

# Quantitative analysis of Staphylococcal enterotoxin B expression and its levels in patients with chronic rhinosinusitis with or without nasal polyps

Nazal polipi olan veya olmayan kronik rinosinüzitli hastalarda Stafilokokal enterotoksin B ekspresyonu ve seviyelerinin kantitatif analizi

Ozan Gökdoğan, MD,<sup>1</sup> Ayşe Kalkancı, MD,<sup>2</sup> Yusuf Kızıl, MD,<sup>3</sup> Utku Aydil, MD,<sup>3</sup>  
İlker Akyıldız, MD,<sup>4</sup> Kayhan Çağlar, MD,<sup>2</sup> Sabri Uslu, MD.<sup>3</sup>

<sup>1</sup>Department of Otolaryngology, Memorial Ankara Hospital, Ankara, Turkey

<sup>2</sup>Department of Microbiology, Medical Faculty of Gazi University, Ankara, Turkey

<sup>3</sup>Department of Otorhinolaryngology, Medical Faculty of Gazi University, Ankara, Turkey

<sup>4</sup>Department of Otorhinolaryngology, Ankara Training and Research Hospital, Ankara, Turkey

## ABSTRACT

**Objectives:** This study aims to investigate the presence and levels of Staphylococcal enterotoxin B (SEB) in chronic rhinosinusitis (CRS) cases with or without nasal polyps.

**Patients and Methods:** The study included 38 patients with CRS. Patients were divided into two groups as CRS group with nasal polyps (group A, n=18) and CRS group without nasal polyps (group B, n=20). The control group (group C) consisted of 15 patients. Staphylococcal enterotoxin B levels were measured in concha supernatants and serum samples in all three groups and in polyp tissues in group A by using enzyme-linked immunosorbent assay. Real-time polymerase chain reaction analysis was used to reveal enterotoxin B gene expression in polyp and concha tissue samples.

**Results:** Staphylococcal enterotoxin B levels were positive in 11 (78.6%) and 11 (55.0%) patients, and in one (6.7%) patient in group A, B, and C, respectively. A statistically significant difference was detected between groups in terms of SEB positivity ( $p<0.0001$ ). When the numbers of SEB gene copies were compared between the groups, there were statistically significant differences between groups A and C ( $p<0.0001$ ), and between groups B and C ( $p=0.002$ ).

**Conclusion:** Higher rates of SEB positivity in serum samples and higher numbers of SEB gene copies in concha samples indicate that Staphylococcal superantigens may be associated with CRS development.

**Keywords:** Enterotoxin B; nasal polyp; rhinosinusitis; *Staphylococcus aureus*; superantigen.

## ÖZ

**Amaç:** Bu çalışmada nazal polipi olan veya olmayan kronik rinosinüzit (KRS) olgularında Stafilokokal enterotoksin B (SEB) varlığı ve seviyesi araştırıldı.

**Hastalar ve Yöntemler:** Çalışmaya KRS'li 38 hasta dahil edildi. Hastalar nazal polipli KRS grubu (grup A, n=18) ve nazal polipsiz KRS grubu (grup B, n=20) olmak üzere iki gruba ayrıldı. Kontrol grubunu (grup C) 15 hasta oluşturdu. Stafilokokal enterotoksin B seviyeleri enzim-bağımlı immünosorbent analiz kullanılarak her üç grupta konka süpernatantlarında ve serum örneklerinde ve grup A'da polip dokularında ölçüldü. Polip ve konka doku örneklerinde enterotoksin B gen ekspresyonunu ortaya koymak için gerçek zamanlı polimeraz zincir reaksiyon analizi kullanıldı.

**Bulgular:** Grup A, B ve C'de SEB seviyeleri sırasıyla 11 (%78.6) ve 11 (%55) hastada ve bir (%6.7) hastada pozitif idi. Gruplar arasında SEB pozitifliği açısından istatistiksel olarak anlamlı derecede farklılık saptandı ( $p<0.0001$ ). Gruplar arasında SEB gen kopyası sayıları karşılaştırıldığında, grup A ile C arasında ( $p<0.0001$ ) ve grup B ile C arasında ( $p=0.002$ ) istatistiksel olarak anlamlı derecede farklılıklar vardı.

**Sonuç:** Serum örneklerinde daha yüksek oranlarda SEB pozitifliği ve konka örneklerinde daha yüksek sayılarda SEB gen kopyası olması, Stafilokokal süperantijenlerin KRS gelişimi ile ilişkili olabileceğini gösterir.

**Anahtar Sözcükler:** Enterotoksin B; nazal polip; rinosinüzit; *Stafilokokus aureus*; süperantijen.



Chronic rhinosinusitis (CRS) is an inflammatory disease of the nasal cavity and paranasal sinuses with persistence of symptoms for more than 12 weeks duration<sup>[1]</sup> and can be classified as CRS with nasal polyps (NP) and CRS without NP. Majority of CRS patients are without NP, and only 20-25% of CRS patients have NP.<sup>[2]</sup> Chronic rhinosinusitis with NP is considered a more severe form of the disease with comorbid diseases and more frequent recurrences despite intensive medical and surgical treatment.<sup>[3]</sup>

Fungi, bacteria, viruses, or their associated products may play a role in initiating or perpetuating abnormal and excessive mucosal inflammation.<sup>[4]</sup> The interaction between *Staphylococcus aureus* (*S. aureus*) and the sinonasal mucosa have been studied previously and invasion of the epithelium of the nasal mucosa by *S. aureus* has been identified by Sache et al.<sup>[5,6]</sup> Whether the microorganism is located within the epithelial cells or not, *S. aureus* is capable of the formation of a T helper 2 (Th2) mediated cytokine pattern which leads to CRS with NP.<sup>[5]</sup> *Staphylococcus aureus* cultured from the nasal cavities of patients with CRS have been shown to produce exotoxins (e.g. TSST-1, Exotoxin B).<sup>[7]</sup>

The superantigen (SAGs) hypothesis has been proposed to comprehend immune modulation in some disease processes as well as to explain NP development.<sup>[4,8]</sup> These proteins have three targets: the T cell receptor (TCR), the B cell receptor (BCR), and the MHC class 2 proteins on antigen presenting cells (APC). Superantigens are the most powerful of all known T-lymphocyte mitogens<sup>[9]</sup> and can stimulate up to 30% of a T cell population while conventional antigens stimulate only 0.001% to 0.01% of a T cell population. After stimulation, massive cytokine release occurs from CD4<sup>+</sup> lymphocytes.<sup>[10]</sup>

These findings suggest a possible causative relation between SAGs and NP development. To the best of our knowledge, quantitative levels of Staphylococcal enterotoxin B (SEB) have not been previously determined in patients with CRS with NP or without NP. The aim of this study is to determine the levels of SEB in patients with two different forms of CRS and compare these with a control group.

## PATIENTS AND METHODS

The performance of this study took place in complete agreement with the declaration of Helsinki and the International Conference on Harmonization (ICH) Good Clinical Practice (GCP) guidelines and was approved by the local ethics committee. Informed consent was obtained from each study and control group patient.

### Patients

There were three groups: group A consisted of patients with CRS with NP, group B consisted of patients with CRS without NP, and group C consisted of patients without any form of CRS. Patients who had symptoms including nasal blockage, facial pain, smelling disorder over 12 weeks with/without endoscopic findings of polyps, mucopurulent drainage from the middle meatus and edema in the middle meatus region with/without CT findings were diagnosed as CRS. Exclusion criteria were history of previous nasal operations and presence of asthma or salicylate intolerance for all groups. Patients with a history of medical treatment within eight weeks were also excluded from groups because of the possible effect of medications on both SA colonization and SEB production. If NP was determined preoperatively, or encountered during surgery, the patient was assigned to group A, otherwise, the patient was assigned to group B. The patients in group C did not have any symptom or sign of CRS. All patients in group A and B had paranasal sinus computed tomography (CT) before endoscopic sinus surgery (ESS) and assigned to the groups according to pre- and intraoperative findings.

All of the patients in groups A and B were evaluated with nasal endoscopy and paranasal sinus computerized tomography (PNCT).

The numbers of patients in groups A, B, and C were 30, 25, and 20 respectively. Tissue specimens from the middle turbinate and 10 mL of serum samples were obtained from all participants in the three groups and polyp samples were obtained from group A patients. All tissue samples were preserved at -85 °C. Laboratory experiments were conducted blinded to the clinical details.

### Quantitative demonstration of SEB by ELISA

The serum samples were preserved at -20 °C. Ridascreen Sets A, B, C, D, E (r-biopharm,

Germany) kits were used for ELISA. 100  $\mu$ L of serum samples were used and optic density (OD) values were read at 450 nm. Supernatants of polyp and concha tissue samples were also studied for detection of enterotoxin B by ELISA. An OD level of 0.960 was accepted as cut-off value according to instructions. OD levels over this value were regarded as positive; the levels below this value were regarded as negative. Positive and negative controls provided by the manufacturer were also included in the assay.

### **Quantitative demonstration of SEB gene expression by real time polymerase chain reaction (PCR)**

#### *1) Messenger Ribonucleic Acid (mRNA) extraction*

The High Pure RNA Tissue Kit (Roche, Diagnostics GmbH, Penzberg, Germany) was used for extraction of RNA from tissue samples. Actual bacterial RNA was also extracted from tissue samples since nasal tissue is already colonized by bacteria. Deoxyribonucleic acid (DNA) contamination was prevented with the use of deoxyribonuclease present in the tissue kit. RNA presence was controlled by measurement of total RNA amount with spectrophotometry (NanoDrop Technologies Inc., Wilmington, DE, USA).

#### *2) Complimentary DNA (cDNA) synthesis by Reverse transcriptase-PCR*

The Transcriptor High Fidelity cDNA Synthesis Kit (Roche, Diagnostics GmbH, Penzberg, Germany) was used for synthesis of complimentary DNA from total RNA. Using with randomized non-specific primer chain, cDNA was obtained after three consequential incubations at 20, 45 and 85 °C.

#### *3) Extraction control by using housekeeping gene.*

Gene expression studies should contain housekeeping gene controls. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) gene for human tissue, 16S genes for bacterial genome were included in the study. The Glyceraldehyde 3-phosphate dehydrogenase Detection kit (Way2Gene Fungi; Genmar, İzmir, Turkey) was used for the control of extraction step.

#### *4) Amplification of enterotoxin B coding gene.*

At least 10 ng cDNA was used for PCR studies. The LightCycler device (Roche Diagnostics,

Tenay, Turkey) and LightCycler software version 3.5 was used for this step. Real time PCR amplification mixture was composed of cDNA, SYBR Green and 100 pmol of each primer (forward 5'-CATTAAACCCCTTGTTGCCAT-3' and reverse 5'-ACAAATCGTTAAAAACGGCG-3') (Apha DNA, Canada). Primers were designed for the amplification of the SEB gene. Amplification was performed in three steps with denaturation (at 95 °C 15 second, temperature change was 20 °C/second), hybridization (at 50 °C 10 second, temperature change was 20 °C/second), and elongation (at 72 °C 10 second, temperature change was 20 °C/second). Analysis was made step-by-step between 65-90 °C (temperature change was 0.2 °C/second). After amplification, melting curve analyses were performed. Results were evaluated as positive or negative on qualitative assessment and as numerical values on quantitative assessment.

Ribonucleic acid amounts were measured as >20 ng/ $\mu$ L before the PCR procedure. cDNA was converted from RNA measured as >125 ng/ $\mu$ L. After controlling RNA and cDNA presence by measuring, the DNA obtained from polyp and concha tissue was confirmed by GAPDH gene augmentation. GAPDH was found positive in all polyp and concha tissue samples.

#### *5) Standard curve formation and quantization*

Six different cDNA concentrations ( $10^1$ ,  $10^2$ ,  $10^3$ ,  $10^4$ ,  $10^5$ ,  $10^6$  copy/mL) were assayed and the calibration curve was formed between the crossing point and DNA amounts of each standard.

Absolute quantification analysis was performed and samples with unknown DNA amount were spotted on the standard curve. *Staphylococcal enterotoxin B* gene expression levels were measured as copy/mL.

### **Statistical analysis**

Power analysis was done in the planning process of the study. Twenty-six patients were planned for inclusion in all groups for significance of study. Our mean sample size was within these numbers.<sup>[11]</sup> The Mann-Whitney U and Kruskal-Wallis tests were used for analyses of quantitative data and analytical methods (Kolmogorov-Smirnov/Shapiro-Wilk's test) to determine whether or not they are normally distributed. Chi-square and Fischer's exact tests

were used for comparison of the three groups. A p-value less than 0.05 was considered significant. The impact value of the study was 0.57 according to mean and standard deviation values. The power of the study was computed at 0.95 with type 1 error taken at  $\alpha=0.05$  and  $n=53$ . The G\*Power 3.1.9.2 software program (Heinrich Heine University, Dusseldorf, Germany) was used for the power analysis of the study.

**RESULTS**

We started the study with 30 patients in group A, 25 patients in group B and 20 patients in group C. These numbers were sufficient for statistical assessment. But during the study, hemolysis of some blood samples decreased our numbers to 18 patients in group A, 20 patients in group B, and 15 patients group C. Twelve patients in group A (40.0%), 11 patients in group B (44.0%) and 13 patients in group C (65.0%) were male. The mean ages of patients in group A, group B, and group C were  $41.9\pm 13.9$ ,  $34.4\pm 11.8$  and  $40.1\pm 12.6$  years respectively. The differences in mean ages were not statistically significant among groups ( $p>0.05$ ).

*a. Staphylococcal enterotoxin B presence and levels in serum samples (ELISA)*

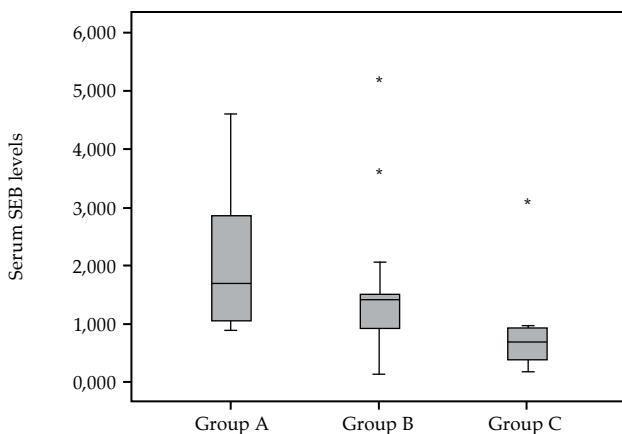
Serum samples of 14 patients in group A, 20 patients in group B and 15 patients in group C were sufficient and could be analyzed by ELISA. SEB was positive in 11 (78.6%), 11 (55.0%), and one (6.7%) patient(s) in groups A, B, and C, respectively. The difference between groups in terms of SEB positivity was statistically significant ( $p<0.0001$ ).

SEB positivity was significantly higher in both group A ( $p=0.001$ ) and group B ( $p=0.005$ ) when compared with the control group. The mean OD values of SEB in serum samples established by ELISA were  $2.06\pm 1.16$ ,  $1.43\pm 1.13$ ,  $0.75\pm 0.68$  in groups A, B, and C, respectively (Figure 1). The OD levels for serum SEB levels were also statistically higher in group A ( $p<0.0001$ ) and group B ( $p=0.006$ ) when compared to the control group. However OD levels in groups A and B were not statistically different ( $p=0.072$ ).

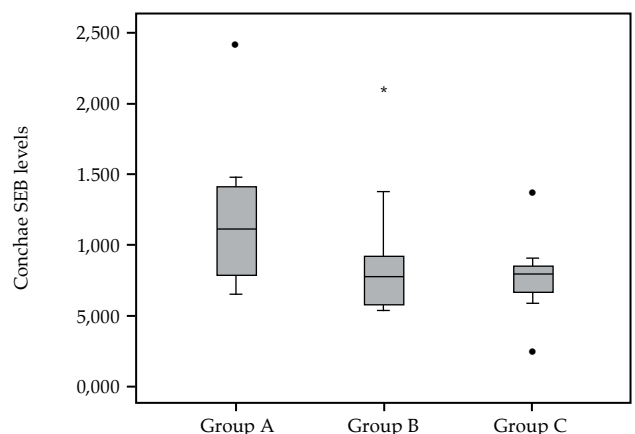
**Staphylococcal enterotoxin B presence and levels in concha supernatants and polyp tissue (ELISA)**

Supernatants of concha tissue samples of nine patients in group A, 10 patients in group B and 11 patients in group C were sufficient and could be analyzed by ELISA for the presence of enterotoxin B. Mean OD levels of SEB measured in concha supernatants were  $1.201\pm 0.543$ ,  $0.916\pm 0.489$ ,  $0.776\pm 0.271$  in patients of group A, B, and C, respectively (Figure 2). SEB positivity in concha supernatants were 66.7% (6/9), 20.0% (2/10) of and 9.1% (1/11) in groups A, B, and C, respectively. The difference between all three groups was not statistically significant in terms of SEB positivity in concha supernatants ( $p=0.08$ ). When OD levels were compared, the differences between groups were also not statistically significant ( $p=0.125$ ).

Supernatants of polyp tissue samples were also evaluated by ELISA for the presence of SEB. The mean OD value of SEB in polyp samples was  $1.043\pm 0.323$  in group A patients.



**Figure 1.** Box and whisker plot showing mean OD values of Staphylococcal enterotoxin B (SEB) in serum samples established by ELISA.



**Figure 2.** Box and whisker plot showing mean optic density values of Staphylococcal enterotoxin B (SEB) in turbinate samples established by ELISA.

### Staphylococcal enterotoxin B gene expression (PCR)

Concha samples of 18 patients in group A, 20 patients in group B and 15 patients in group C were analyzed for quantitative demonstration of SEB gene expression by PCR. The mean numbers of copies of SEB gene in concha samples were  $2,230.56 \pm 1,779.55$ ,  $1,700.00 \pm 1,731.91$  and  $353.00 \pm 525.94$  in groups A, B, and C, respectively. The positivity rates were 55.6% (10/18), 45.0% (9/20), and 6.7% (1/15) in groups A, B, and C, respectively. The difference between all three groups was statistically significant ( $p=0.009$ ). When the numbers of copies of SEB gene were compared, the differences between groups A and C ( $p<0.0001$ ), and groups B and C ( $p=0.002$ ) were statistically significant. However, the difference between groups A and B was not statistically significant ( $p=0.230$ ).

The mean number of copies of SEB gene found in polyp tissue was  $2,157.77 \pm 1,220.39$  in group A patients. The SEB gene was found to be positively expressed in polyp tissues in 77.6% of the patients in group A.

### DISCUSSION

Our results showed that SEB positivity rates and OD levels were significantly higher in patients in group A and group B. When the numbers of copies of SEB gene were compared, the differences between groups A and C, and groups B and C were also statistically significant. These results show that whether NP is present or not, SEB may have a role in CRS development. There was a trend to show that the levels of SEB and mean numbers of copies of SEB were higher in group A than in group B, although the difference was not statistically different. These results could also be interpreted as a more dominant role of SAGs in NP development and these two entities may be different stages of the same disease and for this reason different levels may have been found. We believe that future therapy targeting SAGs may become a new method for the treatment of NP.

The colonization of *S. aureus*, exotoxin release by the microorganism, and the SAGs-related cytokine release have been shown in various airway diseases like allergic rhinitis, asthma, and CRS.<sup>[12]</sup> In patients with CRS, specific immunoglobulin (Ig) E responses to *S. aureus*

exotoxins were detected.<sup>[13]</sup> It was also reported that the SAGs may contribute to glucocorticoid insensitivity.<sup>[14]</sup>

Up to 30% of the nasal cavities of the population are colonized by *S. aureus* and 5-10% of the isolates secrete a SAG: SEB.<sup>[15]</sup> Bachert et al.<sup>[4]</sup> first described SAGs in NPs by demonstrating specific IgE antibodies against *S. aureus* toxins. Bachert et al.<sup>[4]</sup> also showed increased eosinophils and related cytokines as well as antibodies against bacterial toxins in 50% of NP patients.

To better understand the role of *S. aureus* and SAGs in CRS with and without NP, a study aiming to determine presence of SEB in these patients was designed. Since SEB is one of the best known SAGs secreted by *S. aureus*, our hypothesis was that if SAGs have a role in development of CRS with or without NP, SEB levels and SEB gene expression would be higher in these patients when compared to controls. To the best of our knowledge, this is the first study that determines the quantitative SEB and gene expression levels in patients with CRS with or without NP. The gene expression details of our study obtained from RNA work, may add clues to the existing literature.

Our results are in concordance with previous reports. Recently, in a meta-analysis published in 2014, Ou et al.<sup>[16]</sup> demonstrated that *S. aureus* SAGs may be a risk factor for the persistence and severity of CRS with NP, and the presence of these SAGs is related to the disease severity of NP.

The main limitation of this study is the lack of correlation of *S. aureus* enterotoxin B levels with important inflammation markers such as IL-4, IL-5 and IL-13. Demonstration of a possible relationship between *S. aureus* enterotoxin B levels and important inflammation markers may better show the effects of SAGs on CRS development. In case of a proven cause-effect relationship, development of new management strategies for SAGs related diseases such as prevention of *S. aureus* colonization with vaccination may become patients for future studies.

Another limitation of this study is the dropout of study participants due to hemolysis of blood samples, although all nasal tissue specimens were obtained and stored under proper conditions.

Quantitative levels of SEB in patients with two different forms of CRS were evaluated and compared with a control group in the study. We researched on the correlation of SEB levels with the severity of disease and its symptoms, and found a correlation with CRS and SEB levels. Further researches are necessary to demonstrate other SAGs and bacterial toxins. Although we still do not know why CRS presents with polyps in some patients and without polyps in other patients, this study may help future researchers pursue further studies to answer this question.

#### Declaration of conflicting interests

The authors declared no conflicts of interest with respect to the authorship and/or publication of this article.

#### Funding

The authors received no financial support for the research and/or authorship of this article.

#### REFERENCES

1. Fokkens WJ, Lund VJ, Mullol J, Bachert C, Alobid I, Baroody F, et al. European Position Paper on Rhinosinusitis and Nasal Polyps 2012. *Rhinol Suppl* 2012;23:1-298.
2. Meltzer EO, Hamilos DL, Hadley JA, Lanza DC, Marple BF, Nicklas RA, et al. Rhinosinusitis: establishing definitions for clinical research and patient care. *J Allergy Clin Immunol* 2004;114:155-212.
3. Bachert C, Pawankar R, Zhang L, Bunnag C, Fokkens WJ, Hamilos DL, et al. ICON: chronic rhinosinusitis. *World Allergy Organ J* 2014;7:25.
4. Bachert C, Zhang N, Patou J, van Zele T, Gevaert P. Role of staphylococcal superantigens in upper airway disease. *Curr Opin Allergy Clin Immunol* 2008;8:34-8.
5. Sachse F, Becker K, von Eiff C, Metzger D, Rudack C. *Staphylococcus aureus* invades the epithelium in nasal polyposis and induces IL-6 in nasal epithelial cells in vitro. *Allergy* 2010;65:1430-7.
6. Damm M, Quante G, Rosenbohm J, Rieckmann R. Proinflammatory effects of *Staphylococcus aureus* exotoxin B on nasal epithelial cells. *Otolaryngol Head Neck Surg* 2006;134:245-9.
7. Seiberling KA, Conley DB, Tripathi A, Grammer LC, Shuh L, Haines GK, et al. Superantigens and chronic rhinosinusitis: detection of staphylococcal exotoxins in nasal polyps. *Laryngoscope* 2005;115:1580-5.
8. Alouf JE, Müller-Alouf H. Staphylococcal and streptococcal superantigens: molecular, biological and clinical aspects. *Int J Med Microbiol* 2003;292:429-40.
9. Stow NW, Douglas R, Tantilipikorn P, Lacroix JS. Superantigens. *Otolaryngol Clin North Am* 2010;43:489-502.
10. Wang M, Shi P, Yue Z, Chen B, Zhang H, Zhang D, et al. Superantigens and the expression of T-cell receptor repertoire in chronic rhinosinusitis with nasal polyps. *Acta Otolaryngol* 2008;128:901-8.
11. Shiomori T, Yoshida S, Miyamoto H, Makishima K. Relationship of nasal carriage of *Staphylococcus aureus* to pathogenesis of perennial allergic rhinitis. *J Allergy Clin Immunol* 2000;105:449-54.
12. Maher JM, Markey JC, Ebert-May D. The other half of the story: effect size analysis in quantitative research. *CBE Life Sci Educ* 2013;12:345-51.
13. Suh YJ, Yoon SH, Sampson AP, Kim HJ, Kim SH, Nahm DH, et al. Specific immunoglobulin E for staphylococcal enterotoxins in nasal polyps from patients with aspirin-intolerant asthma. *Clin Exp Allergy* 2004;34:1270-5.
14. Wang M, Shi P, Chen B, Shi G, Li H, Wang H. Superantigen-induced glucocorticoid insensitivity in the recurrence of chronic rhinosinusitis with nasal polyps. *Otolaryngol Head Neck Surg* 2011;145:717-22.
15. Bachert C, Gevaert P, Holtappels G, Johansson SG, van Cauwenberge P. Total and specific IgE in nasal polyps is related to local eosinophilic inflammation. *J Allergy Clin Immunol* 2001;107:607-14.
16. Ou J, Wang J, Xu Y, Tao ZZ, Kong YG, Chen SM, et al. *Staphylococcus aureus* superantigens are associated with chronic rhinosinusitis with nasal polyps: a meta-analysis. *Eur Arch Otorhinolaryngol* 2014;271:2729-36.