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Otolaryngology -- Head and Neck Surgery 2011 145: 717 originally published online 4 July 2011
DOI: 10.1177/0194599811413859

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What is This?
Superantigen-Induced Glucocorticoid Insensitivity in the Recurrence of Chronic Rhinosinusitis with Nasal Polyps

Mingming Wang, PhD1*, Peng Shi, PhD2*, Bei Chen, PhD3, Guanggang Shi, MD1, Hong Li, MS1, and Haibo Wang, MD1

Abstract

Objective. To investigate a potential mechanism by which superantigens could induce glucocorticoid insensitivity in chronic rhinosinusitis (CRS) patients.

Study Design. Prospective cohort study.

Setting. Tertiary medical center.

Subjects and Methods. Sinonasal polyps were obtained from CRS patients with nasal polyps (CRSwNP; 20 without recurrence, 18 with recurrent NP followed for 1.5-2.0 years) and nasal mucosa from 16 CRS patients without nasal polyps (CRSsNP). Specimens were tested by enzyme-linked immunosorbent assay for staphylococcal exotoxins (SEs) including SEA, SEB, SEC, SED, and toxic shock syndrome toxin type-1 (TSST-1) and assessed by immunohistochemistry for glucocorticoid receptor (GR) \( \alpha \) and \( \beta \), and the GR\( \beta \)/GR\( \alpha \) ratio was analyzed.

Results. In CRSwNP, 13 of 18 (72.22%) subjects with subsequently recurrent NP, 11 of 20 (55.00%) subjects without NP recurrence, and 1 of 16 (6.25%) CRSsNP subjects with positive reactions for SEs were obtained. There were no positive results in controls. The expressions of GR\( \beta \) in 3 CRS groups and controls were significantly different (all \( P < .05 \)), and a similar increasing tendency of the GR\( \beta \)/GR\( \alpha \) ratio was found among groups besides the comparison of CRSwNP versus recurrent NP groups (\( P = .053 \)). Furthermore, there was a clear trend of increased GR\( \beta \) expression in the enzyme-linked immunosorbent assay (ELISA)–positive samples compared with ELISA-negative samples. Concerning GR\( \alpha \), the expression was enhanced significantly just in toxin-positive recurrent NP versus controls (\( P = .048 \)), but the relative induction of GR\( \beta \) was much higher, thereby leading to a higher GR\( \beta \)/GR\( \alpha \) ratio.

Conclusions. Bacterial superantigens may contribute to glucocorticoid insensitivity through induction of GR\( \beta \), which appears to be a marker of steroid insensitivity in CRSwNP.

Keywords

chronic rhinosinusitis, nasal polyps, superantigens, glucocorticoid resistance, glucocorticoid receptor

Received February 16, 2011; revised May 20, 2011; accepted May 25, 2011.

C

hronic rhinosinusitis (CRS) is the most common chronic disease. In addition to economic burden, it imposes considerable impact on the quality of life of patients. Clinically, CRS can be broadly categorized into CRS without or with nasal polyps (CRSsNP or CRSwNP). CRSsNP with poor disease control would develop to CRSwNP, and CRSwNP with recurrence potential is considered to be a more severe form of the disease and tends to be more refractory to both medical and surgical intervention. Glucocorticoid treatment is widely advocated to control CRS inflammation and growth. However, a subset of patients does not respond to the maximal treatment, showing progression of disease despite steroid treatment. Clinical improvement with potent glucocorticoids is observed only after prolonged periods of treatment. Given these observations, there is a need to decipher the mechanisms and molecular basis of glucocorticoid insensitivity in CRS.

More recently, studies on glucocorticoid resistance have proposed that the abnormal expression level of glucocorticoid receptor (GR), especially the overexpression of the GR\( \beta \) subtype that acts as a dominant inhibitor of functional GR\( \alpha \) to inhibit glucocorticoid action, is functionally involved in
steroid resistance. However, there is controversy regarding the putative role of GRβ. Furthermore, the mechanisms that induce the overexpression of GRβ remain poorly understood. Staphylococcal exotoxins (SEs), known as superantigens, bind directly without antigen processing to MHC class II molecules on antigen-presenting cells and elicit a potent T-cell activation. Recently, T-cell receptor repertoire analysis of peripheral blood mononuclear cells (PBMCs) and in vitro cells from local chronic inflammation demonstrated SEs, as disease modifiers, contributed to the pathogenesis of severe bronchial asthma and CRSwNP. We hypothesized that a similar overexpression might be present in nasal polyp (NP) inflammatory cells and associated with steroid insensitivity. Thus, to better understand persistent glucocorticoid resistance in nasal polyps as well as to assess whether this inflammatory disease is associated with the microbial superantigens and the dysregulation of GRα or GRβ, we analyzed directly the presence of SEs and the entire expressions of GRα and GRβ at the protein level in the NP instead of explant culture or peripheral blood model.

Methods

Study Population

A total of 54 consecutive adult patients with CRS undergoing endoscopic sinus surgery and 12 normal controls were recruited in this study. Only patients who met the published criteria for CRSwNP or CRSsNP with confirmed postoperative histopathologic diagnosis and accepted regularly postoperative glucocorticoid medication (flunisolide for at least 3 months) followed up to 1.5 to 2.0 years were included in the study. The control group consisted of 12 adult volunteers in generally good health with no history of nasal or sinus disease. All subjects failed adequate trials of conservative medical therapy (prolonged antibiotic regimens, nasal and oral steroids, saline irrigations, and decongestants) for control of symptoms. All medications were ceased at least 6 weeks before biopsies. Subjects with anatomic abnormal sinus or any immunodeficiency were excluded. This study was approved by the Clinical Otorhinolaryngology Division of Provincial Hospital affiliated to Shandong University, and informed consent was obtained from all subjects.

Polyp tissue obtained from patients with CRSwNP and sinonasal mucosa samples from the middle meatus with CRSsNP were collected at the time of sinus surgery. Nasal mucosal biopsies from the middle turbinates served as the control tissue from patients in the control group. Duplicate tissue samples were obtained from each patient. A portion was processed for toxin detection using an enzyme-linked immunosorbent assay (ELISA) technique. The second surgical biopsy was immediately fixed in 4% paraformaldehyde for 24 hours, embedded in paraffin, and sectioned into 4-µm slices for the routine hematoxylin and eosin (H&E) staining and immunohistochemical staining.

ELISA Procedures

Freshly obtained tissue specimens from 66 subjects were weighed, and 1 mL of 0.9% NaCl solution was added per 0.1 g of tissue. The tissue was then homogenized with a mechanical homogenizer (B. Braun, Melsungen, Germany) at 1000 rpm (120g) for 5 minutes on ice. After homogenization, the suspensions were centrifuged at 3000 rpm (1560g) for 10 minutes at 4°C, as noted by Bachert et al. All supernatants were assayed for SEs (SEA, SEB, SEC1-C3, SED, and toxic shock syndrome toxin type I [TSST-1]) by means of ELISA with commercially available kits (Gene Tex Inc, San Antonio, Texas) as described by Wang et al. The absorbance was read on a plate reader at 450 nm. Samples with an absorbance more than twice that of the negative control were considered positive for that toxin. The negative control was made of 0.05% PBS-Tween. All control and test samples were run in triplicate.

Immunohistochemistry

Slides were deparaffinized in xylene and then rehydrated through graded levels of ethanol. Endogenous peroxidase activity was neutralized with 3% hydrogen peroxide. The slides were then added to normal goat serum in 1% bovine serum albumin to block nonspecific antibody binding. Commercially available rabbit polyclonal antibodies specific for human GRα or GRβ and no cross-reactivity against GRβ or GRα (Abcam plc, Cambridge, UK) were each added at a concentration of 10 µg/mL, respectively. Slides were incubated overnight at 4°C. After washing in phosphate-buffered saline, the sections were blocked with biotin-labeled anti-rabbit IgG (Zymed Laboratories, San Francisco, California) for 15 minutes at 37°C. The avidin-biotin complex method (Zymed Laboratories) was then used for secondary antibody labeling. Antigen-antibody complexes were visualized using 0.075% 3,3′,3-diaminobenzidine tetrachloride and 0.002% hydrogen peroxide as a chromagen. Slides were lightly counterstained with Harris hematoxylin, dehydrated through graded ethanol, and then coverslipped. Negative control was also evaluated with the use of secondary antibody alone on the study specimens. All of the preparations were counterstained with hematoxylin and mounted prior to examination. One section from each paraffin-embedded specimen was stained with H&E.

To avoid observer bias, slides were coded before analysis and examined in a blinded fashion by 2 independent pathologists using an Olympus microscope (Tokyo, Japan). The graticule (0.2 mm²) was oriented beneath the epithelial basement membrane, and cells positive for GRα or GRβ were counted along the whole length of the biopsy. At least 2 sections in 10 randomly selected fields were counted (final magnification, 400×) for each patient at each time point, and the results were expressed as the mean number of positive cells per mm² ± the standard error of the mean (SEM).

Statistical Analysis

All statistical analyses were completed using SPSS version 16.0 statistical software (SPSS Inc, Chicago, Illinois). Measurements of GRα- and GRβ-positive cells among the study groups were analyzed by 1-way analysis of variance, and the least significant difference (LSD) multiple comparisons were used for testing the differences between each of the 2 groups. SE-positive results among groups were analyzed by χ² test. P values of less than .05 were considered significant.
Results

Baseline Findings
Fifty-four adult patients with CRS were followed for 1.5 to 2.0 years, and 12 healthy individuals were recruited for this study. These 66 subjects were divided into 4 groups: (1) CRSsNP group (n = 16), in which patients remained without NP until the end of the follow-up period; (2) CRSwNP group (n = 20), in which patients remained without recurrence of NP for the duration of the follow-up period of 1.5 to 2.0 years; (3) recurrent NP group (n = 18), in which patients initially presented with CRSwNP and had recurrence of their NP within the follow-up period; and (4) control group (n = 12), in which all subjects were healthy. Table 1 summarizes the demographics of patients in the study.

ELISA Results
A positive response to staphylococcal toxins was detectable in tissue homogenates of 13 of 18 patients (72.22%) with NP recurrence in the subsequent follow-up period. In 11 of 20 patients (55.00%) in the CRSwNP group, and in 1 of 16 (6.25%) patients in CRSsNP group, positive reactions were obtained. Moreover, 3 of those 25 subjects who tested positive for the presence of toxins were positive for 2 toxins (TTST-1 and SEB, SEA and SED). There were no positive findings in the control group.

GRβ and GRα Analysis
GRβ expression in the 3 groups of CRS patients with a different prognosis of disease and controls was quantified. Positive staining for GRβ could be observed in all samples in the cytoplasm and nucleus of epithelium, glandular, and inflammatory cells, especially in the cytoplasm of epithelium and glandular cells (Figure 1). Analysis of the variants by LSD multiple comparisons demonstrated that the numbers of GRβ+ cells were significantly different among the 4 groups (all P < .05; Figure 2). The subjects with CRSwNP and recurrent NP were divided into 2 subgroups respectively on the basis of the results of ELISA: those with toxin-positive results and those with toxin-negative results. LSD multiple comparisons were performed on the variable expression of GRβ+ cells, which demonstrated that the numbers of GRβ+ cells in toxin-positive recurrent NP tissue were significantly higher compared with that in toxin-negative CRSwNP subjects (P = .009). The GRβ expression showed an increasing tendency in patients with toxin-positive CRSwNP paralleling that of toxin-negative CRSwNP, although the difference did not reach statistical significance (P = .099), and a similar increasing tendency was found in the toxin-positive and -negative subgroups of those with recurrent NP (Figure 3). Concerning GRα, except for negative controls, the immunoreactivity could be found in the cytoplasm and nucleus of epithelium, glandular, and inflammatory cells, especially in the cytoplasm of epithelium and glandular cells in all subjects (Figure 4), and there were no statistically significant differences among these 4 groups (all P > .05). However, GRα expression was enhanced significantly in toxin-positive recurrent NP versus controls (P = .048; Table 2).

Analysis of the variable GRβ/GRα ratio of the 4 groups demonstrated that the mean ratios in the CRSsNP and control groups were significantly different compared with that of the CRSwNP or recurrent NP group (all P < .05), and there was no significant difference between CRSwNP versus recurrent NP (P = .53). Further analysis of the variable GRβ/GRα ratio was performed to test the differences between each of the

### Table 1. Baseline Characteristics of Subjects and ELISA Results

<table>
<thead>
<tr>
<th></th>
<th>CRSsNP (n = 16)</th>
<th>CRSwNP (n = 20)</th>
<th>Recurrent NP (n = 18)</th>
<th>Control (n = 12)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Demographics</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean age, y</td>
<td>37.44 (17-68)</td>
<td>41.85 (16-60)</td>
<td>42.50 (17-65)</td>
<td>28.42 (17-45)</td>
</tr>
<tr>
<td>Male sex</td>
<td>9 (56.25%)</td>
<td>14 (70.00%)</td>
<td>13 (72.22%)</td>
<td>7 (58.33%)</td>
</tr>
<tr>
<td>Allergies</td>
<td>6 (37.50%)</td>
<td>9 (45.00%)</td>
<td>10 (55.56%)</td>
<td>0</td>
</tr>
<tr>
<td>Asthma</td>
<td>2 (12.50%)</td>
<td>3 (15.00%)</td>
<td>4 (22.22%)</td>
<td>0</td>
</tr>
<tr>
<td><strong>ELISA</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Positive proportion</td>
<td>6.25% (1/16)</td>
<td>55.00% (11/20)</td>
<td>72.22% (13/18)</td>
<td>0.00% (0/12)</td>
</tr>
<tr>
<td>SEA</td>
<td>0</td>
<td>2</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>SEB</td>
<td>1</td>
<td>6</td>
<td>6</td>
<td>0</td>
</tr>
<tr>
<td>SECl-C3</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>SED</td>
<td>0</td>
<td>1</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>TSST-1</td>
<td>0</td>
<td>4</td>
<td>4</td>
<td>0</td>
</tr>
</tbody>
</table>

Abbreviations: CRSsNP, chronic rhinosinusitis patients without nasal polyps; CRSwNP, chronic rhinosinusitis patients with nasal polyps; ELISA, enzyme-linked immunosorbent assay; NP, nasal polyps; TSST-1, toxic shock syndrome toxin type-1.

*In the CRSwNP group, 2 of those 11 subjects who tested positive for the presence of toxin were positive for 2 toxins (TTST-1 and SEB, SEA and SED). In the recurrent NP group, 1 of those 13 subjects was positive for SEB and SED simultaneously.
2 groups among the 6 groups, including toxin-positive and -negative subgroups of CRSwNP and recurrent NP. The mean ratio in toxin-positive recurrent NP was significantly higher than that in toxin-negative CRSwNP ($P = .03$). However, there were no significant differences between the toxin-positive and toxin-negative subgroups of CRSwNP ($P = .173$), as well as

Figure 1. Immunohistochemical staining for glucocorticoid receptor (GR) $\beta^+$ cells in the nasal tissue in recurrent nasal polyps (NP) with superantigen presence (B1) and superantigen-negative (B2), chronic rhinosinusitis (CRS) patients with nasal polyps (CRSwNP) without recurrence with superantigen-positive (B3) and superantigen-negative (B4), respectively. CRS patients without nasal polyps (CRSsNP) without NP occurred followed up to 1.5 to 2.0 years (B5), and in the controls (B6). Note the increased cells expressing GR$\beta^+$ mainly in the cytoplasm of the epithelium and glandular cells presenting brown grains (original magnification 200×).

Figure 2. Glucocorticoid receptor (GR) $\beta^+$ immunoreactivity in nasal tissue. The numbers of GR$\beta^+$ cells were significantly different among the 4 groups.

Figure 3. Glucocorticoid receptor (GR) $\beta^+$ immunoreactivity in subgroups of chronic rhinosinusitis (CRS) patients with nasal polyps (CRSwNP) and recurrent NP. $^*P < .05$ versus toxin-negative CRSwNP.

Figure 4. Immunostaining for glucocorticoid receptor (GR) $\alpha$ mainly expressing in the cytoplasm of the epithelium and glandular cells with brown grains (original magnification 200×). A1, recurrent nasal polyps (NP), superantigen positive. A2, recurrent NP, superantigen negative. A3, superantigen-positive chronic rhinosinusitis (CRS) patients with NP (CRSwNP) without recurrence. A4, superantigen-negative CRSwNP without recurrence followed up to 1.5 to 2.0 years. A5, CRS patients without NP occurred up to follow-up period end. A6, an example of normal controls.
that in 2 subgroups of recurrent NP ($P = .901$). In addition, there was no statistical difference between CRSsNP and toxin-negative CRSwNP subjects ($P = .192$; Table 2).

### Table 2. Means of GR-Positive Cells in Samples (per mm² ± SEM)*

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>$\text{GR}\alpha^+$ Cells</th>
<th>$\text{GR}\beta/\text{GR}\alpha$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>12</td>
<td>16.47 ± 3.83</td>
<td>0.76 ± 0.28</td>
</tr>
<tr>
<td>CRSsNP</td>
<td>16</td>
<td>16.86 ± 4.63</td>
<td>1.27 ± 0.24</td>
</tr>
<tr>
<td>CRSwNP</td>
<td>20</td>
<td>19.70 ± 4.15</td>
<td>1.54 ± 0.34</td>
</tr>
<tr>
<td>Toxin negative</td>
<td>9</td>
<td>19.09 ± 3.24</td>
<td>1.44 ± 0.29</td>
</tr>
<tr>
<td>Toxin positive</td>
<td>11</td>
<td>20.20 ± 4.87</td>
<td>1.63 ± 0.37</td>
</tr>
<tr>
<td>Recurrent NP</td>
<td>18</td>
<td>19.72 ± 4.92</td>
<td>1.74 ± 0.34</td>
</tr>
<tr>
<td>Toxin negative</td>
<td>5</td>
<td>18.71 ± 4.63</td>
<td>1.75 ± 0.31</td>
</tr>
<tr>
<td>Toxin positive</td>
<td>13</td>
<td>20.11 ± 5.15</td>
<td>1.73 ± 0.36</td>
</tr>
</tbody>
</table>

Abbreviations: GR, glucocorticoid receptor; CRSsNP, chronic rhinosinusitis patients without nasal polyps; CRSwNP, chronic rhinosinusitis patients with nasal polyps; NP, nasal polyps.

*Positive cells with $\text{GR}\alpha$ or $\text{GR}\beta$ were determined by immunohistochemical analysis by using the specific polyclonal antibodies and no cross-reactivity against $\text{GR}\beta$ or $\text{GR}\alpha$. $P$ values of less than .05 were considered significant.

The mean difference compared with all groups is significant.

The mean difference compared with the control group is significant.

The mean difference compared with the toxin-negative group is significant.

### Discussion

The anti-inflammatory and immunosuppressive properties of glucocorticoids are their major pharmacologic benefits and make them the most widely prescribed class of drugs. However, the response and sensitivity to glucocorticoids vary among individuals, tissues, and cell types. In a subgroup of patients, clinical management of CRSwNP and other inflammatory conditions such as asthma and rheumatoid arthritis is becoming a major challenge, considering the resistance to glucocorticoid treatment. Hamilos et al. reported an association between glucocorticoid insensitivity and the increased expression of $\text{GR}\beta$. However, the molecular basis of glucocorticoid resistance and the mechanisms that trigger the overexpression of $\text{GR}\beta$ are not well understood. To date, only 3 reports have appeared in the literature that examined an explant culture and PBMC models to analyze indirectly the relationship between microbial superantigens and $\text{GR}\beta$ in the induction of glucocorticoid insensitivity. In this study, through analyzing directly the presence of superantigens and the expression level of $\text{GR}\beta$ in the nasal polyp, we investigated a potential mechanism by which microbial superantigens contribute to steroid insensitivity.

Understanding the mechanism by which superantigens contribute to poor disease control in superantigen-triggered chronic inflammatory diseases would be of great interest. In the current study, we first investigated directly whether SEs, instead of toxin-producing *Staphylococcus aureus*, presented in sinonasal polyp tissue and mucus of patients with an insensitivity to glucocorticoid treatment. Our data demonstrated that SEs were detectable in 72.22% of patients with recurrent NP and insensitivity to glucocorticoids, which was significantly higher than that in other patients in whom the development of the disease could be controlled by glucocorticoid treatment. In addition, the positive rate (55.00%) in CRSwNP patients without recurrence was more than that in CRSsNP patients (62.5%). This study was designed to detect the 7 most common staphylococcal toxins (ie, SEA, SEB, SEC1-C3, SED, and TSST-1). The incidence of toxins in NP was likely to be higher considering there were at least 19 known staphylococcal superantigens. Our results revealed that glucocorticoid resistance was apt to occur in superantigen-stimulated patients.

Recent studies suggest that $\text{GR}\beta$, as an endogenous inhibitor for the classic $\text{GR}\alpha$, might play an important role in tissue sensitivity to glucocorticoids. GRβ, lacking the ability to bind glucocorticoids, can form heterodimers with hormone-bound $\text{GR}\alpha$ and thereby inhibit glucocorticoid action. High-level expression of $\text{GR}\beta$ would inhibit $\text{GR}\alpha$ binding affinity and transcription of glucocorticoid-responsive genes in a dose-dependent manner. The present study confirmed that $\text{GR}\beta$ expression was significantly higher in the recurrent NP versus the CRSwNP or CRSsNP group, suggesting that steroid resistance seems to be associated with induction of $\text{GR}\beta$ cells. To investigate a potential mechanism by which SEs induce glucocorticoid resistance, we assessed the expression of $\text{GR}\beta$ in SE-positive versus SE-negative nasal tissues, respectively, with glucocorticoid insensitivity or sensitivity. Our results demonstrated that the expression of $\text{GR}\beta$ exhibited an increasing tendency in toxin-positive patients with recurrent NP or CRSwNP paralleling that of toxin-negative recurrent NP or CRSwNP, respectively, although the differences did not reach statistical significance. Furthermore, the expression level of $\text{GR}\beta$ in superantigen-positive tissue of recurrent NP was significantly higher compared with that in superantigen-negative CRSwNP subjects. These findings from subjects with both sensitivity and insensitivity to glucocorticoids indicate that the expression of $\text{GR}\beta$-positive cells would increase relatively in the nasal tissue with superantigens compared with superantigen-negative tissue. This may suggest a potential that microbial superantigens induce the increased $\text{GR}\beta$, resulting in more insensitivity to the effects of glucocorticoids. Further studies comprising abundant cases will be required to identify the presumption.

In addition, previous studies have shown different findings about $\text{GR}\alpha$ expression levels in cases of glucocorticoid resistance. Therefore, we related the expression of $\text{GR}\alpha$ in an immunostaining study. Our results showed that expression of $\text{GR}\alpha$ was not significantly different among these groups. However, $\text{GR}\alpha$ expression was enhanced significantly in toxin-positive recurrent NP versus controls. We subsequently analyzed the variable $\text{GR}\beta/\text{GR}\alpha$ ratio of the same sample in different groups. The results demonstrated that the increase of the ratios was similar to the variable $\text{GR}\beta$. The highest increase of the mean ratio was observed in toxin-positive recurrent NP compared with that of toxin-negative CRSwNP, and there were no significant differences between toxin-positive and toxin-negative subgroups of CRSwNP, as well as in 2 subgroups of recurrent NP.

At present, glucocorticoids represent the most effective therapy to control CRS inflammation and NP growth. However, we
have observed a subgroup of patients whose illness conditions cannot be controlled by glucocorticoids. For these patients, glucocorticoid insensitivity appears to have merit and warrants further research into the potential mechanisms of molecular pathways. Further studies are needed to determine the correlation between GRβ/GRα and superantigens in the model of glucocorticoid insensitivity and to elucidate the mechanisms of inducing steroid insensitivity in inflammatory cells.

**Conclusion**

The present study demonstrates that the role of microbial toxins is more likely to be found in patients with CRSwNP, especially in patients insensitive to glucocorticoids. This indicates that induction of the up-regulated GRβ activity is a potential mechanism by which superantigens may cause glucocorticoid resistance in nasal tissue. Our findings shed new light on the issue relevant to steroid insensitivity in CRSwNP, which may, in turn, offer a promising therapeutic strategy for this disorder.

**Author Contributions**

Mingming Wang, study conduct; Peng Shi, data analysis; Bei Chen, revising the article; Guanggang Shi, data acquisition; Hong Li, data acquisition; Haibo Wang, corresponding author, conception and design.

**Disclosures**

**Competing interests:** None.

**Sponsorships:** None.

**Funding source:** NSFC ID 30801279, financial support and study design.

**References**