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THE EFFECT OF GENETIC PREDISPOSITION AND METABOLIC ABNORMALITIES ON CARDIOVASCULAR RISK IN PATIENTS WITH NONALCOHOLIC FATTY LIVER DISEASE

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ABSTRACT

In recent years, nonalcoholic fatty liver disease is regarded as an independent risk factor for cardiovascular events. The refinement of traditional and non-traditional cardiovascular risk factors in patients with nonalcoholic fatty liver disease is relevant. Therefore, we studied the influence of the adiponectin receptor 2 polymorphic gene and metabolic disorders on the cardiovascular risk patients with nonalcoholic fatty liver disease. The study involved 230 patients with nonalcoholic fatty liver disease. Study results showed association between adiponectin receptor 2 gene polymorphism and development of cardiovascular risk in patients with nonalcoholic fatty liver disease. The results on distribution of genotypes of adiponectin receptor 2 (rs1044471) polymorphism showed association with the degree of cardiovascular risk. Significant differences when comparing the genotypes adiponectin receptor 2 rs767870 in the studied sample depending on the cardiovascular risk were not identified. It was shown that adiponectin receptor 2 rs1044471 CT genotype carriers compared with carriers of TT genotype had potentially greater cardiovascular risk. In nonalcoholic fatty liver disease patients, carriers of adiponectin receptor 2 CT genotype, the proatherogenic lipid profile and significant increase of the intima-media thickness were observed on the background of hypoadiponectinemia.

The obtained results confirm the influence of both metabolic phenotype and genetic factors on the cardiovascular risk in patients with nonalcoholic fatty liver disease. This is probably mediated by the effects of adiponectin receptors in the liver leading to genetic variation in the adiponectin receptor 2 locus and influencing the expression levels of adiponectin receptor 2 and/or their functions.

Keywords: *nonalcoholic fatty liver disease, cardiovascular risk, adiponectin receptor 2 (ADIPOR2) gene polymorphism.*

Introduction

Nonalcoholic fatty liver disease (NAFLD) has multifactorial nature of its onset and progression. High mortality rates of NAFLD patients might indicate that the "fatty" liver can act as an independent risk factor for cardiovascular events. Various studies showed relationship of obesity and insulin resistance to cardiovascular risk (CVR). In the Hoorn Study basal elevation of alanine aminotransferase increased 10-year risk of coronary heart disease (CHD) even after adjustment for the metabolic syndrome components [*Schindhelm R. et al., 2007*].

Cardiovascular risk factors were more common in NAFLD patients compared with individuals without liver steatosis [*Targher G. et al., 2004*], and it is no coincidence. It is due to the fact that "fatty" liver is responsible for the implementation of many metabolic components of CVR, such as very low density lipoproteins, C-reactive protein and components of the blood coagulation system.

The last years inflammatory mechanisms have been shown to play a key role in the initiation, maintenance and development of cardiovascular disease (CVD); furthermore, the association of CVD and obesity is mediated through the secretory activity of adipose tissue. Adiponectin, a key fat-derived protein, exerts its anti-inflammatory anti-atherosclerotic properties under insulin resistance (IR) through receptors ADIPOR1 and ADIPOR2 [*Kumada M. et al., 2003*].

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Adiponectin levels are known to be reduced in patients with obesity, type 2 diabetes mellitus (DM-2), and CHD [*Kumada M. et al., 2003*]. There are publications elucidating the influence of adiponectin levels on the development of adverse outcomes in patients with CHD [*Pilz S. et al., 2006; Schindhelm R. et al., 2007; Dekker J. et al., 2008; Schnabel R. et al., 2008*]. However, the role of adiponectin receptors in IR – regulatory or inhibiting – is not well understood [*Bluher M. et al., 2006; Nannipieri M. et al., 2007*].

Adiponectin receptor 2 (ADIPOR2) gene is located on chromosome 12p13.33 and consists of eight exons. Single nucleotide polymorphism (SNP) of ADIPOR2 is associated with either IR or with hepatic fat accumulation in different populations [*Lopez-Bermejo A. et al., 2008; Halvatsiotis I. et al., 2010*], although not in all studies [*Kathiresan S. et al., 2008*]. Nonetheless, the role of ADIPOR2 genetic variants and cardiovascular risk in NAFLD patients remains a subject of debate.

In this context, the purpose of investigation was to study the influence of ADIPOR2 polymorphic gene and metabolic disorders on cardiovascular risk in NAFLD patients.

MATERIAL AND METHODS

The study involved 230 people with NAFLD, including 148 males and 82 females; mean age: 42.6 ± 3.4 years. Mandatory inclusion criterion was the presence of risk factors such as obesity (BMI not less than 30 kg/m^2), DM-2, hypertension, dyslipidemia. All examined patients were not abusing alcohol (consumption of <50 *g* ethanol/week for men, <30 *g* ethanol/week for women in the last year). Patients had no signs of chronic viral hepatitis associated with hepatitis B, C and D (HBV, HCV, HDV) infection, they were autoimmune, hereditary-mediated and drug-induced hepatitis-free. As to the cardiovascular system, acute myocardial infarction in anamnesis, stroke, coronary artery surgery in medical history were exclusion criteria of the study. The control group consisted of 40 age- and sex-matched volunteers without NAFLD.

According to the SCORE scale, all patients were divided into the groups of high, moderate, and low risk for cardiovascular complications. Amongst the examined cohort 85.4% of patients were overweight; 56.2% patients had diagnosed carbohydrate metabolism disorders in the form of carbohydrate intolerance or DM-2. Clinical signs of hypertension were detected in 54.2% of NAFLD patients, dyslipidemia was recorded in 50%, and CHD was diagnosed in 38.9%.

All procedures carried out in this investigation were performed in accordance with the World Medical Association's Declaration of Helsinki. The study protocol was approved by the Scientific Bioethics Committee. All patients gave the written informed consent.

On the basis of computed tomography all patients under study were diagnosed with hepatic steatosis according to the criteria proposed by B. Birnbaum and co-workers in 2007 [*Ducluzeau P. et al. 2010*].

Molecular genetic DNA testing was performed by polymerase chain reaction (PCR). Genomic DNA was extracted from peripheral blood lymphocytes according to the standard protocol using reagent DIAtom ™ DNA Prep 200 ("Laboratory Isogene" LLC, Russia). The principle of the kit "DIAtom ™ DNA Prep" is based on the use of agent with guanidine thiocyanate, which is designed to disrupt the cellular structure to create a lysate, to cause cell debris solubilization and cellular nucleases denaturation. In the presence of the lysis reagent DNA is actively adsorbed on the NucleoS ™ sorbent, then it is easily washed by alcohol solution to remove salts and proteins. DNA eluted from the sorbent by Extra-Genome ™ or clean water can be studied by various methods.

The methodological basis of genotyping was tetra-primer polymerase chain reaction using two inner allele-specific primers for ADIPOR2 rs767,870 - AD2767F 5'-TCTAAATCATTGGCCAAAATAS -3', 5'-TCTAAGTAAGTATCTGTAAAGTCCA-3' and two external allele-specific primers: AD2767T231 - 5'- GAATTCTGCCAACTCTGC TGAAT-3'; AD2767C66 - 5'-CTGTGCTCTTTTT CCTAGGTTG-3'. For ADIPOR2 rs1044471 we used two relevant internal allele-specific primers: AD2 1044471 RC 270 5'-GCTGGAGATTGCAAGGGCAAG-3', AD2 1044471 F SNP T 140 5'-TTTCTCAGTTATTTTCCTCCT-3', and two external allele-specific primers - AD2 1044471F 5'-GGATCTGGTTCATGGAATTC-3', AD2 1044471 R 5'- ACTGAGTCTTTTGCTCGGTCC-3'.

The method allows to amplify DNA fragments of different lengths corresponding to alternative

alleles. Each external primer ADIPOR2 rs767 870 (T/C) in conjunction with the corresponding inner primer initiated amplification of allele-specific fragments (1 +2 - 297 n.p. – reaction control, 2 + 3 - 231 n.p. – normal value; 1 + 4 - 66 n.p. – mutation). Accordingly, the external primer ADIPOR2 rs1044471 (C/T) in conjunction with the corresponding inner primer initiated amplification of allele-specific fragments (1 +2 -370 n.p. – reaction control, 1+ 3 - 270 n.p. – normal value, 2+ 4 - 140 n.p. – mutation).

Oligonucleotide primers for PCR were designed with the Vector NTI ("Invitrogen", NML Bioinformatics Service, University of Southern California, USA) program and information resource NCBI.

In this work PCR of gene ADIPOR2 rs767870, rs1044471 sequence was performed in an automatic mode on the thermal cyclers "Tertsik" ("DNA technology" Scientific-Industrial Association, Russia), "GeneAmp ® 9700" with a 96-well block ("Applied Biosystems", USA) using a commercial kit reagents "GenePak @ PCR Core" ("IsoGene", Russia) in accordance with the manufacturer protocol.

PCR-products were detected using a horizontal electrophoresis in the plate of 2.5% agarose gel with the addition of ethidium bromide – specific fluorescent intercalating DNA (RNA) dye – using standard trisborate buffer at a field strength of ~ 20 *V/cm* for 30 minutes. Absorbing ultraviolet light with a maximum wavelength of 256 *nm*, ethidium bromide associated with DNA region (amplicon) is capable to fluoresce in accordance with Stokes rule, which is registered in the visible spectrum (610-620 *nm*) as an orange strip. The obtained results of amplicons electrophoresis were evaluated in the ultraviolet light on TFP-M/WL transilluminator ("VILBER LOURMAT", Germany). Results were fixed by standard gel-documenting system using software Vitran Photo.

Liver function was assessed by studying the protein, pigment, enzymatic metabolism according to standard methods. Levels of total cholesterol (TC), high-density lipoprotein (HDL), and triglycerides (TG) were measured in all patients by enzymatic method on "Humalyser" autoanalizer ("Human", Germany). The cholesterol (C) content in the low-density lipoproteins (LDL) was calculated by W. T. Friedewald formula including measuring in *mmol/l* LDL-C = total cholesterol - (HDL-C + TG/2.22). Serum adiponectin was determined using appropriate ELISA kit ("Orgenium Laboratories Anti Biotech Oy", Finland).

Measuring of the intima-media thickness (IMT) of the common carotid artery was performed by the standard method using ultrasonic imaging system ("Phillips IU", USA) [*Stein J. et al., 2008*].

For statistical data processing we used software package of data processing general-purpose "Statistica for Windows" (version 6.0). At the first stage of calculation the methods of descriptive statistics were applied for indicators measured in a quantitative scale including: median and mean value as a measure of the position, the standard deviation and quartiles as a measure of dispersion, the minimum and maximum value as an index of the statistical sampling range. Distribution of all analyzed quantitative indicators was significantly different from the normal (Kolmogorov-Smirnov criterion); therefore, in further discussion of their characteristics we mainly used median (50th percentile) and the 25th and 75th percentiles (lower and upper quartiles). For the description of qualitative variations, the frequency of the trait occurrence was traditionally used. To study effects of the independent variable on the dependent one, nonparametric analogues of dispersion analysis were used: Kruskal-Wallis criterion and the median test. To obtain the differences significance between groups represented as an alternative variation, Fisher's exact test was used.

Results and Discussion

The genotype distributions of polymorphism marker in ADIPOR2 (rs1044471) gene showed an association with the degree of cardiovascular risk (CVR). Thus, the TT genotype was evenly distributed among patients of low and moderate CVR (28.6% and 28.9%, respectively) and was observed in 18.6% of those with high CVR; 57.1% ADIPOR2 rs1044471 heterozygotes had low CVR, 37.8% – moderate and 44.2% – high. Herewith, in the carriers of the CC genotype with the increasing degree of CVR there was an increase in the frequency of its occurrence. The differences in the two groups of homo- and heterozygotes were significant ($p = 0.024$; the χ^2 criterion made 11.23) (Table 1). There were no significant differences at comparing the genotypes ADIPOR2 rs767870 depending on the CVR.

TABLE 1.
Comparative analysis of the genotype distribution of studied polymorphism markers in ADIPOR2 gene depending on CVR

CVR groups	TT	CT	CC
Genotypes rs 1044471 ADIPOR2 gene			
Low risk, %	28.57	57.14	14.29
Moderate risk, %	28.89	37.78	33.33
High risk, %	18.60	44.19	37.21
p=0.024; χ^2 =11.23			
Genotypes rs 767870 ADIPOR2 gene			
Low risk, %	78.57	0.00	21.43
Moderate risk, %	71.11	6.67	22.22
High risk,%	69.77	4.65	25.58
p>0.05			

The received nonlinear dependence of CT genotype of polymorphism marker ADIPOR2 rs1044471 prompted to specify association of alleles with CVR in NAFLD patients (Table 2). The analysis on distribution of ADIPOR2 rs1044471 alleles according to CVR allowed us to identify that the T allele carriers had low CVR in 85.7% cases, moderate – in 66.67%, high – in 62.79%,

TABLE 2.
Comparative analysis of alleles distribution of studied polymorphism marker rs1044471 in ADIPOR2 gene depending on CVR

CVR groups	The frequency of T allele	
	T -	T +
Low risk, %	14.29	85.71 %
Moderate risk, %	33.33	66.67 %
High risk, %	37.21	62.79 %
p=0.021; χ^2 =7.71		
	The frequency of C allele	
	C -	C +
Low risk, %	28.57	71.43
Moderate risk, %	28.89	71.11
High risk, %	18.60	81.40
p>0.05		

and with the increasing degree of CVR there was an increased incidence of NAFLD patients without T allele ($p = 0.021$; χ^2 criterion = 7.71). No significant differences in group C allele of a polymorphic gene ADIPOR2 rs1044471 were received. Furthermore, there were no significant differences in the analysis of allele polymorphism rs767870 ADIPOR2 distribution depending on CVR.

Data obtained on the alleles distribution ADIPOR2 rs1044471 allowed suggesting that, although the dependence between CVR and the T allele was received, at the same time, its presence might prevent the increase of CVR degree. This is clearly shown in the Figure.

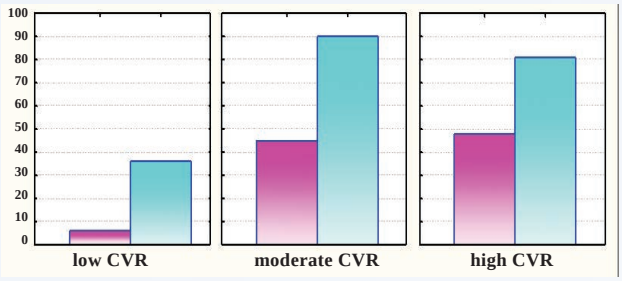

FIGURE. *Histogram showing the distribution of T allele ADIPOR2 rs1044471 according to CVR.*
– absence of T allele; – T allele carrier.

To detect possible relationship between polymorphism marker ADIPOR2 and metabolic disorders determining CVR, we assessed the value of TC, LDL, HDL depending on the genotype ADIPOR2 (rs1044471), adiponectin levels and IMT (Table 3).

Adiponectin concentration in NAFLD patients carriers of different genotypes ADIPOR2 rs1044471 was significantly different from each other. Adiponectin low concentrations, high TC, LDL, HDL, atherogenic ratio (AR) and IMT with a poor metabolic profile were found in NAFLD patients CT genotype carriers; this latter led to association with a high grade CVR. Levels of adiponectin, TC and LDL in NAFLD patients C allele homozygous in comparison with T allele carriers were likely to be associated with a lower degree of CVR. It should be noted that the content of adiponectin in the TT genotype ADIPOR2 rs1044471 carriers was higher than its levels in carriers of the CC genotype more than by 32% confirming the idea that, perhaps, T allele "inhibits" the development of CVR in NAFLD patients. On the other hand, the combination of hypoadiponectinemia with pro-atherogenic indexes in CT genotype car-

Table 3.

Comparative characteristics on contents of metabolic markers associated with CVR in patients with NAFLD depending on the genotype ADIPOR2 rs1044471

Parameter	Statistical Indicators						
	Mean	Median	Minimum	Maximum	Quartiles		Standard deviation
					Lower	Upper	
CT							
Adiponectin	8.96	8.65	3.41	14.73	5.68	12.34	3.22
IMT index	0.87	0.88	0.63	1.12	0.79	0.94	0.12
Total cholesterol	5.78	5.70	4.15	7.44	5.08	6.52	0.88
Low density lipoprotein	3.50	3.59	1.62	5.68	2.91	4.02	0.85
High density lipoprotein	1.41	1.40	0.60	2.18	1.07	1.73	0.42
Atherogenic ratio	3.81	3.47	1.00	9.73	2.63	4.49	1.69
TT							
Adiponectin	10.16	9.87	4.92	16.49	7.47	13.21	3.20
IMT index	0.83	0.87	0.57	1.14	0.70	0.94	0.14
Total cholesterol	5.50	5.61	0.98	7.57	4.80	6.31	1.23
Low density lipoprotein	3.43	3.42	0.38	6.04	2.74	4.20	1.07
High density lipoprotein	1.29	1.25	0.54	3.45	1.04	1.50	0.47
Atherogenic ratio	3.49	3.09	1.12	10.33	2.38	4.23	1.64
CC							
Adiponectin	14.72	14.72	14.45	14.98	13.42	14.64	0.37
IMT index	0.63	0.64	0.60	0.67	0.60	0.65	0.04
Total cholesterol	4.75	4.75	4.23	5.26	4.23	4.89	0.73
Low density lipoprotein	3.21	3.21	2.32	4.10	2.84	3.62	1.26
High density lipoprotein	1.09	1.09	0.77	1.40	0.98	1.24	0.45
Atherogenic ratio	3.33	3.33	2.02	5.83	2.14	4.12	0.69

riers is the proof of hypoadiponectinemia role in the CVR development and the mechanisms associated with the formation of cardiovascular events in NAFLD patients.

Differences between metabolic markers depending on the selected genotypes were significant (Table 4). These data revealed an association between metabolic disorders and CVR in the investigated patients.

We have shown that NAFLD patients carriers of T allele ADIPOR2 rs1044471 compared with C allele carriers are less likely to form high CVR as evidenced not only by the lipid profile, but also the levels of adiponectin and IMT index.

Table 4.

The significance of differences (*p*) of metabolic disorders associated with CVR in patients with NAFLD depending on the genotype TC, TT, CC (Kruskal-Wallis criterion)

Parameter	P – value
Adiponectin	0.0089
IMT index	0.0001
Total cholesterol	0.0000
Low density lipoprotein	0.008
High density lipoprotein	0.0001
Atherogenic ratio	0.0010

The role of genetic factors in the development of NAFLD has remained unknown for a long time. ADIPOR2 belongs to the affinity receptor responsible for "mediating" effects of adiponectin in the liver [*Yamauchi T. et al., 2003*].

Our results are consistent with the conclusions of the I. Halvatsiotis and associates, who showed that ADIPOR2 variants could be crucial in the atherosclerosis development and are dependent on IR, possibly by influence on protein ADIPOR2. The authors also observed significant differences in the allele distribution of rs767870 ADIPOR2 in groups of patients with and without CVD in the Greek population (p = 0.017). There were no significant differences in the allele distribution of polymorphism marker rs1044771 of ADIPOR2 [*Halvatsiotis I. et al., 2010*]. On the other hand, in the Ukrainian population we revealed a significant difference in the allele distribution of rs1044771 ADIPOR2 depending on CVR and its absence for rs767870 ADIPOR2. This is probably due to the ethnic characteristics of the studied samples. In the German cohort study SNP rs767870 ADIPOR2 was not associated with metabolic parameters, anthropometric data and the fat content in liver [*Stefan N. et al., 2005*].

In recent studies, there was no ADIPOR2 locus found to act as a regulator of lipid status [*Kathiresan S. et al., 2008; Willer C. et al., 2008*]. Only one SNP rs2058032 in ADIPOR2 showed the level of significance for HDL (p = 0.017) and TG (p = 0.086) in DM-2 case-control study. Such differences are likely to illustrate the difficulties in identifying specific SNP affecting phenotypic features such as serum lipid profile, which depends on many factors and may be modified by lifestyle. For example, genes responsible for the risk of myocardial infarction, which were originally identified in animal models, were not validated and replicated in the human genome studies [*Samani N. et al., 2007*].

In our study T allele ADIPOR2 rs1044471 was shown to have "protective" properties reducing the possibility of the CVR formation; patients with CT genotype presented a group of NAFLD patients with genetical predisposition to a higher CVR. Despite the limited use of metabolic markers to reflect the CVR, the successive comparisons between ADIPOR2 rs1044471 and the studied indicators (adiponectin, TC, LDL, HDL, AR, IMT) were done and genetic determination of CVR in NAFLD patients was thus confirmed.

Our study evidenced the important role of ADIPOR2 in the CVR formation and associated metabolic phenotype in NAFLD patients; the phenomenon appears to be mediated by the effects of adiponectin receptors in the liver.

The genetic variation in the ADIPOR2 locus can affect expression levels of ADIPOR2 and/or their functions. Any metabolic disorders can change the effects of adiponectin and its receptors in the liver leading to the initiation of the metabolic events cascade and contributing to the CVR formation.

CONCLUSIONS

NAFLD patients, carriers of the CT genotype of polymorphic gene ADIPOR2 rs1044471, have a genetic predisposition to the risk of cardiovascular events formation;

No association of SNP rs767870 in ADIPOR2 with cardiovascular risk in NAFLD patients was found;

Metabolic markers of NAFLD – TC, LDL, HDL, AR – vary depending on the genotype of a polymorphic gene ADIPOR2 and are associated with hypoadiponectinemia;

ADIPOR2 plays an important role in the CVR development in NAFLD thus confirming the influence of genetic factors on the CVR formation.

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