JOURNAL OF CLINICAL MEDICINE OF KAZAKHSTAN (E-ISSN 2313-1519)
Original Article DOI: https://doi.org/10.23950/jcmk/12689

Circulating levels of Bcl-2 and its expression in the nasal mucosa of patients with chronic rhinosinusitis

Iuliia Kalashnyk-Vakulenko[1], Galina Gubina-Vakulyck[2], Anatolii Onishchenko[3], Oksana Nakonechna[4], Tatyana Gorbach[4], Viktoriya Tkachenko[5], Anton Tkachenko[3]

[1]Department of Otorhinolaryngology, Kharkiv National Medical University, Kharkiv, Ukraine
[2]Department of Pathological Anatomy, Kharkiv National Medical University, Kharkiv, Ukraine
[3]Research Institute of Experimental and Clinical Medicine, Kharkiv National Medical University, Kharkiv, Ukraine
[4]Department of Biochemistry, Kharkiv National Medical University, Kharkiv, Ukraine
[5]Department of Microbiology, Virology and Immunology, Kharkiv National Medical University, Kharkiv, Ukraine

Received: 2022-06-23.
Accepted: 2022-11-18

J Clin Med Kaz 2022; 19(6):63-67

Corresponding author:
Anton Tkachenko.
E-mail: as.tkachenko@knmu.edu.ua;
ORCID: 0000-0002-1029-1636

Abstract

Aim: To evaluate expression of anti-apoptotic Bcl-2 protein in the nasal tissue and its levels in blood serum of patients with chronic rhinosinusitis with (CRSwNP) and without nasal polyps (CRSsNP).

Material and methods: Expression of Bcl-2 in the sinonasal tissue and its levels in blood serum of patients with CRSsNP and CRSwNP were evaluated immunohistochemically and using ELISA, respectively.

Results: In patients with CRSsNP, Bcl-2 was overexpressed in nasal epithelial cells mainly in the atrophic regions. However, its upregulation was also observed in regions with epithelial cell proliferation. Immunostaining for Bcl-2 was stronger both in the stroma and epithelial lining compared with control subjects. The level of Bcl-2 in blood serum was elevated in both forms of chronic rhinosinusitis with a more pronounced increase in CRSwNP.

Conclusion: CRSsNP and especially CRSwNP are associated with overexpression of anti-apoptotic Bcl-2 in nasal epithelial cells and in the lamina propria against the background of elevated circulating concentrations of Bcl-2.

Key words: nasal polyps, nasal epithelial cells, apoptosis, B-cell lymphoma 2

Introduction

Chronic rhinosinusitis (CRS) is an inflammation of the nasal tissue and paranasal sinuses lasting for at least 12 consecutive weeks and affecting up to 10-12% of population [1,2]. This debilitating disease significantly affects the quality of life and is characterized by enormous economic costs for the healthcare system. Morphologically, two forms of CRS have been reported. Chronic rhinosinusitis with nasal polyps (CRSwNP) is accompanied by the development of noncancerous soft outgrowths in the nasal and paranasal tissue. They are referred to as nasal polyps. Another form is called chronic rhinosinusitis without nasal polyps (CRSsNP). In its case, sinonasal inflammation occurs without the formation of polyps [3,4]. Despite numerous hypotheses offered to explain the etiopathogenesis of both forms of the disease, it still remains not fully elucidated. However, converging lines of evidence demonstrate that some factors contribute to the development of both CRSwNP and CRSsNP: genetic predisposition, defects of the innate immunity, features of sinonasal microbiota, epigenetic factors, abnormal mucociliary clearance, etc. [5-7]. Although both subtypes of CRS share common characteristics, CRSwNP and CRSsNP have numerous distinct features [8]. In particular, inflammation in CRSwNP has been reported to be Th2-mediated, whereas in CRSsNP Th1 cell subset prevails [9]. The search for dissimilarities between CRSsNP and CRSwNP may help find novel targets for therapeutic interventions specific for a particular subtype of CRS.

It has been reported that cell death is of crucial importance for the regulation of any kind of inflammation, including the sinonasal one [10]. One of cell death modes is apoptosis, which occurs in response to a wide variety of stimuli. However, pro-apoptotic signaling pathways are counterbalanced by anti-apoptotic signaling, and B-cell lymphoma 2 (Bcl-2) protein is a key anti-apoptotic regulator in the cells [11]. It provides pro-survival signaling by inhibiting the intrinsic mitochondrial apoptotic pathway via regulating mitochondrial outer membrane permeabilization (MOMP), which is believed to be a point-of-no-return in the intrinsic pathway [12,13]. MOMP results in the release of cytochrome c from mitochondria, followed

by the formation of apoptosome and activation of caspases [14]. Since Bcl-2 provides selective survival advantage for cells, it is of huge importance for neoplasms. It has been reported that overexpression of Bcl-2 is observed in a wide range of tumors and premalignant lesions, which allows suggesting its important role at early stages of tumorogenesis [15,16].

Features of Bcl-2 expression in cells of sinonasal mucosa and the role of this anti-apoptotic protein in CRS have been under investigation with some promising results reported recently [17]. We have hypothesized that Bcl-2 expression may be altered in CRS. Exploration of Bcl-2 involvement in the pathogenesis of CRS may contribute to the emergence of Bcl-2-targeted therapy. Thus, our study may shed extra light on the pathogenesis of CRS and to broaden our knowledge about the differences between CRSwNP and CRSsNP.

The aim of the research was to assess and compare circulating levels of Bcl-2 and features of its expression in the nasal mucosa of patients with CRSsNP and CRSwNP.

Material and methods

Patients

A total of 30 patients with CRS were recruited from the Kharkiv Regional Clinical Hospital (Kharkiv, Ukraine). Fifteen of them were diagnosed with CRSsNP (9 males and 6 females). Their age ranged from 21 to 60 years with the mean age of 37.44±4.21 years. In other 15 patients, the diagnosis of CRSwNP was verified (8 males and 7 females). Their age varied from 20 to 58 years of age. They mean age reached 34.98±3.41 years. The control group consisted of 15 healthy subjects (7 males and 8 females) of 19 to 53 years of age. These patients underwent a correction of deviated septum (septoplasty) under combined general and regional anesthesia. The mean age of this group was 31.85±4.02 years. The control subjects did not show any clinical signs of inflammation in the upper airways. ‘‘EPOS 2012: European Position Paper on Rhinosinusitis and NPs 2012” guidelines were used to verify the diagnosis [18].

The exclusion criteria were smoking, acute or exacerbation of chronic inflammatory diseases, cystic fibrosis, allergic diseases and asthma, administration of glucocorticoids at least 1 month prior to the collection of biological materials, pregnancy, and endocrine diseases.

Collection of blood samples and determination of circulating levels of Bcl-2 by ELISA

In order to assess the circulating level of Bcl-2, we collected blood samples immediately after hospitalization. Blood was used to prepare serum by centrifugation. Bcl-2 values in blood serum were determined by ELISA test using Human Bcl-2 Platinum ELISA Kits (eBioscience, Austria). The level of Bcl-2 was expressed in ng/ml.

Immunohistochemical study of Bcl-2 expression in the nasal tissue

Nasal tissue samples were obtained exclusively during surgery if there were indications for it. Briefly, formalin-fixed and paraffin-embedded tissue samples were obtained and used for immunohistochemical evaluation of Bcl-2 expression. Four-µm-thick sections of nasal tissues were immunostained with anti-Bcl-2 antibodies (Thermo Fischer Scientific, UK). Staining was completed with 3,3′-Diaminobenzidine (DAB) with the formation of brown coloration indicating Bcl-2 expression.

Bioethics

The revised Helsinki Declaration (2000) and “Bioethical Expertise of Preclinical and Other Scientific Researches Conducted on Animals” (Kyiv, 2006) were strictly followed. The experiment protocol was approved by the Ethical Committee of Kharkiv National Medical University (minutes No 6 dated June 6, 2018). Informed consent was carefully read and signed by all patients and control subjects enrolled in this study.

Statistical analysis

Numerical values of circulating Bcl-2 levels were analyzed with the help of the non-parametric Kruskal-Wallis test, since three unmatched groups were compared. As a post-hoc test, we used the Dunn’s multiple comparison test. Data are represented as the medians and interquartile ranges. Differences were considered statistically significant at $p < 0.05$. Statistical analysis was performed using GraphPad Prism 5.0 (GraphPad software, USA).

Results

Analysis of Bcl-2 expression in the nasal mucosa of control subjects showed that its expression was observed in some cells of the lamina propria and submucosa. Bcl-2 expression was also revealed in some nasal epithelial cells (NECs).

Figure 1 - Immunostaining for Bcl-2 in the nasal mucosa of a control individual. Brown staining shows Bcl-2 expression (red arrows). Some Bcl-2 positive cells are found in the lamina propria. 400x.

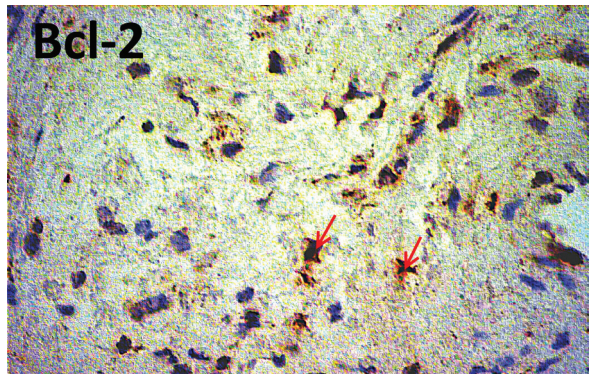


Figure 2 - Immunostaining for Bcl-2 in patients with CRSsNP. Strong staining is found in nasal epithelial cells. The number of Bcl-2-labelled nasal epitheliocytes is much higher compared with the control group (A, C, D; yellow arrows). Atrophic regions were characterized with a lower number of Bcl-2 positive cells in the lamina propria (D; red arrows). Bcl-2 expression was week in the glandular epithelial cells in regions with no signs of damage (B). In some regions, extracellular matrix cells strongly expressed Bcl-2 (B; red arrows). Figures 2a, 2c, 2d - 100x; Figure 2b – 400x.

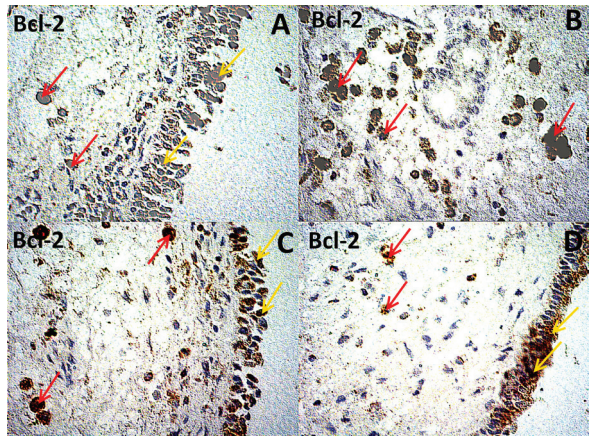


Figure 3 - Immunostaining for Bcl-2 in patients with CRSwNP. The amount of Bcl-2-positive cells in the lamina propria is visually higher than in the control group (A, B; red arrows). Strong Bcl-2 expression is observed in nasal epithelial cells that cover the polyps (B, C, D; yellow arrows). 400x

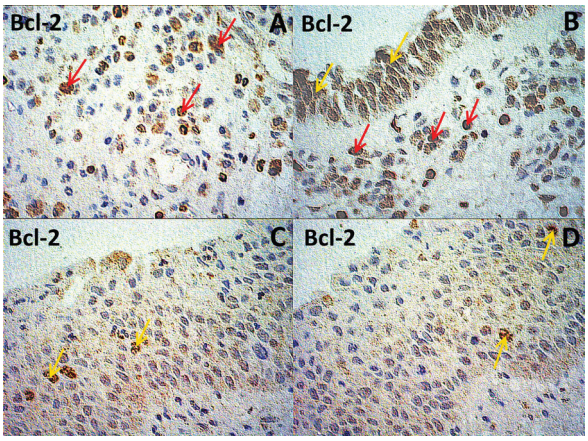
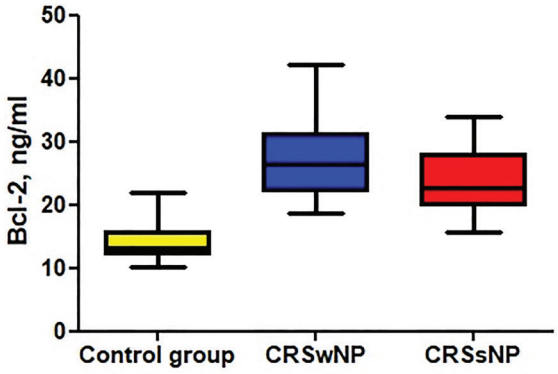


Figure 4 - Circulating levels of Bcl-2 in patients with CRSwNP and CRSsNP. Both forms of chronic rhinosinusitis are associated with statistically significant elevation of Bcl-2 in blood serum compared with healthy controls. Circulating Bcl-2 concentration increases 1.7–fold in CRSsNP ($p < 0.0001$), while in patients with CRSwNP its level is twice higher ($p < 0.0001$) compared with the control group. The statistical difference between Bcl-2 levels in blood serum in CRSsNP and CRSwP was insignificant ($p > 0.05$).



The mucosa of patients with CRSsNP was characterized by the presence of regions with either atrophic or regenerating epithelia. It is important to note that in the latter case the epithelial layer had several rows of NECs. This fact is of huge importance, since Bcl-2 was found to be significantly upregulated in NECs in atrophy. No or weak Bcl-2 expression was detected in the mucosal glandular epithelial cells in regions with no signs of damage. However, cells of the extracellular matrix (probably fibroblasts) were characterized by strong Bcl-2 expression.

NECs that cover polyps in patients with CRSwNP significantly express Bcl-2, excluding regions where excessive proliferation of NECs was observed. Under the epithelial layer in the extracellular matrix a higher number of Bcl-2-positive cells were found compared with the control group. They were either oval-shaped or spherical, i.e. these could be both fibroblasts and macrophages. Bcl-2 was overexpressed both in the lamina propria and NECs in patients with CRSwNP compared with control subjects.

The development of CRSwNP is accompanied by a more pronounced Bcl-2 expression in the epithelial layer that covers polyps and in their stroma than in the inflamed nasal mucosa of patients with CRSsNP.

All patients and control subjects were evaluated for Bcl-2 circulating concentrations on admission to the hospital. Serum levels of Bcl-2 in patients with both forms of CRS were found to be statistically significant ($p < 0.0001$) elevated compared with the control group (Figure 4). It is important to note that serum concentrations of Bcl-2 were twice elevated in CRSwNP compared with control subjects (26.33 [22.17; 31.12] ng / ml against 13.23 [12.14; 15.67] ng / ml in controls), while in patients with CRSsNP (22.67 [19.89; 27.90] ng / ml) this parameter was only 1.7-fold higher than in the control group. However, statistical analysis showed no significant difference ($p > 0.05$) between circulating Bcl-2 levels in patients with different morphological forms of CRS.

Discussion

It has been reported that NECs play an important role in inflammatory responses, which is not restricted to their passive barrier function. In addition to their barrier role, NECs release cytokines [19]. Thus, these cells are directly involved in the regulation of inflammatory response and their survival is important for maintaining nasal homeostasis during inflammation. The fate of NECs depends on the balance between numerous pro-apoptotic and anti-apoptotic signaling. There is strong evidence that CRS is associated with the activated NEC apoptosis [20]. We believe that the pattern of Bcl-2 expression in NECs of patients with CRSsNP, namely more pronounced overexpression of anti-apoptotic and pro-survival Bcl-2 protein in atrophic regions observed in this study, aims at providing the survival of remaining NECs to maintain the integrity of damaged epithelial lining.

However, overexpression of Bcl-2 in multi-row, hyperplastic epithelium covering nasal polyps in patients with CRSwNP may be involved in sustaining proliferating NECs. Our hypothesis is consistent with other data that support the involvement of Bcl-2 in sustaining inflammation-induced hyperplasia of airway epithelial cells [21-23]. Nevertheless, the role of Bcl-2 overexpression in CRSwNP seems to be more important in damaged mucosal areas than in the proliferating ones, evidenced by a higher upregulation of Bcl-2 in NECs in regions with signs of damage to epithelial lining. Since the strongest Bcl-2 immunostaining in this study (both for CRSsNP and CRSwNP) was observed in the NECs where the damage to epithelia was the most significant and weak expression within NECs was found in undamaged areas, we believe that its upregulation is compensatory. It increases the viability of cells, which is crucial for preventing the extra involvement of nasal microbiota to the inflammatory process due to the loss of epithelial barrier.

Our findings are consistent with experimental data provided by Suo et al who demonstrated that nasal inflammation in rats is accompanied by overexpression of Bcl-2 mRNA and Bcl-2 protein in mucosal epithelia [24].

We speculate that Bcl-2 elevation in blood serum of patients with CRS found in this study is due to its release from damaged tissues. This can be partially confirmed by the trend, albeit statistically insignificant, towards a more pronounced increase in circulating Bcl-2 in CRSwNP compared with CRSsNP against the background of its higher expression in the nasal mucosa. However, this hypothesis has to be tested experimentally.

Taken together, our findings suggest that chronic nasal and paranasal inflammation may affect Bcl-2, mostly in CRSwNP, which can be considered an adaptive response to prevent cell loss.

Intense surface epithelial cell proliferation has been reported earlier in CRSwNP, evidenced by overexpression of proliferation markers such as Ki67 and proliferating cell nuclear antigen (PCNA) in the nasal mucosa. According to Mumbuc et al, such NEC proliferation in nasal polyps may contribute to the development of inverted papillomas [25]. We believe that Bcl-2 overexpression found in our study may also be involved in this process, since Bcl-2 is known to facilitate oncogenesis by inhibiting apoptosis [26]. However, more studies are required to study the role of Bcl-2 oveexpression in the development of tumors in the nasal and paranasal tissues.

This study has some limitations. Firstly, we did not analyze cell-specific markers in the lamina propria, which prevented us from identifying accurately the cells with overexpressed Bcl-2. Secondly, we did not have to opportunity to evaluate Bcl-2 mRNA expression. Nevertheless, immunohistochemical studies provided clear evidence of Bcl-2 overexpression in the nasal mucosa of patients with CRS. Thirdly, we had a limited number of patients. However, we managed to obtain statistically significant results. Fourthly, we could not determine circulating levels of pro-apoptotic BAX protein to assess the BAX/Bcl-2 ratio.

Conclusions

Both CRSwNP and CRSsNP are associated with overexpression of anti-apoptotic Bcl-2 in NECs and stroma against the background of elevated circulating levels of Bcl-2 protein. In CRSwNP upregulation of Bcl-2 in the nasal mucosa is more pronounced than in CRSsNP, while concentrations in blood serum don't differ. The study encourages the more detailed research on evaluating diagnostic and therapeutic implications of Bcl-2 in CRS.

Disclosures: There is no conflict of interest for all authors.

Acknowledgements: None.

Funding: None.

References

1. Sedaghat AR. Chronic rhinosinusitis. *Am Fam Physician.* 2017;96(8):500-506.
2. DeConde AS, Soler ZM. Chronic rhinosinusitis: Epidemiology and burden of disease. *Am J Rhinol Allergy.* 2016;30(2):134-9. https://doi.org/10.2500/ajra.2016.30.4297
3. Dennis SK, Lam K, Luong A. A review of classification schemes for chronic rhinosinusitis with nasal polyposis endotypes. *Laryngoscope Investig Otolaryngol.* 2016;1(5):130–134. https://doi.org/10.1002/lio2.32
4. Bachert C, Zhang L, Gevaert P. Current and future treatment options for adult chronic rhinosinusitis: Focus on nasal polyposis. *J Allergy Clin Immunol.* 2015;136(6):1431-1440. https://doi.org/10.1016/j.jaci.2015.10.010
5. Kim JY, Kim DK, Yu MS, Cha MJ, Yu SL, Kang J. Role of epigenetics in the pathogenesis of chronic rhinosinusitis with nasal polyps. *Mol Med Rep.* 2018;17(1):1219–1227. https://doi.org/10.3892/mmr.2017.8001
6. Tan BK, Min JY, Hulse KE. Acquired immunity in chronic rhinosinusitis. *Curr Allergy Asthma Rep*. 2017;17(7):49. https://doi.org/10.1007/s11882-017-0715-0
7. Tan BK, Chandra RK, Pollak J, Kato A, Conley DB, Peters AT, et al. Incidence and associated premorbid diagnoses of patients with chronic rhinosinusitis. *J Allergy Clin Immunol.* 2013;131(5):1350-60. https://doi.org/10.1016/j.jaci.2013.02.002
8. Cho SW, Kim DW, Kim JW, Lee CH, Rhee CS. Classification of chronic rhinosinusitis according to a nasal polyp and tissue eosinophilia: limitation of current classification system for Asian population. *Asia Pac Allergy.* 2017;7(3):121–130. https://doi.org/10.5415/apallergy.2017.7.3.121
9. Stevens WW, Ocampo CJ, Berdnikovs S, Sakashita M, Mahdavinia M, Suh L, et al. Cytokines in chronic rhinosinusitis. Role in eosinophilia and aspirin-exacerbated respiratory disease. *Am J Respir Crit Care Med*. 2015;192(6):682-94. https://doi.org/10.1164/rccm.201412-2278oc
10. Yang Y, Jiang G, Zhang P, Fan J. Programmed cell death and its role in inflammation. *Mil Med Res.* 2015;2:12. https://doi.org/10.1186/s40779-015-0039-0
11. Opferman JT, Kothari A. Anti-apoptotic BCL-2 family members in development. *Cell Death Differ.* 2018;25(1):37–45. https://doi.org/10.1038/cdd.2017.170
12. Kale J, Osterlund EJ, Andrews DW. BCL-2 family proteins: changing partners in the dance towards death. *Cell Death Differ*. 2018;25(1):65–80. https://doi.org/10.1038/cdd.2017.186
13. Hata AN, Engelman JA, Faber AC. The BCL2 Family: Key Mediators of the Apoptotic Response to Targeted Anticancer Therapeutics. *Cancer Discov.* 2015;5(5):475–487. https://doi.org/10.1158/2159-8290.cd-15-0011
14. Kalkavan H, Green DR. MOMP, cell suicide as a BCL-2 family business. Cell Death Differ. 2018;25(1):46–55. https://doi.org/10.1038/cdd.2017.179
15. Arya V, Singh S, Daniel MJ. Clinicopathological correlation of Bcl-2 oncoprotein expression in oral precancer and cancer. *J Oral Biol Craniofac Res.* 2016;6(1):18–23. https://doi.org/10.1016/j.jobcr.2015.12.011
16. Shukla S, Dass J, Pujani M. p53 and bcl2 expression in malignant and premalignant lesions of uterine cervix and their correlation with human papilloma virus 16 and 18. *South Asian J Cancer.* 2014;3(1):48–53. https://doi.org/10.4103/2278-330x.126524
17. Morawska-Kochman M, Śmieszek A, Marcinkowska K, Marycz KM, Nelke K, Zub K, et al. Expression of Apoptosis-Related Biomarkers in Inflamed Nasal Sinus Epithelium of Patients with Chronic Rhinosinusitis with Nasal Polyps (CRSwNP)-Evaluation at mRNA and miRNA Levels. *Biomedicines.* 2022;10(6):1400. https://doi.org/10.3390/biomedicines10061400
18. Fokkens WJ, Lund VJ, Mullol J, Bachert C, Alobid I, Baroody F, et al. EPOS 2012: European position paper on rhinosinusitis and nasal polyps 2012. A summary for otorhinolaryngologists. *Rhinology*. 2012;50(1):1–12. https://doi.org/10.4193/Rhino12.000
19. Ozturk AB, Bayraktar R, Gogebakan B, Mumbuc S, Bayram H. Comparison of inflammatory cytokine release from nasal epithelial cells of non-atopic non-rhinitic, allergic rhinitic and polyp subjects and effects of diesel exhaust particles in vitro. *Allergol Immunopathol* (Madr). 2017;45(5):473-481. https://doi.org/10.1016/j.aller.2016.10.015

20. Basinski TM, Holzmann D, Eiwegger T, Zimmermann M, Klunker S, Meyer N, et al. Dual nature of T cell-epithelium interaction in chronic rhinosinusitis. *J Allergy Clin Immunol.* 2009;124(1):74-80.e1-8. https://doi.org/10.1016/j.jaci.2009.04.019
21. Chand HS, Harris JF, Tesfaigzi Y. IL-13 in LPS-induced inflammation causes Bcl-2 expression to sustain hyperplastic mucous cells. *Sci Rep*. 2018;8(1):436. https://doi.org/10.1038/s41598-017-18884-9
22. Chand HS, Harris JF, Mebratu Y, Chen Y, Wright PS, Randell SH, et al. Intracellular insulin-like growth factor-1 induces Bcl-2 expression in airway epithelial cells. *J Immunol.* 2012;188(9):4581-9. https://doi.org/10.4049/jimmunol.1102673
23. Harris JF, Fischer MJ, Hotchkiss JA, Monia BP, Randell SH, Harkema JR, et al. Bcl-2 sustains increased mucous and epithelial cell numbers in metaplastic airway epithelium. *Am J Respir Crit Care Med.* 2005;171:764–772. https://doi.org/10.1164/rccm.200408-1108OC
24. Suo L, Zhao C, An Y, Zhang X, Tao Z. Expression of Bcl-2 mRNA and Bcl-2 protein in nasal mucusa of rat in allergic rhinitis model. *Lin Chuang Er Bi Yan Hou Ke Za Zhi.* 2004;18(2):105-7 [in Chinese]
25. Mumbuc S, Karakok M, Baglam T, Karatas E, Durucu C, Kibar Y. Immunohistochemical analysis of PCNA, Ki67 and p53 in nasal polyposis and sinonasal inverted papillomas. *J Int Med Res.* 2007;35(2):237-41. https://doi.org/10.1177/147323000703500208
26. Campbell KJ, Tait SWG. Targeting BCL-2 regulated apoptosis in cancer. *Open Biol.* 2018;8(5):180002. https://doi.org/10.1098/rsob.180002