



**CLINICAL AND IMMUNOLOGICAL FEATURES OF PATIENTS WITH CHD  
ASSOCIATED WITH TNF-A**

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Article Received on 10/09/2017

Article Revised on 01/10/2017

Article Accepted on 22/10/2017

**ABSTRACT**

The study included 101 patients with coronary heart disease aged from 34 to 76 years ( $56.9 \pm 0.9$ ), of them 64.4% were men and 35.6% - women. We have shown the role of TNF- $\alpha$  gene (-308G> A) in the development of CHD-allele A, the A/G and A/A genotypes are characterized as risky variants in the development of unstable (progressive) stenocardia in the Uzbek population. In the group of unstable stenocardia with genotype profile A/G+A/A of TNF- $\alpha$  gene (-308G> A), the levels of TNF- $\alpha$ , IL-6, IL-18, CRP and fibrinogen were reliably higher in comparison with SS and control groups. Despite the fact that we have shown the role of TNF- $\alpha$  (-308G> A) in the development of CHD in the Uzbek population, further study of this SNP on much more material is necessary, in connection with the already known risk factors to establish its potential causal relationship.

**KEYWORDS:** CHD, tumor necrosis factor, TNF $\alpha$ , interleukins, Uzbek population.

**INTRODUCTION**

In Uzbekistan, as in majority of developing, industrialized countries, cardiovascular diseases (CVD) occupy the first place in the structure of disability and mortality. In addition, these diseases lead to an escalation of health care costs. At the same time coronary heart disease (CHD) determined 50.1% of CVD mortality and consequently, it causes half of all deaths of patients with cardiovascular pathology.<sup>[1]</sup> This is an average of 3-3.5 times higher than in Western Europe, the US and Japan. Prevention of these diseases is one of the main tasks of scientific and practical medicine.

CHD is a multifactorial and polygenic disease and the highest number of full genomic (GWAS) studies was conducted precisely with this pathology. The majority of GWAS studies presented to date has been performed in CHD and totaled more than 27,000 studies in a wide range of different forms of the disease.<sup>[2-17]</sup> When searching for the GWAS catalog (January 2017) with a severe restriction ( $P < 10^{-8}$ ), 83 significant loci for coronary heart disease and 31 for myocardial infarction were presented.<sup>[2,4-16]</sup> Despite the great number of large-

scale full-genomic studies in CHD, understanding the mechanisms for implementing the majority of GWAS loci in atherosclerotic cardiovascular diseases is very limited. Although some of these loci overlap with known risk factors, suggesting a potential causal link, most of them remain still completely unknown, and require further investigation.

The inflammatory genesis of atherosclerosis is distinguished separately, where signs of inflammation are present together with the accumulation of lipids in the arterial wall. For example leukocytes, mediators of the immune system and inflammation are found in places of atherosclerosis formation in the early stages of both experimental animals and humans. A recent detailed analysis of a significant number of studies on the association of CHD and genetic markers of genes whose products are involved in the development of inflammatory processes has shown a reliable association CHD and gene such as CRP, IL6 (interleukin 6) and SELP (P selectin).<sup>[18]</sup>

Based on the bulk of all these data, in the present article, as candidate genes we chose TNF genes that encode tumor necrosis factor (TNF), a cytokine belonging to the group of proinflammatory cytokines and is synthesized mainly in macrophages. The coding gene is located on chromosome 6 in the 6p21.3 region within the main histocompatibility complex (MHC).<sup>[19]</sup> TNF is a multifunctional cytokine that acts on lipid metabolism, processes of blood coagulation and glucose utilization as well as endothelial function. TNF is found in atherosclerotic plaques<sup>[20]</sup>, so many authors believe that it can make a significant contribution to the progression of plaques enhancing the local inflammatory effect.<sup>[21]</sup>

### The aim of the research

In connection with the above-mentioned the aim of our study was to determine the relationship of certain clinical and immunological parameters with the genotypic profile of the TNF gene (-308G>A, rs1800629) in patients with unstable and stable stenocardia in the Uzbek population.

### MATERIALS AND METHODS

The study included 101 patients with coronary heart disease aged from 34 to 76 years (mean age  $56.9 \pm 0.9$  years), of them 64.4% were men (65 patients) and 35.6% - women (36 patients). The average age for men was  $55.48 \pm 0.88$  years, for women:  $60.5 \pm 0.76$  years. Indications for the undergone myocardial infarction (MI) had 36% of patients. CHD was on a background of arterial hypertension (AH) in 93% of patients; in 27% of cases it was combined with type 2 diabetes. The duration of CHD disease at the time of inclusion to the study was  $8.06 \pm 0.74$  years on average. According to clinical variants of CHD patients were formed into groups of unstable (progressive) stenocardia and stable exertional stenocardia.

Group of unstable (progressive) stenocardia (US) II FC on Braunwald E. (1989) comprised 50 patients with an average age of  $55.7 \pm 1.32$  years. Among them 72% were men (36 patients) and 28% were women (14 patients). Group of stable exertional stenocardia II-III FC comprised 51 patients whose mean age was  $58.1 \pm 1.22$  years, of them 29 (56.9%) were men and 22 (43.1%) female patients. The diagnosis of stable exertional stenocardia II-III FC was verified according to the bicycle ergometry, according to the recommendations of the SCRF (Society of cardiology of Russian Federation) / WHO (2004), classification of the Canadian Society of Cardiovascular Diseases (1989).

The thermostable DNA Taq polymerase, manufactured by the 'DNA technology' (Moscow, Russia) was used in

the study. The oligonucleotide primers - *tggaagttagaaggaaacagac* and *acacaagcatcaaggatacc*, were synthesized at the RSCI MH RUz (Tashkent, Uzbekistan). The venous blood from the ulnar vein, 3-5 ml in volume was a material for obtaining DNA, for blood sampling were used vacutainers with an anticoagulant / preservative-15% K3 EDTA. A two-stage method for lysing blood cells in a modernized form was used to obtain genomic DNA.<sup>[22]</sup> The DNA concentration was measured by a NanoDrop™ Lite spectrophotometer (Thermo Fisher Scientific, USA) and all DNA samples were 100 ng /  $\mu$ l. HRM-qPCR (Stratagene M \* 3005P, Agilent Technologies, Germany, DT-Prime, DNA-Technology, Russia) and microchip PCR detection (MCE 202 MultiNA) were used for typing the polymorphic variants of TNFa (-308G>A, rs1800629), Zhimadzu, Japan).

The statistical processing of study results was made using software packages: "SPSS v14.0", "R", "PLINK" and "Haploview 4.2". The distribution of studied polymorphisms genotypes was checked for conformance to expected at Hardy-Weinberg equilibrium by the Fisher exact test (Weir, 1995). To compare the frequencies of alleles and genotypes between the analyzed groups were used the Pearson  $\chi^2$  criterion with the Yates correction or the Fisher exact test. To evaluate associations of genes polymorphisms with pathological phenotype the indicator "odds ratio" - OR was calculated. To determine the nature of obtained data distribution the Shapiro-Wilk test was used. For each polymorphism and genotype OR, P value, and 95% confidence interval were calculated. Differences were considered statistically significant at  $P < 0.05$ .

### RESULTS AND DISCUSSION

Analysis of distribution of alleles frequency and genotypes by polymorphisms rs1800629 in the group with SS exertion II-III FC and in the control sample did not reveal statistically significant differences between them ( $\chi^2 < 0.99$ ,  $P > 0.32$ ).

In a comparative analysis of the US group with the SS groups and control, we found significant differences. Thus, the odds risk (OR) of allele A of rs1800629 polymorphism when comparing US groups and control equaled 18.47 ( $P < 0.004$ ), respectively, and the most significant genotype was A/G (OR=14.75,  $P < 0.02$ ) (Table 1). The genotype A/A was also detected only in the US group (OR=3.06,  $P < 0.02$ ). The reliable frequency of occurrence of A allele, A/G and A/A genotypes characterizes them as risk variants in the development of unstable (progressive) stenocardia.

**Table 1. Comparative analysis of TNF $\alpha$  (-308G>A, rs1800629) in patients with unstable stenocardia and control group.**

Alleles/ Genotypes	US	Controls	$\chi^2$	p	OR	
	n = 50	n = 50			value	95% CI
Allele A	0.080	0.000	8.33	0.004	18.47	1.05 – 324.51
Allele G	0.920	1.000			0.05	0.00 – 0.95
Genotype A/A	0.020	0.000	7.53	0.02	3.06	0.12 – 76.95
Genotype A/G	0.120	0.000			14.75	0.81 – 269.35
Genotype G/G	0.860	1.000			0.06	0.00 – 1.03

In addition, a comparative analysis of the distribution of TNF genotypes among patients with stable (SS) and unstable stenocardia has also revealed differences in distribution (Table 2). Thus, the OR value of the allele A of the TNF polymorphism was 8.78 (P <0.02),

respectively. Genotypes A/G and A/A showed a trend of significance, OR=6.82 and OR=3.12, respectively (P <0.08). When studying the dominant inheritance model, it was revealed that the genotypes A/A+A/G had OR=8.14 (P <0.03).

**Table 2. Comparative analysis of TNF $\alpha$  (-308G>A, rs1800629) in patients with stable and unstable stenocardia**

Alleles/ Genotypes	US	SS	$\chi^2$	p	OR	
	n = 50	n = 51			value.	95% CI
Allele A	0.080	0.010	5.84	0.02	8.78	1.08 – 71.58
Allele G	0.920	0.990			0.11	0.01 – 0.93
Genotype A/A	0.020	0.000	5.09	0.08	3.12	0.12 – 78.46
Genotype A/G	0.120	0.020			6.82	0.79 – 58.85
Genotype G/G	0.860	0.980			0.12	0.01 – 1.04

Comparative evaluation of clinical parameters among patients with US and SS relative to the distribution of genotypes rs 1800629 showed that when comparing G/G genotypes, SS patients significantly differed in the duration of coronary artery disease onset (p <0.01) relative to US group, however, in the obesity group US was 5 times higher than the SS group, which has reliable nature (p <0.001) (Table 3). A comparative analysis of the initial data of SBP showed its highest level in carriers of genotype A of TNF- $\alpha$  (-308G>A) in US patients, which was 150.0  $\pm$  6.9 mm Hg. and was 14.9% (p <0.05)

significantly higher relative to carriers of the G/G genotype in US patients. Comparative analysis of the initial DBP data showed its highest level, as well as the level of SBP, in carriers of genotype A of TNF- $\alpha$  gene (-308G>A) in US patients, which was 90.0  $\pm$  3.78 mm Hg. and was 11.9% (p <0.05) significantly higher relative to carriers of the genotype G/G in US patients. There were no significant differences in the genotypes of TNF between the SS and US group in sex, age, BMI, history of AH, CD2 and postinfarction cardiosclerosis.

**Table 3. Comparative evaluation of clinical parameters of patients with US and SS relative to distribution of genotypes rs 1800629) (M  $\pm$  m, n (%))**

Indication	US		SS	Control (n=40)
	Genotype G/G, (n=43)	Genotype A/G+A/A (n=7)	Genotype G/G (n=51)	
Median age, years	55,37 $\pm$ 1,51	57,71 $\pm$ 1,51	58,50 $\pm$ 1,26	55,80 $\pm$ 0,76
CHD duration, years	5,88 $\pm$ 0,50	7,29 $\pm$ 0,92	10,31 $\pm$ 1,22**	-
Obesity	21 (48,8%)*	3 (42,9%)	5 (9,8%)	-
Heart rate, bpm	77,7 $\pm$ 1,91^^	73,4 $\pm$ 4,56	75,6 $\pm$ 1,13^^	67,4 $\pm$ 1,1
SBP mm Hg	130,6 $\pm$ 3,63^	150,0 $\pm$ 6,90^A^^	127,5 $\pm$ 2,56^	120,7 $\pm$ 1,8
DBP mm Hg	80,4 $\pm$ 1,83	90,0 $\pm$ 3,78^A^^	82,7 $\pm$ 1,38^^	78 $\pm$ 0,75

^P<0,05, ^^P<0,01, ^^^P<0,001- reliability with respect to the control group;

\*P<0,05, \*\*P<0,01, \*\*\*P<0,001- the reliability between US and SS groups with the genotypes of the G/G of TNF- $\alpha$  (-308 G/A); ^P<0,05, ^^P<0,01, ^^^P<0,001- the reliability within the US group between the genotypes G/G and A/G+A/A of the TNF- $\alpha$  (-308 G/A).

We analyzed the parameters of clinical and immunological biomarkers with respect to the

distribution of the genotypes of TNF- $\alpha$  gene (-308G>A, rs 1800629) in patients with CHD (Table 4).

**Table 4. Comparative evaluation of clinical and immunological biomarkers of inflammation in patients with US and SS with respect to the distribution of genotypes of TNF- $\alpha$  gene (-308G> A, RS 1800629) (M  $\pm$  m)**

Indications	US		SS	Control (n=40)
	Genotype G/G, (n=43)	Genotype A/G+A/A (n=7)	Genotype G/G (n=51)	
TNF- $\alpha$ , pg/ml	13,4 $\pm$ 1,2 <sup>^^^</sup>	60,6 $\pm$ 17,2 <sup>^^^</sup>	12,5 $\pm$ 2,1 <sup>^^^</sup>	4,58 $\pm$ 0,81
IL-6, pg/ml	17,9 $\pm$ 2,3 <sup>^^^</sup>	57,3 $\pm$ 14,8 <sup>^^^</sup>	14,5 $\pm$ 1,8 <sup>^^^</sup>	3,42 $\pm$ 0,28
IL-18, pg/ml	155,0 $\pm$ 10,65 <sup>^^</sup>	197,7 $\pm$ 23,9 <sup>^^</sup>	133,69 $\pm$ 9,14	106,3 $\pm$ 11
CRP, mg/l	6,65 $\pm$ 0,8 <sup>^^^</sup>	7,25 $\pm$ 1,69 <sup>^^^</sup>	4,48 $\pm$ 0,31 <sup>^^^</sup>	0,9 $\pm$ 0,03
Fibrinogen, g/l	2,98 $\pm$ 0,1 <sup>^^^</sup>	3,62 $\pm$ 0,25 <sup>^^^</sup>	3,15 $\pm$ 0,18 <sup>^^</sup>	2,5 $\pm$ 0,09
Leucocytes, $\times 10^9/l$	6,47 $\pm$ 0,26 <sup>*</sup>	6,30 $\pm$ 0,38	5,72 $\pm$ 0,16	5,2 $\pm$ 1,1
Lymphocytes, %	31,9 $\pm$ 0,93 <sup>*</sup>	29,3 $\pm$ 1,55	28,41 $\pm$ 1,15	
ESR, mm/s	10,2 $\pm$ 1,17 <sup>*</sup>	11,1 $\pm$ 4,14	6,37 $\pm$ 0,45	6,5 $\pm$ 1,9

<sup>^</sup>P<0,05, <sup>^^</sup>P<0,01, <sup>^^^</sup>P<0,001- reliability with respect to the control group;

<sup>\*</sup>P<0,05, <sup>\*\*</sup>P<0,01, <sup>\*\*\*</sup>P<0,001- the reliability between US and SS groups with the genotypes of the G/G of TNF- $\alpha$  (-308 G/A); <sup>^</sup>P<0,05, <sup>^^</sup>P<0,01, <sup>^^^</sup>P<0,001- the reliability within the US group between the genotypes G/G and A/G+A/A of the TNF- $\alpha$  (-308 G/A).

In all study groups was a significantly increased level of proinflammatory cytokine TNF- $\alpha$  in the blood serum relative to the control group, in particular, a 2.8-times increase (p <0.001) in patients with the G/G genotype and 13-time increase in patients with genotype G/A (p <0.01). When comparing the genotypes within the US group, it was found that in patients with the A/G+A/A genotype, the level of TNF- $\alpha$  was significantly higher in 4.5 times (p <0.01) relative to patients with the G/G genotype. When comparing the US and SS group to the G/G genotype there was no significant difference, but there was a tendency to increase in US patients. Our data coincide with the studies of number of authors, where they showed that in carriers of haplotype of TNF $\alpha$ -308A, protein synthesis occurs 3 times more active than in persons with the -308G genotype.<sup>[32,38]</sup> Also M. Addela et al. (2012) showed that - AA and GA variants of the TNF $\alpha$  gene (-308G / A) are associated with an atherosclerotic plaque<sup>[23]</sup>, which coincides with our data, since in US patients with A/G+A/A genotype we observed significantly higher value (by 16%) of thickness of RCC artery intima-media complex (1.07  $\pm$  0.06, p <0.05) compared to patients with G/G genotype (0.92  $\pm$  0.04). Consequently, more significant progression of atherosclerosis was observed in US patients with genotype A/G+A/A of the TNF- $\alpha$  gene (-308G> A) that corresponds with literature data.<sup>[37]</sup>

A comparative analysis of proinflammatory cytokine IL-6 serum level in relation to healthy subjects showed a significant increase in the US group in carriers of the G/G genotype in 5.2 times (p <0.001), in carriers of the G/A+A/A genotype in 16.8 times (p <0.001), and in the SS group with genotype G/G in 4.2 times (p <0.001). When comparing the level of IL-6 in US patients was observed significant increase in 3.2 times (p <0.05) in patients with genotype G/A+A/A relative to the carriers of G/G genotype of the TNF- $\alpha$  gene (-308G> A), which confirms the literature data that TNF- $\alpha$  stimulates the expression of production of IL-1 $\beta$ , IL-6, IL-8.<sup>[24,29]</sup>

Comparative evaluation of the clinical inflammation markers between US and SS groups with the G/G genotype showed significantly increased values of hsCRP in 1.5 times (p <0.05), leukocyte counts in 1.1 times (p <0.05), lymphocyte count in 1.1 times (p <0.05) and ESR in 1.6 times (p <0.05) in patients with US. It should be noted that the highest levels were concentrated in US group with the genotype G/A+A/A, but were not verifiable when compared to US patients with genotype G/G. The level of fibrinogen was also significantly increased in the US group with the genotype G/A+A/A in 1.2 times (p <0.05) relative to patients with the G/G genotype.

The data we obtained are consistent with the results of meta-analysis conducted in 2011<sup>[33]</sup>, where european carriers of allele A had 1.5 times greater risk of developing CVD, compared to carriers of the GG genotype (AG+AA vs. GG, OR=1.50, 95 % CI=1.23-1.77). The level of TNF in the blood plasma is elevated in patients with CVD and may increase the risk of cardiac pathologies through effects on the vascular endothelium.<sup>[34]</sup> It should also be noted that carriers of allele A have higher transcription level of the TNF gene.<sup>[35,36]</sup> The molecular mechanism of such differences is not completely clear, since there was no difference in the strength of interaction of transcription factors with two allelic variants of the polymorphic marker G (-308) A of the TNF gene, at least for the Raji cell line. Perhaps, as a result of differences in DNA folding in the polymorphic region, the interaction of transcription factors with this site differs in the carriers of different alleles, which leads to increased transactivation of TNF gene. Despite the fact that polymorphic marker lies in the binding sequence of transcription factor AP2, an increase in the level of AP2 binding under in vitro conditions with the polymorphic region was not revealed.<sup>[35]</sup>

Identification of significant genetic variants associated with CVD risk has not yet been convincingly introduced into routine clinical medicine, however, clarifying the biological role of proteins in the atherosclerosis process

will lead to a better understanding of the pathways involved in pathophysiology of CVD. In addition, these pathways may become a target for the development of future therapeutic and prophylactic compositions.

### CONCLUSIONS

– We have shown the role of TNF- $\alpha$  gene (-308G> A) in the development of CHD-allele A, the A/G and A/A genotypes are characterized as risky variants in the development of unstable (progressive) stenocardia in the Uzbek population.

– In the group of unstable stenocardia with genotype profile A/G+A/A of TNF- $\alpha$  gene (-308G> A), the levels of TNF- $\alpha$ , IL-6, IL-18, CRP and fibrinogen were reliably higher in comparison with SS and control groups.

– Despite the fact that we have shown the role of TNF- $\alpha$  (-308G> A) in the development of CHD in the Uzbek population, further study of this SNP on much more material is necessary, in connection with the already known risk factors to establish its potential causal relationship.

### ACKNOWLEDGEMENT

The studies were carried out with the financial support of the Uzbek Foundation for Applied Research (project No. FA-A10-T010).

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