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ПРОФИЛИ ЭКСПРЕССИИ ГЕНОВ ФАКТОРОВ ВРОЖДЕННОГО ИММУНИТЕТА У ПАЦИЕНТОВ С АТОПИЧЕСКИМ ДЕРМАТИТОМ

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Резюме. Атопический дерматит – многофакторное генетически детерминированное воспалительное заболевание кожи, характеризующееся зудом, хроническим течением, возрастными особенностями локализации и морфологии очагов поражения. Патогенез атопического дерматита сложен и включает эпигенетические изменения, вовлеченные в геномную адаптацию, реакции иммунного ответа и дисфункцию эпителиального барьера, которые в совокупности запускают развитие этого заболевания. Целью данного исследования является определение уровня экспрессии генов *IL4*, *IL13*, *IL33*, *TLR2*, *TLR9* у пациентов с атопическим дерматитом.

Таргетные гены для дальнейшей оценки экспрессии были выбраны в соответствии с нашими предыдущими результатами полногеномного исследования метилирования ДНК. Нами были определены сигнальные пути с дифференциально метилированными генами, которые, скорее всего, имеют место в патогенезе атопического дерматита. Поэтому мы исследовали уровни экспрессии генов *IL4*, *IL13*, *IL33*, *TLR2*, *TLR9* в коже, мононуклеарных клетках периферической крови, а также в цельной крови с помощью ПЦР-РВ у 55 детей и 26 здоровых людей, а также у 50 пациентов старшего возраста. Статистический анализ проводился с использованием H-критерия Краскела–Уоллиса и U-критерия Манна–Уитни.

Анализ экспрессии генов показал, что в образцах кожи уровень экспрессия *TLR9* и *IL4* был в 12 раз ниже ($p < 0,0001$, $p < 0,0005$) в пораженной коже; а в случае *TLR2* – в 6 раз ($p < 0,01$); результаты для мононуклеарных клеток крови отличались, и уровни экспрессии для тех же цитокинов были значительно выше до лечения. Мы также обнаружили, что эти различия были сильно выражены в старшей возрастной группе (12-18 лет). Изучение экспрессии гена *IL33* в образцах цельной крови у

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взрослых пациентов показало, что его уровень достоверно выше у больных со среднетяжелой формой АтД. Кроме того, мы пришли к выводу, что локально в пораженной коже может доминировать воспалительный иммунный ответ; в мононуклеарных клетках, по-видимому, имеет место Th2-иммунный ответ.

Для понимания патогенеза атопического дерматита необходимо учитывать действие иммунологических факторов, а также связь между ними. Описанные гены и их белковые продукты могут являться потенциальными лекарственными мишенями, а также способствовать формированию тактики ведения пациентов с атопической патологией.

Ключевые слова: атопический дерматит, цитокины, Toll-подобные рецепторы, метилирование, врожденный иммунитет, экспрессия генов

INNATE IMMUNE FACTOR GENE EXPRESSION PROFILES IN PATIENTS WITH ATOPIC DERMATITIS

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Abstract. Atopic dermatitis is a multifactorial genetically determined inflammatory skin disease characterized by itching, chronically relapsing dermatitis, age-related features of localization and morphology of lesions. The pathogenesis of atopic dermatitis is complex and includes epigenetic alterations, involved in the genomic adaptation, immune response reactions and dysfunction of the epithelial barrier that together trigger the development of atopic dermatitis. The aim of this study is to detect the expression level for *IL4*, *IL13*, *IL33*, *TLR2*, *TLR9* genes in the biological materials of atopic patients.

The targeted genes for further expression evaluation were selected according to our previous findings on genome-wide methylation study. We detected the cascades with the differentially methylated genes that are most likely to take place in atopic dermatitis. Thus, we investigated expression levels for the *IL4*, *IL13*, *IL33*, *TLR2*, *TLR9* genes in the skin, peripheral blood mononuclear cells and whole blood cells using RT-PCR on 55 pediatric patients and 26 healthy volunteers, and on 50 adult patients. Statistical analysis was performed with the use of Kruskal–Wallis H test and Mann–Whitney U test. Targeted expression analysis revealed that in the skin samples the expression of *TLR9* and *IL4* was 12 times significantly lower ($p < 0.0001$, $p < 0.0005$) in the lesional skin; and there was a 6-fold decrease in case of *TLR2* ($p < 0.01$). The results for blood mononuclear cells differed and expression levels for most of the assessed targets were significantly higher before treatment. We have also found out that those differences were strongly pronounced especially in an elder age group (12-18 y.o.). Studying the *IL33* gene expression in the whole blood samples of adults revealed that its level was significantly higher in case of patients with moderate form of AD. Besides, we concluded that locally in the affected skin inflammatory immune response may dominate; in the mononuclear cells Th2 immune response apparently takes place. New insights on immunological markers and links among them may shed a light on atopic dermatitis pathogenic mechanisms. The detected molecules could play role as potential therapeutic targets and form a management approach for patients with atopic dermatitis.

Keywords: atopic dermatitis, cytokines, Toll-like receptors, methylation, innate immunity, gene expression

Introduction

Atopic dermatitis (AD) is a multifactorial genetically determined inflammatory skin disease characterized by itching, chronically relapsing dermatitis, age-related features of localization and morphology of lesions. Frequently the onset of AD occurs in childhood, and in some cases may become a chronic lifelong disease. The prevalence of the disease among the child population reaches 20%, among the adult population about 3% worldwide [7].

The pathogenesis of atopic dermatitis is complex and includes dysfunction of the epithelial barrier (gene mutations as well, e.g. *FLG* gene), epigenetic alterations, involved in the genomic adaptation, and immune response reactions. In most cases of AD, Th2 immune response dominates. However, depending on

the course of the disease, Th17 and Th22 responses may also take place [1]. In moderate-to-severe course of AD, an increase in the number of T and B cells is observed both at the systemic level in the blood cells and locally in the skin [3]. Depending on specific cytokines' presence and expression of their genes, it is possible to determine the exact phase of the AD pathogenesis.

Besides the cytokine release, some innate immune structures could play an important role as triggers of the AD development. AD is often complicated by recurrent bacterial or viral infection. In this case, pattern recognition receptors (PRRs) that recognize certain microbial molecules (known as PAMPs) become activated and trigger innate immune responses. Thus, gene polymorphisms of some PRRs (for example) TLRs are involved in the pathogenesis of various autoimmune and inflammatory diseases, including AD.

The aim of this study is to detect the expression level for *IL4*, *IL13*, *IL33*, *TLR2*, *TLR9* genes in the biological materials of atopic patients.

Materials and methods

The study was conducted in accordance with the WMA Declaration of Helsinki – Ethical Principles for Medical Research Involving Human Subjects, 2013. The study was approved by the local ethics committee at the Mechnikov RIVS. All patients signed a voluntary informed consent to participate in the study.

Fifty-five pediatric patients from 6 to 18 years of age and 50 adult patients from 18 to 60 years old with the verified diagnosis of atopic dermatitis were enrolled in the study. According to the SCORAD score we divided patients into two groups: moderate, SCORAD 25-50 (n = 38 children; n = 26 adults) and severe, SCORAD ≥ 50 (n = 17 children; n = 24 adults) forms. Biological materials such as biopsies of the affected skin and blood samples were taken from patients in the Medical Research Center for Children's Health; 26 healthy volunteers formed a control group. Whole blood samples were taken from adult patients in the Clinic for Skin and Venereal Diseases named after V. A. Rakhmanov.

The targeted genes for further expression evaluation were selected according to our previous findings on genome-wide methylation study. After the enrichment analysis we got canonical signaling pathways, including IRAK-TRAF6-NFκB cascade that may be triggered by different types of TLRs and influence the production of cytokines; and the IgE-dependent cascade that also have an effect on cytokine profile.

RNA was extracted from PMBCs of patients and healthy donors using Extract RNA reagent as per the manufacturer (Evrogen, RF). Total RNA from whole blood samples from adult patients was extracted using the AmpliPRIME RIBO-sorb kit (NextBio, Russia), rRT-PCR was performed using the SYBR Green Syntol kit (Syntol, RF), and oligonucleotide primers for *TLR2*, *TLR9*, *IL4*, *IL13*, *IL33*, and *ACTB* genes were synthesized by Syntol (Syntol, RF). The reaction was carried out under the following conditions: 1 cycle at 95 °C for 5 min; 40 cycles at 95 °C for 15 s and 60 °C (or 58 °C) for 50 s; melting. Beta-actin was used as the reference gene for the analysis of target genes. The $2^{(- c(t))}$ method was used for analysis of the obtained data. Statistical analysis was performed with the use of Kruskal-Wallis H test and Mann–Whitney U test.

Results and discussion

The first set of questions aimed to *IL4* and *IL13* cytokine expression profiles in the PBMCs. All results considered to be significant at the $p \leq 0.05$ levels. It must be mentioned that all significant differences were found in case of adolescents (12-18 years of age). Figure 1 provides an example of the experimental data on *IL4* gene expression. There was a significant increase of *IL4* relative expression level (median for elder group of pediatric patients = 1717.3, median for control group = 0.4, $p < 0.0001$; median for patients of 6-12 y.o. = 29.4). Similar results were obtained regarding *IL13* gene: median for adolescent group = 380.0, median for control group = 0.3, $p < 0.0001$; median for younger group of patients was 3.5).

Further analysis revealed the expression levels for *TLR2* and *TLR9* genes in the PBMCs. Comparing the data for patients of childhood years and healthy controls again did not show any significant results. Figure 2 shows an example of the results on *TLR9* gene expression. It can be seen from the graph that there was an increase of this gene expression in comparison with control group ($p < 0.0001$). Medians for 6-12 age group, 12-18 age group, and healthy donors were equal 82.7, 1478.6, and 1.3 respectively. Similar situation is for *TLR2* gene: medians for children = 33.0, adolescents = 10226.3, and healthy donors = 8.3, $p < 0.0001$.

Changes in *IL33* gene expression in the whole blood samples were assessed in the adult group of patients. As can be seen from the Figure 3, patients with moderate form of AD reported significantly higher expression level of *IL33* than patients with severe form. Median for the moderate AD group equals 57.7 and for severe AD group = 4.4 ($p < 0.01$).

Our study showed differences between groups of atopic patients and healthy donors concerning the expression profile of *IL4, IL13* cytokines and pattern-recognition receptors *TLR2* and *TLR9* in the blood mononuclear cells. Increased levels of all suggested molecules may be an evidence of Th2 immune response enhancement in the acute phase of the dermatitis, which is, in fact, an acknowledged

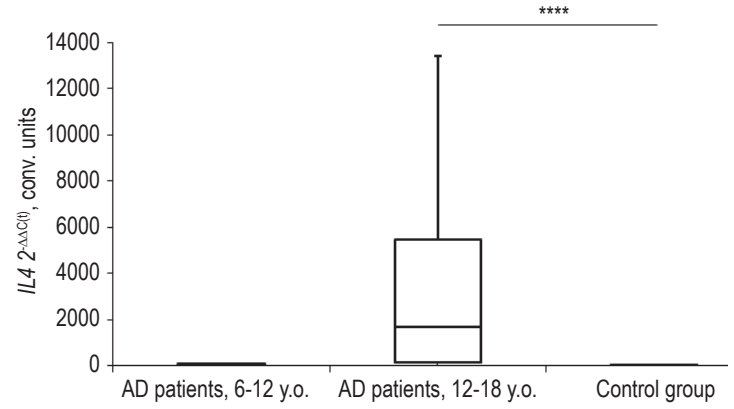

Figure 1. *IL4* gene expression level in the PBMCs of pediatric ad patients compared to the control group
Note. ****, p < 0.0001.

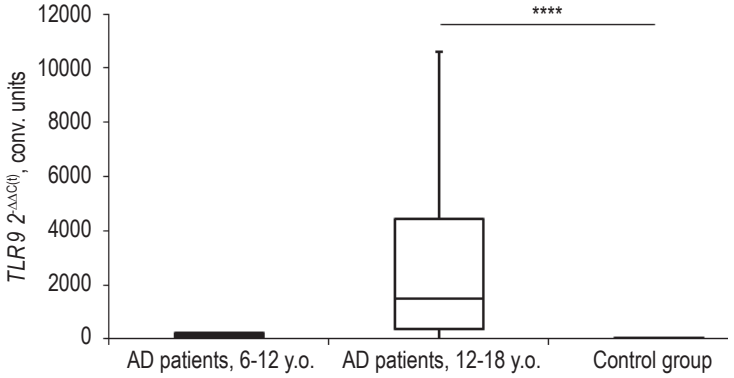

Figure 2. *TLR9* gene expression level in the PBMCs of pediatric ad patients compared to the control group
Note. ****, p < 0.0001.

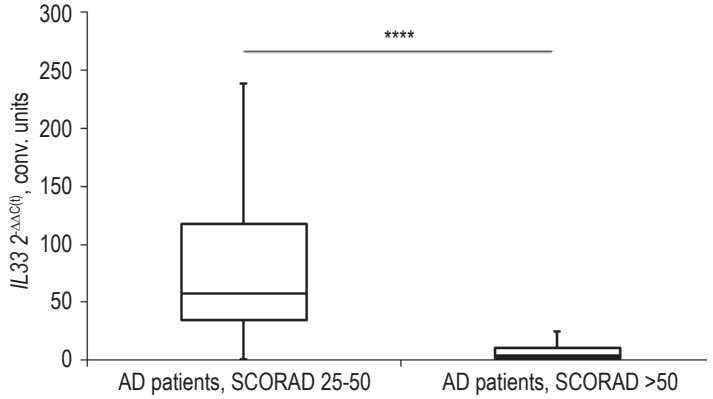

Figure 3. *IL33* gene expression level in the whole blood samples of adult ad patients
Note. ****, p < 0.0001.

classic model of the systemic AD pathogenesis. Nevertheless, our previous studies showed that at the same time there might be a difference at the local level (in the skin), where the inflammation takes place and anti-inflammatory mechanisms prevail [2]. Targeted expression analysis revealed that in the skin samples the expression of *TLR9* and *IL4* was 12 times significantly lower ($p < 0.0001$, $p < 0.0005$) in the lesional skin; and there was a 6-fold decrease in case of *TLR2* ($p < 0.01$). Such differences may occur due to different factors, epigenetic alterations in particular.

The findings on *IL33* expression level suggested that in case of moderate form of AD in adults, where the chronic form of the disease often takes place, this cytokine may prevail on systemic level. Presumably, in case of severe form *IL33* may be released locally, in the affected skin, so its systemic effect reduces. Thus, further studies are required.

There is much research that emphasis the importance of *IL4* and *IL13* cytokines in the pathogenesis and management of atopic dermatitis. These cytokines are proven targets for treatment of moderate-to-severe forms of AD with a systemic upregulation of the mentioned interleukins. Dupilumab is the targeted biologic agent approved in many countries that inhibits the *IL4*-receptor α, the shared subunit of *IL4* and *IL13* [4]. Due to the mechanism of this medical agent, it is important to prescribe it not only regarding the symptoms and severity of AD, but also the inadequate levels of Th2 type cytokines (e.g., via blood or serum analyses).

IL-33 belongs to the IL-1 inflammatory cytokine family. IL-33 is one of the inflammatory cytokines associated with innate immunity. It can activate group 2 innate lymphoid cells without antigen stimulation to induce type 2 cytokines. There is evidence that it may be produced in keratinocytes of the lesional skin and even become a trigger for itch-scratch cycle [5]. But there is lack of studies explaining its systemic role in the AD pathogenesis.

TLR2 is a PRR family member, which is also known to be expressed on immune cells. *TLR2* is able to recognize variety of microbial components and form a link between innate and adaptive immunity. Iwamoto et al. suggested that in situ analysis of isolated Langerhans cells and inflammatory dendritic epidermal cells there was a decreased level of *TLR2* expression and this could partly contribute to the immune deviation in AD [6]. In another study conducted by Yangyang et al., an excessive chemokine mRNA expression (CCL5, CCL8, CCL13, CCL18, and CCL22) in PBMCs was shown to be induced by *TLR2* activation, which was associated with the AD development [9]. All in all, our findings agree with the studies as due to different biological material (local tissue or systemic level cells) the results may differ.

TLR9 is identified as a CpG DNA sensing receptor expressed in professional innate immune cells such as dendritic cells and macrophages. It was proved that polymorphisms of the *TLR9* gene in the promoter region with significantly increased activity were associated with the development of AD. In a study by Moriwaki et al., it was shown that *S. aureus* in AD was captured by keratinocyte lysosomes, which led to the secretion of the proinflammatory cytokine IL-1α via *TLR9* [8]. Our study did not show any activation of this receptor locally in the skin, although its higher expression in the blood may contribute to inflammation maintaining.

Conclusion

New insights on immunological markers and links among them may shed a light on atopic dermatitis pathogenic mechanisms. The detected molecules could play role as potential therapeutic targets and form a management approach for patients with atopic dermatitis.

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