Expression of interleukin-5 in different pathologic types of nasal polyp tissues

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Abstract: Interleukin-5 (IL-5) exhibits aberrantly high expression in nasal polyps (NP) tissues, which is closely associated with the formation and development of NP. Antagonizing IL-5 is recognized as a promising strategy for NP patients. However, the expression of IL-5 in different pathologic types of NP has not yet been completely understood. In order to explore immunohistochemical IL-5 expression in NP, compare the results in different NP types, 52 cases of NP tissues and 20 cases of inferior turbinate mucosa tissues as healthy control were collected. Hematoxylin-eosin staining was adopted to detect four NP types: inflammatory infiltrated (15.38%), adenocystic (11.54%), edematous (57.69%) and fibrous polyp (15.38%). Immunohistochemical analysis confirmed that the expression of IL-5 in NP patients (73.08%) were significantly increased compared with controls ($\chi^2 = 19.871$, $p<0.001$). IL-5 expression exhibited obvious difference in different pathological types of NP ($\chi^2 = 47.333$, $p<0.001$). We did not detect IL-5 expression in adenocystic polyp tissues. Among patients with IL-5 positive expression, the levels of IL-5 in inflammatory, edematous and fibrous polyp were weak (+), moderate (+ for 2, ++ for 13, and +++ for 15 patients) and strong (+++) respectively. Collectively, IL-5 was overexpressed in NP tissues in general, but varied notably in different NP types, the stronger expression of IL-5 indicating the more severe NP condition. Our data support IL-5 as a potential target for the treatment of NP, but different types of polyps should be dependent on different approaches.

Keywords: Nasal polyps, interleukin-5, inflammatory infiltrated, adenocystic, edematous, fibrous

Introduction

Nasal polyps (NP) accounts for 1-4% in the adult population and derives from the abnormal mucosal protrusions on nasal mucosa or paranasal sinuses along with mucosal epithelial hyperplasia, infiltration of inflammatory cells, neo-vascularization, and remarkable edema [1, 2]. The etiology and pathophysiology of NP are still controversial. 80-90% of the NPs are characterized by prominent eosinophilia (especially the activated eosinophils) in the middle and inferior turbinates bones [3]. The recruitment of mucosal eosinophils facilitated by cytokines and chemokines reflects a more extensive disease and a decreased surgical success rate [4, 5]. Besides, the released cytokines and chemokines also contribute to the accumulation of other inflammatory cells, such as B cells, plasma cells, macrophages, and neutrophils and induce the development of NP [6, 7]. Currently, intranasal glucocorticoids is the most common strategy for NP, and endoscopic sinus surgery is reserved for cases refractory to medical treatment [8, 9], but still remains 10-40% recurrence rate [10]. Hence, finding the key molecules involved in NP is crucial for revealing the pathological progression and developing novel approaches for NP.

Interleukin-5 (IL-5), an interdigitating homodimeric glycoprotein, is conserved among hematopoietic cytokines responsible for regulating proliferation, differentiation, maturation and migration, as well as preventing apoptosis by combining with IL-5R on target cells [11]. Eosinophils and their precursors are the main target cells expressing IL-5R [12]. Moreover, IL-5 could promote the release of histamine and leukotrienes from basophils to improve the activity of basophils. IL-5 also stimulates the secretion of IgA by inducing the differentiation of B cells [12]. Previous studies have found that IL-5 was involved in various diseases, such as
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asthma and atopic diseases [13], helminth infections [14] and drug hypersensitivity [15]. In NP, the expression of IL-5 in polyp tissues was increased significantly at both mRNA and protein levels, which was closely related to the formation of edema and development of NP [16, 17]. Apart from that, Xu et al. reported that the expression of IL-5 in eosinophils was remarkably stronger during the progression of NP compared with the healthy turbinate mucosa [18]. Neutralizing IL-5 with humanized anti-IL-5 monoclonal antibodies suppressed the inflammatory reaction and reduced the size of NP in half of the patients with NP [19-22]. However, limited attention has been directed towards the expression of IL-5 in patients with different types of NP.

In the present study, clinical research on tissues from 52 NP patients and 20 healthy control, hematoxylin-eosin (HE) staining, immunohistochemistry, quantitative real time RT-PCR and Western blotting assay were performed to explore the types of NP and the expression of IL-5 in different NP types. We found that the expression of IL-5 in NP patients were notably increased compared with controls, IL-5 expression varied in different NP types, and the higher expression of IL-5 pointed to the more severe NP condition.

Materials and methods

Patients

We studied NP tissues from 52 patients who underwent endoscopic sinus surgery between January 1999 and March 2009 at the Ear-Nose-Throat (E.N.T) department of Dongying People’s Hospital, Shandong, China, and a total of 20 healthy nasal mucosa tissue samples (15 cases received correction of deviated nasal septum surgery and 5 cases received dacryocystorhinostomy) were taken as healthy control. All tissue specimens were obtained with permission from the Medical Ethics Committee of Dongying People’s Hospital. The median age of all patients was 35.6 years (range, 18-69 years); 29 cases were male, and 23 cases were female. There were 14 cases patients experienced the NP course for 5 months to 2 years, 15 cases for 2 years to 4 years and 23 cases for over 4 years. NP diagnosis was based on a CT examination, endoscopic sinus surgery and postoperative pathological examination, which was complied with new diagnostic criteria of nasal polyps formulated by E.N.T textbook. None of the patients suffered from cystic fibrosis or received any related drugs before surgery. Patients and controls with asthma, aspirin specific reactive bronchial dilation, chronic obstructive pulmonary and autoimmune diseases were excluded. All participants were without acute upper respiratory tract infection or steroid drug history before 4 weeks of surgery, and agreed to participate in our study and signed an informed consent.

Specimens

Fresh tissues removed from the operating room were immediately sectioned. One part of them was fixed in 10% formaldehyde at room temperature for 4-12 h and washing with phosphate buffered saline (PBS, pH 7.2-7.4). The other part of tissues was preserved in liquid nitrogen tank. The fixed tissues were then dehydrated through graded ethanol solutions (70% for 2 h, 80% overnight, 90% for 2 h and 100% for 2 h), followed by post-fixed into dimethylbenzene for 30 min and embedded in paraffin. 5 µm thick sections were cut subsequently and placed onto slides and dried in a 70°C chamber for 40 min. Thereafter, sections were dewaxed by ethanol and soaked into PBS for further HE staining and immunohistochemical analysis.

Hematoxylin-eosin (HE) staining

Sections were soaked in sequence with hematoxylin (Solarbio, Beijing, China) for 5-8 min, ddH_2O for 5 min and 1% hydrochloric ethanol for 3 s. After rinsing with ddH_2O for 20 min, sections were stained with 1% eosin for 5 min and dehydrated by a graded ethanol series (75%, 95%, 95%, and 100%) for 5 min respectively, followed by permeabilizing twice with dimethylbenzene for 10 min. Then sections were mounted with neutral gum and observed using a microscope (Olympus, Tokyo, Japan).

Immunohistochemical analysis

After deparaffinized and rehydrated, sections were exposed to 5 µl methanol containing 0.3% H_2O_2 for 10 min to block endogenous peroxidase and autoclaved at 121°C for 10 min in citrate buffer (10 mM sodium citrate, pH 6.0) for antigen retrieval. Streptavidin-biotin-peroxidase complex (SABC) technique was performed for immunohistochemical staining after block-
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ing with normal goat serum. In brief, sample sections were incubated with mouse anti-IL-5 monoclonal antibody (1:50 diluted) (Abcam, Cambridge, MA, USA) overnight at 4°C and stained with UltraSensitive™ SP (Mouse) IHC Kit (Maixin Biotech, Fuzhou, China) strictly following the manufacturer’s instruction. Diaminobenzidine (DAB) was applied as the chromogen, and nuclei were stained by hematoxylin.

Result determination

The results were evaluated separately by two pathologists who were blinded to the clinical parameters. For each section, 5 high-power fields that contained at least 100 cells for each field were selected randomly. Results were determined by the percentage of IL-5 positive staining cells (0% = -, <30% = +, 31-80% = ++, ≥81% = +++).

Quantitative real time RT-PCR (qRT-PCR)

Total RNA was extracted from each group using Trizol reagent (TaKaRa) according to manufacturers’ protocols. Real-Time PCR Kit (DBI) was used to carry out reverse transcription to obtain the cDNA; Expression of IL-5 was examined using SYBR® Premix Ex TaqTM II (TaKaRa) and GAPDH was served as internal reference; IL-5 upstream primer sequence: 5’-ACCTGGC- ACTGTTTTCTACTC-3’; downstream primer sequence: 5’-GGTTTACTCTCCGTCTTCTCTC-3’; GAPDH upstream primer sequence: 5’-ATGGG- ATGGAGTGTGGCTCAT-3’, downstream primer sequence: 5’-GTTTTAATCTCCGTCTTCTCTC-3’; The PCR conditions were 94°C for 2 min, and then 40 cycles of 94°C for 20 sec, 58°C for 20 sec, and 72°C for 20 sec, and a final extension at 72°C for 10 min. All experiments were performed in duplicate. Results are represented as fold induction using the 2-ΔΔCt method.

Western blotting assay

Total protein was extracted from 40-100 mg tissues with an appropriate cold lysis buffer supplemented with 1 mM phenylmethylsulfonyl fluoride (PMSF), and the protein concentration was determined by a BCA protein assay Kit (Amresco, USA). Samples were subjected to 10% SDS-PAGE and transferred to a PVDF membrane (Millipore, USA). Membranes were blocked and incubated overnight at 4°C with the primary antibodies IL-5 (1:300; Abcam) or GAPDH (1:1,000; ABGENT), the membrane was incubated with HRP-conjugated secondary antibody at room temperature for 1 h; proteins were detected using an enhanced chemiluminescence (ECL) kit (Pierce). The protein expression were adjusted to correspond to the IOD value of target protein versus IOD of correspond internal reference.

Statistical analysis

Experimental results were expressed as mean ± SD. Statistical analysis was performed with SPSS 19.0 software. One-way ANOVA was used for the analysis of the differences between the groups. The categorical data was analyzed by the chi-square test. Differences between IL-5 expressions in positive NP pathologic types were assessed by Friedman rank sum test. p<0.05 or p<0.01 was considered to be statistically significant difference.

Results

Pathologic types in NP tissues

We employed HE staining to identify the pathologic types among 52 NP tissues. The results were shown in Table 1 and Figure 1, four different histopathologic types were detected according to the observed structural characteristics. 8 patients (15.38%) were detected as inflammatory infiltrated type of NP, 6 patients (11.54%) were showed as adenocystic type of NP, 30 patients (57.69%) were demonstrated as edematous type of NP, and 8 patients (15.38%) were proved to be fibrous type of NP.

As shown in Figure 1, for inflammatory infiltrated type of NP, there were a large number of inflammatory cells densely infiltrated in the lamina of mucous membrane, which was dominated by eosinophils, but without fibroblast proliferation (Figure 1B). Mucous gland hyperplasia with gland expansion and mucus retention, slightly inflammatory cell infiltration in stroma without fibroblast proliferation, and squamous epithelium were visualized in adenocystic polyp

<table>
<thead>
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(Figure 1C). The edematous polyp is morphologically characterized by pseudostratified ciliated columnar epithelium, thickening of the basement membrane, edema even present-ing the edema pool, scattered cells with numerous leukocytes, predominantly eosinophils, tiny amounts of fibroblast proliferation (Figure 1D). The last histological type is fibrous polyp characterized by local or total fiber tissue proliferation, elevated fibroblast cells, and collagen fiber deposition (Figure 1E).

Enhanced expression of IL-5 in NP tissues

Immunohistochemistry was carried out to analysis the expression of IL-5 in NP tissues. We observed a slightly expression of IL-5 in control tissues, which accounted for 15% (3/20 cases), and a dispersed expression of IL-5 in NP stroma, which accounted for 73.08% (38/52 cases) (Table 2). Chi-square test revealed a statistically significant difference between NP tissues and control tissues ($\chi^2 = 19.871, p<0.001$), which indicating that the expression of IL-5 in NP tissues was increased significantly compared with control tissues.

Expression of IL-5 in different NP pathologic types

We invited two pathologists to evaluate the expression of IL-5 in different NP pathologic types. As shown in Table 3. Among the 8 cases of inflammatory infiltrated polyp tissues, only one case (12.5%) showed positive IL-5 expression. All adenocystic polyp tissues exhibited negative IL-5 expression. All edematous and fibrous polyp tissues were 100% IL-5 positive rate. Chi-square test demonstrated that the expression of IL-5 in different pathological types differed from each other ($\chi^2 = 47.333, P<0.001$). We further verified these findings by immunohistochemical analysis, quantitative real time RT-PCR and western blotting assay. As shown in Figure 2A, IL-5 positive cells which stained yellow or tan can be observed in most nasal polyps tissues and a few control tissues. And the IL-5 positive staining part was mainly distributed in fibroblasts cytoplasmic. In different histopathologic types, IL-5 was highly expressed in the edema-

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The expression of IL-5 in nasal polyp tissues was compared with control tissues. The expression of IL-5 in inflammatory infiltrated polyp and adenocystic polyp tissues was significantly increased compared with control tissues (P<0.01). Meanwhile, adenocystic polyp tissues were also higher than control tissues (P<0.05). The quantitative real-time RT-PCR results demonstrated the relative mRNA expression of IL-5 in edematous polyp, fibrous polyp tissues were significantly increased compared with control tissues (P<0.01).
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**Table 4. Immunohistochemical intensity of IL-5 in positive NP types**

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In this study, we found that IL-5 was positively stained in inflammatory infiltrated, edematous and fibrous polyps but negatively stained in adenocystic polyp, suggesting that the expression of IL-5 was associated with the pathologic type of NP. Bachert et al. revealed that IL-5 was secreted by antigen activated T cells at the initial stage of NP, then the activated eosinophils replaced T cells as the main source of IL-5 with the development of NP [17]. So one possible explanation of our result is that there were eosinophilia and eosinophil infiltration in the three positive NP types, but with less eosinophils sequenced in adenocystic polyp which may lead to the decreased distribution of IL-5. On the other side, Zhang et al. and Cao et al. both point out that NPs in Chinese patients are clinically indistinguishable from polyps of their white counterparts, but part of them lack IL-5 and eotaxin expression in NP tissue, indicating different pathologic processes in them [24, 25], which is similar with the adenocystic polyp in this study. Furthermore, except for eosinophils infiltration, mast cells and neutrophils infiltration also play an important role in the progression of NP. We therefore speculate that adenocystic polyp may exhibit a pathologic mechanism different from other three NP types. Our experiment also indicates that different types of polyps require different treatments based on the respective pathophysiology.

Prior studies pointed out that eosinophil-fibroblast interactions facilitated the development of eosinophil-associated diseases, eosinophils express at least 2 potent mediators (IL-1β and TGF-β) to induce a fibrogenic fibroblast phenotype in asthma and allergen-induced subepithelial and peribronchial fibrosis [26, 27]. Rudack et al. proved that fibroblasts secreted GM-CSF prolonged eosinophil survival in vitro combined with IL-3 and IL-5 produced by eosinophils in NP development [28]. Apart from that fibroblasts act as important immune regulatory cells via their ability to cross-talk with T cells, it can promote Th2 polarization in Th-cell

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responses [29]. So fibroblast proliferation is related to a serious degree of NP, moreover, both Th2 and eosinophils are the main source of IL-5. The present study observed a weakly stained IL-5 in inflammatory infiltrated polyp, moderate stained IL-5 (positive were range from weak to strong) in edematous polyp, and high stained IL-5 in all fibrous polyp, indicating that the pathological type of NP was gradually become serious along with the increased expression of IL5.

In summary, our present study demonstrated that the increased expression of IL-5 was varied in different NP types, and the higher expression of IL-5 indicated the more serious NP type except for the non-IL-5 expressed adenocystic polyp. Our data support anti-IL-5 as a promising candidate for the treatment of NP, but different types of polyps should dependent on different approaches.

Disclosure of conflict of interest

None.

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References

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