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NORMAL AND PATHOLOGICAL PHYSIOLOGY

Bukach O., Ventoniuk V., Fratsiian M., Tanasesku D., Shkvarchuk V.
INTRODUCTION OF *T-786C* ENDOTHELIAL *NO*-SYNTHASE FOR INTERRUPTIONS AND DEVELOPMENT OF RHEUMATOID ARTHRITIS.....72

PEDAGOGY

Pashayeva P.
CULTURE OF SPEECH AND LISTENING IN THE PROCESS OF BUSINESS COMMUNICATION 77
Kovalenko T.
LEADERSHIP QUALITIES OF STUDENTS: THEORETICAL ANALYSIS 80
Mazur S.
FREEDOM AND SELF-SUFFICIENCY OF THE INDIVIDUAL IN THE FORMATION OF POSITIVE MORAL AUTONOMY 83
Pashayeva P.
EFFECTIVE USE OF INTEGRATION IN CONDUCTING LANGUAGE CLASSES.....85
Koldoshev M., Toychiev Sh.
VALUE ATTITUDE TO THE HORSE AS A FACTOR OF EDUCATION OF KYRGYZ YOUTH BASED ON ANCESTRAL TRADITIONS (ON THE EXAMPLE OF THE KYRGYZ FOLK HEROIC EPIC «MANAS») 87

PHILOLOGY

Ahmadova S.
A COMPARATIVE QUALITY ASSESSMENT OF CHATGPT MODELS AND HUMAN TRANSLATION OF SCIENTIFIC TEXTS.....90
Karimova A.
GREAT MASTER OF TURKISH POETRY.....95

PHYSICS

Khalilov S.
STUDU OF ELECTRICAL CONDUCTIVITY AND DIELECTRIC PROPERTIES OF POLYUETHYLENEFMINE CURED OLIGOSTYNE EPOXY RESIN.....97

NORMAL AND PATHOLOGICAL PHYSIOLOGY

INTRODUCTION OF *T-786C* ENDOTHELIAL *NO*-SYNTHASE FOR INTERRUPTIONS AND DEVELOPMENT OF RHEUMATOID ARTHRITIS

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Abstract

Rheumatoid arthritis is a systemic autoimmune disease characterized by a chronic course, causing erosive and destructive changes in the joints and involving the internal organs and systems of the body in the pathogenesis.

The etiology of rheumatoid arthritis is still not fully understood, but both genetic and environmental factors contribute to the development of the disease. Several genetic features have been linked to rheumatoid arthritis, as have several environmental factors, such as cigarette smoke, dust exposure, and the microbiome. Other environmental factors, as well as hormonal influences, may explain the higher risk of developing this disease in women.

Keywords: rheumatoid arthritis, *T-786C* of endothelial *NO*-synthase, *T*-allele and *C*-allele of the *eNOS* gene, patterns of inheritance of rheumatoid arthritis.

Introduction. Patients with rheumatoid arthritis (RA) have a higher risk of mortality compared to the general population. Despite the reduction of this indicator after the improvement of the treatment strategy and the deepening of knowledge about the course of the disease, studies during the last 50 years have consistently observed the risk of mortality in patients with RA 1.5 times higher than in the general population [7].

The pathogenesis of RA is based on autoimmune inflammation with an unknown primary antigen, which can be viruses, bacteria or even hypothermia, which causes T-lymphocyte immunodeficiency and leads to uncontrolled synthesis by B-lymphocytes of antibodies directed to the synovial membrane of the affected joint, which are immunoglobulins G, A, M. They combine with the antigen in a complex antigen, which independently damage the synovial membrane or undergo phagocytosis in the synovial fluid. After being absorbed by phagocytes, they activate lysosomal enzymes that, after exiting, damage the synovial tissues of the joint, starting a nonspecific inflammation [5, 13].

As a result of damage to the synovial membrane of the joint, fragments of proteins are formed, which the body perceives as foreign objects, as a result of which antibodies are produced, closing the vicious circle [18, 16].

A key role in the pathogenesis of RA is played by genetic factors in interaction with the environment and the individual's lifestyle, they play an important role in changing gene expression and the clinical realization of inherited or acquired pathology, including multifactorial. That is why the study of genetic mutations and their association with a certain pathology is becoming important today not only from a scientific point of view, but also for practical health care [1, 2].

Despite a number of studies establishing the role of the above genetic factors in the occurrence of certain vascular, infectious and autoimmune diseases, as well as RA [9, 12], individual immunological mechanisms of the development of RA remain unstudied and require detailed targeted research. Also, information on the role of the *T-786C* polymorphism of the *eNOS* gene promoter in the formation of RA in the available sources of literature is limited, and the available data are contradictory and relate to individual nosologies and their pathogenetic links [20].

In this regard, it was considered necessary to study the frequencies of alleles and genotypes of the *T-786C* polymorphism of the *eNOS* gene in patients with RA and their association with the severity of the course of RA.

Material and methods. The clinical diagnosis of RA was verified according to the criteria of the American College of Rheumatology and the European Antirheumatic League (ACR/EULAR 2010) [3].

To study the *T-786C* polymorphism of the *eNOS* gene promoter, 2 groups of patients were selected: a control group, n=20 and an experimental group (RA patients, n=60).

Genomic DNA for molecular genetic research was isolated from peripheral blood using the commercial

test system "innuPREP Blood DNA Mini Kit" (Analytik Jena, Germany) using centrifugal filters.

To determine polymorphic variants of the *eNOS* gene (*T-786C*) (rs2070744) [4], modified protocols with oligonucleotide primers using the PCR method and subsequent analysis of restriction fragment length polymorphism (RFP) were used. The studied regions of the genes were amplified using specific primers ("Metabion", Germany), indicated in the table. 1.

Table 1

Oligonucleotide primers

Gene (polymorphism)	Primer sequence (5′ – 3′)	The size of the amplified DNK region
eNOS (T-786C)	TGGAGAGTGCTGGTGTACCCA - *forward* GCCTCCACCCCCACCCTGTC - *reverse*	180 p.n.

Specific fragments of the eNOS gene (T-786C) were amplified using the commercial DreamTaq Green PCR Master Mix (2x) kit (Thermo Scientific, USA) (Table 2).

Table 2

Composition of amplification mixtures

A fragment of a gene	Reagents	Number
eNOS (T-786C)	DreamTaq Green PCR Master Mix	25 μl
	Primer F	100 pmol (1 μl)
	Primer R	100 pmol (1 μl)
	DEPC-treated Water	18 μl
	DNK	5 μl
The total volume of the mixture		50μl

Amplification products of DNA fragments of the *eNOS* gene (*T-786C*) were subjected to hydrolytic cleavage with the MspI FastDigest restriction endonuclease (Thermo Scientific, USA). The state of the restriction fragments was analyzed in a 4% agarose gel (agarose from Cleaver Scientific, Great Britain) with the addition of ethidium bromide and further visualization using a transilluminator. The resulting image was processed in the Vitran program.

Statistical processing was carried out using Microsoft Office Excel® 2010™, IBM SPSS Statistics® 23.0 applications.

The difference in the distribution of genotypes of the *T-786C* polymorphism of the *eNOS* gene in the control group and patients for the conformity of the distribution of genotypes to the Hardy-Weinberg law was tested using the $\chi 2$ test with 2 degrees of freedom, and in the control group - using the $\chi 2$ test with 1 degree of freedom, without using Yeats corrections.

Criteria for the inclusion of patients in the study: the age of patients over 18 years old, the diagnosis of RA according to the ACR/EULAR 2010 criteria, the duration of the disease is at least 6 months, with a low, moderate and high degree of activity according to DAS28; signed informed consent of the patient to participate in the study.

Criteria for excluding patients from the study: the patient's age is younger than 18 years, pregnancy or lactation, acute coronary syndrome, acute cerebrovascular accident, acute heart failure, chronic heart failure of classes II-IV according to NYHA, the presence of mental disorders, malignant neoplasms and infectious diseases in the stage exacerbation or unstable remission, alcohol and drug addiction.

Research results and discussion.

The study of genetic mutations and their association in patients with RA is gaining importance today, as it remains one of the unsolved medical and social problems.

Considerable attention is paid to the study of *NO* metabolism, which is a mediator of synovial fluid cell apoptosis in the pathogenesis of RA [10]. In patients with RA, *NO* contributes to the body's immune defense, acting as an immunoregulator, which is caused by the action of cytokines that stimulate the synthesis of *NO* [19, 6]. As the disease progresses, the concentration of *NO* increases, which causes its cytotoxic effect and complicates the course of RA [11].

Among the 453 allelic variants of the eNOS gene (according to the NCBI database), one of the most studied is the T-786C polymorphism of the eNOS gene, which is located on the 7th chromosome (7q 35-36), consists of 26 exons and encodes an mRNA of 4052 nucleotides [17]. The T-786C polymorphism in the promoter region is associated with a decrease in eNOS expression [14], which leads to endothelial dysfunction [8].

As a result of the above, endothelial dysfunction and systemic inflammation occur, which are important pathological processes in RA and lead to the progression of the underlying disease.

In our study, the relative frequencies of the *T*-allele and *C*-allele of the *eNOS* gene in patients with RA and in the control group (Table 3) probably did not differ. However, the *T*-allele occurred more often in the experimental group than the *C*-allele (by 35.0%; $\chi 2=29.40$, $p<0.001$), without a statistically significant difference in the control. In general, among the 160 isolated alleles in the examined population, the *T*-allele dominated over the *C*-allele (by 30.0%, $\chi 2=28.80$, $p<0.001$).

Table 3

Frequencies of T-786C alleles of the eNOS gene polymorphism (rs 2070744) in patients with rheumatoid arthritis

Research groups, n alleles	*T* alleles, n (%)	*C* alleles, n (%)	OR [95% CI]	χ^2
Research group, n=120	81 (67,5)	39 (32,5)	4,31 [2,51-7,40]	χ^2=29,40 p<0,001
Control group, n=40	23 (57,5)	17 (42,5)	1,83 [0,74-4,44]	χ^2=1,80 p>0,05
OR [95% CI]	1,54 [0,74-3,20]	0,65 [0,31-1,36]	-	-
χ^2 p	χ^2=1,32; p>0,05		-	-
In general, n=160	104 (65,0)	56 (35,0)	3,44 [2,18-5,46]	χ^2=28,80 p<0,001

Note. OR - Odds ratio, n (%) is the absolute (relative) number of alleles.

The distribution of genotypes in the healthy group does not correspond to the Hardy-Weinberg law with a probable excess of heterozygosity (F=-0.33; χ2=4.49; p=0.034), which, however, overlapped with the normal distribution in the experimental group and generally formed a normal population equilibrium without a probable difference between the expected on the actual heterozygosity (Table 4).

Table 4

Indicators of heterozygosity of the T-786C polymorphism of the eNOS gene (rs 2070744) in patients with rheumatoid arthritis

Групи	Alleles, n (%)		P_T	P_C	H_O	H_E	F	χ^2	P
	T	*C*							
Research group, n=120	81 (67,5)	39 (32,5)	0,67	0,33	0,38	0,44	0,13	<1,0	>0,05
Control group, n=40	23 (57,5)	17 (42,5)	0,57	0,43	0,65	0,49	-0,33	4,49	0,034
In general, n=160	104 (65,0)	56 (35,0)	0,65	0,35	0,45	0,45	0,01	<1,0	>0,05

Notes: 1. P_T is the relative frequency of the T allele; P_C is the relative frequency of the C allele. 2. H0 – actual heterozygosity; H_E – expected heterozygosity; F is the inbreeding coefficient. 3. χ2p is a criterion for the validity of the "null" hypothesis between actual and expected heterozygosity. 4. n (%) is the absolute number (percentage) of observations.

Frequencies of *T-786C* genotypes of the *eNOS* gene polymorphism in patients with RA are shown in table. 5. The analysis proved a probable superiority of the relative frequency of *TC* of the polymorphic variant in the control group over that in patients (by 26.67%, χ2=4.31, p=0.038). Also, the *TC* genotype dominated over the *CC* genotype both in the group of patients and in the control: by 25.0% (χ2=14.52, p<0.001) and 55.0% (χ2=16.13, p <0.001) respectively. Among patients with RA, carriers of *TT* alleles occurred more often than those with the *CC* variant (by 35.0%, χ2=23.84, p<0.001). And among practically healthy individuals, the relative frequency of *TC* carriers prevailed over that with a favorable *TT* variant (by 40.0%, χ2=7.11, p=0.008).

The probability of the model according to the Akaike information criterion (AIC) is 16.01 (OR=9.0; 95% CI=4.40-18.41; χ2=40.0; p<0.001).

Table 5

Frequencies of T-786C genotypes of the eNOS gene polymorphism in patients with rheumatoid arthritis

№	Genotypes of the eNOS gene	Research groups, n=80		OR [95% CI]	χ^2 p
		RA, n=60 (%)	Control , n=20 (%)		
1	*TT*- genotypes, n=34	29 (48,33)	5 (25,0)	2,81 [0,91-8,70]	χ^2=3,34 p=0,067
2	*TC*- genotypes, n=36	23 (38,33)	13 (65,0)	2,99 [1,04-8,59]	χ^2=4,31 p=0,038
3	*CC*- genotypes, n=10	8 (13,33)	2 (10,0)	1,38 [0,27-7,14]	χ^2<1,0 p>0,05
OR [95% CI] *TT- genotypes against TC;* χ^2 p		1,59 [0,73-3,45] χ^2=1,38 p>0,05	0,15 [0,03-0,64] χ^2=7,11 p=0,008	-	-
OR [95% CI] *TT*- genotypes against *CC;* χ^2 p		13,14 [4,34-39,75] χ^2=23,84 p<0,001	6,25 [0,61-63,54] χ^2<1,0 p>0,05	-	-
OR [95% CI] *TC*- genotypes against *CC;* χ^2 p		8,27 [2,65-25,79] χ^2=14,52 p<0,001	42,25 [5,15-148,9] χ^2=16,13 p<0,001	-	-
The probability of the co-dominant model of the higher education system [95% CI] χ^2; p		9,0 [4,40-18,41] χ^2=40,0; p<0,001		-	-

Note. OR – odds ratio; 95% CI is a 95% confidence interval.

Racial and population analysis (Table 6) showed that the frequency of the *CC* genotype of the *eNOS* gene in the population of the Bukovyna region examined by us (10.0% - in the control, 13.33% - in the experimental group) does not reliably differ from that of Caucasian populations and individual populations of the equatorial race, including by allelic distribution (P_T =0.57-0.68 and P_C =0.32-0.43 versus P_T =0.50-1.0 and P_C =0.33- 0.59; p>0.05), indicating high heterogeneity and racial non-specificity. At the same time, the frequency of the *T*-allele in our studies is slightly lower, and the frequency of the *C*-allele is higher than those for Asian populations (P_T =1.0; p<0.05 and P_C =0) [15, 18].

Table 6

Racial and population differences in frequencies of genotypes, T-786C alleles of the eNOS gene polymorphism

Races, populations	TT- genotypes	TC- genotypes, %	CC - genotypes, %	T-алель	C-алель
The results we received (Bukovyna)	0,25-0,48	0,38-0,65	0,10-0,13	0,57-0,68	0,32-0,43
Equatorial race (African Americans)	0,39-1,0	0,17-0,50	0-0,10	0,50-1,0	0,08-0,50
European race (Caucasians)	0,13-1,0	0-0,56	0-0,30	0,41-1,0	0,33-0,59
Asian race	0,97-1,0	0-0,43	0-0,012	1,0	0

Conclusion. Therefore, this gene in a homozygous state is found in 12.5% of the examined residents of Bukovyna: in 13.33% of patients with RA, and in 10.0% of healthy people, respectively (p>0.05). According to the allelic distribution of the T-786C polymorphism of the eNOS gene in the population in general, the T-allele dominates over the C-allele (65.0% vs. 35.0%; χ2=28.80; p<0.001): in patients with RA – by 35 .0% [OR=4.31, 95%CI=2.51-7.40, p<0.001], in the control - by 15.0% (p>0.05) with a probable excess of heterozygosity (F=- 0.33; χ2=4.49; p=0.034). The TT genotype of the eNOS gene occurs in 48.33% of patients with RA. The frequency of the C-allele of the eNOS gene in the population of the Bukovyna region examined by us probably does not differ from that for Caucasians and certain populations of the equatorial race (P_T =0.57-0.68 and P_C =0.32-0.43 vs. P_T =0.50 - 1.0 and P_C =0.33-0.59; p>0.05), indicating high heterogeneity and racial non-specificity.

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