The Pharma Innovation Journal 2017; 6(2): 116-120

www.ThePharmaJournal.com

The Pharma Innovation

ISSN: 2277- 7695
TPI 2017; 6(2): 116-120

www.thepharmajournal.com
Received: 19-12-2016
Accepted: 20-01-2017

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Association of T-786C polymorphism of endothelial oxide synthase gene with rheumatoid arthritis

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Abstract
The aim of the study was to analyze the frequency of alleles/genotypes of eNOS gene T-786C polymorphism in patients with rheumatoid arthritis (RA), Arterial Hypertension (AH), Obesity (AO), type 2 Diabetes Mellitus (DM2), and their association with severity and RA clinical manifestation. The eNOS T-786C gene polymorphism was analyzed by polymerase chain reaction in 60 patients with RA and 20 healthy individuals. Wild T-allele dominates over mutant C-allele in the population by 30% (p<0.001). Frequency of comorbid pathology (AO, DM2 and AH) in patients with RA didn't depend on analyzed gene polymorphic variants. Among T-allele carriers there were more patients with a painful joints number >10 by 37.94% (p=0.004) and 73.92% (p<0.001); among the TC-variant carriers there were more subjects with swelling joints number >10 by 47.82% (p=0.001); all CC-genotype patients complained of heart pain; morning stiffness with duration of ≥6 hours didn't depend on analyzed gene polymorphism.

Keywords: eNOS gene T-786C, rheumatoid arthritis, risk

1. Introduction
Cardio-vascular diseases are one of the most frequent causes of death in economically developed countries. While myocardial infarction is a frequent cause of death among people under 55 [1, 2], a high rate of cardio-vascular comorbidity, especially due to ischemic heart disease (IHD), is the most sufficient predictor of untimely death of patients with rheumatoid arthritis (RA) [3, 4]. Certain investigations have detected relations between RA and IHD through the systemic inflammatory response [5], and suggested that IHD is a sign of extra-articular RA provoked by active inflammatory process, endothelial dysfunction (ED), drug effect, or other secondary factors [6, 7]. Systemic inflammatory response plays a key role in the formation of ED, and inflammatory markers are considered as an independent predictor of cardio-vascular risk [8, 9]. At the same time peroxide oxidation products and free radicals change the balance between protective and damaging effects in the vascular wall, and they are certain traps for nitrogen oxide (NO) molecules. NO is a primary physiological transmitter synthesized primarily by the endothelium in case of sufficient activity of NO synthase of type 3 (NOS3). It plays a comprehensive role in the body as a vasodilator with various anti-atherogenic effects, influences on the activity and adhesion of platelets, leukocytes, proliferation of smooth muscles cells and inhibits inflammation [10, 11]. Therefore reduced expression of NOS3 decreases biological availability of NO, and additionally an excessive formation of oxygen active forms in the vascular wall becomes an important cause of endothelial dysfunction and inflammation. Expression of the above mentioned enzyme depends on genetic factors as well. NOS3 gene contains a number of polymorphous sights including single nucleotide polymorphism (*SNP*), variable number tandem repeat (*VNTR*) sequences, etc. Among 453 allele variants of this gene (according to NCBI base) the most studied and functionally connected are polymorphisms: *T-786C* in 5'UTR region of the 7th chromosome (7q 35-36), *G894T* – in the 7th exon (7q35-36) and 27 functional tandem repeats of nucleotide pairs (*VNTR*) in the 4th intron (4a/b) of this chromosome respectively [9, 12-14]. According to a number of studies the above mentioned types of polymorphism of endothelial NOS gene (eNOS) can be accompanied by lesions of NO biological availability, dysregulation of vasoconstriction/vasodilation processes, ED, possess pro-thrombogenic and pro-inflammatory effects playing a key role in pathogenesis of IHD, RA, diabetes mellitus (DM), heart failure and arterial hypertension (AH) [9, 11, 14, 15].

According to the above mentioned facts it is considered to be essential to conduct analysis of T-786C polymorphism of eNOS gene in the structure of RA patients and to find probability of its effect on comorbid diseases occurrence.

1.1 The aim of the research: to analyze the frequency of alleles and genotypes of eNOS gene T-786C polymorphism (rs2070744) in patients with RA including those with comorbid AH, obesity, type 2 DM, and their association with severity and clinical manifestation of RA.

2. Materials and Methods

2.1 Compliance with bioethics

Study was performed in compliance with the Council of Europe Convention on Human Rights and Biomedicine and recommendations of the Committee on Bioethics of the Ministry of Health of Ukraine. Patients' Examination Cards and Patients' Informed Consent Forms were approved by the Biomedical Ethics Commission of Bukovina State Medical University, Ministry of Health of Ukraine (Chernivtsi, Ukraine). All enrolled patients were treated in the Regional Clinical Hospital (Chernivtsi, Ukraine) during 2014-2016 y.y. Genetic bench study performed at the laboratory of the State institution "Reference centre of molecular diagnostics of the Ministry of Health of Ukraine" (Kyiv) and at the laboratory of Medical Biology and Genetics Department of Bukovina State Medical University. After screening (matching inclusion/exclusion criteria) 60 patients with exacerbation of Rheumatoid Arthritis (RA), or acute RA, persistent erosive RA were selected for further examination. The control group included 20 practically healthy individuals who were not relatives with the patients, without reliable differences of sex and age.

2.2 Inclusion / Exclusion criteria.

2.2.1 Inclusion criteria: Patients with RA typical symptoms for 6 weeks according to ACR/EULAR Classification Criteria for Rheumatoid Arthritis [16] (joint involvement refers to any swollen or tender joint on examination). Categories of joint distribution were classified according to the location and number of the involved joints, with placement into the highest category possible based on the pattern of joint involvement. "Large joints" refers to shoulders, elbows, hips, knees and ankles. "Small joints" refers to the MTP joints, proximal interphalangeal (PIP) joints, second to fifth MTP joints, thumb interphalangeal joints and wrists. "Symmetric" were defined as bilateral involvement of at least one region. In the category ">10 joints", at least one of the involved joints must be a small joint; the other joints could include any combination of large and additional small joints, as well as other joints not specifically listed elsewhere (eg, temporomandibular, acromioclavicular, sternoclavicular, etc). Also were included patient with at least one joint with definite clinical synovitis (swelling). Classification Criteria included additionally serology tests (rheumatoid factor (RF) information and anti-citrullinated protein antibody (ACPA)), acute-phase reactants (normal / abnormal C-reactive protein (CRP); erythrocyte sedimentation rate (ESR)) and duration of symptoms (patient self-report of the duration of signs or symptoms of synovitis (eg, pain, swelling, tenderness) of joints that are clinically involved at the time of assessment, regardless of treatment status. Determination of definite RA (either active or inactive) was based on scoring system: ≥6 points out of possible 10.

2.2.2 Exclusion criteria

We excluded patients who were not matched to the ACR/EULAR Classification Criteria for Rheumatoid Arthritis; younger than 18 y.o and older than 80 y.o.; pregnant women; those who had any localization of oncological process; persons with psychological disorders; additionally distal interphalangeal joints, first carpometacarpal joints and first metatarsophalangeal (MTP) joints were excluded from assessment; patients with undifferentiated inflammatory arthritis.

2.3 Diagnosis of Rheumatoid Arthritis.

Selection of patients and their distribution into groups was performed according to the classifications of Ukrainian and ACR/EULAR Classification Criteria [16, 17]. The diagnosis of RA was made on the basis of the criteria of the acting national and international recommendations [16, 17]. All patients were examined comprehensively: general clinical, laboratory and instrumental examination (Roentgen of involved joint).

2.4 Genotyping of the eNOS gene T-786C polymorphism

Genomic DNA was extracted from peripheral blood leukocytes using the "DNA-sorb-B" test system, with primers specific to the genes' alleles [18]. Detection of T-786C polymorphism of eNOS gene was performed by the multiplex polymerase chain reaction (PCR) according to the manufacturer's protocol. Allele-specific primers were used in the PCR (Table 1). PCR amplification was conducted in a total volume of 50 µl containing: 200 ng of isolated DNA, 65 mM Tris-HCl pH=8.9, 0.05% Tween20; 16 mM $(NH_4)_2SO_4$, 3.5 мМ $MgCl_2$), 0.8×SYBR Red, 0.2 mM of each dNTPs, 1 µM of each primer (tab. 1) and 25 µl DreamTaq Green PCR Master Mix (Thermo Scientific, USA). The amplification conditions were subjected to initial denaturation at 95°C for 2 min; 35 cycles consisting of denaturation at 95°C for 30 s, primer annealing at 63°C for 30 s and DNA elongation at 72°C for 30 s; the final DNA extension was at 72°C for 2 min. The PCR products were digested by restriction endonuclease MspI FastDigest for C-allele+ (Thermo Scientific, USA) at 37°C for 1 hour. The PCR products (genotype TT: 138 and 42 bp; genotype TC: 138, 92, 46 and 42 bp; genotype CC: 92, 46 and 42 bp) were separated by horizontal electrophoresis on 4% agarose gels (Cleaver Scientific, Great Britain), stained with 4 µl of ethidium-bromide and visualized in the presence of molecular mass ladder (40-1000 bp) using a UV transilluminatior and Vitran® computer based program.

Table 1: Primer sequences for eNOS T-786C gene SNP and size of fragments

SNP locus	Primers	Primer sequences (5'-3')	Size of fragments, bp
eNOS 7q 35-36	Forward	5′-TGGAGAGTGCTGGTGTAC CCA-3′	*Genotype TT*: 138 and 42 bp; *Genotype TC*: 138, 92, 46 and 42 bp; *Genotype CC*: 92, 46 and 42 bp.
	Reverse	5′-GCCTCCACCCCCACCCTG TC-3	

bp – base pair

2.5 Statistical analysis

Statistical analysis was performed using Statistica 7.0 (StatSoft Inc, USA) software. P value and odds ratio (OR), with 95% confidence interval (CI) using a chi-square test were determined for the calculated frequencies of each allele and genotypes. Risk ratios (RR) were estimated by OR. Adjusted OR and 95%CI were estimated for association between age, specific / non-specific tuboovarium inflammation and genetic polymorphism. P values <0.05 were considered statistically significant.

3. Results and Discussions

Distribution of polymorphous variants of eNOS gene T-786C polymorphism in observed groups was the following: TT-genotype was found in 48.33% (29) patients with RA, an in 25.0% (5) in the control ($p>0.05$); *TC*-genotype – in 38.33% (23) and 65.0% (13) [OR=2.99; $\chi^2=4.31$; p=0.038] respectively; CC-genotype – in 13.33% (8) and 10.0% (2) ($p>0.05$), respectively. In general wild T-allele dominates over mutant C-allele in the population (65.0% against 35.0%; $\chi^2=28.80$; $p<0.001$): in patients with RA – 35.0% higher [OR=4.31; 95%CI OR=2.51-7.40; $p<0.001$], in the control – 15.0% higher ($p>0.05$) with a reliable excessive heterozygosity (F=-0.33; $\chi^2=4.49$; p=0.034). Excessive heterozygosity in the control is exceeded by a normal distribution in the study group and generally it does not disturb expected *Hardy-Weinberg* equilibrium in general.

Distribution of *eNOS* gene (rs2070744) genotypes in RA patients including comorbid and polymorbid pathology is presented in Table 2. A relative frequency of subjects in the examined population with RA and comorbid AH was reliably less than those with RA only – by 18.33% ($\chi^2=5.05$; p=0.025), or RA, AH and AO – by 16.66% less ($\chi^2=4.22$; p=0.04), as well as RA, AH, AO and DM2 – by 18.33% less ($\chi^2=5.05$; p=0.025) respectively. Statistically valuable dependence of comorbidity frequency in RA patients depending on the analyzed gene genotypes has not been found.

Table 2: Polymorphic variants of eNOS gene (rs 2070744) distribution in patients with Rheumatoid Arthritis depending on comorbidity conditions

Observed groups, n (%)	Genotypes of eNOS gene, n (%)			Total, n=60 (%)
	TT	TC	CC	
Patients with RA	8 (44.44)	8 (44.44)	2 (11.11)	18 (30.0)
RA+AH	3 (42.86)	3 (42.86)	1 (14.29)	7 (11.67)
χ^2 p_1	$\chi^2<1.0$ $p_1>0.05$	$\chi^2<1.0$ $p_1>0.05$	$\chi^2<1.0$ $p_1>0.05$	$\chi^2=5.05$ $p_1=0.025$
RA+AH+AO	9 (52.94)	7 (41.18)	1 (5.88)	17 (28.33)
χ^2 p_1	$\chi^2<1.0$ $p_1>0.05$	$\chi^2<1.0$ $p_1>0.05$	$\chi^2<1.0$ $p_1>0.05$	$\chi^2<1.0$ $p_1>0.05$
χ^2 p_2	$\chi^2<1.0$ $p_2>0.05$	$\chi^2<1.0$ $p_2>0.05$	$\chi^2<1.0$ $p_2>0.05$	$\chi^2=4.22$ $p_2=0.04$
RA+AH+AO+DM2	9 (50.0)	5 (27.78)	4 (22.22)	18 (30.0)
χ^2 p_1	$\chi^2<1.0$ $p_1>0.05$	$\chi^2<1.0$ $p_1>0.05$	$\chi^2<1.0$ $p_1>0.05$	$p_1>0.05$
χ^2 p_2	$\chi^2<1.0$ $p_2>0.05$	$\chi^2<1.0$ $p_2>0.05$	$\chi^2<1.0p_2>0.05$	$\chi^2=5.05$ $p_2=0.025$
χ^2 p_3	$\chi^2<1.0$ $p_3>0.05$	$\chi^2<1.0$ $p_3>0.05$	$\chi^2<1.0$ $p_3>0.05$	$\chi^2<1.0$ $p_3>0.05$

Notes: 1. RA – Rheumatoid Arthritis; AH – arterial hypertension; AO – abdominal obesity; DM 2 – type 2 diabetes mellitus.
2. p_1 – reliability of differences concerning rheumatic patients; p_2 – reliability of differences concerning rheumatic patients with arterial hypertension; p_3 – reliability of differences concerning rheumatic patients with arterial hypertension and abdominal obesity.

Clinical symptoms in RA patients depending on eNOS gene (rs 2070744) polymorphous variants are presented in Table 3. Among the T-allele carriers were found patients with a number of painful joints >10 joints by 37.94% more often (p=0.004) and 73.92% ($p<0.001$); and among the TC-variant carriers the persons with swelling of joints (>10 swelling joints) prevailed – by 47.82% (p=0.001). Morning stiffness with duration of ≥6 hours was found in the majority of patients and did not depend on the analyzed gene polymorphism. All CC-genotype patients complained of pains in the heart, that was relatively more often than among T-allele patients – by 72.41% and 60.87% ($\chi^2=13.63$; p=0.001). Frequency of complaints on headache, dry mouth, polyuria, quick tiredness did not depend on eNOS gene polymorphic variants.

Table 3: Clinical symptoms in patients with Rheumatoid Arthritis depending on eNOS gene (rs2070744) polymorphic variants

Clinical symptoms		Genotypes of eNOS gene			χ^2	p
		TT, n=29	TC, n=23	CC, n=8		
Number of painful joints, n (%)	<10,n=16	9 (31.03)	3 (13.04)	4 (50.0)	$\chi^2=4.69$	$p>0,05$
	≥10, n=44	20 (68.97)	20 (86.96)	4 (50.0)		
χ^2 p		$\chi^2=8,34$ p=0,004	$\chi^2=25.13$ $p<0.001$	-	-	-
Number of swelling joints, n (%)	<10,n=28	14 (48.28)	6 (26.09)	8 (100.0)	$\chi^2=13.09$	p=0,001
	≥10, n=32	15 (51.72)	17 (73.91)	0		
χ^2 p		$\chi^2<1,0$ $p>0,05$	$\chi^2=10.52$ p=0.001	-	-	-
Duration of morning stiffness, n (%)	2-5 год, n=15	9 (31.03)	5 (21.74)	1 (12.50)	$\chi^2=1.36$	$p>0,05$
	≥6 год, n=45	20 (68.97)	18 (78.26)	7 (87.50)		
χ^2 p		$\chi^2=8,34$ p=0,004	$\chi^2=14.70$ $p<0.001$	p=0.005	-	-
Pains in the heart, n=25 (%)		8 (27,59)	9 (39.13)	8 (100.0)	$\chi^2=13.63$	p=0.001
Headache, n=28 (%)		13 (44,83)	9 (39,13)	6 (75,0)	$\chi^2=3,15$	$p>0,05$
Dry mouth, n=18 (%)		9 (31,03)	5 (21,74)	4 (50,0)	$\chi^2=2,29$	$p>0,05$
Polyuria, n=18 (%)		9 (31,03)	5 (21,74)	4 (50,0)	$\chi^2=2,29$	$p>0,05$
Quick tiredness, n=43 (%)		18 (62,07)	20 (86,96)	5 (62,50)	$\chi^2=4,29$	$p>0,05$

Analysis of pain syndrome intensity by visual-analogous scale (VAS) gradation (<4 cm – mild pain, 4-6 cm – moderate pain, ≥6 cm – severe pain) was performed. The severe joints ache in the majority of patients did not depend clearly on eNOS gene polymorphic variants (Table 4). Similar manifestation was observed according to disease activity sign (DAS 28): DAS 28 was 5.1 s.u. and higher in 83.33% patients and indicated to a high RA activity, without dependence on eNOS gene alleles condition (rs2070744).

Table 4: Pain syndrome intensity and disease activity index in patients with Rheumatoid Arthritis depending on polymorphous variants of eNOS gene (rs2070744)

Clinical symptoms		Genotypes of eNOS gene			χ^2	p
		TT, n=29	TC, n=23	CC, n=8		
Visual-analogous scale, n (%)	<6, cm n=11	6 (20.69)	4 (17.39)	1 (12.50)	χ^2<1.0	p>0.05
	≥6, cm n=49	23 (79.31)	19 (82.61)	7 (87.50)		
χ^2 p		χ^2=19,93 p<0,001	χ^2=19.57 p<0.001	p=0.005	-	-
DAS 28 – disease activity index, s.u., n (%)	≤5,1 n=10	8 (27.59)	1 (4.35)	1 (12.50)	χ^2=5.10	p>0.05
	>5,1 n=50	21 (72.41)	22 (95.65)	7 (87.50)		
	χ^2 p	χ^2=11.66 p<0.001	χ^2=38.35 p<0.001	p=0.005	-	-

The risk of RA occurrence in the population considering genetic component, as well as comorbid pathology (Obesity, AH, type 2 DM) are presented in Table 5. Alleles of the analyzed gene were not found to be additional risk factors of RA occurrence and above mentioned comorbid pathology. Although in case of TC-genotype probability of RA in the population is reliably lowest [OR=0.33; 95% CI OR: 0.12-0.96; p=0.038].

Table 5: Genotypes and alleles variants of eNOS (rs2070744) gene as risk factors of Rheumatoid Arthritis and Comorbid conditions in observed population

Groups		Potential Risk Factors				
		TT	TC	CC	C-allele	T-allele
Rheumatoid arthritis	RR	1.93	0.59	1.33	0.76	1.17
	OR	2.81	0.33	1.38	0.65	1.54
	95% CI RR	0.87-4.32	0.37-0.93	0.31-5.77	0.49-1.19	0.87-1.57
	95% CI OR	0.91-8.70	0.12-0.96	0.27-7.13	0.31-1.36	0.74-3.20
	p	>0.05	0.038	>0.05	>0.05	>0.05
Risk Factors		**TT**	**TC- + CC- genotypes**			
RA + Obesity	RR	1.33	0.77			
	OR	1.74	0.57			
	95% CI RR	0.77-2.32	0.47-1.25			
	95% CI OR	0.62-4.88	0.20-1.60			
	p	>0.05	>0.05			
RA + Hypertension	RR	1.25	0.83			
	OR	1.50	0.67			
	95% CI RR	0.67-2.32	0.52-1.34			
	95% CI OR	0.50-4.45	0.22-1.98			
	p	>0.05	>0.05			
RA+Diabe-tes Mellitus	RR	1.12	0.9			
	OR	1.25	0.8			
	95% CI RR	0.56-2.25	0.48-1.67			
	95% CI OR	0.34-4.64	0.22-2.97			
	p	>0.05	>0.05			

Note: RelR - relative risk; RR – Risk Ratio; OR – Odds Ratio; 95%CI RR, OR – 95% confidence interval of RR, OR.

Racial and population analysis indicated that minor CC-genotype frequency of eNOS gene among the examined population of Northern Bukovyna region (10.0% - in the control, 13.33% - in the study group) did not differ reliably from that in Caucasian populations and some populations of equatorial race including allele distribution (P_T=0.57-0.68 and P_C=0.32-0.43 against P_T=0.50-1.0 and P_C=0.33-0.59; p>0.05). Our results showed a high heterogeneity and race non-specificity of eNOS gene alleles (rs2070744) distribution. At the same time the frequency of T-allele in our study is a little bit less, and C-allele – greater than in certain populations of Asian race (P_T=1.0; p<0.05 i P_C=0) [19].

4. Conclusion

Mutation of eNOS gene (rs 2070744) in 5'UTR region of the 7th chromosome (7q 35-36) in homozygotic condition is found among the residents of Northern Bukovyna in 12.5% cases: in patients with RA – in 13.33%, among practically healthy – in 10.0% respectively (p>0.05). Wild T-allele dominates over mutant C-allele in the population (65.0% against 35.0%; χ^2=28.80; p<0.001). Frequency of comorbid pathology (AO, DM2 and AH) in RA patients did not depend on the analyzed gene polymorphic variants. Genotypes and alleles of eNOS gene (rs2070744) are not additional risk factors of RA occurrence as well as comorbid AO, type 2 DM and AH in observed population of Northern Bukovyna.

Clear unidirectional dependence of RA clinical symptoms on eNOS gene alleles condition was not found: among the T-allele carriers there were found more patients with a number of painful joints >10 – by 37.94% more (p=0.004) and 73.92% (p<0.001); and among the TC-variant carriers the individuals with swelling joints number >10 prevailed by 47.82% (p=0.001); all patients with unfavorable CC-genotype complained of heart pains; morning stiffness with duration of

≥6 hours was found in the majority of patients and did not depend on analyzed gene polymorphism.

4.1 Limitations of the Study: The present study was limited by a number of enrolled subjects.

4.2 Conflict of Interest: None declared.

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