesis and progression of CRSwNP.

High mobility group box 1 (HMGB1) protein, one of a group of non-histone chromatin binding nuclear proteins in the nucleus, is essential for DNA recombinant,

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reparation, differentiation, transcription, and extracellular signaling.^{8–10} It is reported in most recent studies that extracellular HMGB1 acts as the class of “alarmins” when it is actively secreted by immune competent cells (eg, leukocyte, T-cell, and dendritic cell) or passively released from necrotic or damaged cells.^{8,11–13} Elevated expression of HMGB1 protein has been shown to participate in the pathogenesis of several chronic inflammatory and autoimmune diseases such as sepsis, chronic obstructive pulmonary disease (COPD), systemic lupus erythematosus (SLE), and rheumatoid arthritis (RA).^{12,14–18}

In the extracellular environment, HMGB1 protein has been found involved in varieties of biological processes, such as recruitment of inflammatory cells,^{9,12} stimulation of other proinflammatory cytokines, including tumor necrosis factor α (TNF- α), interleukin-1 β (IL-1 β), IL-6, and IL-8,^{16,19–21} differentiation and proliferation of cells, and tissue repair.^{22,23} Thereby HMGB1 protein can be regarded as a bridge, linking innate and adaptive immune responses.²⁴

HMGB1 was found to be upregulated in the nasal mucosa with allergic rhinitis and in asthmatics with sputum eosinophilia.^{25,26} A recent report showed augmented HMGB1 expression in the nasal polyps as well as IL-5, IL-8, and TNF- α , which indicate that HMGB1 may be an important mediator of chronic inflammation in CRSwNP.²⁷ However, the pathogenetic role of HMGB1 in CRSwNP with different endotypes remains unclear. Therefore, the aim of the present study was to evaluate HMGB1 expression levels by immunohistochemical analysis in the nasal polyps with and without eosinophilic infiltration. Although several studies indicate that there is an increased expression of IL-5, IL-8, and TNF- α protein and mRNA in nasal polyps, little is known about the relationships between the mRNA levels of HMGB1 and the cytokines (IL-5, IL-8, and TNF- α) in nasal polyps, as well as between HMGB1 expression levels (as mRNA) and clinical signs. Our study may also contribute to a better understanding of the underlying mechanisms of intractable diseases such as eosinophilic CRSwNP.

Patients and methods

This study was approved and supervised by the ethics committee of Chinese People's Liberation Army (PLA) General Hospital. All patients gave their informed consent before recruitment into this study.

Study subjects and sample collection

In this study, nasal polyps from 41 patients with CRSwNP who underwent functional endoscopic sinus surgery (FESS) were obtained together with nasal mucosal biopsies from 9 non-CRS controls in the Department of Otorhinolaryngology Head and Neck Surgery of Chinese PLA General Hospital.

CRSwNP was confirmed by medical history, nasal endoscopy, and computed tomography (CT) scans of the

paranasal cavities according to the European Position Paper on Rhinosinusitis and Nasal Polyps 2012 guidelines.¹ Control subjects were 5 patients with cerebrospinal fluid rhinorrhea, 2 fracture of optic canal, 1 intracranial cholesteatoma, 1 encephalocele, and no history of sinus disease or any respiratory illness. Biopsies of the uncinate process and ethmoidal mucosa were obtained during surgery because of anatomic variations. None of these patients had received oral or nasal glucocorticoids and antibiotics for at least 4 weeks prior surgery. Patients with both noninvasive fungal sinusitis and invasive fungal disease, COPD, or systemic diseases such as congenital mucociliary diseases, cystic fibrosis, aspirin-exacerbated respiratory disease (AERD), or severe asthma were excluded.

CRS symptoms analysis

Individual CRS symptoms,¹ including nasal congestion, nasal discharge or postnasal drip, headache or facial pain, and hyposmia, were evaluated by using a scale of 0 to 3 (0 = no symptoms, 1 = mild symptoms, 2 = moderate symptoms, and 3 = severe symptoms) before surgery.

Nasal endoscopic examination

The presence and size of nasal polyps were graded as: 0, absence of polyps; 1, polyps in middle meatus only; 2, polyps beyond middle meatus but not blocking the nose completely; and 3, polyps completely obstructed the nose. It was calculated bilaterally for each patient.¹

CT scans of paranasal cavities

CT scans of the sinus opacification was graded with the Lund-Mackay stage classification: 0, no opacification; 1, partial opacification; and 2, total opacification. The maxillary sinus score (MS), anterior ethmoid sinus score (AES), posterior ethmoid sinus score (PES), sphenoid sinus score (SS), frontal sinus score (FS), ostiomeatal complex score (OMCS), and the total of the ethmoid sinus (ES), except for the OMCS were calculated bilaterally for each subjects with CRSwNP.²⁸

Skin prick tests

Allergic rhinitis patients were defined as those individuals with a characteristic history of intermittent or persistent rhinitis symptoms (eg, nasal itching, paroxysmal sneezing, nasal congestion, rhinorrhea) and a positive skin prick test to at least 1 of the following common inhalant allergens: house dust mites, pollens of ragweed, plantain, grasses, rye, trees, birch, *Alternaria*, *Hormodendrum*, cats, and dogs, based on the Allergic Rhinitis and Its Impact on Asthma (ARIA) document.²⁹

Sampling of tissue

All tissue samples were divided into 3 parts: (1) one part was immersion-fixed in 4% paraformaldehyde in phosphate buffered saline (PBS) at 4°C overnight. Then specimens

were embedded in paraffin (4 μ m thick) for hematoxylin-eosin (H&E) and immunohistochemistry or embedded in tissue-freezing medium; (2) frozen samples were kept at -80°C for further study; (3) the biopsies were transferred into RNAlater (R0901, Sigma, St. Louis Missouri, U.S.A.) or immediately snap-frozen and stored in liquid nitrogen for later quantitative reverse-transcription polymerase chain reaction (qRT-PCR) experiments.

Histologic analysis

Paraffin sections were processed routinely and stained with H&E. The number of the infiltrating eosinophils was counted and then averaged in 10 high power (HP) fields randomly using microscopy ($\times 400$ magnification) by 2 independent individuals who were blind to the clinical data and the diagnosis.

Among 41 nasal polyps, 20 samples having more than 10 eosinophils and 21 samples having fewer than 10 eosinophils per HP field were tentatively categorized as eosinophilic CRSwNP (Eos CRSwNP) and non-eosinophilic CRSwNP (non-Eos CRSwNP), respectively (Table 1).³⁰

Immunohistochemistry

Briefly, after deparaffinization and rehydration, antigen retrieval was carried out in 0.01 M citrate buffer (pH 6.0) for 15 minutes at 95°C in a microwave oven to increase the sensitivity and specificity. Endogenous peroxidase activity

was inhibited with 3% hydrogen peroxidase for 10 minutes at room temperature. The normal serum of secondary antibody was used to block nonspecific binding for 20 minutes. Slides were incubated with HMGB1 rabbit polyclonal antibody (Abcam, Cambridge, MA, U.K.; ab18256, dilution 1:300), IL-5 mouse monoclonal antibody (Abcam, Cambridge, MA, U.K.; ab25034, dilution 1:400) or TNF- α mouse monoclonal antibody (Abcam, Cambridge, MA, U.K.; ab1793, dilution 1:100) at 4°C overnight in a humidified chamber. The slides were rinsed in PBS 3 times and then incubated with PV-9000 Kit or PV-9003 Kit Two-step immunohistochemistry (IHC) Detection Reagent following the manufacturer's protocol (ZhongShan Goldenbridge Bio, Beijing, China). Antigen-antibody complexes were visualized with 3,3'-diaminobenzidine (DAB) (ZhongShan Goldenbridge Bio) and counterstained with Mayer's hematoxylin (Sigma, St. Louis, Missouri, U.S.A.).

The frozen specimens for IL-8 goat polyclonal antibody (Abcam; ab10769, dilution 1:100) staining were sectioned (5 μ m) in a cryostat (Leica, Germany), mounted onto gelatinized slides, allowed to dry at room temperature and fixed in 4% formaldehyde. The sections were then incubated in PBS-0.3% Triton X-100, pH 7.4 for 20 minutes. The subsequently treatments were the same with the paraffin immunohistochemistry.

Negative control slides were performed by the normal goat serum or isotype-matched antibodies under the same experimental conditions. The degree of HMGB1 protein expression was evaluated by dividing the stained sections

TABLE 1. Subjects' clinical characteristics

	Control subjects	CRSwNP		<i>p</i> ^a
		Eos	non-Eos	
Subjects, n	9	20	21	—
Gender (male/female), n	7/2	14/6	16/5	NS, NS, NS
Age, years, mean (range)	37 (21–58)	45 (25–61)	41 (17–70)	NS, NS, NS
Bilateral polyps, n	0	20	16	—
Recurrent history, n	0	13	8	—
Positive skin prick test (Y/N/U)	0/9/0	5/15/0	2/19/0	NS, NS, NS
Increased eosinophils, n (%) ^b	0 (0)	15 (75)	2 (10)	<0.001, NS, <0.001
Increased lymphocytes, n (%) ^b	0 (0)	2 (20)	1 (9.1)	NS, NS, NS
Methodology used				
IHC n, Gender (male/female)	8 (7/1)	20 (14/6)	21 (16/5)	NS, NS, NS
Age, years, mean (range)	35 (21–46)	45 (25–61)	41 (17–70)	NS, NS, NS
PCR, Western blotting n, Gender (male/female)	9 (7/2)	20 (14/6)	21 (16/5)	NS, NS, NS
Age, years, mean (range)	37 (21–58)	45 (25–61)	41 (17–70)	NS, NS, NS

^a*p* value: Control vs Eos CRSwNP, Control vs non-Eos CRSwNP, and Eos CRSwNP vs non-Eos CRSwNP, respectively.

^bIn peripheral blood.

CRSwNP = chronic rhinosinusitis with nasal polyps; Eos = eosinophilic; IHC = immunohistochemistry; N = no; NS = not significant; PCR = polymerase chain reaction; U = unknown (not performed); Y = yes.

in 4 portions: nucleus of epithelial cells, cytoplasm of epithelial cells, focal extracellular infiltration, and inflammatory cells. HMGB1 protein positive staining was assessed as a percentage of the total staining in each portion per HP field with Image-Pro Plus 6.0 software. For determining the distribution of IL-5, IL-8, and TNF- α staining, only the percentage of intracellular staining in inflammatory cells was estimated. Ten fields were randomly selected and scored by 2 independent individuals as in the histologic study.

qRT-PCR

RNA isolation from tissue samples was performed using Total RNA Isolation Kit (Quantobio Co., Ltd., Peking, China) based on the manufacturer's protocols. RNA was quantified with Nanodrop 8000. Total RNA was reverse-transcribed into complementary DNA (cDNA) using Oligo dT with M-MLV Reverse Transcriptase (Promega Corp., USA) (70°C for 10 minutes; 50°C for 50 minutes; and 70°C for 15 minutes) in 20- μ L reaction. Quantitative PCR was performed on the ABI 7900. Each PCR mixture contained 10 μ L 2 $\times$ qPCR Mix, 0.4 μ L FastStart Universal Probe Master (ROX), 0.3 μ L cDNA, 2 μ L of each primer, 5.3 μ L RNase-free water, forming a final volume of 20 μ L. The conditions for PCR were an initial denaturation at 95°C for 5 minutes, followed by 40 cycles of denaturation for 15 seconds at 95°C, annealing for 15 seconds at 55°C or 50°C (IL-5), and extension for 60 seconds at 72°C. The specific primers were designed from published cDNA sequences as follows: HMGB1, forward 5'-CCC GCT CTC ACA GCC ATT GCA GTA-3', reverse 5'-GCT CCT CCC GAC AAG TTT GCA C-3'; IL-5, forward 5'-AAT CAC CAA CTG TGC ACT GAA G-3', reverse 5'-ATC CAC TCG GTG TTC ATT ACA C-3'; IL-8, forward 5'-CGT GGC TCT CTT GGC AGC CTT-3', reverse 5'-CTG TGT TGG CGC AGT GTG GTC-3'; TNF- α , forward 5'-TCC ACC CAT GTG CTC CTC AC-3', reverse 5'-GTT GGA TGT TCG TCC TCC TCA C-3'; β -actin, forward 5'-AGC GAG CAT CCC CCA AAG TT-3', reverse 5'-GGG CAC GAA GGC TCA TCA TT-3'. β -Actin was used as a housekeeping gene for normalization. The fold change of target gene mRNA expression in the patients with CRSwNP to relative to control was quantified using the $2^{-\Delta\Delta C_t}$ method.³¹

Western blotting

Total proteins from tissue samples were lysed in radioimmunoprecipitation assay (RIPA) buffer with fresh protease inhibitors and the concentration of the proteins in the supernatant was measured photometrically using a bicinchoninic acid (BCA) protein assay kit (Pierce, Rockford, IL). Proteins were separated on 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) for HMGB1 and β -actin, and transferred to a polyvinylidene difluoride membrane. After nonspecific blocking, the membrane was probed overnight with HMGB1 mouse monoclonal antibody (Abcam; ab77302, dilution 1:1000 and

Santa Cruz Biotechnology, Santa Cruz, CA; sc47778, dilution 1:1000) in 5% nonfat dry milk/mixture of Tris-Buffered Saline and Tween 20 (TBST) overnight at 4°C. After washing in TBST the membrane was then incubated with secondary antibodies and treated with enhanced chemiluminescence detection reagents. The specific blotting band was recorded on film.

Statistical analysis

All statistical analysis was performed using SPSS version 13.0 statistical analysis software. Results were presented as the means $\pm$ standard deviation (SD), or medians and interquartile ranges (25th-75th percentiles) when data were not normally distributed. Data were expressed in box-and-whisker plots. Comparisons between unpaired groups were performed using the Student *t* test and 1-way analysis of variance (ANOVA) with post hoc tests. Results were assessed first using parametric tests assuming normality. For nonparametric data, comparisons among the 3 groups were used by the nonparametric Kruskal-Wallis test, and Mann-Whitney U test was used to assess significant variability between groups. Correlations between different variables were analyzed by the Spearman coefficient. A chi-squared test was used for differences in proportions between groups. Significance was accepted at a *p* value <0.05.

Results

Subjects' clinical characteristics

The clinical characteristics of all study subjects are summarized in Table 1. They were a total of 37 males and 13 females, aged from 17 to 70 years, with a median age of 43 years. Subjects in the various subgroups were similar in age and gender. History of recurrence was found in 13 Eos CRSwNP patients and 8 non-Eos CRSwNP patients. Positive skin prick test was evidenced in 5 Eos CRSwNP patients and 2 non-Eos CRSwNP patients. None of the patients had fungal sinusitis, asthma, or aspirin sensitivity. Differences of CT scans and symptoms scores between the Eos CRSwNP and non-Eos CRSwNP groups are shown in Table 2. Significantly higher symptom scores were found for nasal congestion, headache, and loss of smell in patients with Eos CRSwNP compared to patients with non-Eos CRSwNP. Similarly, patients with Eos CRSwNP also had a significantly higher bilateral CT score than patients with non-Eos CRSwNP. Histological findings of nasal polyps in different study groups are shown in Figure 1. The percentage (%) of peripheral blood eosinophils correlates significantly with infiltrating eosinophil counts in nasal polyps ($r_s = 0.667$; $p < 0.001$) in all subjects ($n = 50$).

Western blotting and immunohistochemistry for HMGB1

Western blotting confirmed that HMGB1 protein is present in the nasal polyps and controls (Fig. 2A, B). Higher levels

TABLE 2. CT scans, nasal endoscopic examination, and symptoms scores analysis in Eos CRSwNP and non-Eos CRSwNP

	Eos CRSwNP	non-Eos CRSwNP	<i>p</i>
MS	2.3 ± 1.0	2.1 ± 1.1	NS
AES	3.4 ± 0.9	2.3 ± 1.3	0.004
PES	2.7 ± 1.1	1.4 ± 1.3	0.003
SS	2.2 ± 1.6	1.0 ± 1.3	0.016
FS	2.2 ± 1.5	1.3 ± 1.4	NS
OMCS	3.3 ± 1.2	2.2 ± 1.5	0.02
ES	6.0 ± 1.8	3.8 ± 2.5	0.02
Lund-Kennedy	3.6 ± 1.1	2.5 ± 1.4	0.01
Nasal congestion	7.5 ± 1.2	6.2 ± 2.5	0.049
Rhinorrhea	5.9 ± 2.4	4.2 ± 3.2	NS
Headache	4.4 ± 3.0	1.8 ± 2.1	0.002
Hyposmia	5.8 ± 3.4	3.2 ± 3.7	0.003
VAST	23.2 ± 5.6	15.3 ± 8.1	0.001

Values are mean ± SD.

AES = anterior ethmoid sinus score; CT = computed tomography; CRSwNP = chronic rhinosinusitis with nasal polyps; Eos = eosinophilic; ES = total ethmoid sinus score; FS = frontal sinus score; MS = maxillary sinus score; NS = not significant; OMCS = ostiomeatal complex score; PES = posterior ethmoid sinus score; SD = standard deviation; SS = sphenoid sinus score; VAST = total visual analogue scale.

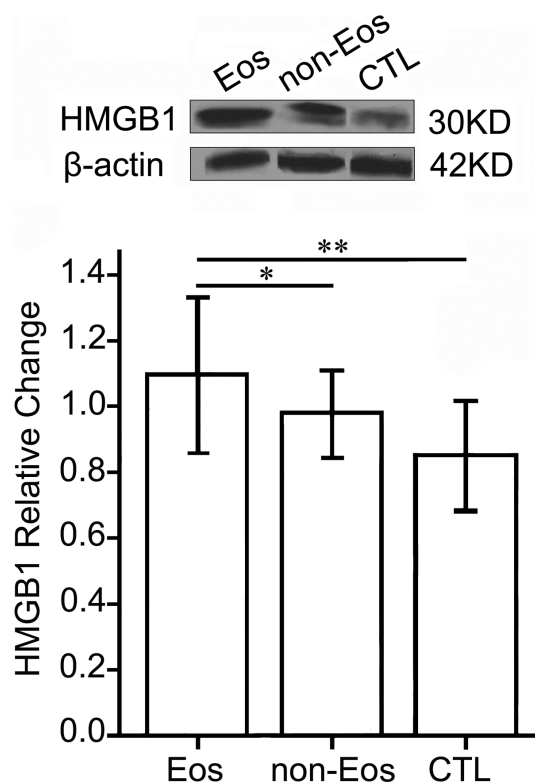


FIGURE 2. HMGB1 protein in healthy control and nasal polyps. Data were presented as means ± SD, and comparisons were made by 1-way ANOVA. **p* < 0.05, ***p* < 0.01, ###*p* < 0.01. ANOVA = analysis of variance; CTL = control; Eos = eosinophilic chronic rhinosinusitis with nasal polyps; non-Eos = non-eosinophilic chronic rhinosinusitis with nasal polyps; SD = standard deviation.

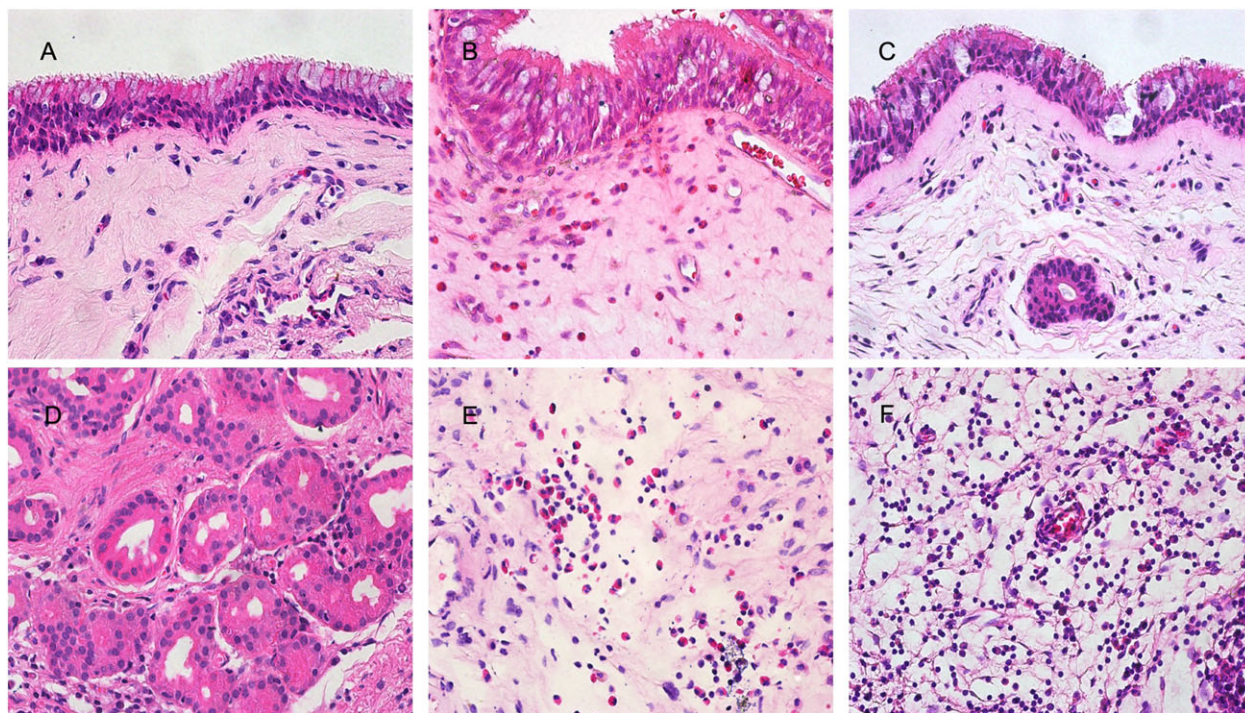


FIGURE 1. Histology of the healthy mucosa and nasal polyps. The sections were stained with hematoxylin-eosin in the healthy control (A, D), Eos CRSwNP (B, E) and non-Eos CRSwNP (C, F), respectively. Original magnification ×400. CRSwNP = chronic rhinosinusitis with nasal polyps; Eos = eosinophilic.

of HMGB1 were seen in the Eos CRSwNP group, compared with those in the non-Eos CRSwNP group and controls ($p = 0.004$ and $p = 0.001$, respectively). No statistical significance was found between the non-Eos CRSwNP patients and controls. After immunohistochemical staining, HMGB1 protein was widely detectable in all subjects and predominantly seen in the nucleus of epithelium and also in inflammatory cells (Fig. 3A–D). Figure 4 showed that the proportion of epithelial cells with HMGB1-positive nucleus was significantly higher in both patients with Eos CR-

SwNP and patients with non-Eos CRSwNP than in controls ($p < 0.001$ and $p < 0.001$, respectively). The proportion of epithelial cells with cytoplasmic HMGB1 immunostaining was significantly augmented in the Eos CRSwNP patients and the non-Eos CRSwNP patients, compared with controls ($p = 0.009$ and $p = 0.04$, respectively). The number of focal infiltrating inflammatory cells containing HMGB1 was further elevated in Eos CRSwNP patients and non-Eos CRSwNP patients ($p < 0.001$, respectively, vs controls). The level of extracellular HMGB1 was significantly higher

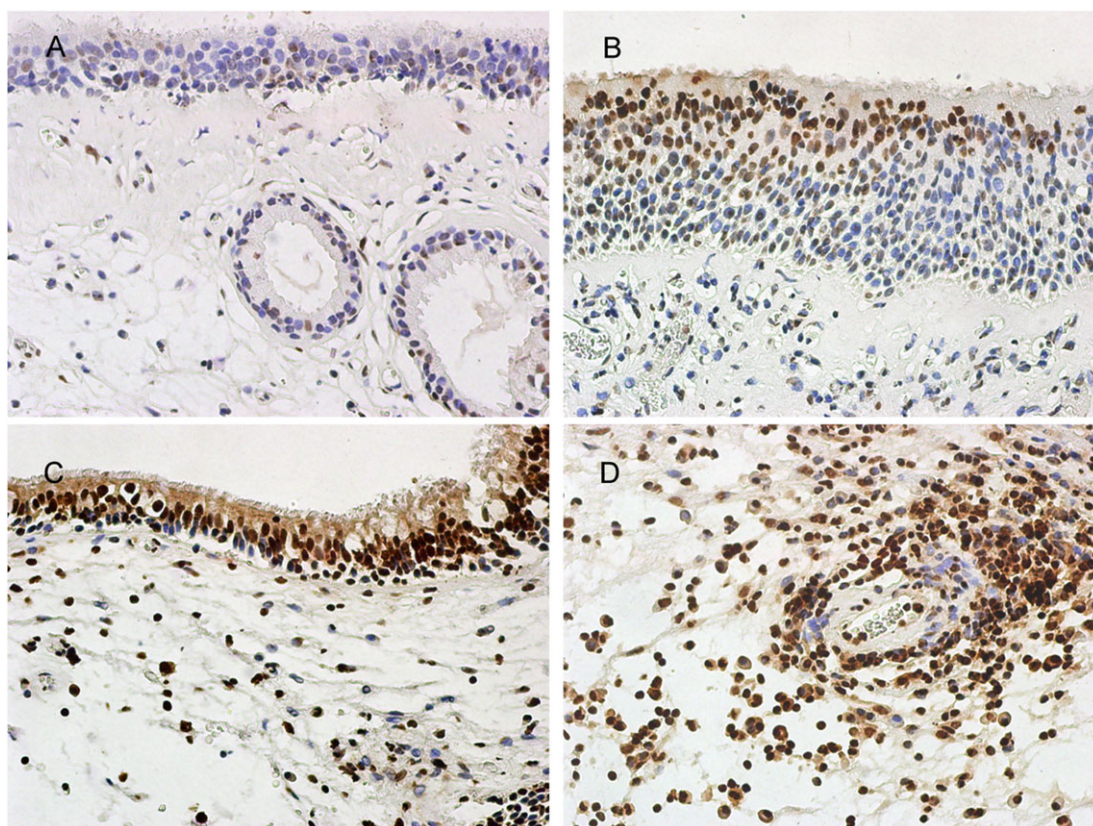


FIGURE 3. Immunohistochemical locations of HMGB1 in the nasal polyps and healthy nasal mucosa ($\times 400$). Representative immunostaining for HMGB1 within the epithelium from a control subject (A), a non-Eos CRSwNP patient (B), and Eos CRSwNP patient (C), and within the submucosa from a non-Eos CRSwNP patient (D). HMGB1 expression (brown) in epithelial cells, infiltrating inflammatory cells, and among infiltrating inflammatory cells. HMGB1 expression in patients with CRSwNP is higher than in the healthy control. CRSwNP = chronic rhinosinusitis with nasal polyps; Eos = eosinophilic; HMGB1 = high-mobility group protein box 1.

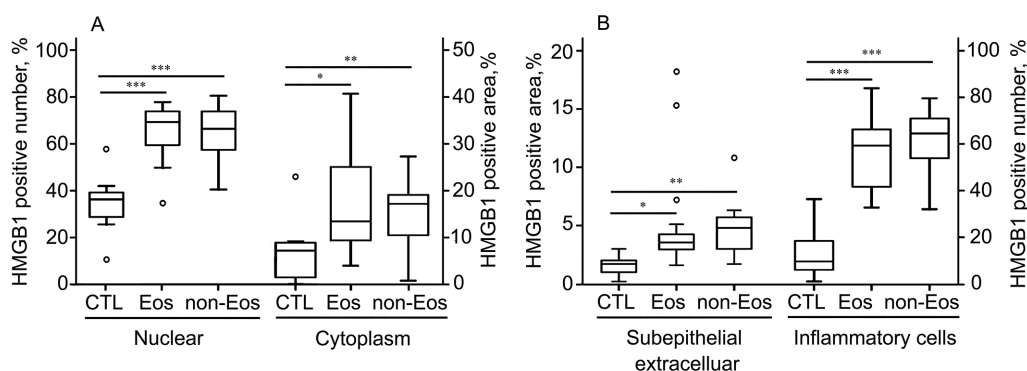


FIGURE 4. Semiquantitative analysis of HMGB1 positive number (%), proportion of epithelial cells with cytoplasm HMGB1 immunostaining, and extracellular HMGB1 among infiltrating inflammatory cells (A, B). Results were presented as box-and-whisker plots, and comparisons were made by 1-way ANOVA and Mann-Whitney U test. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. ANOVA = analysis of variance; HMGB1 = high-mobility group protein box 1.

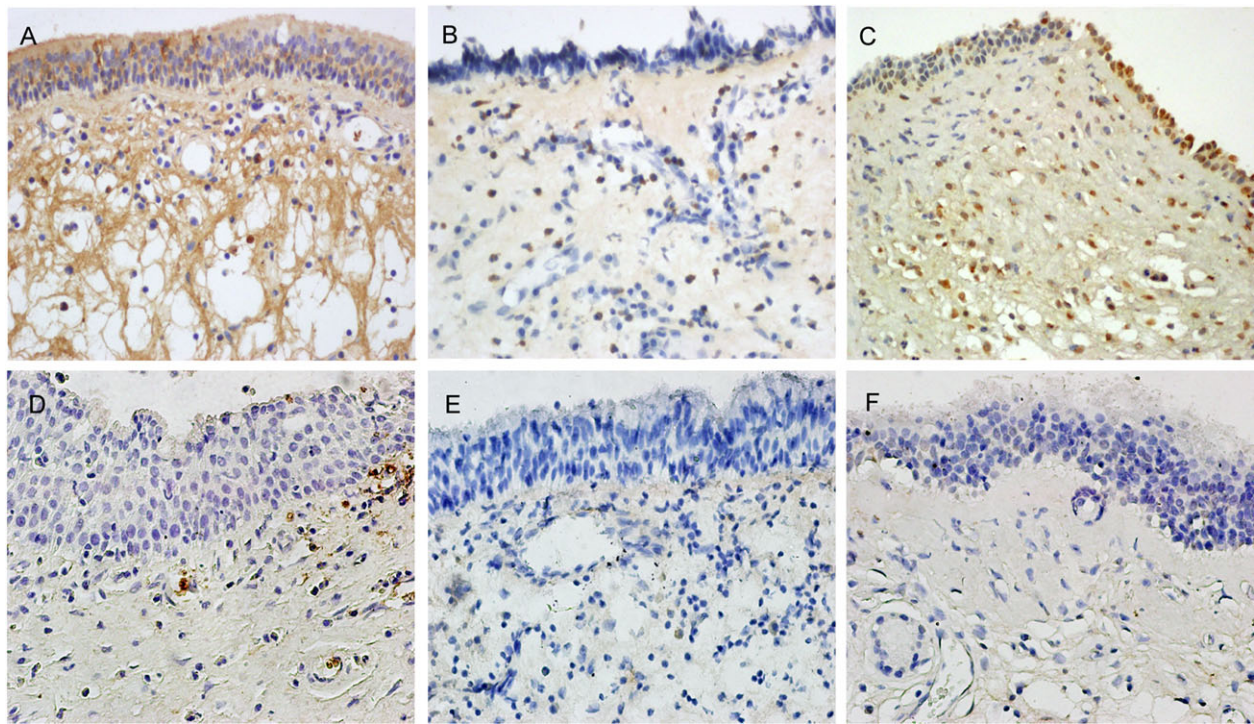


FIGURE 5. Immunohistochemical locations of IL-5, IL-8, and TNF- α in the nasal polyps and healthy nasal mucosa ($\times 400$). IL-5 (A), IL-8 (B), and TNF- α (C) positive cells (brown) was shown in nasal polyps. Absence of immunostaining in a healthy control (D–F). IL = interleukin; TNF = tumor necrosis factor.

in the Eos CRSwNP patients and the non-Eos CRSwNP patients than controls ($p = 0.011$ and $p = 0.013$, respectively). But no statistical significant difference was found for the HMGB1-positive inflammatory cells and extracellular HMGB1 staining between polyps from Eos CRSwNP patients and non-Eos CRSwNP patients ($p = 0.22$ and $p = 0.994$, respectively).

Immunohistochemistry for IL-5, IL-8, and TNF- α

As shown in Figure 5A–C, the cellular expressions of IL-5, IL-8, and TNF- α was observed in the submucosal inflammatory cells in specimen from CRSwNP subjects, and the extracellular expressions of IL-5, IL-8, and TNF- α were detected among infiltrating inflammatory cells. In comparison with the controls, the expressions levels of cellular IL-5, IL-8, and TNF- α were significantly higher in patients with Eos CRSwNP and non-Eos CRSwNP ($p < 0.001$, respectively) (data not shown). Furthermore, the expression levels of IL-5 in Eos CRSwNP was higher than those in non-Eos CRSwNP ($p = 0.001$), but no statistical significance for IL-8 and TNF- α (Fig. 6A, B) was found.

qRT-PCR

Figure 7 shows the concentration of mRNA for HMGB1, IL-5, IL-8, and TNF- α determined in nasal polyps and controls using reverse transcription PCR. HMGB1 mRNA expression was significantly upregulated in Eos CRSwNP patients than both non-Eos CRSwNP patients and controls ($p = 0.006$ and $p = 0.01$, respectively) (Fig. 7A), as well

as for IL-5 mRNA ($p = 0.001$ and $p = 0.018$, respectively) (Fig. 7B), IL-8 ($p = 0.01$ and $p = 0.002$, respectively) (Fig. 7C), and TNF- α ($p < 0.001$ and $p = 0.001$, respectively) (Fig. 7D).

Correlation between HMGB1, IL-5, IL-8, TNF- α , and clinical data

Overall, there was a significant correlation of mRNA expression levels between HMGB1 and all markers of IL-5 ($r_s = 0.665$; $p < 0.001$), IL-8 ($r_s = 0.771$; $p < 0.001$), and TNF- α ($r_s = 0.724$; $p < 0.001$) in both nasal polyps and control tissues. There was a slightly positive correlation between HMGB1 mRNA and the number of infiltrating eosinophils in all specimen ($r_s = 0.149$; $p = 0.012$) or the blood eosinophils count ($r_s = 0.225$; $p = 0.001$) in all subjects (Fig. 8). Figure 8 reports the correlation of mRNA expression levels between HMGB1 and IL-5, IL-8, TNF- α , infiltrating eosinophils number, peripheral blood-eosinophil percentage. Correlation between different variables was measured by Spearman's rank order method. R sq, correlation coefficient; solid lines, 50 subjects included. However, HMGB1 expression level (mRNA) was not associated with the CT parameters and the clinical symptom scores (data was not shown) in all subjects.

Discussion

In this study, the expression levels of HMGB1 protein in Chinese patients with CRSwNP with or without eosinophilia were investigated. Immunostaining

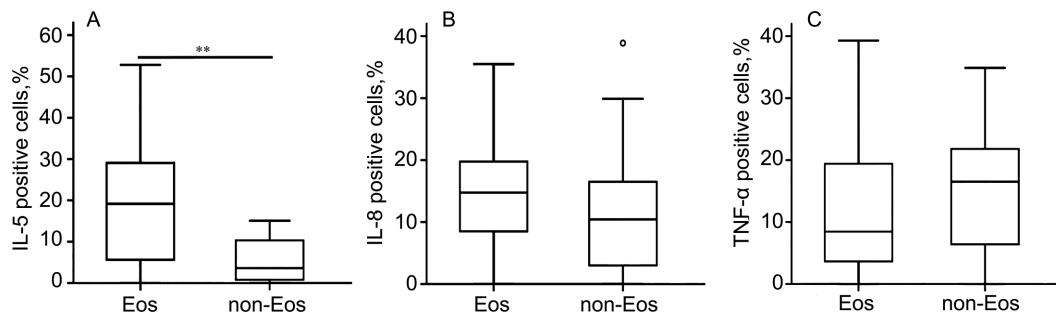


FIGURE 6. Numbers of IL-5, IL-8, and TNF- α positive cells, in nasal polyps with different endotypes (A–C). Data are presented as box-and-whisker plots, and Student t test and Mann-Whitney U test were used for statistical analysis. * $p < 0.05$. IL = interleukin; TNF = tumor necrosis factor.

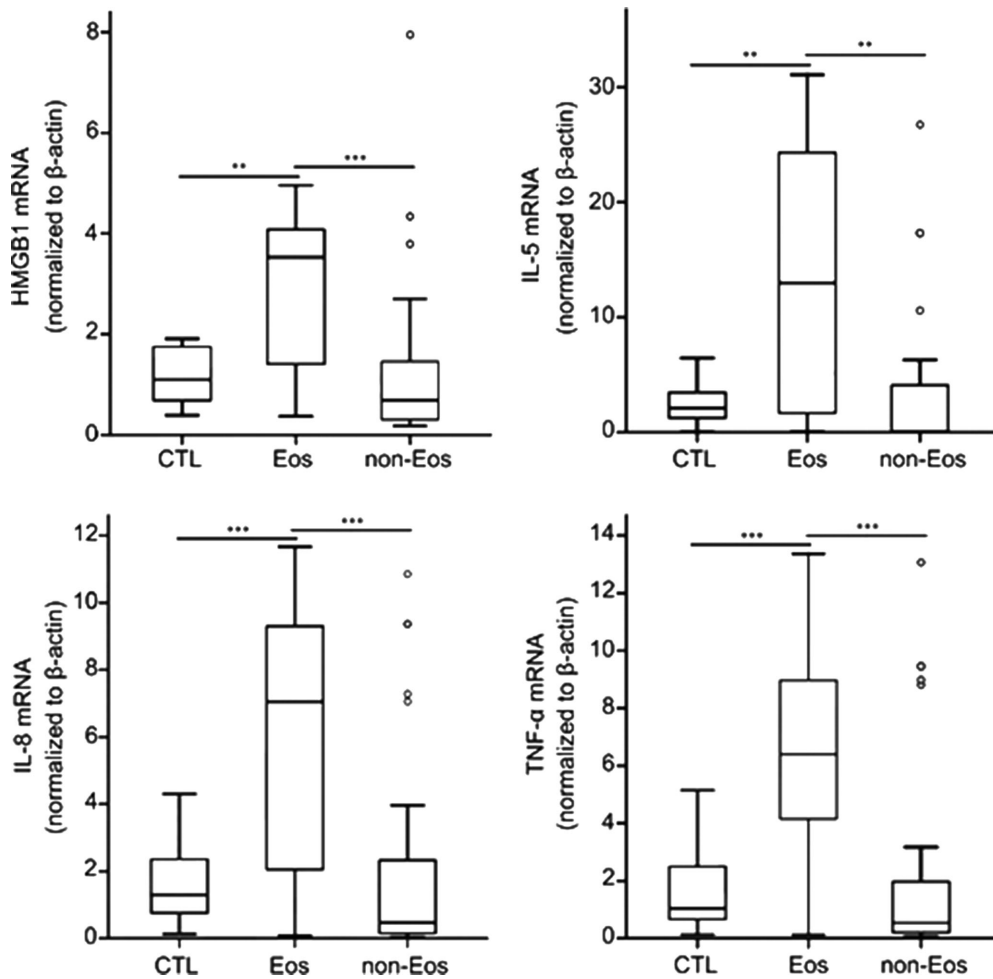


FIGURE 7. mRNA expression of HMGB1, IL-5, IL-8, and TNF- α in healthy control subjects and patients with CRSwNP. mRNA expression of HMGB1 (A), IL-5 (B), IL-8 (C), and TNF- α (D) were significantly increased in Eos CRSwNP patients compared with those in non-Eos CRSwNP and control subjects. Data are presented as box-and-whisker plots, and comparisons were made by Kruskal-Wallis test and Mann-Whitney U test. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. CRSwNP = chronic rhinosinusitis with nasal polyps; Eos = eosinophilic; HMGB1 = high-mobility group protein box 1; IL = interleukin; mRNA = messenger RNA; TNF = tumor necrosis factor.

results demonstrated that nasal polyps specimens with different endotypes had higher expression levels of HMGB1 in the nucleus predominantly, as well as in the cytoplasm of nasal epithelial cells, than the mucosal controls. HMGB1 was also localized in the nucleus and in the cytoplasm of the infiltrating inflammatory cells, including eosinophils, neutrophils, and lymphocytes. Furthermore, extracellular

HMGB1 was found in the area of HMGB1-positive inflammatory cells infiltrated. These results, together with a previous report on levels of HMGB1 in CRSwNP,²⁷ are in agreement with several studies in other inflammatory diseases or autoimmune disorders, such as COPD, sepsis, rheumatoid arthritis, cutaneous lupus erythematosus, and asthma,^{5,14–18,26} in which an increased expression level of

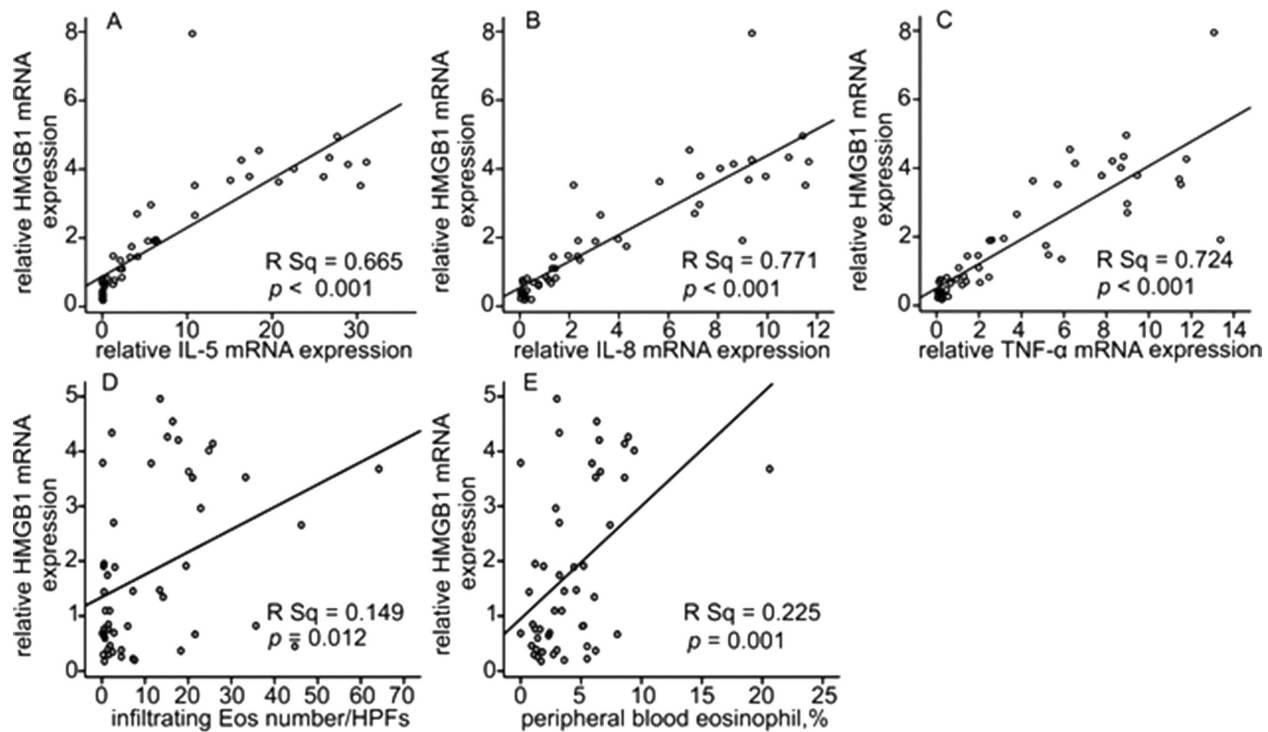


FIGURE 8. Correlation of mRNA expression levels between HMGB1 and IL-5, IL-8, TNF- α , infiltrating eosinophils number, peripheral blood eosinophil percentage. Correlation between different variables was measured by Spearman's rank order method. R sq, correlation coefficient; solid lines, 50 subjects included.

HMGB1 in epithelial cells and in inflammatory cells were investigated. HMGB1-positive cells may participate in elevation of the secreted HMGB1 and extracellular HMGB1, a proinflammatory cytokine that may act as an enhancer of the pathogenesis of nasal polyps.

Previous studies showed that HMGB1 is secreted actively by monocytes/macrophages and stimulated by a number of proinflammatory cytokines including TNF- α , IL-1 β , IL-5, IL-6, and IL-8.^{8,20,25,32} Besides, as airway chronic diseases, such as rhinosinusitis and asthma, have been recently recognized as characterized by an impaired airway epithelium immune barrier function,³³ in a very recent study, the HMGB1 released through *Escherichia Coli* cell wall lipopolysaccharide (LPS) from the epithelial cells in patients with CRSwNP was analyzed. The study has documented the presence of HMGB1 immunostaining in the nuclei of human nasal epithelial (HNE) cells from nasal polyps; after LPS stimulation, translocation of HMGB1 from nuclei to cytoplasm was observed, suggesting that HNE cells represent a potential source of the secreted form of HMGB1 in the airways.³⁴

HMGB1 also promotes secretion of other proinflammatory cytokines, such as TNF- α , IL-1 β , IL-6, and IL-17.^{13,15,27,35} It is well known that several multifunctional cytokines and chemokines, such as IL-1 β , IL-5, IL-8, IL-13, TNF- α , TNF- β , and "regulated on activation, normal T cell expressed and secreted" (RANTES), may contribute to the development of nasal polyps.^{1,2,4} The results of the present study confirmed higher HMGB1, IL-5, IL-8, and TNF- α expression levels in nasal polyps than the

control nasal mucosa, which were consistent with the literature findings. In a recent study, the levels of HMGB1 mRNA positively correlated with mRNA levels of IL-5 and TNF- α in sputum supernatant of asthmatic patients.²⁶ The synthesis of IL-5, IL-8, and TNF- α could be induced by overexpression of HMGB1 in the inflammatory cells, which is a ligand for the receptor for advanced glycation end products (RAGE), as well as the pattern recognition receptors toll-like receptors (TLR)-2 and TLR-4.^{27,36-38} In the broadest sense, inflammation should be considered as a physiologically protective response to infection and injuries of a different nature, where infiltration of inflammatory cells and release of a range of mediators and cytokines are responsible for new inflammatory cells recruitment and survival, leading to the amplification of the inflammatory process.³⁹ In CRSwNP, HMGB1 together with IL-5, IL-8, and TNF- α may form a proinflammatory loop promoting chronic inflammation of nasal and paranasal sinus mucosa.

Differing from white patients, Chinese and Japanese CRSwNP patients display different patterns of sinus mucosal inflammation and more than one-half of CRSwNP patients presented neutrophilic CRSwNP.^{4,40} In this study, in Eos CRSwNP patients, increased levels of both HMGB1 protein and mRNA in the nasal polyps were observed. These differences reflect a distinguished eosinophil and neutrophil eukaryotic activation bias in Eos CRSwNP and non-Eos CRSwNP patients. Eos CRSwNP patients had a higher degree of total inflammatory cells together with eosinophils driven infiltration than non-Eos CRSwNP patients.

It has been reported that HMGB1 levels are significantly higher in the eosinophilic airway inflammation and HMGB1 also enhances survival of human eosinophils and acts as a chemoattractant.^{21,25,26} Further, Th2 skewing, and IL-5 in particular might be involved in the increased activation of eosinophils, which may attribute to the over-expression and release of HMGB1.

The limitation of this study is that the expression of cytokines was not detected in nasal polyps and controls. In addition, the control tissues used were the ethmoid or uncinate process mucosa obtained from patients with cerebrospinal fluid rhinorrhea, fracture of optic canal, or with a skull base benign tumor. Although one could argue that these tissues are not exactly the same origin as compared to nasal polyps, it is, however, the most appropriate specimen

we could use. Further studies are needed in order to elucidate the biological effects of HMGB1 in CRSwNP, and its involvement in Eos CRSwNP and non-Eos CRSwNP in depth.

Conclusion

This study demonstrates clearly an increased expression level of HMGB1 proteins in Chinese CRSwNP patients, which is especially more pronounced in Eos CRSwNP. This finding may contribute to a better understanding of the pathogenesis of CRSwNP along with IL-5, IL-8, and TNF- α . The eosinophilia classification together with higher HMGB1 expression may be a better defining subgroup than “polyps/no polyps.”

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