Медицинская иммунология
2021, Т. 23, № 4,
стр. 895-902

Краткие сообщения
Short communications

Medical Immunology (Russia)/
Meditsinskaya Immunologiya
2021, Vol. 23, № 4, pp. 895-902

РОЛЬ ПОЛИМОРФИЗМА ГЕНОВ TLR2, TLR4 В ФОРМИРОВАНИИ МИКРОСОСУДИСТЫХ ОСЛОЖНЕНИЙ У ПОДРОСТКОВ С САХАРНЫМ ДИАБЕТОМ 1-ГО ТИПА

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Резюме. Отсроченные осложнения сахарного диабета 1-го типа (СД1) у детей и подростков являются важной проблемой современной медицины. В последние годы активно обсуждается роль иммунных механизмов, в частности хронического воспаления в развитии как СД1, так и его микрососудистых осложнений. Активация Toll-like рецептора 2 (TLR2) и Toll-like рецептора 4 (TLR4) приводит к индукции синтеза провоспалительных цитокинов, хемокинов, молекул адгезии, участвующих в формировании диабетических микрососудистых осложнений. При этом наличие генетического полиморифизм TLR2 и TLR4 изменяет иммунное реагирование в ответ на их стимуляцию эндогенными лигандами, что может повышать риск возникновения диабетических микроангиопатий. Цель исследования – изучить распределение частот генотипов и аллелей генов TLR2 и TLR4 и определить содержание TNFα, IL-1, VCAM-1, фракталкин, эндотелин-1 у подростков с СД1 и микрососудистыми осложнениями. Обследовано 139 подростков с СД1 от 14 до 18 лет и 56 здоровых подростков. Пациенты с СД1 распределены на две группы: I группа – пациенты с декомпенсацией СД1, (HbA1C > 9,0%), (n = 64); II группа – пациенты с удовлетворительным гликемическим контролем СД1 (HbA1C ≤ 9,0%), (n = 75). В зависимости от наличия осложнений выделены 4 подгруппы: Iа (n = 49), IIа (n = 38) – подростки с микрососудистыми нарушениями: Iб (n = 15), IIб (n = 37) – с неосложненным течением заболевания. Определение аллельных вариантов генов TLR проводили с использованием тест-систем ГосНИИ генетика (Москва). Определение содержания цитокинов в сыворотке крови осуществлялось методом твердофазного иммуноферментного анализа BIOSCIENCE. Обработка данных проводилась с использованием пакетов прикладных программ Statistica 6.0. При изучении распределения полиморфизмов TLR2 (Arg753Gln) и TLR4 (Thr399Ile) не было выявлено достоверных различий между исследуемыми подгруппами и контролем. Отмечено, что вариант Asp299Gly в Iа и IIа подгруппах (с осложненным течением) встречался достоверно реже, чем в Iб, IIб подгруппах и контролем. Установлено, что наличие аллеля Gly в гене TLR4 влияет на уровень экспрессии TNFα и VCAM-1 в сыворотке крови и может считаться протективным по развитию микрососудистых осложнений.

Ключевые слова: сахарный диабет 1-го типа, подростки, микрососудистые осложнения, полиморфизм генов TLR-рецепторов, цитокины

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А.А. Воропай, М.А. Левкович, Г.А. Галкина, М.В. Комкова, М.В. Морозова «Роль полиморфизма генов TLR2, TLR4 в формировании микрососудистых осложнений у подростков с сахарным диабетом 1-го типа» // Медицинская иммунология, 2021. Т. 23, № 4. С. 895-902. doi: 10.15789/1563-0625-ROT-2271

For citation:

For citation: A.A. Voropai, M.A. Levkovich, G.A. Galkina, M.V. Komkova, M.V. Morozova "Role of TLR2, TLR4 gene polymorphism in developing microvascular complications in adolescents with type 1 diabetes mellitus", Medical Immunology (Russia)/Meditsinskaya Immunologiya, 2021, Vol. 23, no. 4, pp. 895-902. doi: 10.15789/1563-0625-ROT-2271

DOI: 10.15789/1563-0625-ROT-2271

ROLE OF TLR2, TLR4 GENE POLYMORPHISM IN DEVELOPING MICROVASCULAR COMPLICATIONS IN ADOLESCENTS WITH TYPE 1 DIABETES MELLITUS

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Abstract. Long-term complications of type 1 diabetes mellitus (T1DM) in children and adolescents are an important problem in modern medicine. Recently, the role of immune mechanisms, in particular, chronic inflammation, in the development of both T1DM and its microvascular complications has been actively discussed. Activation of Toll-like receptor 2 (TLR2) and Toll-like receptor 4 (TLR4) leads to hyperproduction of proinflammatory cytokines, chemokines, adhesion molecules involved in the formation of diabetic microvascular complications. At the same time, TLR2 and TLR4 gene polymorphism alters the immune susceptibility to the endogenous ligands, which may increase the risk of diabetic microangiopathies. The aim of this study is to evaluate the frequency of genotypes and alleles of TLR2 and TLR4 genes distribution and to determine the content of TNFα, IL-1, VCAM-1, fractalkine, endothelin-1 in adolescents with T1DM with microvascular complications. We examined 139 adolescents with T1DM from 14 to 18 years old and 56 healthy teenagers. Patients with T1DM were divided into two groups: Group I – patients with poor glycemic control (HbA1C > 9.0%), (n = 64); Group II – patients with satisfactory glycemic control of T1DM (HbA1C ≤ 9.0%), (n = 75), including adolescents with optimal (HbA1C < 7.5%) and suboptimal glycemic control (7.5% ≤ HbA1C ≤ 9.0%) (ISPAD clinical practice consensus guidelines 2014). According to the presence of microvascular complications, the groups were subdivided into subgroups: Ia (n = 49), IIa (n = 38) – adolescents with verified microvascular disorders: diabetic retinopathy, nephropathy and neuropathy; Ib (n = 15), IIb (n = 37) – without microvascular complications. Allelic variants of TLR genes were determined using test systems GosNII genetics (Moscow). The content of cytokines in blood serum was carried out by the method of enzyme-linked immunosorbent assay "BIOSCIENCE". Data were analyzed using software packages Statistica version 6.0. The assessment of TLR2 (Arg753Gln) and TLR4 (Thr399Ile) polymorphism distribution did not reveal significant differences between the observed subgroups and the control. In Ia and IIa subgroups (with complications) Asp299Gly variant was noted to be significantly less common when compared to subgroups Ib, IIb and controls. The presence of Gly allele in TLR4 gene was found to disrupt the expression of TNFα and VCAM-1 and can be considered protective for the development of microvascular complications.

Keywords: type 1 diabetes mellitus, adolescents, microvascular complications, TLR receptors gene polymorphism, cytokines

Introduction

Long-term microvascular complications affecting both the quality of life and life expectancy of patients with type 1 diabetes mellitus (T1DM) are the primary issues in modern medicine. It should be noted that the incidence of microvascular complications 2-5 years after the onset of T1DM in adolescents is higher than predicted compared to other age groups [10]. Optimal metabolic control during childhood and adolescence is critical for the prevention of prematurely formed vascular complications in T1DM [4]. Many studies report risk factors such as arterial hypertension, microalbuminuria, dyslipidemia, aggravating existing disorders [2]. At the same time, a significant part of adolescents with T1DM, despite having close to the target glycemic profile, show the initial signs of microvascular complications soon after the onset of the underlying disease, which suggests the presence of other concomitant factors, such as environmental, hormonal, immunological, genetic factors, creating the prerequisites for their early formation.

Current studies highlight an important role of vascular inflammation in development of diabetic nephro-, neuro-, retinopathy. Identification of the primary links in cascade of proinflammatory reactions in the vascular wall is of particular interest for developing new diagnosing strategies and early etiopathogenetic treatment, as well as identifying risk groups for microangiopathies. The most promising approach is to examine innate immunity receptors – Toll-like receptors (TLRs) and their genetic poly-

morphism. It is assumed that TLRs recognizing not only exogenous ligands (bacterial, viral, and other infectious agents), but also endogenous pathogens (acute phase proteins, oxidized lipoproteins), formed under the influence of metabolic changes existing in T1DM, cause an immune-inflammatory process and activate genes of adaptive response [5]. TLR2 and TLR4 are the main components of innate immunity, that stimulate subsequent gene expression of cytokines and other signal peptides. Moreover, TLR4 dysregulation contributes to multiple pathophysiological changes responsible for impaired glucose homeostasis, breaking the vicious circle of metabolic and immune abnormalities [1].

In the literature, an increased risk of diabetic nephropathy and retinopathy in T1DM patients with Arg753Gln TLR2 variant is reported, and the Asp299Gly polymorphism of the TLR4 gene is currently considered to be responsible for the development of retinopathy due to inflammation-induced angiogenesis [14, 16]. Thus, the study of TLR2 and TLR4 gene polymorphisms can be useful in predicting microvascular complications in patients with diabetes mellitus. At the same time, there are few studies that assessed modulating influence of different genetic variants of TLR2 and TLR4 on early manifestation of specific diabetic complications in adolescents with T1DM with different glycemic control.

Purpose of the work is to investigate the frequency of genotypes and alleles of the TLR2 and TLR4 genes distribution and to determine level of TNFα, IL-1, VCAM-1, fractalkin, endothelin-1 in adolescents with T1DM with microvascular complications.

Materials and methods

139 adolescents aged 14 to 18 years old, treated in the pediatric endocrinology department of Rostov State Medical University during the years 2012-2015 were examined. Adolescents with clinical and laboratory signs of acute inflammatory diseases or exacerbation of chronic infection at the time of the study were excluded. The control group consisted of 56 healthy adolescents with normal parameters of weight and height, without disorders of carbohydrate metabolism, without burdened by diabetes mellitus heredity.

All participants and their parents signed informed consent to be enrolled to the study. The study protocol was approved by the ethics committee.

The adolescents with T1DM underwent a number of tests to assess the metabolic control, early diagnosis of diabetic complications and determine immunological and genetic parameters. Screening for diabetic complications included examination by a neurologist, ophthalmologist according to the standard technique, ophthalmoscopy to detect fundus microcirculation disorders.

To verify diabetic nephropathy, we studied twice the level of microalbumin in the sediment of the morning portion of urine, serum urea and creatinine. In addition, the levels of glycosylated hemoglobin (HbA1c) were determined in the examined patients by latex inhibition of agglutination (Randox, Great Britain).

The diagnostics of specific complications of type 1 diabetes and assessment of quality of glycemic control by HbA1c level was carried out according to 2014 ISPAD clinical practice consensus guidelines [6].

All patients with T1DM were divided into two groups: Group I – patients with poor glycemic control (HbA1C > 9.0%), (n = 64); Group II – patients with satisfactory glycemic control of T1DM (HbA1C ≤ 9.0%), (n = 75), including adolescents with optimal (HbA1C < 7.5%) and suboptimal glycemic control (7.5% ≤ HbA1C ≤ 9.0%). According to the detected microvascular complications, the groups were subdivided into subgroups: Ia (n = 49), IIa (n = 38) – adolescents with verified microvascular disorders: diabetic retinopathy, nephropathy and neuropathy; Ib (n = 15), IIb (n = 37) – without microvascular complications.

Determination of allelic variants genes TLR-2, TLR-4 was carried out using PCR method with subsequent restriction analysis by using a commercial test system for molecular genetic analysis GosNII genetics (Moscow).

The level of serum TNFα, IL-1, VCAM-1, fractalkine, endothelin-1 was determined by the method of enzyme-linked immunosorbent assay using "BIOSCIENCE" reagents.

To form the database and conduct a statistical study of empirical data, we used the capabilities of the Excel 2003 spreadsheet processor and application packages (Megastat and Statistica 6.0). To determine the statistical validity of the differences between the studied groups, the Mann–Whitney test was used for independent groups with the maximum allowable level of type I error equal to p = 0.05. The statistical significance of the differences between the compared groups or subgroups for frequencies of genotypes and alleles of the studied genes was assessed using the Fisher test or the chi-square by using the standard formula, taking into account Yates' correction for paired comparisons.

Results and discussion

The clinical characteristics of the patients did not reveal differences in anthropometric parameters, gender, the prevalence of comorbidity among adolescents with T1DM in the studied subgroups. While

verifying the specific complications of T1DM, it was found that 77.6% of adolescents with poor glycemic control and half patients with satisfactory glycemic control of T1DM had microvascular complications.

In the observed group of adolescents with T1DM, most common were peripheral neuropathy (I group – 43.8%, II group – 33.3%), early stages of diabetic retinopathy (mild to moderate non-proliferative retinopathy) (I group – 43.8%, II group – 30.7%) and nephropathy (i.e. microalbuminuria) (I group – 20.3%, II group – 9.3%). At the same time, patients in I and II groups did not statistically differ in the prevalence of microangiopathies.

There were no significant differences in distribution of TLR2 (Arg753Gln) and TLR4 (Thr399Ile) gene polymorphisms between control and T1DM subgroups with and without complications (Table 1).

However, studying the distribution of TLR4 (Asp299Gly) gene polymorphisms, the frequency of the A/G genotype in subgroups Ia and IIa compared to subgroups Ib and IIb was lower by 4.1 and 2.3 times, respectively, ($p < 0.05$), and lower than in the

TABLE 1. FREQUENCY DISTRIBUTION OF GENOTYPES AND ALLELES OF TLR2 AND TLR4 GENES IN ADOLESCENTS WITH TYPE 1 DIABETES MELLITUS

			Clinical groups				Control (n = 56)
			I (n = 64)		II (n = 75)		
			Ia (n = 49)	Ib (n = 15)	IIa (n = 38)	IIb (n = 37)	
TLR2 (Arg753Gln)	Genotype	Arg/Arg	42 (85.7%)	14 (93.3%)	35 (92%)	32 (86.5%)	53 (94.6%)
		Arg/Gln	7 (14.3%)	1 (6.7%)	3 (7.9%)	5 (13.5%)	3 (5.4%)
		Gln/Gln	0	0	0	0	0
	Alleles	Arg	91 (92.9%)	29 (96.7%)	73 (96.1%)	69 (93.3%)	109 (97.3%)
		Gln	7 (7.1%)	1 (3.3%)	3 (3.9%)	5 (6.8%)	3 (2.7%)
TLR4 (Thr399Ile)	Genotype	Thr/Thr	37 (75.5%)	13 (86.7%)	30 (78.9%)	32 (86.5%)	48 (85.7%)
		Thr/Ile	12 (24.5%)	2 (13.3%)	8 (21.1%)	5 (13.5%)	8 (14.3%)
		Ile/Ile	0	0	0	0	0
	Alleles	Thr	86 (87.8%)	28 (93.3%)	68 (89.5%)	69 (93.2%)	104 (92.9%)
		Ile	12 (12.2%)	2 (6.7%)	8 (10.5%)	5 (6.8%)	8 (7.1%)
TLR 4 (Asp299Gly)	Genotype	Asp /Asp	45 (91.8%)*▲	10 (66.7%)	34 (89.5%)*Δ	26 (70.3%)	35 (62.5%)
		Asp /Gly	4 (8.2%)*▲	5 (33.3%)	4 (10.5%)*	9 (24.3%)	16 (28.6%)
		Gly/Gly	0	0	0	2 (5.4%)	5 (8.9%)
	Alleles	Asp	94 (95.9%)*▲	25 (83.3%)	72 (94.7%)*Δ	61 (82.4%)	86 (76.8%)
		Gly	4 (4.1%)*▲	5 (16.7%)	4 (5.3%)*Δ	13 (17.6%)	26 (23.2%)

Note. Difference from the control: *, $p < 0.05$; difference from Ib subgroup: ▲, $p < 0.05$; difference from IIb subgroup: Δ, $p < 0.05$.

TABLE 2. CYTOKINE PRODUCTION IN ADOLESCENTS WITH TYPE 1 DIABETES MELLITUS WITH TLR-4 ASP299GLY GENE POLYMORPHISM

pg/ml	Clinical groups			
	Ia		IIa	
	Patients with genotype Asp/Asp	Patients with genotype Asp/Gly	Patients with genotype Asp/Asp	Patients with genotype Asp/Gly
TNFα	293,2* (255.3-300.4)	176,5 (150.2-185.9)	8,5 (6.4-50.9)	6,1 (6.4-50.9)
IL-1	21,1 (17.5-27.6)	14,7 (13.6-17.2)	1,8 (0.9-7.3)	1,5 (0.9-7.3)
VCAM-1	357,2* (320.4-370.3)	275,1 (230.4-300.1)	298,4 (237.7-418.4)	321,1 (237.7-418.4)
Fractalkine	0,89 (0.55-0.94)	0,75 (0.72-0.88)	0,9 (0.8-1.0)	1,1 (0.8-1.0)
Endothelin-1	1,87 (1.77-1.95)	1,51 (1.44-1.88)	0,9 (0.5-1.1)	0,8 (0.5-1.1)

Note. Difference from the patients with genotype Asp/Gly: *, $p < 0.05$.

control by 3.5 and 2.7 times, respectively, ($p < 0.05$) was noted.

Thus, according to the distribution of TLR4 Asp299Gly gene polymorphism, subgroups with microvascular disorders, both with poor and satisfactory glycemic control, were distinguished. In their genotype, the frequency of the Gly allele was significantly lower (subgroup Ia – 4.1%; subgroup IIa – 5.3% and 23.2%).

It is noteworthy that homozygous Gly299Gly TLR4 gene was found only among healthy individuals and patients with T1DM without complications. From our data, it appears that the absence of the Gly allele in the genotype increases the risk of complications (Ia/Ib – OR = 4.7; IIa/IIb – OR = 3.14) both in T1DM groups with poor and satisfactory glycemic control.

Our data are consistent with the results on contribution of polymorphic variants Asp299Gly and Thr399Ile to changes in TLR4 functioning [10] showing that presence of Asp299Gly polymorphism in cells interacting with endogenous and exogenous ligands results in inhibition of developing proinflammatory regulatory peptides compared to those without analogous mutations.

A number of studies revealed [18] an increase in IL-1β and TNFα levels creating conditions for enabling inflammatory component of specific diabetic complications at the microcirculatory level in tissues of different locations.

Since the polymorphism in TLR2 and TLR4 genes is associated with changes in serum cytokine concentration and correlates with the degree of capillary wall damage, it is of great interest to assess the effect of Asp299Gly polymorphism on the level of cytokine production in adolescents with poor and satisfactory glycemic control. The data obtained presented in Table 2 demonstrated the relationship between the Asp/Gly TLR4 genotype and parameters of body nonspecific defense.

In the group with poor glycemic control the level of TNFα and VCAM-1 in patients with the Gly allele of the TLR4 gene in a heterozygous state were found to be significantly lower than in individuals with T1DM without this polymorphism ($p < 0.05$). Meanwhile, there were no significant differences in the level of the studied cytokines and adhesion molecules between adolescents with Asp299Gly and Asp299Asp variants in satisfactory control group, that confirms the hypothesis that the hyperglycemic environment in T1DM is a key trigger activating TLR4, responsible for the initiation of inflammatory damage to target organs. At the same time, the presence of Asp299Gly polymorphism TLR4 gene is currently considered to be a protective genotype downregulating the proinflammatory pathological cascade and the risk of diabetic microangiopathies [18].

Our data complement published data showing that the Asp299Gly TLR4 gene polymorphism disrupts the TLR4 signaling pathway, modulating the production of TNFα and adhesion molecules, in particular VCAM-1 [3].

In previous studies [7, 16], we showed that the main inflammatory mediator TNFα in hyperglycemia

and dyslipidemia environment triggers a sequence of biological events, that culminate in formation of retinopathy and nephropathy [8]. Moreover, experiments carried out in vitro have established that TNFα-mediated apoptosis of endothelial cells underlies vascular complications of T1DM [12]. At the stages preceding overt structural organ changes, dysfunction of nephrons and retinal capillaries is initiated by constant leukocyte infiltration and firm attachment to endothelial cells as a result of the stimulating effect of TNFα on the expression of chemokines, adhesive molecules, both on endothelial cells and leukocytes. It results in production of multiple angiogenic, inflammatory and fibrogenic factors that contribute to the rupture of intercellular connections, damage and apoptotic death of the endothelium and retinal pericytes [13]. Long before the onset of overt complications, the accumulation of monocytes causes leukostasis in the retinal [7, 13] and kidneys capillaries [11], which obstructs blood flow and increases damage.

Thus, the presence of the Asp299Gln polymorphism in the TLR 4 gene affects the serum TNFα and VCAM-1 levels and increases the risk of microvascular complications in patients with different glycemic control.

The study of genetic associations and interactions not only for TLRs, but also TLR-dependent signaling molecules, can elucidate their role in the formation of diabetic microvascular complications in a new way, as well as explain the differences in the timing of manifestation and the severity of their clinical symptoms. This approach allows to use the obtained individual characteristics to justify a personalized approach to patients.

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Поступила 15.03.2021
Отправлена на доработку 31.05.2021
Принята к печати 08.06.2021

Received 15.03.2021
Revision received 31.05.2021
Accepted 08.06.2021