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The association of rs4696480 polymorphism in *TLR2* gene and *Staphylococcus aureus* skin colonization in children with atopic dermatitis

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Topicality. Up to 90% of patients with atopic dermatitis are colonized with *S. aureus,* with *S. aureus* predominance being unique to atopic dermatitis. *TLR2* play a role in the presentation of *S. aureus* antigens in a course of atopic dermatits.
Purpose — to investigate the association of rs4696480 polymorphism in *TLR2* gene and *S. aureus* skin colonization.
Materials and methods. The study included 101 patients with eczema and 84 healthy children. Skin swab cultures were taken. Subjects were classified as carriers if the cultures were positive, while those with culture found to be negative were classified as non-carriers. Genotyping for *TLR2* rs4696480 was performed by using Real-time PCR.
Results. We determined the prevalence of *S. aureus* carriage in a cohort study of atopic dermatitis patients in Ukraine. Skin culture for the presence of *S. aureus* was performed in 82 patients: 45.1% children had positive culture for *S. aureus,* 54.9% had a negative result. SCORing Atopic Dermatitis (SCORAD) index was significantly higher in *S. aureus* caarriers ($p<0.001$). There was no difference in genotype distribution among patients and control group (OR=1.021 (95% CI 0.507–2.054) for AT genotype, OR=0.880 (95% CI 0.398–1.947) for TT genotype, $p>0.05$). AA genotype was significantly more frequent among *S. aureus* carriers (OR=2.745 (95% CI 0.865–8.708) for AT genotype, OR=7.000 (95% CI 1.852–26.462) for TT genotype. To our knowledge, the association of T16934A (rs4696480) and *S. aureus* colonization of lesion skin in children with atopic dermatitis have not been studied before.
Conclusions. AA variant of *TLR2* rs4696480 polymorphism predisposes to *S. aureus* colonization of skin in children with atopic dermatits.
The research was carried out in accordance with the principles of the Helsinki Declaration. The study protocol was approved by the Local Ethics Committee of the participating institution. The informed consent of the patient was obtained for conducting the studies.
No conflict of interests was declared by the author.
Keywords: children, atopic dermatitis, Single Nucleotide Polymorphism SNP, *TLR2, S. aureus.*

Асоціація поліморфізму rs4696480 гена *TLR2* та колонізації шкіри *Staphylococcus aureus* у дітей з атопічним дерматитом

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Актуальність. До 90% пацієнтів з атопічним дерматитом колонізовані *S. aureus,* причому переважання *S. aureus* є характерним лише для атопічного дерматиту. *TLR2* відіграють роль у презентації антигенів *S. aureus* у перебігу атопічного дерматиту.
Мета — вивчити зв'язок поліморфізму rs4696480 у гені *TLR2* та колонізації шкіри *S. aureus.*
Матеріали та методи. До дослідження залучено 101 хворого на екзему та 84 здорові дитини. Взято посів мазків шкіри. Хворих класифіковано як носіїв, якщо культури були позитивними, а тих, у кого культура виявилася негативною, — як неносіїв. Генотипування для *TLR2* rs4696480 проведено за допомогою полімеразної ланцюгової реакції в реальному часі.
Результати. Визначено поширеність носійства *S. aureus* у когортному дослідженні пацієнтів з атопічним дерматитом в Україні. Посів шкіри на наявність *S. aureus* проведено у 82 пацієнтів: 45,1% пацієнтів мали позитивний посів на *S. aureus,* 54,9% — негативний. Бал SCORing Atopic Dermatitis (SCORAD) був достовірно вищим у носіїв *S. aureus* ($p<0,001$). Не було різниці в розподілі генотипу між пацієнтами та контрольною групою (ВШ=1,021 (95% ДІ: 0,507–2,054) для АТ-генотипу, ВШ=0,880 (95% ДІ: 0,398–1,947) для ТТ-генотипу, $p>0,05$). АА-генотип достовірно частіше зустрічався серед носіїв *S. aureus* (ВШ=2,745 (95% ДІ: 0,865–8,708) для АТ-генотипу, ВШ=7,000 (95% ДІ: 1,852–26,462) для ТТ-генотипу). Наскільки нам відомо, асоціація T16934A (rs4696480) та *S. aureus* колонізації ураженої шкіри у дітей з атопічним дерматитом раніше не досліджувалася.
Висновки. АА-варіант поліморфізму *TLR2* rs4696480 схиляє до колонізації шкіри *S. aureus* у дітей з атопічним дерматитом.
Дослідження виконано відповідно до принципів Гельсінської декларації. Протокол дослідження ухвалено Локальним етичним комітетом зазначеної в роботі установи. На проведення досліджень отримано інформовану згоду батьків дітей.
Автор заявляє про відсутність конфлікту інтересів.
Ключові слова: діти, атопічний дерматит, однонуклеотидний поліморфізм SNP, *TLR2, S. aureus.*

Introduction

Atopic dermatitis (AD) is a chronic skin condition caused by multiple genetic, immune and environmental factors [11]. AD patients have skin barrier dysfunction, which is demonstrated by increased trans-epidermal water loss, increase of pH, reduced stratum corneum hydration, and altered microbiota. The skin microbiome is strongly associated with pathogenesis of AD with an over growth of *Staphylococcus aureus (S. aureus)* at the infected lesions. This may be due to the virulence factors and ability to evade the cutaneous immunity of patients with AD. *S. aureus* fibronectin has a special affinity for type 2 inflammation [3,13]. In addition, *S. aureus* produces enterotoxins — superantigens, that are known to impair the skin barrier and induce T-helper 2 (TH2) inflammation [3]. Increased

percutaneous sensitization from microbial products produces a vicious cycle stimulating host immunity with resulting AD symptoms [4,13].

Toll-like receptors (TLRs) play an important role in activating the immune system by regulating the production of antimicrobial peptides and inflammatory cytokines. TLR2 are involved in the innate immune response to gram-positive bacteria, a kind of immune sensor for components of the cell wall of gram-positive bacteria. The colonization of *S. aureus* on lesional skin of AD patients resulted in enhanced expression of Th2 cytokines, such as interleukin (IL)-4, IL-13, and thymic stromal lymphopoietin (TSLP) [5]. When incubated with *S. aureus*-derived peptidoglycan, TLR2 induced chemokine (C-C motif) ligand 5, 17 and 22 (CCL5, CCL17, and CCL22) production in Langerhans cells (LC) from AD patients [5]. This suggests that TLR2-mediated chemokine production may be a crucial process that protects against skin bacterial infection but, on the other hand, it can contribute to the development of AD. Futhermore, increased levels of chemokines, which regulate the immune response by attracting leukocytes, lead to the infiltration of skin by immune cells, such as eosinophils, T cells, mast cells, monocytes, and LC. So, increased serum levels of chemokines are closely related to the disease severity of AD [9].

Polymorphisms in TLR2 impair the body's immune response, which is associated with the development of bacterial infection. M. Żukowski et al. studied the frequency of TLR2 R753Q (rs5743708) and T16934A (rs4696480) polymorphisms in patients to determine the association between polymorphisms and the *S. aureus* nasal carriage, but no correlation was found for rs4696480 polymorphism [15]. As TLR2 plays a role in the presentation of *S. aureus* antigens in a course of AD, and has been shown to be associated with worsening of AD [8], we decided to investigate the association of rs4696480 polymorphism and *S. aureus* skin colonization in AD patients.

Purpose of the study — to investigate the association of rs4696480 polymorphism in TLR2 gene and *S. aureus* skin colonization for the potential identification of the risk group among children with AD regarding the development of *S. aureus* infection and the use of further therapy.

Materials and methods

The study included patients with AD (n=101); aged 6 month to 18 years, median 6 [3;10] from department of allergy at Kyiv City Children Clinical Hospital No.2 (group AD) and 84 children aged 12 month to 18 years old (6 [5;9]) without allergic disease at the time of examination or according to history data (control group). This study was approved by the ethical committee of the Bogomolets National Medical University; all patients / parents of affected children gave informed consent to participate.

Outcome measures. The diagnosis of AD was established according to the criteria of Hanifin & Rajka [14], by the patient's history. Clinical parameters of patients included age, gender, age of onset and severity of eczema. Scoring of AD severity and skin sampling to determine *S. aureus* were performed at the same visit.

Assessment of atopic dermatitis severity. The severity of AD was assessed using SCORing Atopic Dermatitis (SCORAD) index. A SCORAD <25 indicated mild AD, a SCORAD between 25 and 50 indicated moderate AD, and a SCORAD >50 (with a maximum index of 103) indicated severe AD [1].

Skin culture. Skin swabs were taken by wiping the skin with a sterile cotton swab for 5 seconds on the flexural (antecubital fossa) surface of the hand in the affected area of skin in children with AD. Swabs with the selected material were immersed in a test tube with Amies transport medium and transported to the laboratory. Blood agar (CA) and yolk-salt agar (CSA) were inoculated from the swab. The plates with the material were incubated in a thermostat at 37°C for 24 hours. In the absence of visible colonies, incubation was continued for another 24 hours under the same conditions. Gram-stained smears were prepared from the growing colonies. For further study, colonies were selected, which, according to the results of bacterioscopy, were formed by Gr + cocci. These colonies were then tested for catalase (the ability to break down hydrogen peroxide to form water and gaseous oxygen). Subsequent identification of catalase-positive colonies was performed on a Vitek2compact bacteriological analyzer. Subjects were classified as carriers if the cultures were positive, while those with culture found to be negative were classified as non-carriers.

Selection of Single Nucleotide Polymorphism (SNP). An SNP ***TLR2*** rs4696480 (A>T), which was located in intron region, was selected for genotyping, as it can affect expression of ***TLR2*** and is reported to be common in European populations.

DNA extraction. Buccal epithelium was taken by using buccal brushes with the following

Table 1

Characteristics of the group AD

Parameters	*S. aureus* carriers (n=37)	*S. aureus* non-carriers (n=45)	P
Age [mediana]	5 (2.5;8)	6 (4;12)	>0.05
Male / Female, n (%)	19 (51.4) / 18 (48.6)	22 (48.9) / 23 (51.1)	>0.05
SCORAD, mean	48.9	29.1	
Genotype			
AA	14	6	1.00
AT	17	20	2.745 (95% CI 0.865–8.708)
TT	6	18	7.000 (95% CI 1.852–26.462)
Dominant model			
AA	14	6	1.00
AT+TT	23	38	3.855 (95% CI 1.299–11.438)
Recessive model			
AA+AT	31	26	1.00
TT	6	18	3.577 (95% CI 1.238–10.333)

freezing of samples and their storage at -20°C. DNA for genotyping was extracted from the samples by using NeoPrep 100 DNA (Neogen, Ukraine) according to manufacturer's protocol. The concentration of total DNA was determined by using a NanoDrop spectrophotometer ND1000 (NanoDrop Technologies Inc., USA).

qPCR Genotyping. Amplification reactions were performed by using a 7500 Fast Real-time PCR System («Applied Biosystems», USA) in a final reaction volume of 20 µl, which contained 2X TaqMan Universal Master Mix («Applied Biosystems», USA), assay C__27994607_10 and the template DNA. The thermal cycling conditions involved a denaturation step at 95°C for 20 s, followed by 40 cycles of amplification at 95°C for 3 s and 60°C for 30 s. Analysis of the data was carried out with 7500 Fast Real-Time PCR Software. The primer sequences of rs4696480 TLR2 polymorphism were follows: For TLR2-F 5' AACAGAAATTTATCCATTCATGGTT 3', Rev TLR2-R 5' AGCAGTTTATTGTGAGAATGAGTTT 3' (https://www.ncbi.nlm.nih.gov/SNP/).

Statistical analysis. Since the distribution of most of the sample characteristics differed from the Gaussian (normal) distribution, the statistical sample was heterogeneous, and therefore non-parametric statistical methods were used. Quantitative data for each of the study groups were presented as median — Me [QI; QIII], categorical (dichotomous qualitative) variables — as the frequency of each of the values (n) and the percentage (%) in the group. Mann—Whitney U-test was used to compare differences between SCORAD in *S. aureus* carriers and non-carriers.

Statistical processing was performed using EZR (Easy R) software version 1.32 (graphical interface R (version 2.13.0)).

SNPAnalyzer (web-based software) was used to examine Hardy—Weinberg equilibrium. χ^2 test was performed to investigate if there was any difference in the frequency of the genotype and the allele between the group AD and the healthy control group.

Results

101 children between 6 months and 18 years old of age were investigated in our study. The average age of children in the main group was 6 [3;10] years, including 52 boys and 49 girls. Skin culture for the presence of *S. aureus* was performed in 82 patients of the group AD. Among them 37 (45.1%) patients had positive culture for *S. aureus*, 45 (54.9%) patients had a negative result. There was no difference in age or sex distribution among *S. aureus* carriers and non-carriers. SCORAD was significantly higher in *S. aureus* carriers (p<0.001) (Table 1).

Genotyping. We found that SNP was in Hardy—Weinberg equilibrium. There was no difference in genotype distribution among patients and control group (OR=1.021 (95% CI 0.507–2.054) for AT variant, OR=0.880 (95% CI 0.398–1.947) for TT variant, p>0.05) (Table 2).

We investigated the association of the TLR2 rs4696480 polymorphism with *S. aureus* colonization. AA genotype was significantly more frequent among patients with *S. aureus* positive culture (OR=2.745 (0.865–8.708) for AT genotype, OR=7.000 (95% CI 1.852–26.462) for TT genotype) (Table 1).

Table 2

The destribution of rs4696480 polymorphisms in *TLR2* gene in group AD and control group

Genotype	Group AD	Control group	OR (CI)	p
AA	27	23		
AT	46	40	1.021 (95% CI 0.507–2.054)	>0.05
TT	28	21	0.880 (95% CI 0.398–1.947)	>0.05
Dominant model				
AA	27	23	1.00	
AT+TT	74	61	0.968 (95% CI 0.505–1.856)	>0.05
Recessive model				
AA+AT	73	63	1.00	
TT	28	21	0.869 (95% CI 0.450–1.679)	>0.05

There were statistically significant differences in the distribution of genotypes in patients with *S. aureus* colonization of the skin and among non-carriers (χ^2=8.905; p=0.012). AA genotype rs4696480 in the TLR2 gene predisposes to *S. aureus* carriage or infection, while the T allele seems to have a protective value.

Studies demonstrated that the progression and severity of AD are related to the ability of *S. aureus* to penetrate through skin barrier, stimulating the chronic inflammation in skin [3]. Up to 90% of patients with AD are colonized with *S. aureus,* with *S. aureus* predominance being unique to AD. We determined the prevalence of *S. aureus* carriage in a cohort study of AD patients in Ukraine. Skin culture of 82 patients with AD was performed. 45.1% participants of our study were colonized with *S. aureus.* Meta-analysis of 95 observational studies of culture-based methods reported that the prevalence of *S. aureus* carriage by patients with AD was 70% on lesional skin compared with 39% on nonlesional skin or healthy control skin within the same patient [10]. The rate of *S. aureus* colonization in this meta-analysis was related severity of AD [10].

Proinflammatory staphylococcal lipoproteins induce TSLP expression in primary human keratinocytes in a TLR2/TLR6-dependent manner, identifying another possible mechanism how *S. aureus* induces a Th2 response [12]. M. Żukowski et al. [15] aimed to investigate the frequencies of TLR2 gene polymorphisms R753Q (rs5743708) and T16934A (rs4696480) in cardiac surgery patients and to explore the association between the polymorphisms and the nasal carriage of *S. aureus* in patients undergoing cardiac surgery operations, but no correlation was found for rs4696480. Polymorphism rs4696480 was studied in AD individuals, with conflicting data have been received. Our study did not reveal difference in genotype distribution among patients and control group. To our knowledge, the association of T16934A (rs4696480) and *S. aureus* colonization of lesion skin in children with AD have not been studied before. Our data demonstrated, that AA genotype rs4696480 in the TLR2 gene is significantly associated with a higher risk of *S. aureus* carriage in children with AD, while the T allele seems to have a protective value. Undoubtedly, many factors, both environmental factors and the immune status of the host, play a crucial role in determining the skin or infection of the *S. aureus* carrier.

Conclusions

TLR2 rs4696480 polymorphism was not found to play a role in the development of atopic dermatitis in children in Ukraine. However, the AA variant of TLR2 rs4696480 polymorphism is significantly associated with a higher risk of *S. aureus* colonization of the skin in children with atopic dermatitis, thus possibly predisposing to a more severe disease phenotype. Carriers of variant AA may be considered as a risk group for the development of *S. aureus* infection and potentially subject to the use of further therapy. At that time, the T allele can play a protective role. The role of other predisposing factors, such as disease severity and host immune status, in *S. aureus* infection and carriage must also be considered.

Further research perspectives. There remains a need for further study of the exact mechanisms by which pattern recognition receptors, and TLR2 in particular, and their polymorphisms influence the recognition of microbial antigens and disrupt the normal immune response to them. In particular, there is a need to conduct such research for

S. aureus colonization of the skin in children with atopic dermatitis.

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