Original Article

May zonula occludens proteins regulate the pathogenesis of allergic rhinitis?

Özge Yılmaz[1], Esra Toprak Kanık[1], Ercan Pınar[2], Ahmet Türkeli[1], Elgin Türköz Uluer[3], Sevinç İnan[3], Hasan Yüksel[1]

[1]*Department of Pediatric Allergy and Pulmonology, Celal Bayar University Medical Faculty, Manisa, Turkey*
[2]*Department of Otorhinolaryngology, Atatürk Training and Research Hospital, Izmir, Turkey*
[3]*Department of Histology and Embryology, Celal Bayar University Medical Faculty, Manisa, Turkey*

ABSTRACT

Objectives: This study aims to investigate the expression pattern of zonula occludens (ZO) proteins, namely occludin, claudin-1, tricellulin, junctional adhesion molecules (JAM), and ZO-1, -2, and -3 in nasal mucosal biopsies of individuals with and without allergic sensitization.

Patients and Methods: Between August 2011 and August 2012, a total of 69 patients (38 males, 31 females; mean age 28.0 years, range, 18 to 61 years) who underwent surgery for nasal septum deviation were included in this cross-sectional study. All patients underwent skin prick test with environmental allergen mixtures. Cup forceps biopsy samples were obtained from the inferior turbinate during septoplasty. These samples were stained immunohistochemically for occludin, claudin-1, tricellulin, JAM, and ZOs. Staining intensity was graded semi-quantitatively using the H-Score.

Results: Of all patients, 14 were atopic. Occludin, claudin-1, and JAM scores were significantly lower in the mucosal samples from atopic patients, compared to the non-atopic patients (median 142.5 vs. 288, 153 vs. 296, and 156 vs. 312, respectively; $p<0.001$ for all). The ZO-1, -2, and -3 proteins were significantly lower in atopic patients ($p<0.001$ for all). The tricellulin, located at the intersection of three epithelial cells, was not significantly different between the two groups (208.5 vs. 195, respectively; $p=0.686$).

Conclusion: Expression of the structural proteins of ZO decreases in the upper airways of asymptomatic atopic patients. These findings indicate that ZO may be an important determinant of atopic sensitization and, therefore, may be a potential target in the treatment of allergic rhinitis.

Keywords: Allergic rhinitis, atopy, tricellulin, zonula occludens.

Airway epithelial barrier integrity and selective permeability related to the epithelial barrier function (EBF) and dysfunction (EBD) throughout the respiratory tract is the main focus of researches on pathogenesis of allergic inflammation and form the potential new therapeutic modality. Epithelial barrier is composed of three major components from apical to basal levels of intercellular adherence: tight junctions (TJs), adherent junctions (AJs), and desmosomes (D). Tight junctions, also called zonula occludens (ZO), located at the apical surface of epithelial barrier between the adjacent epithelial cells, determine solute

Received: May 23, 2019 **Accepted:** July 30, 2019 **Published online:** August 09, 2019

Correspondence: Özge Yılmaz, MD. Celal Bayar Üniversitesi Tıp Fakültesi Çocuk Alerji Kliniği, Solunum Birimi, 45030 Yunusemre, Manisa, Turkey.
e-mail: oyilmaz_76@hotmail.com

Doi: http://dx.doi.org/10.5606/Tr-ENT.2019.64936

Citation:
Yılmaz Ö, Toprak Kanık E, Pınar E, Türkeli A, Türköz Uluer E, İnan S, et al. May zonula occludens proteins regulate the pathogenesis of allergic rhinitis?. Tr-ENT 2019;29(2):100-106.

permeability and form the strongest and major component of mechanical EBF.[1] It has been demonstrated that both allergens and T helper 2 cytokines influence the integrity of epithelial TJs, thus the epithelial permeability.[2,3] In one of our previous studies, we demonstrated that TJ proteins decreased in the lower airway epithelium in animal models of asthma.[4] Similarly, epithelium and EBF are the first-line of defense in the upper airways, and ZO regulate the passage of antigens and other molecules through the paracellular space.[5]

Zonula occludens is composed of different proteins: integral membrane proteins that are occludin, claudins, and junctional adhesion molecules (JAMs) and peripheral membrane proteins that are called ZO-1, -2, and -3. Among these, occludin which is the first discovered protein of ZO junction, is the most sensitive marker of TJs. The JAMs belong to the immunoglobulin superfamily and are important for ZO assembly. The claudin family with more than 20 members participates in the formation of ZO strands. The ZO-1 and -2 participates in the polymerization of claudin molecules. Similarly, tricellulin found at the intersection of three epithelial cells contribute to the barrier structure and function.[5] Thus, ZO and JAM proteins are the most important proteins which influence the epithelial permeability and are expected to play an important role in the pathogenesis of allergic rhinitis (AR) by interaction of inhaled allergens with antigen-presenting cells and sub-mucosal immune system.

In the present study, we aimed to compare the expression pattern of ZO proteins, namely occludin, claudin-1, tricellulin, JAM, ZO-1, -2, and -3 in nasal mucosal biopsies of atopic and non-atopic individuals.

PATIENTS AND METHODS

This cross-sectional study was conducted at Yesilyurt Teaching Hospital between August 2011 and August 2012. A total of 69 patients (38 males, 31 females; mean age 28 years, range, 18 to 61 years) who underwent surgery for nasal septum deviation were included. *Exclusion criteria were as follows:* The use of topical or systemic anti-inflammatory treatment such as nasal steroids in the previous month and having an evidence of acute upper respiratory tract infection. A written informed consent was obtained from each patient. The study protocol was approved by the Celal Bayar University Institutional Review Board (Date: 22.06.2011/No. 210). The study was conducted in accordance with the principles of the Declaration of Helsinki.

All patients were interviewed by one of the researchers at enrollment before they underwent surgery. During this interview, age and gender of all the patients were recorded and they were questioned for the presence of upper respiratory tract infection in the previous month and use of nasal anti-inflammatory treatment. All patients underwent skin prick test with environmental allergen mixtures, as described below.

Cup forceps biopsy samples were obtained from the inferior turbinate from all patients during septoplasty. These nasal mucosa samples were put in formaldehyde solution for transfer to the Histology and Embryology Department.

Allergen skin prick test

Allergen skin prick test was performed in accordance with the European Academy of Allergy and Clinical Immunology (EAACI) guidelines.[6] Negative control, histamine as positive control, and allergen extracts were applied to the skin. Allergen mixtures consisted of dermatophagoides farinae, dermatophagoides pteronyssinus, grass pollen, Olea europeae pollen, and mold mixture extracts (Allergopharma Ltd., Reinbek, Germany).

Sample preparation for immunohistochemistry

Routine paraffin embedding procedures were performed. In brief, tissue samples were fixed in 10% formalin solution, dehydrated in a graded ethanol series, cleared in xylene, embedded in paraffin wax, and 5-μm-thick sections were cut. The tissue blocks were, then, chosen carefully for histological examination of the sections stained with hematoxylin and eosin (H&E, Hematoxylin acc. to Gill III, Cat. No. 1.05174, Eosin Y solution 0.5% alcoholic, Cat. No. 1.02439, Merck, Darmstadt, Germany).

Immunohistochemical staining of ZO proteins

For immunohistochemical staining, the sections were incubated at 60°C overnight and, then, held in xylene and rehydrated through a series of ethanol solutions. The sections were washed with distilled water and phosphate-buffered saline (PBS, P4417, Sigma-Aldrich, St. Louis, Mo, USA) for 10 min and, then, treated with 0.1 % trypsin (T7409, Sigma-Aldrich) at 37°C for 10 min and washed with PBS. The sections were delineated with a Dako PAP pen (S2002, Dako, Glostrup, Denmark) and incubated in a solution of 3% H2O2 for five min to inhibit the endogenous peroxidase activity.

After washing in PBS, the sections were incubated with non-immune serum for one h and were incubated with primary antibodies: rabbit anti-occludin polyclonal antibody (bs-1495R, Bioss, Massachusetts, USA), anti-claudin-1 antibody (sc-166338, Santa Cruz Biotechnology, Heidelberg, Germany), anti-ZO-1 antibody (bs-1329R, Bioss, Massachusetts, USA), anti-ZO-2 antibody (sc-11448, Santa Cruz Biotechnology, Heidelberg, Germany), anti-ZO3 (36-400, Invitrogen, Camarillo, CA, USA), anti-TSLP antibody (bs-2964R, Bioss, Massachusetts, USA), anti-tricellulin (488400, Invitrogen, Camarillo, CA, USA), and anti-JAM-A antibody (sc-25629, Santa Cruz Biotechnology, Heidelberg, Germany) 1:100 dilution overnight at 4°C in a humidity chamber.

The sections were washed three times, five min each with PBS, followed by incubation with biotinylated secondary antibody and, then, with streptavidin conjugated to horseradish peroxidase in PBS for 30 min each (Histostain-plus-Peroxidase-kit, 85-9043, Zymed, San Francisco, CA, USA). After washing three times with PBS, the sections were incubated with diaminobenzidine (DAB) for five min for immunostaining. After washing with distilled water, the sections were counterstained with the Mayer's hematoxylin and washed with alcohol and xylene.

The sections were mounted with Entellan® (Merck Co., Darmstadt, Germany) and were observed with an Olympus BX 40 bright-field microscope (Olympus, Tokyo, Japan). The presence of a red-brown precipitate indicated positive findings for the primary antibodies. The negative control samples were processed in an identical manner and the same type of immunoglobulin G was used as primary antibodies.

Interpretations of immuno-stained materials

Two observers, blinded to clinical information of the patients, evaluated the staining scores independently. Staining intensity was graded semi-quantitatively using the H-Score to compare the control and patient samples with the following equation:

H-Score= ∑ Pi (i+1), where i = intensity of staining with a value of (±), (+), (++) or (+++) (minimal, mild, moderate, or strong, respectively) and Pi is the percentage of cells stained with each intensity, varying between 0 and 100%.

Table 1. H-Scores of zonula occludens proteins in nasal mucosa samples of allergic and non-allergic patients

	Atopic (n=14)		Non-atopic (n=55)		
Zonula occludens proteins*	Median	25th to 75th percentile	Median	25th to 75th percentile	*p***
Occludin	142.5	120-156	288	272-312	<0.001
Claudin-1	153	135-165	296	280-312	<0.001
Tricellulin	208.5	180-240	195	180-240	0.686
Junctional adhesion molecule	156	144-168	312	296-320	<0.001
Zonula occludens-1	211.5	160-228	268	240-304	<0.001
Zonula occludens-2	154	130-195	192	154-204	0.036
Zonula occludens-3	142	130-156	240	195-268	<0.001

* H-scores are given in median (25th to 75th percentile); ** Mann-Whitney U test.

Statistical analysis

Statistical analysis was performed using the SPSS version 15.0 software (SPSS Inc., Chicago, IL, USA). Morphometric data of immunohistochemistry were expressed in median (25^{th} to 75^{th} percentile), while descriptive data were expressed in mean ± standard deviation (SD) or in number and frequency Continuous variables in atopic and non-atopic groups were compared using the Mann-Whitney U test. A *p* value of $p<0.05$ was considered statistically significant.

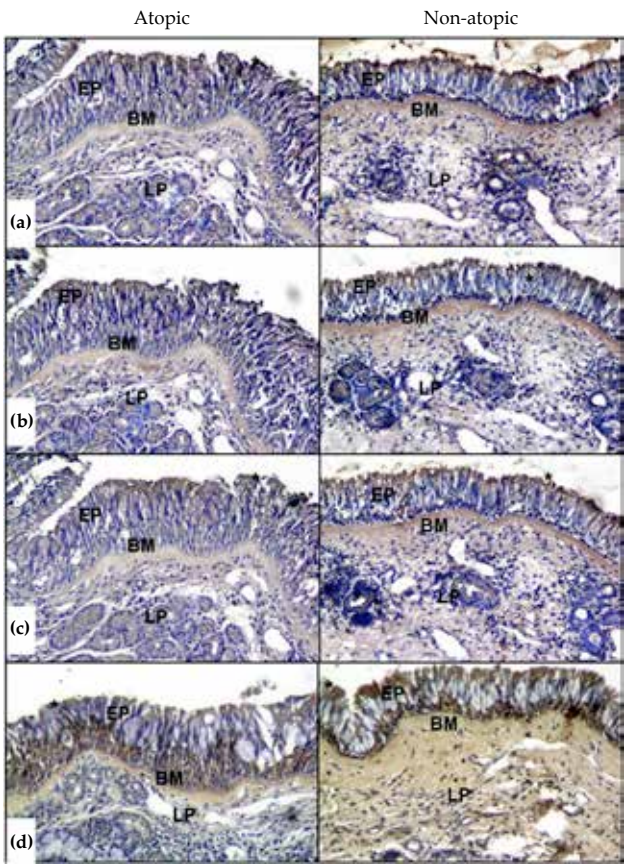

*Figure 1. Light microscopic sections of the nasal specimens from atopic and non-atopic patients, indirect immunohistochemical stained with **(a)** anti-occludin, **(b)** anti-claudin, **(c)** anti-JAM and **(d)** anti-tricellulin staining. Epithelium on BM and LP are visualized. Immunoreactivity (*) of the stained zonula occludens protein located at the apical part of the epithelium is demonstrated. There is a significant decrease in immunoreactivity for occludin, claudin and JAM, but not tricellulin, as calculated by the H-score.*
EP: Epithelium; BM: Basal membrane; LP: Lamina propria; JAM: Junctional adhesion molecules.

RESULTS

Of a total of 60 patients, 14 were atopic and 46 were non-atopic. There was no significant difference in the age and gender between atopic and non-atopic patients ($p=0.56$ and $p=0.37$, respectively).

Occludin scores were significantly lower in mucosal samples from atopic patients compared to the non-atopic patients (median 142.5 vs. 288, respectively; $p<0.001$). Similarly, claudin-1 H-scores were significantly lower in the atopic group (median 153 vs. 296, respectively; $p<0.001$) (Table 1, Figure 1).

In addition, ZO proteins were significantly lower in atopic patients. The median expression levels of ZO-1, -2, and -3 in the mucosa samples from atopic patients were 211.5, 154, and 142, respectively and 268, 192, and 240, respectively in non-atopic patients ($p<0.001$ for all) (Table 1, Figure 2).

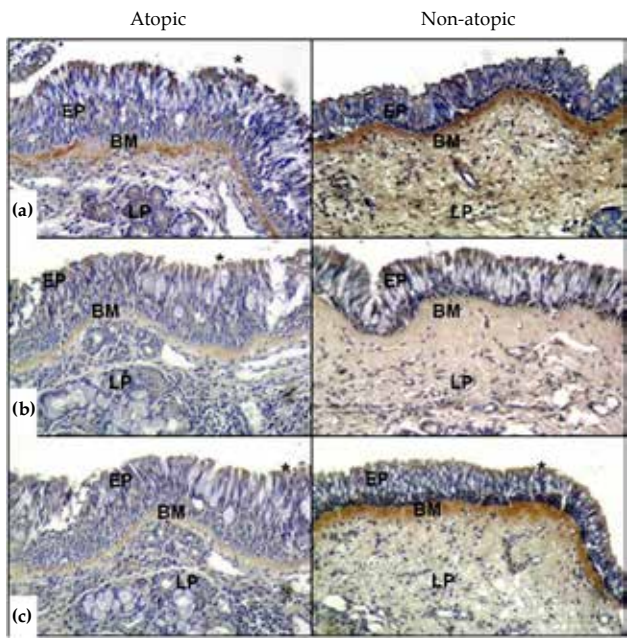

*Figure 2. Light microscopic sections of the nasal specimens from atopic and non-atopic patients, indirect immunohistochemical stained with **(a)** anti-ZO-1, **(b)** anti-ZO-2, **(c)** anti-ZO-3 staining. Epithelium on BM and LP are visualized. Immunoreactivity (*) of the stained zonula occludens protein located at the apical part of the epithelium is demonstrated.*
EP: Epithelium; BM: Basal membrane; LP: Lamina propria.

The expression of another ZO protein, JAM, was found to be 156 in the atopic group compared to the H-score of 312 in the non-atopic group ($p<0.001$) (Table 1, Figure 1).

The only ZO protein which was not significantly different among the two groups was tricellulin located at the intersection of three epithelial cells (208.5 vs. 195, respectively; $p=0.686$) (Table 1, Figure 1).

DISCUSSION

Zonula occludens is the most apically located inter-epithelial junction which is responsible to regulate paracellular antigen transfer, suggesting an important role in the pathogenesis of allergic polarization. In this study, we examined the expression of various proteins of this junction such as occludin, claudin-1, and ZO and demonstrated that all were lower in patients with atopic sensitization to inhalant allergens, except for tricellulin which plays a role in the intersection of three cell membranes.

Zonula occludens is a strictly selectively permeable interepithelial junction which is composed of heteropolymers of claudins, TJ-associated marvel proteins composed of occludin, tricellulin and marvelD3, as well as JAMs.[7] The defect in the barrier function of this structure has been proposed to play an important role in the initiation of the allergic cascade and disease.[3] The EBF is disrupted, leading to allergen sensitization and allergens with protease activities cleave ZO proteins, disrupting the barrier function, which contributes to allergic polarization.[8,9] It has been demonstrated that pollen allergens with protease activity can disrupte ZO proteins, occludin, claudin-1, and E-cadherin.[10] Expression of ZO proteins such as occludin, claudin, and ZO-1 are under epigenetic control, such as histone deacetylases. A recent therapeutic research has discovered that histone deacetylase inhibitors increase the expression of these proteins, thereby, improving the epithelial barrier integrity.[11] Thus, in the present study, we investigated the expression of different proteins of ZO in the upper airways of atopic and non-atopic individuals.

Occludin functions to tighten the strand component of ZO.[7] It has been demonstrated that modulation of occludin expression in epithelium modulates permeability.[12] In addition, a previous study with bilayer culture model of human bronchial epithelial-derived cells showed that increased expression of occludin messenger ribonucleic acid (mRNA) and protein was associated with increased trans-epithelial resistance.[13] In recent study, it was reported that the decrease in occludin expression in patients with house dust mite-induced AR, was associated with decreased barrier function, indicating the role of occludin in the epithelial barrier formation.[14] On the other hand, occludin along with tricellulin plays a role in the barrier formation only in the presence of inflammation, which makes it an important component of epithelial barrier in the presence of allergic inflammation.[15] Our finding of decreased occludin expression in nasal mucosa of atopic individuals compared to the non-atopic ones also supports the role of decreased interepithelial ZO function in development of atopic sensitization.

Claudins are the major proteins which determine permeability of ZO and some contributes to the sealing, while some contributes to the pore formation of the junction.[7] Claudins interact with the claudin molecules in the adjoining epithelial cells and connect to cellular actin via the scaffold proteins, such as ZO-1 and -2. Moreover, claudin expression is influenced by JAM-A.[16] Claudins are a large family of proteins and different claudins have different functions. Claudin-1 that we assessed in this study is necessary for the formation and stability of ZO.[15] Based on our study results, we found that claudin-1 expression decreased in nasal epithelium in atopic patients.

Junctional adhesion molecules are type 1 transmembrane proteins that are the members of the immunoglobulin superfamily. These molecules are expressed on endothelium, contributing to the formation of ZO.[6] The JAM-A interacts with cytoplasmic scaffold proteins such as ZO-1 and -2 to aid assembly of claudins into the ZO.[17] The findings of our study demonstrated decreased expression of JAM-A expression in nasal epithelial samples of atopic individuals, similar to many other proteins of ZO.

Tricellulin is a component of the ZO which is present between the corners of three epithelial cells and is considered the potentially weak point of the barrier.[7] In several studies, tricellulin has been shown to be expressed in human nasal mucosa at the tricellular and bicellular borders in the ZO structure. In addition, expression of this protein in epithelial cells is more stable than other bicellular border elements of ZO.[18] Our study results demonstrated that expression of tricellulin was not significantly different among atopic and non-atopic nasal mucosa samples. There are potential explanations for this. First, tricellulin does not play a significant role in the decrease of the EBF in allergic pathogenesis. Second, expression of tricellulin is more stable, compared to the other proteins. Additionally, tricellulin plays a role in barrier formation only in the presence of inflammation, as described above, and this might have been one of the reasons that we found no significant difference between the two groups, considering that these patients were not symptomatic at the time of biopsy.[15]

Zonula occludens-1, -2, and -3 are among the plaque proteins and located in the cytoplasm beneath the ZO. They function in nuclear signaling and membrane scaffolding similar to other plaque proteins.[7] In addition, these proteins link the intracellular domains of the ZO components to the actin-binding proteins.[19] The ZO-1 expression, similar to occludin expression, decreased in patients with AR, and this decrease was associated with an increased epithelial permeability. Also, steroids have been shown to improve this expression, while IL-4 may impair.[14] Our findings are supportive of these findings in demonstration of decreased ZO-1, -2, and -3 expression in AR patients.

The findings of this study provide an overall insight to the expression of all the major proteins of the ZO in the upper airway of atopic and non-atopic individuals and, thus, valuable in providing an overall view. However, the main limitation is the lack of functional evaluation, such as trans-epithelial resistance. This needs to be investigated in further researches.

In conclusion, the expression of many structural proteins of ZO, such as occludin, claudin, JAM, and ZO proteins, decreases in the upper airways of atopic patients. This finding suggests the current hypothesis of the role of EBD in allergy pathogenesis. Nonetheless, these findings warrant further research investigating the role of future therapeutic agents which target these proteins of ZO in the treatment of upper airway allergic disease.

Declaration of conflicting interests

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

Funding

This research was funded by Celal Bayar University Research Fund.

REFERENCES

1. Förster C. Tight junctions and the modulation of barrier function in disease. Histochem Cell Biol 2008;130:55-70.
2. Henriquez OA, Den Beste K, Hoddeson EK, Parkos CA, Nusrat A, Wise SK. House dust mite allergen Der p 1 effects on sinonasal epithelial tight junctions. Int Forum Allergy Rhinol 2013;3:630-5.
3. Wise SK, Laury AM, Katz EH, Den Beste KA, Parkos CA, Nusrat A. Interleukin-4 and interleukin-13 compromise the sinonasal epithelial barrier and perturb intercellular junction protein expression. Int Forum Allergy Rhinol 2014;4:361-70.
4. Yuksel H, Yilmaz O, Karaman M, Firinci F, Turkeli A, Kanik ET, et al. Vascular endothelial growth factor antagonism restores epithelial barrier dysfunction via affecting zonula occludens proteins. Exp Ther Med 2015;10:362-8.
5. Kojima T, Go M, Takano K, Kurose M, Ohkuni T, Koizumi J, et al. Regulation of tight junctions in upper airway epithelium. Biomed Res Int 2013;2013:947072.
6. Dreborg S, Frew A. EAACI subcommittee on skin tests. Position paper. Allergen standardization and skin tests. Allergy 1993;48:49-61.
7. Brandner JM, Schulzke JD. Hereditary barrier-related diseases involving the tight junction: lessons from skin and intestine. Cell Tissue Res 2015;360:723-48.
8. Tai HY, Tam MF, Chou H, Peng HJ, Su SN, Perng DW, et al. Pen ch 13 allergen induces secretion of mediators and degradation of occludin protein of human lung epithelial cells. Allergy 2006;61:382-8.
9. Wan H, Winton HL, Soeller C, Taylor GW, Gruenert DC, Thompson PJ, et al. The transmembrane protein occludin of epithelial tight junctions is a functional target for serine peptidases from faecal pellets of Dermatophagoides pteronyssinus. Clin Exp Allergy 2001;31:279-94.
10. Vinhas R, Cortes L, Cardoso I, Mendes VM, Manadas B, Todo-Bom A, et al. Pollen proteases compromise the airway epithelial barrier through degradation of transmembrane adhesion proteins and lung bioactive peptides. Allergy 2011;66:1088-98.

11. Wawrzyniak P, Wawrzyniak M, Wanke K, Sokolowska M, Bendelja K, Rückert B, et al. Regulation of bronchial epithelial barrier integrity by type 2 cytokines and histone deacetylases in asthmatic patients. J Allergy Clin Immunol 2017;139:93-103.
12. Everett RS, Vanhook MK, Barozzi N, Toth I, Johnson LG. Specific modulation of airway epithelial tight junctions by apical application of an occludin peptide. Mol Pharmacol 2006;69:492-500.
13. Chowdhury F, Howat WJ, Phillips GJ, Lackie PM. Interactions between endothelial cells and epithelial cells in a combined cell model of airway mucosa: effects on tight junction permeability. Exp Lung Res 2010;36:1-11.
14. Steelant B, Farré R, Wawrzyniak P, Belmans J, Dekimpe E, Vanheel H, et al. Impaired barrier function in patients with house dust mite-induced allergic rhinitis is accompanied by decreased occludin and zonula occludens-1 expression. J Allergy Clin Immunol 2016;137:1043-53.
15. Georas SN, Rezaee F. Epithelial barrier function: at the front line of asthma immunology and allergic airway inflammation. J Allergy Clin Immunol 2014;134:509-20.
16. Overgaard CE, Mitchell LA, Koval M. Roles for claudins in alveolar epithelial barrier function. Ann N Y Acad Sci 2012;1257:167-74.
17. Schlingmann B, Molina SA, Koval M. Claudins: Gatekeepers of lung epithelial function. Semin Cell Dev Biol 2015;42:47-57.
18. Ohkuni T, Kojima T, Ogasawara N, Masaki T, Ninomiya T, Kikuchi S, et al. Expression and localization of tricellulin in human nasal epithelial cells in vivo and in vitro. Med Mol Morphol 2009;42:204-11.
19. Rezaee F, Georas SN. Breaking barriers. New insights into airway epithelial barrier function in health and disease. Am J Respir Cell Mol Biol 2014;50:857-69.

