ANALYSIS OF TLRs GENES EXPRESSION AND *DEFB1* POLYMORPHISMS ASSOCIATION IN CHILDREN WITH BRONCHIAL ASTHMA

Zaitseva MA[1]✉, Bragvadze BG[1], Svitich OA[1,2], Namazova-Baranova LS[3], Gankovskaya LV[1]

[1] Department of Immunology, Biomedical Faculty,
Pirogov Russian National Research Medical University, Moscow, Russia

[2] Laboratory of Molecular Immunology,
Mechnikov Research Institute of Vaccines and Sera, Moscow, Russia

[3] Scientific Center of Children's Health, Moscow, Russia

Bronchial asthma (BA) is one of the most common respiratory system diseases. The role of innate immunity components in the pathogenesis of bronchial asthma is studied widely, with particular focus on the antimicrobial peptides. Those include beta defensins that prevent pathogen intrusion into the respiratory tract mucosa, the most active of such pathogens being β-defensin-1 (human beta defensin-1, HBD-1) encoded by the *DEFB1* gene. We studied the association of three single nucleotide polymorphisms in the 5'- untranslated region of the gene, namely, *rs11362*, *rs1799946* and *rs1200972*, with bronchial asthma in children. We also evaluated gene expression of toll-like receptors *TLR2*, *TLR4* and *TLR9*. The experimental group included 48 patients of 3 to 7 years of age with BA and 70 healthy children. The AA genotype of the *rs11362* polymorphism and the CC genotype of the *rs1799946* polymorphism were reliably associated with the disease, while the GG genotype of the *rs1799946* polymorphism and the AA genotype of the *rs120097* polymorphism were found protective. Also, the AA genotype of the *rs11362* polymorphism was associated with the reduced expression of *DEFB1*, the human beta defensin-1 encoding gene, while the AG genotype was associated with its increased expression. In children with BA, *TLR2* expression increased 19.5 times in comparison with the controls; *TLR9* expression increased 9.5 times, while *TLR4* expression increased 8.3 times.

Keywords: bronchial asthma, human beta defensin-1, toll-like receptors, *DEFB1*, *TLR2*, *TLR4*, *TLR9*, single nucleotide polymorphism, polymorphic marker

✉ **Correspondence should be addressed:** Margarita Zaitseva
ul. Svyazistov, d.10, kv. 68, Krasnoznamensk, Moscow oblast, Russia, 143090; astice@list.ru

Recieved: 14.06.2016 **Accepted:** 23.06.2016

АНАЛИЗ ЭКСПРЕССИИ ГЕНОВ TLRs И АССОЦИАЦИИ ПОЛИМОРФИЗМОВ ГЕНА *DEFB1* У ДЕТЕЙ С БРОНХИАЛЬНОЙ АСТМОЙ

М. А. Зайцева[1]✉, Б. Г. Брагвадзе[1], О. А. Свитич[1,2], Л. С. Намазова-Баранова[3], Л. В. Ганковская[1]

[1] Кафедра иммунологии, медико-биологический факультет,
Российский национальный исследовательский медицинский университет имени Н. И. Пирогова, Москва

[2] Лаборатория молекулярной иммунологии,
Научно-исследовательский институт вакцин и сывороток имени И. И. Мечникова, Москва

[3] Научный центр здоровья детей, Москва

Бронхиальная астма (БА) — одно из наиболее распространенных заболеваний органов дыхания. Активно исследуется роль элементов врожденного иммунитета в патогенезе бронхиальной астмы, в частности, противомикробных пептидов. К ним относятся β-дефенсины, предотвращающие вторжение патогенов в слизистую оболочку респираторного тракта, наиболее активным из которых является β-дефенсин-1 (human beta defensin-1, HBD-1), кодируемый геном *DEFB1*. В исследовании была изучена ассоциация трех однонуклеотидных полиморфизмов в 5'-нетранслируемой области гена — *rs11362*, *rs1799946* и *rs1200972* — с бронхиальной астмой у детей. Также оценивали уровень экспрессии генов toll-подобных рецепторов *TLR2*, *TLR4* и *TLR9*. В опытную группу включили 48 пациентов в возрасте 3–7 лет с БА и 70 здоровых детей. Генотип АА полиморфизма *rs11362* и генотип СС полиморфизма *rs1799946* достоверно ассоциированы с заболеванием, а генотип GG полиморфизма *rs1799946* и генотип АА полиморфизма *rs120097* являются протективными. Генотип АА полиморфизма *rs11362* также ассоциирован с пониженной экспрессией, а генотип AG — с повышенной экспрессией гена β-дефенсина-1 *DEFB1*. У детей с БА выявили повышение уровня экспрессии гена *TLR2* в сравнении с контрольной группой в 19,5 раз, *TLR9* — в 9,5 раз, *TLR4* — в 8,3 раза.

Ключевые слова: бронхиальная астма, β-дефенсин-1, toll-подобные рецепторы, *DEFB1*, *TLR2*, *TLR4*, *TLR9*, однонуклеотидный полиморфизм, полиморфный маркер

✉ **Для корреспонденции:** Зайцева Маргарита Алексеевна
143090, Московская область, г. Краснознаменск, ул. Связистов, д. 10, кв. 68; astice@list.ru

Статья получена: : 14.06.2016 **Статья принята в печать:** 23.06.2016

Bronchial asthma (BA) is a chronic inflammatory disease of the upper respiratory tract accompanied by bronchial obstruction and hyperresponsiveness. It manifests itself through shortness of breath, wheezing, coughing and choking episodes. It's prevalence is increasing fast in high- and middle-income countries. According to the Russian Respiratory Society, asthma affects as many as 10 million people in Russia; over 20 % of them are children [1].

It was observed that respiratory infections have a more severe course in patients with BA than in healthy individuals [2, 3]. Acute infections of the upper respiratory tract frequently trigger asthma exacerbations: about 85 % of exacerbations in children and 50 % in adults are caused by respiratory viruses [2]. Pathogens damage ciliated epithelium of the respiratory tract mucosa making it more vulnerable for allergens and toxins and maintaining bronchial hyperresponsiveness. Acute exacerbations can be life-threatening regardless of the BA grade of severity [3].

A lot of contemporary research studies focus on the in-depth analysis of BA pathogenesis, including the role of innate immunity components. Of particular interest is a new class of effector molecules (antimicrobial peptides), such as β-defensins. Antimicrobial properties of the latter are due to the electrostatic interactions between negatively charged surface components of the bacterial membrane, such as lipopolysaccharides of gram-negative bacteria and teichoic or lipoteichoic acids of gram-positive bacteria, and a positively charged β-defensin molecule. Critical concentrations of β-defensin on the surface of the target cell trigger pore formation in its membrane followed by cell lysis. Besides, β-defensins exhibit immunoregulatory activity, participating in chemotaxis and adaptive immunity activation, inducing dendritic cell maturation, etc. [4].

The key role in protecting respiratory tract mucosa is played by human β-defensin-1 (HBD-1) synthesized by epithelial cells [5]. β-defensin-1 is encoded by the *DEFB1* gene located on the short arm of chromosome 8 (8p23.1) in a highly polymorphic cluster. Due to gene mutations, its expression can be decreased; in turn, insufficient secretion of β-defensins facilitates bacterial adhesion to and invasion of the mucosa and triggers inflammation [6, 7].

Toll-like receptors (TLRs) of the epithelial cells of the respiratory tract mucosa are another important element of the innate immunity. They recognize pathogen-associated molecular patterns (PAMP) of microorganisms and their metabolic byproducts, transmit the signal into the cell and boost leukocyte functional activity, increase pro-inflammatory cytokine and interferon gene expression. The majority of bacterial and viral pathogens are recognized by TLR2, TLR4, and TLR9 that can activate the local mucosal immunity in the respiratory tract.

The aim of this work was to give a comprehensive assessment of the innate immunity markers, namely, the level of expression of the *TLR2*, *TLR4*, *TLR9* and *DEFB1* genes, and to study the association of some single nucleotide polymorphisms (SNPs) in the 5'-untranslated region of the *DEFB1* gene with bronchial asthma in children. Three SNPs were studied: *rs1799946*, *rs1800972* and *rs11362*. They are associated with HIV infection and infections caused by *Candida albicans*, *Pseudomonas aeruginosa* and other microorganisms and sepsis development [8, 9], but there are no reports on their association with allergies.

METHODS

The study was carried out in patients of the Rehabilitation

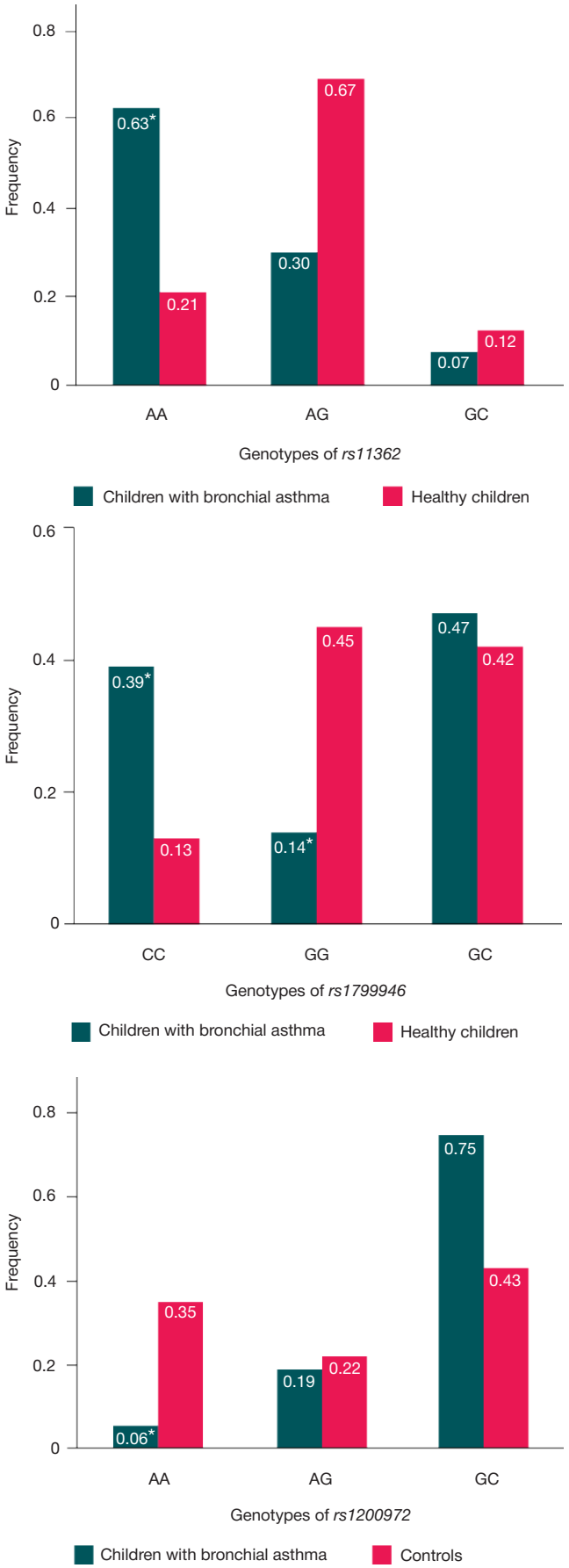

Fig.1. Genotype frequency distribution of single nucleotide polymorphisms *rs11362*, *rs1799946* and *rs1200972* in the *DEFB1* gene in asthmatic children (* — p <0.05, compared to the controls)

Care Unit for Children with Allergies and Respiratory Tract Diseases of the Scientific Center of Children's Health (Moscow). The study included 48 asthmatic children aged 3–7 years. The control group included 70 children without respiratory conditions, inflammatory and infectious diseases and allergies. Nasal scrapes were collected at the time of BA exacerbations that were accompanied by an acute respiratory infection.

For DNA extraction, the AmpliPRIME Ribo-sorb kit (InterLabService, Russia) was used. The real time PCR assay was conducted using SYBR Green I PCR Kit by Syntol, Russia. Data were statistically processed in MO Excel 2007 with Statistica 10.0 software (StatSoft, USA). Pearson's chi squared and Odd Ratio were computed (OR >1 indicated genotype association with BA, OR <1 indicated a genotype protective against BA) [10].

Expression of the *DEFB1*, *TLR2*, *TLR4* and *TLR9* genes was compared to β-actin gene expression. For RNA extraction, the AmpliPRIME Ribo-sorb kit was used. Reverse transcription was performed with the OT-1 kit by Syntol, real time PCR was carried out using the SYBR Green I PCR Kit. For statistical processing, Mann-Whitney test was applied ($p < 0.05$).

The study was approved by the Ethics Committee of Pirogov Russian National Research Medical University. Participants' parents gave their informed consent.

RESULTS

Genotype frequency distribution of *rs1799946*, *rs1800972* and *rs11362* polymorphisms of the *DEFB1* gene showed that the following genotypes are associated with the risk of asthma in children: AA of *rs11362* and CC of *rs1799946*, while genotypes GG and AA of *rs1799946* and *rs1200972* are protective against BA (fig. 1). Distribution of *DEFB1* alleles was alike in both groups.

Expression of the *DEFB1* gene was 3.5 times lower in children with bronchial asthma, compared to healthy children (fig. 2). A single nucleotide polymorphism in the promoter region can affect the level of gene expression and the amount of the produced protein. We divided patients of the experimental group into 3 subgroups based on the level of β-defensin-1 expression: low expression (>10,000 times higher than β-actin expression), moderate (10,000–30,000 times higher than β-actin expression) and high (>30, 000 times higher than β-actin expression). It was found that AG genotype of *rs11362* polymorphism is associated with the increased level of β-defensin-1 expression in epithelial cells. For example, the frequency of AG genotype in subgroups with high and low expression of *DEFB1* was 0.67 and 0.30, respectively. Genotype AA is associated with reduced expression of the β-defensin gene. Other genotypes of the studied polymorphisms are not associated with changes in the β-defensin gene expression. Patients with bronchial asthma showed a 19.5 times increased expression of the *TLR2* gene compared to the controls; *TLR9* expression was 9.5 times higher, *TLR4* expression was 8.3 times higher. Results are presented in the table below.

DISCUSSION

The obtained data can indicate that chronic inflammation of the bronchial mucosa in asthmatic children is partially associated with mutations in the 5'-untranslated region of *DEFB1*. Having assessed the expression of *DEFB1*, *TLR2*, *TLR4* and *TLR9*, we made a supposition that β-defensin-1 participates in BA pathogenesis. Antimicrobial peptides produced by epithelial cells of the respiratory tract mucosa prevent the invasion of pathogens into the mucosa. However, if antimicrobial peptide production is decreased and bacterial load is high, pathogens are recognized by TLRs of epithelial cells, which triggers a cascade of pro-inflammatory reactions, including synthesis of IL-1β, IL-6 and IL-12, INF-α, INF-β and chemokynes. Besides, through the activation of epithelial TLRs, production of thymic stromal lymphopoietin and IL-33 is induced. The latter interact with dendritic cells, boost activity of CD40 and CD80 costimulatory molecules, regulate Th0 and Th2 differentiation, and come into contact with mast cells inducing their degranulation [11, 12]. It facilitates the development of chronic inflammation (fig. 3).

CONCLUSIONS

Genotype AA of *rs11362* and genotype CC of *rs1799946* polymorphisms located in the 5'-untranslated region of the

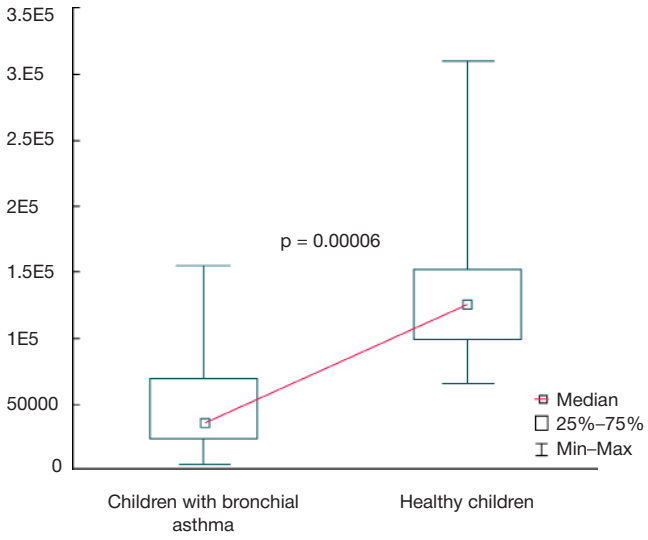

Fig.2. Expression of *DEFB1* in the epithelial cells of nasal mucosa in children with bronchial asthma and healthy children (compared to the expression of the β-actin gene).

Expression of *TLR2*, *TLR4*, *TLR9* and *DEFB1* in the epithelial cells of nasal mucosa in children with bronchial asthma and healthy children

Gene	Children with bronchial asthma	Healthy children
TLR2	130 000 (27 000; 270 000)	6 500 (5 000; 7 000)
TLR4	150 (30; 450)	18 (17; 120)
TLR9	30 000 (5 000; 50 000)	3 300 (3 000; 4 500)
DEFB1	35 000 (25 000; 68 000)	125 000 (100 000; 150 000)

Note: data are presented as a median (25 %; 75 %) of cDNA copies per 1 million copies of cDNA of β-actin, $p < 0.05$

DEFB1 gene are reliably associated with bronchial asthma in children. Genotype GG of *rs1799946* and genotype AA of *rs120097* polymorphisms are protective against asthma. Genotype AA of *rs11362* polymorphism is also associated with the reduced expression of the β-defensin-1 gene *DEFB1*. Thus, some mutations in *DEFB1* cause imbalances in the nasal mucosal innate immunity resulting in frequent exacerbations of BA in the setting of respiratory infections.

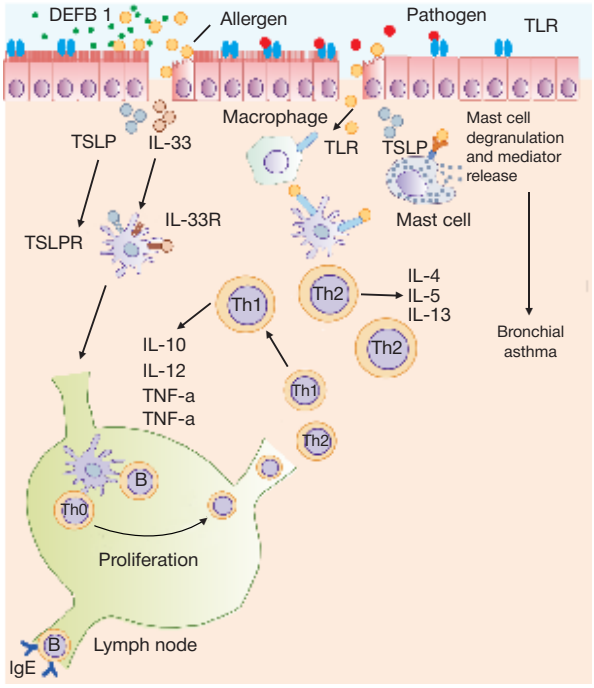

Fig. 3. Mechanism of chronic inflammatory response in bronchial asthma

When an allergen first comes in contact with the mucosa, it damages the epithelial barrier, which triggers cytokine secretion, including TSLP, IL-25 and IL-33. In the presence of cytokines, the secondary contact with the allergen induces maturation of dendritic cells (DCs) and their migration to lymph nodes, where DCs in collaboration with major histocompatibility complex molecules (MHC-II) "report" the allergen to Th0 cells (T-helpers) initiating their proliferation and differentiation into Th2 cells. Activated allergen-specific Th2s produce a wide range of cytokines: IL-4 (increases proliferation of B-lymphocytes and serves as their growth and differentiation factor, induces B-cell class switching to IgE), IL-5 (stimulates proliferation of eosinophils and facilitates release of the major basic protein) and IL-9 (activates mast cells). Allergen-specific IgE antibodies bind to high-affinity receptors (FceRI) of mast cells and basophils and to low-affinity receptors (FceRII) of eosinophils and macrophages. In case of a repeated allergen invasion, IgE of mast cell membranes binds to the allergen, thus ensuring its degranulation. Not all pathogens can be eliminated by antimicrobial peptides if bacterial load is high. Part of them is recognized by epithelial TLRs of the respiratory tract sustaining bronchial inflammation.

References

1. Chuchalin AG, Ospel'nikova TP, Osipova GL, Lizogub NV, Gervazieva VB, Krivitskaya VZ, et al. Rol' respiratornykh infektsii v obostreniyakh bronkhial'noi astmy. Pul'monologiya. 2007; (5): 32–4. Russian.
2. Wark RA, Johnston SL, Bucchieri F, Powell R, Puddicombe S, Laza-Stanca V, et al. Asthmatic bronchial epithelial cells have a deficient innate immune response to infection with rhinovirus. J Exp Med. 2005 Mar 21; 201 (6): 937–47.
3. Cirillo I, Marseglia G, Klersy C, Ciprandi G. Allergic patients have more numerous and prolonged respiratory infections than nonallergic subjects. Allergy. 2007 Sep. 62 (9): 1087–90.
4. Koval'chuk LV, Gankovskaya LV, Meshkova RYa. Klinicheskaya immunologiya i allergologiya s osnovami obshchei immunologii. Moscow: GEOTAR-Media; 2011. p. 148–65. Russian.
5. Svitich OA, Gankovskaya LV, Rakhmanova IV, Zaitseva IA, Gankovskii VA. Assotsiatsiya polimorfnykh markerov, lokalizovannykh v 5'-netransliruemoi oblasti gena β-defensina DEFB1, s gipertrofiei adenoidnykh vegetatsii. Vestnik RGMU. 2012; (3): 59–62. Russian.
6. Noutsios GT, Floros J. Childhood asthma: causes, risks, and protective factors; a role of innate immunity. Swiss Med Wkly. 2014 Dec 24; 144: w14036.
7. Arslan F, Babakurban ST, Erbek SS, Sahin FI, Terzi YK. Chronic tonsillitis is not associated with beta defensing 1 gene polymorphisms in Turkish population. Int J Pediatr Otorhinolaryngol. 2015 Apr; 79 (4): 557–60. doi: 10.1016/j.ijporl.2015.01.028. Epub 2015 Jan 30.
8. Milanese M, Segat L, Pontillo A, Arraes LC, de Lima Filho JL, Crovella S. DEFB1 gene polymorphisms and increased risk of HIV-1 infection in Brazilian children. AIDS. 2006 Aug 1; 20 (12): 1673–5.
9. Gankovskaya OA, Bakhareva IV, Gankovskaya LV, Zverev VV. Assotsiatsiya polimorfnykh markerov G(-20)A, C(-44)G i G(-52)A gena DEFB1 s razvitiem prezhdevremennykh rodov i vnutriutrobnym infitsirovaniem ploda. Russ J Immunol. 2011; 5 (1): 26–33. Russian.
10. Glantz S. [Primer of biostatistics]. Moscow: Praktika, 1999. 459 p. Russian.
11. Spann KM, Baturcam E, Schagen J, Jones C, Straub CP, Preston FM, et al. Viral and host factors determine innate immune responses in airway epithelial cells from children with wheeze and atopy. Thorax. 2014 Oct. 69 (10): 918–25.
12. Islam SA, Luster AD. T cell homing to epithelial barriers in allergic disease. Nat Med. 2012 May 4; 18 (5): 705–15.

Литература

1. Чучалин А. Г., Оспельникова Т. П., Осипова Г. Л., Лизогуб Н. В., Гервазиева В. Б., Кривицкая В. З. и др. Роль респираторных инфекций в обострениях бронхиальной астмы. Пульмонология. 2007; (5): 32–4.
2. Wark PA, Johnston SL, Bucchieri F, Powell R, Puddicombe S, Laza-Stanca V, et al. Asthmatic bronchial epithelial cells have a deficient innate immune response to infection with rhinovirus. J Exp Med. 2005 Mar 21; 201 (6): 937–47.
3. Cirillo I, Marseglia G, Klersy C, Ciprandi G. Allergic patients have more numerous and prolonged respiratory infections than nonallergic subjects. Allergy. 2007 Sep. 62 (9): 1087–90.
4. Ковальчук Л. В., Ганковская Л. В., Мешкова Р. Я. Клиническая иммунология и аллергология с основами общей иммунологии. М.: ГЭОТАР-Медиа; 2011. с. 148–65.
5. Свитич О. А., Ганковская Л. В., Рахманова И. В., Зайцева И. А., Ганковский В. А. Ассоциация полиморфных маркеров, локализованных в 5'-нетранслируемой области гена β-дефенсина DEFB1, с гипертрофией аденоидных вегетаций. Вестн. РГМУ. 2012; (3): 59–62.
6. Noutsios GT, Floros J. Childhood asthma: causes, risks, and protective factors; a role of innate immunity. Swiss Med Wkly. 2014 Dec 24; 144: w14036.
7. Arslan F, Babakurban ST, Erbek SS, Sahin FI, Terzi YK. Chronic tonsillitis is not associated with beta defensin 1 gene polymorphisms in Turkish population. Int J Pediatr Otorhinolaryngol. 2015 Apr; 79 (4): 557–60. doi: 10.1016/j.ijporl.2015.01.028. Epub 2015 Jan 30.
8. Milanese M, Segat L, Pontillo A, Arraes LC, de Lima Filho JL, Crovella S. DEFB1 gene polymorphisms and increased risk of HIV-1 infection in Brazilian children. AIDS. 2006 Aug 1; 20 (12): 1673–5.
9. Ганковская О. А., Бахарева И. В., Ганковская Л. В., Зве-

рев В. В. Ассоциация полиморфных маркеров G(-20)A, C(-44) G и G(-52)A гена DEFB1 с развитием преждевременных родов и внутриутробным инфицированием плода. Рос. иммунол. журн. 2011; 5 (1): 26–33.
10. Гланц С. Медико-биологическая статистика. М.: Практика, 1999. 459 с.
11. Spann KM, Baturcam E, Schagen J, Jones C, Straub CP, Preston FM, et al. Viral and host factors determine innate immune responses in airway epithelial cells from children with wheeze and atopy. Thorax. 2014 Oct. 69 (10): 918–25.
12. Islam SA, Luster AD. T cell homing to epithelial barriers in allergic disease. Nat Med. 2012 May 4; 18 (5): 705–15.