ASSOCIATION OF BIOCHEMICAL AND LIPID BLOOD PARAMETERS WITH t894g POLYMORPHISM OF ENDOTHELIAL NITROGEN OXIDE SYNTHASE GENE IN PATIENTS WITH HYPOTHYROIDISM AND CONCOMITANT CHRONIC NONCALCULOUS CHOLECYSTITIS

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Rezumat. Asociaţia parametrilor biochimici al lipidelor din sânge cu t894g gena polimorfismului endoepitelial oxid nitric la pacienţii cu hipotireoză şi colecistită cronică acalculoasă concomitentă

A fost studiată asocierea T894G a polimorfismului genei eNOS cu lipide şi unii parametri biochimici ai sângelui la pacienţii cu hipotireoză şi colecistita cronică acalculoasă concomitentă. Pacienţii cu hipotireoză şi colecistita cronică acalculoasă concomitentă la purtătorii T-alelei a fost observată cu 19,2% (p=0,02) mărirea colesterolului lipidelor cu densitate joasa în comparaţie cu cele ale pacienţilor cu GG-genotip. Astfel de particularităţi a profilului lipidic la pacienţii cu T-alela a genei eNOS, a contribuit la creşterea indicelui aterogenic cu 14,4% (p<0,05) în comparaţie cu parametrii corespunzători a pacienţilor purtători a GG-genotipului.

Cuvinte-cheie: hipotireoză, colecistită cronică, gena sintetazei endoteliale a oxidului nitric

Summary

Association of T894G eNOS gene polymorphism with certain biochemical and lipid blood parameters in patients with hypothyroidism and concomitant chronic noncalculous cholecystitis was investigated. Patients with hypothyroidism and concomitant chronic noncalculous cholecystitis T-allele carriers were characterized by 19,2% (p = 0,02) higher cholesterol of low density lipoprotein blood level compared to the appropriate indicator in patients with GG-genotype. Such peculiarities in lipid profile in patients with T-allele of eNOS gene led to the increased value of atherogenic index by 14,4% (p < 0,05) compared with proper parameter in patients GG-genotype carriers.

Key words: hypothyroidism, chronic cholecystitis, endothelial nitric oxide synthase gene

Резюме. Ассоциация биохимических и липидных показателей крови с t894g полиморфизмом гена эндотелиальной синтазы оксида азота у больных гипотиреозом и сопутствующим хроническим некалькулезным холециститом

Исследована ассоциация T894G полиморфизма гена eNOS с определенными биохимическими и липидными показателями крови у больных гипотиреозом и сопутствующим хроническим некалькулезным холециститом. У пациентов с гипотиреозом и сопутствующим хроническим некалькулезным холециститом носителей Т-аллеля наблюдали на 19,2% (p = 0,02) более высокий уровень холестерина липопротеинов низкой плотности по срав-

нению с соответствующим показателем у больных с GG-генотипом. Такие особенности липидного профиля у пациентов с Т-аллелем гена eNOS привели к увеличению у них индекса атерогенности на 14,4% (p <0,05) по сравнению с надлежащим параметром у пациентов носителей GG-генотипа.

Ключевые слова: гипотиреоз, хронический холецистит, ген эндотелиальной синтеза окиси азота

Introduction

Gene polymorphism of endothelial nitrogen oxide synthase (eNOS) has been the object of scientific interest for many years, since it plays a principal role in the regulation of vascular tone [6]. eNOS gene encodes the synthesis of the eNOS enzyme, that is responsible for the NO production in the vascular walls – an essential vasodilatator, antioxidants antymitogen and antiplatelet agent [5]. NO plays a key role in vascular relaxation, reducing of migration and proliferation of vascular smooth muscle cells, inhibition of platelet adhesion of leukocytes to the endothelium, inhibiting of low-density lipoprotein oxidation [4]. eNOS reduced activity, as a result of certain minor allelic variants of proper gene leads to a decrease in the NO-synthase expression and deficiency and NO blood concentration. Described changes result in endothelial dysfunction manifestation [3], leading to the atherogenesis development [8]. In particular, it was found out, that minor T-allele carriers of the eNOS gene show reduced activity of the eNOS enzyme [14] and decreased NO blood level [9]. Numerous studies indicate an association of minor T-allele of eNOS gene (T894G) with a higher incidence of heart stroke and arterial hypertension occurrence [1, 2, 12, 13]. Nozaki Y et al., in experimental studies on mice have shown a significant role of deficiency eNOS-derived NO blood concentration in the hepatobiliary system diseases development, in particular non-alchoholic fatty liver disease, due to the impact on the redistribution of fat in the liver of mice occurred due to the hepatic tissue blood flow violation [10]. We have not found any data about the peculiarities of chronic cholecystitis development depending on the eNOS gene (T894G) polymorphism, especially in patients with hypothyroidism who are prone to the development of this pathology.

The *objective* of the study was to investigate a possible association of T894G eNOS gene polymorphism with certain biochemical and lipid blood parameters in patients with hypothyroidism and concomitant chronic noncalculous cholecystitis.

Material and methods

The study involved 52 patients with hypothyroidism and concomitant chronic noncalculous cholecystitis (average age 46,1 ± 14,4 years), which were signed in research group. Disease duration since the diagnosis of hypothyroidism ranged from 1 to 10 years, chronic cholecystitis between 1 to 5 years respectively. The control group consisted of 20 practically healthy individuals correlative by their age and gender to the groups examined. Blood samples were obtained before the morning food intake from antecubital vein before the appointment of treatment. 5% solution of disodium salt of ethylene diamine tetraacetate was performed as an anticoagulant. The protocol of the study was done in accordance with the revised Helsinki Declaration (2008) and was approved by the local medical ethics committee. Written informed agreements were signed by all of the participants.

The range of indicators of biochemical blood analysis included: total bilirubin and its fractions, total protein and albumin, urea, creatinine, plasma enzyme activity (aspartate aminotransferase (AST), alanine aminotransferase (ALT), lactate dehydrogenase (LDH), gamma-glutamyl transferase (GGT), alkaline phosphatase (AP)). Lipid profile of the blood was studied by measuring the content of cholesterol, triacylglycerols, cholesterol of high density lipoproteins (HDL), cholesterol of low density lipoproteins (LDL), cholesterol of very low density lipoproteins (VLDL) in plasma. Atherogenic index was calculated on the base of received data. Biochemical studies were performed on the blood biochemical analyzer "Accent-200" ("Cormay SA", Poland).

Investigation of T894G polymorphism of eNOS gene was carried out in the state institution "Reference Center for Molecular Diagnostics of the Ministry of Public Health of Ukraine" (Kyiv, Ukraine). To determine the polymorphic variants of eNOS gene (G894T) (rs1799983) modified protocols with specific oligonucleotide primers ("Metabion", Germany) were used (**Table 1**) [11] using the method

Table 1

Oligonucleotide primers

Gene (polymorphism)	Primer sequences (5'-3')	Allele calling (size of fragments, bp)
eNOS *(G894T)*	AAGGCAGGAGACACTGATGGA- *forward* CCCAGTCAATCCCTTTGGTGCT- *reverse*	248 bp

of polymerase chain reaction (PCR) and subsequent analysis of restriction fragment length polymorphism.

The amplification products (amplicons) of eNOS gene (G894T) were evaluated in gel electrophoresis and only if proper DNA fragment of 248 bp was investigated subsequent analysis of restriction fragment length polymorphism was performed. For this purpose, amplicon obtained hydrolytic cleavage using restriction enzyme MboI ("Thermo Scientific", USA). Amplified fragments were analyzed by 2% agarose gel ("Cleaver Scientific", UK), with the addition of ethidium bromide, molecular weight marker GeneRuler 50 bp DNA Ladder ("Thermo Scientific", USA). Obtained results were visualized in transilluminator by the computer program Vitran.

To determine the type of data distribution, comparing the arithmetic mean and Wilcoxon-Shapiro test were used. To investigate the statistical differences between two independent groups Mann-Whitney test was used. Hardy-Weinberg equilibrium was calculated by a χ-square test. p values < 0,05 were considered statistically significant.

Results and discussion

Polymorphism (T894G) of eNOS gene was studied in 52 patients with hypothyroidism and concomitant chronic noncalculous cholecystitis and 20 healthy volunteers. The distribution of genotypes of the eNOS gene polymorphism is shown in **table 2**.

In the group of healthy individuals it was found: 1 (5,0%) person with TT-genotype, 10 (50,0%) heterozygous carriers and 9 carriers of GG-genotype (45,0%). T-allele of eNOS gene among healthy individuals was observed in 12 (30,0%) of 40 selected alleles, G-allele – in 28 (70,0%), respectively. Accordance of genotypes distribution with the Hardy-Weinberg equilibrium in the control group was tested using χ-square test with 1 degree of freedom, without Yates correction. As the result of this calculation it was found, that genotypes distribution in the control group complies with the Hardi-Weinberg equilibrium.

Among patients with hypothyroidism and concomitant chronic noncalculous cholecystitis 4 (7,7%) had TT genotype, 20 (38,5%) – TG-genotype, 28 (53,8%) – GG-genotype. T-allele of eNOS gene was observed in 28 (26,9%) of 104 cases selected alleles, G-allele – in 76 (73,1%), respectively. Using the χ-square test with 2 degrees of freedom, we found no statistically significant differences in the distribution of genotypes among observed patients and healthy people.

The method of determining the odds ratio (OR) established that frequency of T- and G-allele did not differ significantly in patients with hypothyroidism and concomitant chronic noncalculous cholecystitis and a group of healthy individuals OR = 0,86 (95% CI 0,39 – 1,92).

The analysis of biochemical blood indicators of the patients according to the T894G polymorphism of the eNOS gene was provided (**Table 3**). As the number of TT-genotype carriers was limited (n = 4), we conducted an analysis of the parameters studied for the presence of minor T-allele. As a result of this analysis, any statistically significant differences between the rates of biochemical blood analysis and different allelic polymorphism of the eNOS gene (T894G) in the observed patients were found.

The analysis of the possible association between options of lipid profile and allelic polymorphism of the eNOS gene (T894G), established significantly higher levels of cholesterol of low density lipoproteins in the patients T-allele carriers. In this group of patients it was in 19,2% (p = 0,02) higher compared to the appropriate indicator in patients with GG-genotype (**Table 4**). These findings confirm data of other investigators [4, 7], who postulate that NO plays an important role in the metabolism of low density lipoproteins. The mechanisms of such association require further investigations.

These changes causes a significant increase in atherogenic index in patients with T-allele in which

Table 2

Distribution of the eNOS gene polymorphism (T894G) in patients with hypothyroidism and concomitant chronic noncalculous cholecystitis and healthy individuals

eNOS gene genotype	Patients with hypothyroidism and concomitant chronic noncalculous cholecystitis (n = 52)		Healthy volunteers (n = 20)	
	Absolute quantity, n	Percentage	Absolute quantity, n	Percentage
TT	4	7,7	1	5,0%
TG	20	38,5	10	50,0%
GG	28	53,8	9	45,0%

Table 3

Biochemical blood parameters in patients with hypothyroidism and concomitant chronic noncalculous cholecystitis according to T894G polymorphism of the eNOS gene

Plasma level	Healthy volunteers, n = 20	Patients with hypothyroidism and concomitant chronic noncalculous cholecystitis, n = 52	
		GG-genotype carriers, n = 28	T-allele carriers, n = 24
Glucose, mmol/l (N= 3,9-6,0 mmol/L)	4,7 ± 0,09	5,1 ± 0,28	5,2 ± 0,31
Total bilirubin, mkmol/L (N=5,0-20,5)	12,0 ± 1,46	10,9 ± 1,19	10,4 ± 0,68
Direct bilirubin, mkmol/L (N=0,5-5,0)	3,6 ± 0,60	2,6 ± 0,28	2,4 ± 0,25
Albumin, g/L (N=35-50)	44,3 ± 0,57	45,3 ± 0,65	44,1 ± 0,45
Total protein, g/L (N=65-85)	68,5 ± 0,88	71,4 ± 1,18	69,8 ± 0,79
Urea, mmol/L (N=2,4-8,3)	4,4 ± 0,40	4,6 ± 0,32	4,6 ± 0,30
Creatinine, mkmol/L (N=40-110)	80,5 ± 2,72	80,8 ± 2,13	81,1 ± 2,65
Aspartate aminotransferase, units of action/L (N<37)	18,1 ± 1,42	22,4 ± 1,82 $p_1 = 0,02$	22,3 ± 1,38 $p_1 = 0,01$
Alanine aminotransferase, units of action/l (N<32)	14,7 ± 2,01	23,0 ± 2,90 $p_1 = 0,01$	23,2 ± 3,06 $p_1 = 0,01$
Lactate dehydrogenase, units of action/L (N=210-420)	378,4 ± 20,04	524,7 ± 19,99 $p_1 < 0,0001$	539,7 ± 21,96 $p_1 < 0,0001$
Alkaline phosphatase, units of action/L (N=42-141)	68,5 ± 4,35	91,5 ± 6,32 $p_1 = 0,003$	85,8 ± 3,28 $p_1 = 0,01$
Gamma-glutamyl transferase, units of action/L (N=10-50)	17,0 ± 0,92	27,6 ± 4,80 $p_1 = 0,03$	26,2 ± 3,62 $p_1 = 0,01$

Comment: p_1 – significance of differences compared with figures in the group of healthy people.

Table 4

Lipid profile in patients with hypothyroidism and concomitant chronic noncalculous cholecystitis according to T894G polymorphism of the eNOS gene

Plasma level	Healthy volunteers, n = 20	Patients with hypothyroidism and concomitant chronic noncalculous cholecystitis, n = 52	
		GG-genotype carriers, n = 28	T-allele carriers, n = 24
Cholesterol, mmol/l	4,32 ± 0,15	5,38 ± 0,24 $p_1 < 0,0001$	5,85 ± 0,30 $p_1 < 0,0001$
Triacylglycerols, mmol/l	0,74 ± 0,05	1,63 ± 0,20 $p_1 < 0,0001$	1,54 ± 0,20 $p_1 < 0,0001$
Cholesterol HDL, mmol/l	1,44 ± 0,06	1,49 ± 0,05	1,50 ± 0,05
Cholesterol LDL, mmol/l	2,32 ± 0,06	3,02 ± 0,12 $p_1 < 0,0001$	3,60 ± 0,24 $p_1 < 0,0001$ $p_2 = 0,02$
Cholesterol VLDL, mmol/l	0,36 ± 0,03	0,77 ± 0,09 $p_1 < 0,0001$	0,78 ± 0,10 $p_1 < 0,0001$
Atherogenic index	2,14 ± 0,21	2,64 ± 0,14 $p_1 = 0,01$	3,02 ± 0,15 $p_1 < 0,0005$ $p_2 < 0,05$

Comment: p_1 – significance of differences compared with the figures in the group of healthy people; p_2 – significance of differences compared with rates in patients with the GG-genotype.

it was by 14,4% ($p < 0,05$) elevated compared with corresponding figure in patients GG-allele carriers. No significant differences in other parameters of lipid profile were found.

Conclusions

1. Patients with hypothyroidism and concomitant chronic noncalculous cholecystitis T-allele carriers were characterized by 19,2% ($p = 0,02$) higher cholesterol of low density lipoprotein blood level compared to the appropriate indicator in patients with GG-genotype.
2. Such peculiarities in lipid profile in patients

with T-allele of eNOS gene lead to the increased value of atherogenic index by 14,4% ($p < 0,05$) compared with proper parameter in patients GG-genotype carriers.

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