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Expression profiles of CD11b, galectin-1, beclin-1, and caspase-3 in nasal polyposis*

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Background/aim: Nasal polyposis is a chronic inflammatory disease affecting the paranasal sinuses and nasal mucosae. It is thought that genetic and molecular mechanisms in inflammatory and apoptotic pathways are the main factors in the etiopathogenesis of nasal polyposis. The aim of this study was to investigate the expression patterns of CD11b, galectin-1, beclin-1, and caspase-3 in nasal polyps.

Materials and methods: The mRNA expression levels of CD11b, galectin-1, beclin-1, and caspase-3 protein and western blot analysis of caspase-3 protein were evaluated in inferior turbinate mucosae and nasal polyp tissues.

Results: CD11b expression was markedly higher in nasal polyp tissues when compared to turbinate mucosae (5.5 times higher, $P < 0.05$). Expression of galectin-1 was not statistically higher in nasal polyp tissues when compared to the controls. Beclin-1 expression in nasal polyp tissues was lower than in controls (17 times lower, $P < 0.05$). Caspase-3 expression was significantly lower in nasal polyp tissues than in controls (5.5 times lower, $P < 0.05$).

Conclusion: Inflammation, apoptosis, and hyperproliferation are the major cellular processes in nasal polyposis and these proteins may take part and play some important roles in formation of this disease and the targeting of new treatment protocols.

Key words: Nasal polyp, CD11b, galectin-1, beclin-1, caspase-3

1. Introduction

Nasal polyposis (NP) is a type of chronic rhinosinusitis, and its treatment is one of the most difficult challenges in clinical rhinological practice. Although the pathogenesis of NP has received much attention recently, its etiopathogenesis and underlying molecular mechanisms remain controversial. NP is a multifactorial disease and its genetic causes have not been fully defined (1). Hyperproliferation, apoptosis, subepithelial chronic inflammation, and local immunological dysregulation may contribute to the development and progression of NP (2–4). Considerable evidence shows that apoptosis and proliferation induce secondary changes in chronic inflammation, including epithelial hyperplasia and tissue remodeling (5). Therefore, investigating hyperproliferation and apoptosis in inflammatory processes is important. The expression profiles of proteins that are involved in these processes could provide crucial information for understanding the pathogenesis of NP (6).

In this study, the expression of CD11b, galectin-1, beclin-1, and caspase-3 was measured in NP tissues and normal nasal mucosae. CD11b, a protein subunit known as integrin alpha M (ITGAM), is expressed on the surface of leukocytes in the innate immune system (7). It plays a major role in cell adhesion, inflammation, chemotaxis, cellular activation, and phagocytosis and is involved in systemic lupus erythematosus according to genome-wide association studies (8). Galectin-1 is a galactosidase-binding protein that is thought to play a role in immunotolerance during pregnancy. Galectin-1 modulates cell–cell and cell–matrix interactions and is important for cell proliferation, acting as an autocrine negative growth factor (9). Beclin-1 regulates autophagy and plays an important role in cellular development and tumorigenesis. It has important effects on cell proliferation and is involved in neoplastic and neurodegenerative diseases (10). Caspase-3 is involved in different apoptotic pathways and plays a central role in the execution of apoptosis.

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In a cell, native caspase-3 exists as procaspase, which is activated by a biochemical change (11). In addition to the mRNA expression analyses of these proteins, caspase-3 was studied via western blot analyses. In summary, in this study, we investigated molecules that have important roles in apoptosis, proliferation, and inflammation in order to help elucidate the etiopathogenesis of NP.

2. Materials and methods

This study was approved by the local institutional ethical committee (approval number: 024; date: 18 January 2012), and informed consent was obtained from all participants before samples were collected.

2.1. Sample collection and preparation

NP tissue samples were obtained from 21 subjects (13 men and 8 women) undergoing endoscopic sinus surgery as treatment for chronic rhinosinusitis with NP. All subjects received systemic steroids and antibiotics prior to surgery. Inferior turbinate control tissues were obtained from 20 controls (10 men and 10 women) undergoing surgery for either septorhinoplasty or septoplasty. All samples were obtained during surgery. Control subjects had no allergies or chronic rhinosinusitis.

For preparation of RNA studies and western blot analysis, NP tissues were washed with saline in the operating room. Tissues were stored at –80 °C with frozen liquid nitrogen.

2.2. Total RNA extraction and cDNA preparation

Total RNA was extracted using the TriPure reagent (Roche, Darmstadt, Germany) and some arrangements were planned to avoid DNA contamination according to the manufacturer's instructions. After homogenization of tissues and incubation in 100 mg/mL TRIzol for 5 min, separation of RNA was done using chloroform (0.2 mL/1 mL TRIzol). After 15 min of centrifugation at 12,000 × *g* at 4 °C, the aqueous phase of the sample was transferred to a new fresh tube. Sedimentation of RNA material was done by isopropyl alcohol (0.5 mL/1 mL TRIzol). Incubation and centrifugation was repeated as above and the pellet was washed with 75% ethanol. Centrifugation of the mixture was applied for 5 min at 7500 × *g* at 4 °C. The air-dried pellet was dissolved again in DEPC-treated water. RNA yield, efficiency, and standards were determined by measuring the absorbance at 260 and 280 nm using a spectrophotometer (NanoDrop ND-1000; Montchanin, DE, USA). An A260/280 ratio of <2.0 and an A260/230 ratio in the range of 1.8–2.2 were accepted as measures of RNA purity in the analysis. Reverse-transcription of total RNA (1 µg) was applied in a reaction mixture of 20 µL using random hexamers and a Transcriptor First Strand cDNA Synthesis Kit (Roche Diagnostics GmbH, Mannheim, Germany). The procedure was done according to the manufacturer's instructions.

2.3. Quantitative real-time PCR analysis

BECN1, *CASP3*, *CD11b*, and *galectin-1* mRNA expression levels were measured by real-time PCR as described above previously (12). Sets of primers and probes were designed using the Probe Finger Design Assay Centre. The primers and probe numbers for the *CD11b*, *BECN1*, *galectin-1*, *CASP3*, and *GAPDH* genes are described in the Table. The reaction mixture of 10 µL contained 1X LightCycler Probe Master mix, 2.5 pmol of each primer, 1 pmol of UPL probe, 4 mM $MgCl_2$, and 1 µM cDNA prepared in 96-well plates. All PCR reactions were performed in a LightCycler 480 thermocycler (Roche Diagnostics). The housekeeping gene, *GAPDH*, was used for normalizing quantitative reverse transcriptase PCR results. Each sample was tested in triplicate. Amplification efficiencies and yields of the target genes and *GAPDH* were approximately equal. The results were reported as mean ± standard deviation of at least three independent experiments (Table).

2.4. Protein extraction and western blot analysis

Western blot analysis of caspase-3 was performed as described previously in a recent study (13). Briefly, the cells were lysed in lysis buffer solution (Cell Signaling Technology, Inc., Danvers, MA, USA) containing 1 mM phenylmethanesulfonyl fluoride (Sigma-Aldrich). Equal amounts of protein were loaded and separated by 12% SDS-

Table. Primers and probe numbers for *BECN1*, *CD11b*, *galectin-1*, *CASP3*, and *GAPDH*.

Gene	Forward primer	Reverse primer	UPL probe
BECN1	5'-GGATGGTGTCTCTCGCAGAT-3'	5'-TTGGCACTTTCTGTGGACAT-3'	#20
CD11b	5'-GGCATCCGCAAAGTGGTA-3'	5'-GGATCTTAAAGGCATTCTTTCG-3'	#9
Galectin-1	5'-CGCCAGCAACCTGAATCT-3'	5'-CAGGTTCAGCACGAAGCTCT-3'	#80
CASP3	5'-CTGGTTTTCGGTGGGTGT-3'	5'-CCACTGAGTTTTCAGTGTTCTCC-3'	#34
GAPDH	5'-AGCCACATCGCTCAGACAC-3'	5'-GCCCAATACGACCAAATCC-3'	#60

UPL: Universal Probe Library, GAPDH: glyceraldehyde 3-phosphate dehydrogenase, A: adenine, G: guanine, C: cytosine, T: thymine.

PAGE and a transfer process was done to a polyvinylidene difluoride membrane (Thermo Fisher Scientific, Inc., Waltham, MA, USA). For blockage of the membrane, 5% w/v nonfat milk or 5% w/v bovine serum albumin in Tris-buffered saline with 0.1% Tween-20 was used. After blocking, the membrane was incubated for 24 h at 4 °C with a rabbit antihuman caspase-3 polyclonal antibody (Cell Signaling Technology), and a rabbit antihuman β-actin monoclonal antibody (Cell Signaling Technology) was used as the loading control. All primary antibodies were diluted in a ratio of 1/1000. The incubation process was applied with a goat antirabbit horseradish peroxidase (HRP)-conjugated secondary antibody (Cell Signaling Technology) for 2 h at room temperature. Visualization of proteins was performed using a Kodak Gel Logic 2200 imaging system (Kodak, Rochester, NY, USA) with a Luminata Crescendo Western HRP substrate (EMD Millipore, Billerica, MA, USA).

2.5. Statistical analyses

The relative expression was described according to the expression ratio of the target gene to a reference gene. This approach is usually adequate for evaluations of physiological processes and changes in gene expression levels. The results are affected by the reference gene and a normalization procedure should be used. Mathematical models have been developed to calculate relative expression ratios for genetic and molecular studies. The Pfaffl equation is the most common and convenient mathematical model for these purposes (Eq. (1)). Gene expression analyses of *IkBα* and *CASP3* were performed using the Relative Expression Software Tool (REST v 2009, Technical University, Munich, Germany) (14). $P < 0.05$ was considered to be statistically significant.

$$\text{Ratio} = [(E_{target})^{\Delta CPtarget(control - sample)}] / [(E_{ref})^{\Delta CPref(control - sample)}] \quad (1)$$

3. Results

3.1. Demographic and clinical characteristics of the subjects

A total of 41 subjects were included in two groups. The NP group contained 21 subjects [13 (62%) males and eight (38%) females; median age: 43.3 ± 14.08 years] and the control group contained 20 subjects [ten (50%) males and ten (50%) females; median age: 28.1 ± 10.39 years].

3.2. RT-PCR analysis of *BECN1, CD11b, galectin-1,* and *CASP3* expression

The mRNA levels were quantified by RT-PCR (Figure 1). *BECN1* expression was 17 times lower in NP samples ($P <$

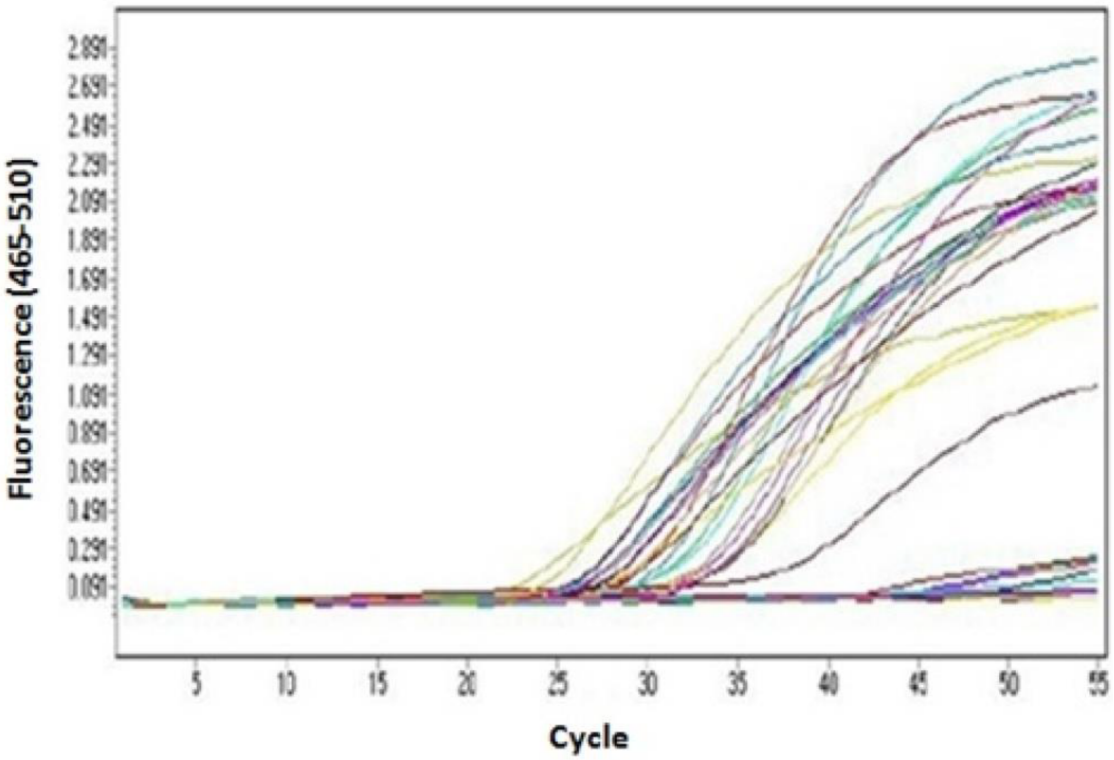

Figure 1. Amplification curves of the quantitative mRNA levels of the genes studied. Quantitative amplification curves *of BECN1*, *CD11b*, *galectin-1*, and *CASP3*. The x-axis represents Cp levels of RT-PCR reactions and the y-axis represents fluorescent signals.

0.05). *CD11b* expression was 5.5 times higher in NP tissues ($P < 0.05$), while *CASP3* expression was 5.5 times lower ($P < 0.05$). *Galectin-1* expression was not significantly higher in NP tissues. The expression profiles of the investigated genes are presented in Figure 2.

3.3. Western blot analyses of caspase-3 expression

Caspase-3 expression was semiquantitatively measured by western blotting and using the REST software (2009). The caspase-3 protein was expressed in both normal nasal turbinate mucosae and NP tissues. Caspase-3 expression was significantly higher in turbinate mucosae than in NP tissues. This correlated with reduced *CASP3* mRNA expression (Figure 3).

4. Discussion

NP is chronic disease affecting the nose and paranasal sinuses and the main pathological characteristic is inflammation (15). Apoptosis and hyperproliferation could be observed in asthma and allergic rhinitis, although the main role of these cellular processes could not be defined well in NP (16,17). Caspase-3 and beclin-1 are the main key proteins in apoptotic pathways; results of expression profiles in this study are statistically significant and this points to the importance of these processes in NP. Besides this, the results of CD11b expression are also significant and show the eosinophilic migration and inflammation in NP; this result is a new and different insight into the chronic inflammatory pathways of NP.

CD11b is a protein subunit expressed on surface integrins of inflammatory cells. Lim et al. showed that CD11b expression affects the migration of eosinophils from the blood stream to local tissues. Steroid receptor activation reduces CD11b expression in eosinophils (18). Furthermore, eosinophils in bronchoalveolar fluid are

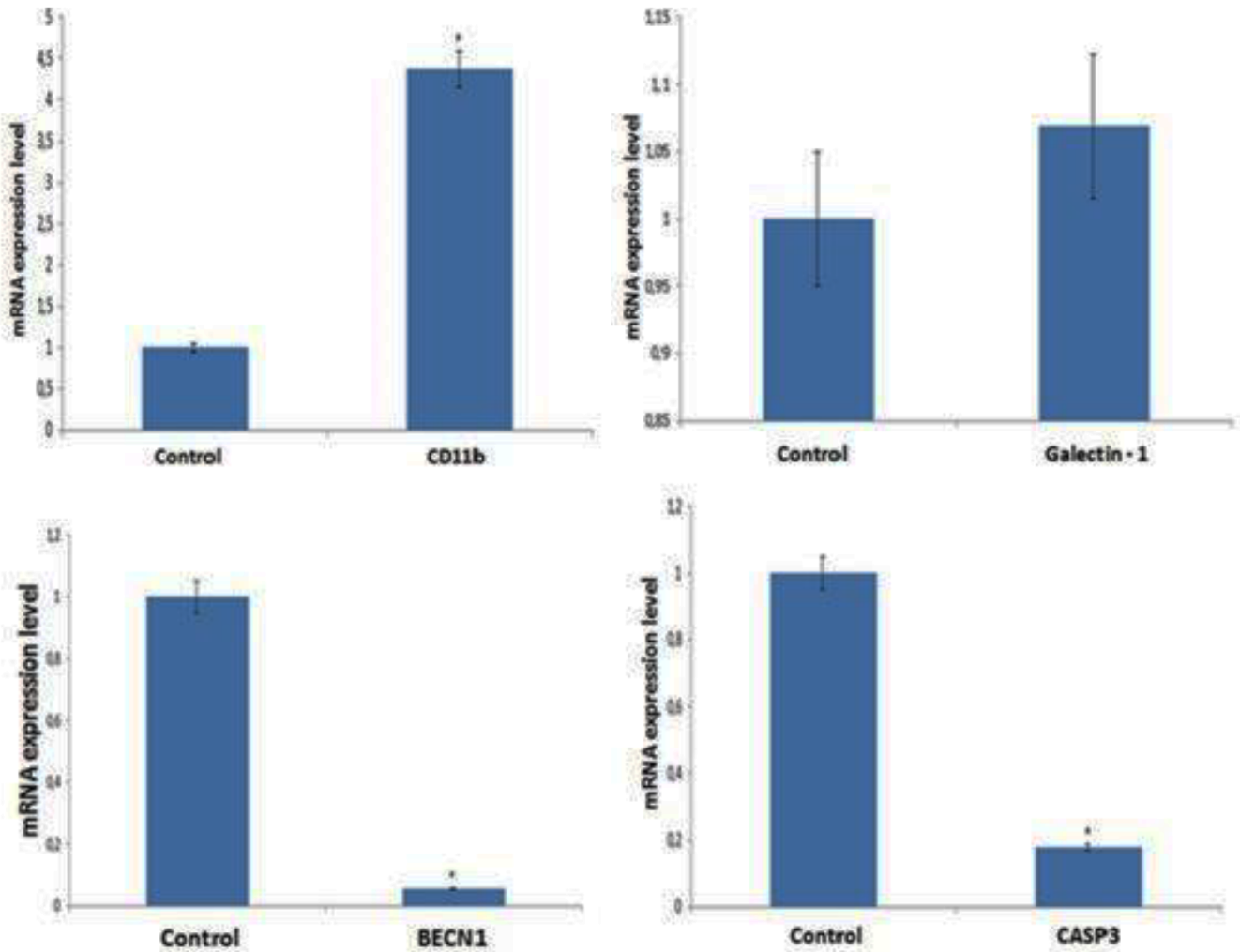

Figure 2. Quantitative mRNA expression profiles of *BECN1*, *CD11b*, *galectin-1*, and *CASP3* in NP samples and normal inferior turbinate mucosae. The differences in *BECN1*, *CD11b*, and *CASP3* expression were statistically significant.

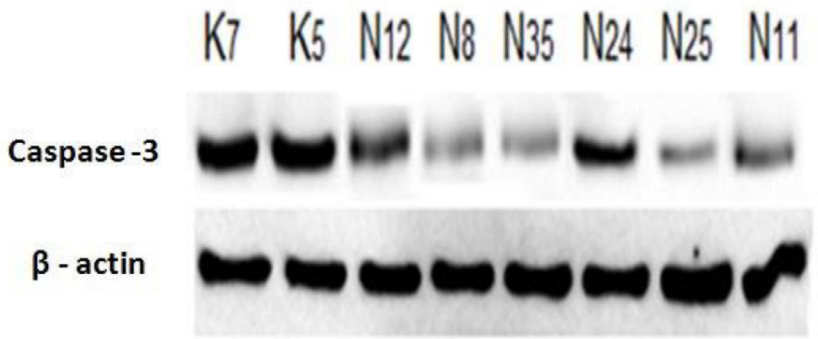

Figure 3. Reduced caspase-3 protein expression in nasal polyp tissues, as analyzed by western blot.

activated by lung fibroblasts expressing CD11b, which highlights the importance of CD11b (19). Eosinophils in NP tissues express higher levels of the chemotaxin eotaxin (20). CD11b may contribute to eosinophilic activation by affecting cytokines like eotaxin. Eotaxin upregulates CD11b expression in integrins and the increased expression of CD11b has been related to oxidative burst and cell–cell interactions by eosinophils (21). Oxidative stress in NP tissues may be caused by CD11b, which would suggest altered CD11b expression in NP tissues. We observed higher CD11b expression in NP tissues in the present study. In eosinophilic migration and eosinophilic activation, CD11b may have a key role and therefore it may be an important protein in adhesion and activation of inflammatory cells.

Galectins have a wide range of functions including cell–cell and cell–matrix interactions, apoptosis, and T-cell receptor activation in the immune response. Galectin-1 contributes to apoptosis and is upregulated during an immune response (22). Galectin-3 is expressed in head and neck carcinomas, where it influences apoptosis (23). Similarly, galectin-9 is a selective eosinophil chemoattractant that may contribute to the development of NP, asthma, or allergic rhinitis (24). Delbrouck et al. detected a higher expression of galectin-1 and galectin-3 in NP tissues than in controls and showed that these galectins influenced polyp growth and immunoregulation (25). Galectin-1 expression was also found to be positively correlated with allergen status (26). Increased galectin-1 expression should be expected in allergic diseases such as asthma, allergic rhinitis, and NP. However, an association between NP and allergic rhinitis has not been clearly demonstrated to date. Galectin overexpression has also been reported in aspirin-induced asthma (27). Galectin-1 expression was not significantly higher in NP tissues in the present study. This was not surprising, considering that NP is not related to allergen status. Previous studies have shown that galectin-1 may be related to atopic immunological processes and allergic responses; therefore, tissues need to be analyzed from allergic and nonallergic NP patients in order to elucidate the role of galectin-1 in NP. Effects and relations of allergic and immunologic substructure in NP etiopathogenesis are still unclear. Progression of molecular studies focusing on these issues may help in identifying the role of galectin-1 in NP formation.

Apoptosis is caused by environmental and developmental factors. Resistance to apoptosis prolongs cell survival and causes hyperproliferation (28). Activation of antiapoptotic pathways promotes cell survival, causing hyperplastic and neoplastic development. Hyperproliferation of cells in NP tissues may thus be explained by apoptosis-related processes. Küpper et al. evaluated the influence of apoptosis on NP by observing the expression of p53 and caspase proteins in NP tissues (29). Apoptotic agents like methotrexate and mitomycin can resolve NP symptoms (30). Beclin-1 has major roles in autophagy, differentiation, antiapoptosis, and the development and progression of neoplastic processes. Its regulation of the autophagic pathway and contribution to cell differentiation and apoptosis in the cell cycle have been well established. It was one of the first autophagy proteins to be related to malignancies (31). Beclin-1 functions as a tumor suppressor in breast and ovarian cancers and glioblastomas and is associated with tumorigenesis in colorectal and gastric cancers (32–34). Because beclin-1 can influence apoptosis and cellular hyperproliferation, it may have a role in NP. However, beclin-1 expression has not been investigated in NP tissues. In the present study, we observed significantly reduced beclin-1 expression in NP tissues compared with that in controls. This result indicates that apoptosis and cell hyperproliferation are important for NP etiopathogenesis and chronic inflammation.

Apoptosis in inflammatory cells may reduce inflammation. Glucocorticoids increase the apoptosis of polymorphonuclear cells such as eosinophils, which improves disease progression (35). Caspases play an

important role in apoptosis. Caspase-3 plays a central role in the caspase cascade and affects the execution of cellular apoptosis, causing protein proteolysis (36). Cho et al. found no significant differences in caspase-3 expression in NP tissues compared with that in controls (37). However, Lin et al. reported caspase-3 downregulation together with second mitochondria-derived activator of caspases (Smac) in NP tissues. In the present study, the expression of caspase-3 mRNA and protein was reduced in NP tissues compared with that in controls. This indicated decreased apoptosis, possibly caused by increased inflammation in NP. However, more molecular analyses are required to elucidate the role of caspase-3 in the development of NP (38).

Beclin-1-dependent apoptosis is defined as programmed cell death type II, while caspase-dependent apoptosis is defined as programmed cell death type I (39). The altered expression levels of beclin-1 and caspase-3 may promote NP development by affecting cell proliferation in the subepithelial area and affecting the survival of inflammatory cells localized in NP tissues. NP is a chronic inflammatory disease that affects the paranasal sinuses and is exacerbated by these proteins. Significant results of beclin-1 and caspase-3 expressions may show the importance of hyperproliferation and apoptosis in NP besides the immunologic and inflammatory pathways. In future treatment protocols for NP, agents that block hyperproliferative cascades and agents that induce apoptosis of inflammatory cells may be used for effective management.

There are some limitations of this study that may lead to misleading results while evaluating the etiology of NP. One of the important limitations is the age difference between patient and control subjects. It may have a possible effect on expression profiles of genes; therefore, age differences must be taken into consideration in this study. Although the subjects in the two groups were selected carefully and matched according to clinical characteristics, our study is not without limitations. Another important limitation of the study is the small sample size. Studies with high enough numbers of subjects focusing on this topic can help identify the etiologic and pathological basis of NP.

Pezato et al. described the different response of interstitial hydrostatic behavior during a saline infusion between the inferior turbinate and middle turbinate mucosae (40). There can be an effect on inferior turbinate tissue as a control compared to the middle turbinate. In spite of different mechanical and physical characteristics of the inferior and middle turbinates, it may have a slight effect on expression patterns of proteins in this study. Results of this study should be evaluated while these limitations are kept in mind. In the future, NP tissue might be compared to middle turbinate tissue of completely healthy controls in molecular studies because of the molecular and mechanical similarities of these two tissues.

In conclusion, CD11b, beclin-1, and caspase-3 expressions were altered in NP tissues, while no changes in galectin-1 expression were observed. Chronic inflammation and cell proliferation are important processes in NP formation. Apoptotic pathways may also contribute to NP development by inducing hyperproliferation in the basal membrane and by controlling the lifespan of inflammatory cells. Inhibiting these processes may prevent the formation and recurrence of NP. Therefore, future studies should focus on these processes to identify inhibitory agents. Medications targeting apoptotic processes may represent future treatment protocols.

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