Ref: Ro J Med Pract. 2019;14(2)
DOI: 10.37897/RJMP.2019.2.8

Analysis of polymorphic options of the *eNOS, PNPLA3, CD14* genes at alcoholic liver disease

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Abstract

Introduction. *The development and progression of alcoholic liver disease (ALD) is conditioned and modified by genetic polymorphism and the interaction of genes with numerous factors, among which the important part is the amount of alcohol consumed, the presence of a viral lesion of the liver and the concomitant diseases of the patients.*

Objective. *Evaluation of the frequency of polymorphism genotypes eNOS (rs2070744), PNPLA3 (rs738409), CD14 (rs2569190) at ALD.*

Methods. *Polymorphic variants of eNOS (T-786C), PNPLA3 (C10109G), CD14 (C-159T) genes were analyzed by a polymerase chain reaction in 99 patients with alcoholic liver disease and 21 subjects in the comparison group.*

Results. *The frequency of polymorphous variants of the eNOS (T-786C) gene in the examined groups was: the TT-genotype was found in 21.6% of patients with alcoholic hepatitis (AH), in 48.4% of patients with alcoholic liver cirrhosis (ALC), in 28.6 % of practically healthy persons; TS-genotype – at 64.9%; 40.3%; 61.9% respectively; CC-genotype – in 13.5%; 11.3% and 9.5% respectively. The frequency of polymorphous variants of the PNPLA3 (C10109G) gene: CC-genotype – 54.0%, 48.4%, 61.9% respectively; CG-genotype – 21.6%, 22.6%; 19.05% respectively; GG-genotype – in 24.4%, 29.0%; 19.05% respectively. The frequency of polymorphic variants of the CD 14 (C-159T) gene: CC-genotype – in 32.4%, 40.3%; 23.81% respectively; the CT-genotype – in 48.6%, 35.5%, 52.38%, respectively; the TT-genotype – in 18.9%, 24.2%, 23.81%, respectively.*

Conclusion. *Association between the -786 TT genotype on the e-NOS gene and the development of alcoholic cirrhosis of the liver, as well as between -786 TC as a genotype for the e-NOS gene and the development of alcoholic hepatitis at prolonged alcohol abuse have been established. The absence of a specific one-nucleotide substitution in the e-NOS gene results in the development of a heavier liver injury, despite the same duration of alcohol abuse. Polymorphic variants of PNPLA3 (C10109G), CD14 (C-159T) genes are not additional risk factors for alcoholic liver disease in the examined patients.*

Keywords: eNOS gene (rs2070744), PNPLA3 gene (rs738409), CD14 gene (rs2569190), alcoholic hepatitis, alcoholic liver cirrhosis

Abbreviations
AH – alcoholic hepatitis, ALC – alcoholic liver cirrhosis, ALD – alcoholic liver disease

INTRODUCTION

A number of works during the last decade (1,2,3) was dedicated to the definition of genetic markers for the risk of alcoholic liver disease (ALD). Existing interest in conducting these studies is due to both the spread of this progressive disease in different populations and the development of the

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Article History:
Received: 6 May 2019
Accepted: 28 May 2019

irreversible process course of the toxic liver damage by alcohol that leads to cirrhosis of the liver and reduces the life expectancy of persons of working age in many industrialized countries.

The development and progression of ALD are conditioned and modified by genetic polymorphism and the interaction of genes with numerous factors, among which the important part is the amount of alcohol consumed, the presence of a viral lesion of the liver and the concomitant diseases. The presence of the viral lesion and concomitant diseases, according to some authors, does not always determine the phenotype of progressing liver damage. Genotype features of patients associated with faster development of cirrhosis were revealed (1-4). But an isolated analysis of these potential biological markers is not capable of answering all practical questions including the possibility of predicting the risk of development and the course of ALD.

MATERIAL AND METHODS

Prior to our genetic study, 99 patients with ALD who were treated in the department during 2012-2015 (main group), and 21 persons in the comparison group. The presence of patients with alcoholic hepatitis or alcoholic cirrhosis of the liver were the criteria for inclusion in the genetic study. Criteria for exclusion from the study were patients with diseases of internal organs with relapsing and organ failure, and detected chronic viral infection (herpes viruses, hepatitis, etc.). In 37 (37.37%) of 99 patients in the main group, alcoholic hepatitis (AH) was confirmed by additional laboratory and instrumental methods, and in 62 (62.62%) – alcoholic cirrhosis of the liver (ALC).

The genomic DNA for molecular genetic studies was isolated from peripheral blood using a commercial "innuPREP Blood DNA Mini Kit" test system (Analytik Jena, Germany) using centrifuge filters.

To determine the polymorphic variants of *CD14 (C-159T)*, rs2569190 gene and *PNPLA3 (C10109G)*, rs738409 gene, modified protocols with oligonucleotide primers were used using the PCR method and the subsequent analysis of restriction fragment length polymorphism (RFLP) (5,6). To determine the *eNOS (T-786C)*, rs2070744 mutation, a protocol with oligonucleotide primers was used with the allelic-specific PCR method (7). The investigated genes were amplified using specific primers (Metabion, Germany).

Specific fragments of *CD14 (C-159T)*, *PNPLA3 (C10109G)* and *eNOS* (T-786C) genes were amplified using the commercial DreamTaq Green PCR Master Mix (Thermo Scientific, USA).

To assess the distribution of genotypes and alleles between groups, the two-sided Pearson Chi-square test (χ^2) was used. The calculations were made using the Statistica software, version 10.0.

STUDY RESULTS

The baseline clinical response was evaluated in all patients and individuals in the comparison group (Table 1). This table shows that we did not find any significant differences in the investigated parameters.

TABLE 1. *Baseline clinical response of the patients and individuals in the comparison group*

Clinical parameters	Pa ts with AH (n=37)	Pa ts with ALC (n=62)	Comparison group (n=31)
Gender (male/female)	22/15	36/26	17/14
Age, years (M±m)	50.29±12.69	54.24±12.61	47.38±11.75
Weight, kg (M±m)	68.41±11.73	67.14±16.70	73.45±12.50
Height, cm (M±m)	172.27±7.16	170.63±8.10	170.58±7.07
IMT (M±m)	23.02±4.07	22.93±5.15	25.33±3.56

Alcohol consumption prior to the detection of ALD and the duration of the disease were analyzed in patients with AH and ALC. There was no significant difference in the duration of alcohol consumption prior to the detection of the AH or ALC. The daily dose of alcohol (in ml) did not differ significantly in the groups of patients with AH and ALC. Almost half of the patients in both groups most often used vodka (Table 2).

Among patients with AH, there was an anamnesis of the use of vodka with other alcoholic beverages (surrogates, wine, beer) in 16.21% of cases, as well as ALC patients combined vodka consumption in 14.51% (surrogates, wine, beer). The other most used alcoholic beverage was a surrogate, which consumption did not differ significantly in two groups – 29.73% and 24.19% respectively. Surrogates with other alcoholic beverages were more likely to be combined by patients with AH compared to ALC patients (5.4% and 3.23%, respectively). However, patients with AH were significantly more likely to use vodka with beer than patients with ALC (10.81% and 1.61%, respectively, χ^2=4.08, p=0.027). The least used alcoholic drink for the patients of both groups was wine (Table 2).

TABLE 2. *Indicators of alcohol consumption prior to the detection of ALD and duration of illness in patients with AH and ALC*

The parameters studied	Patients with AH (n=37)	Patients with ALC (n=62)
Dura ohol consump ears, (M±m)	9.27±2.62	9.24±2.07
Daily dose of alcohol consumed, ml (M±m)	279.73±69.18	285.48±73.77
Alcohol drinks, n (%):		
Vodka	17 (45.95%)	33 (53.22%)
Surrogate	11 (29.73%)	15 (24.19%)
Wine	2 (5.4 %)	2 (3.23%)
Vodka/surrogate	1 (2.7 %)	7 (11.29%)
Vodka/wine	1 (2.7 %)	1 (1.61 %)
Vodka/beer	4 (10.81%)*	1 (1.61 %)
Surrogate/wine	1 (2.7 %)	2 (3.23%)
Surrogate/beer	1 (2.7 %)	-
ALD disease experience, n (%):		
less than 1 year	6 (16.22%)	6 (9.68%)
1-1.5 year	20 (54.05%)	9 (14.52%)
2.-2.5 years	11 (29.73%)	47 (75.80%)

We also studied the duration of the use of various alcoholic beverages (in years) before diagnosing the ALD in the general group. The average duration of alcoholic beverage consumption did not differ in patients in case of excessive consumption of vodka (9.20 ± 1.89), surrogate (9.26 ± 2.49), beer (9.33 ± 2.34), but these indicators were significantly lowered (p <0,05) compared with patients who consumed wine (11.00 ± 3.67). For patients who consumed different beverages, the duration of abuse (9.95±2.39) was somewhat lower in relation to wine consumption, but without a significant difference.

Taking into consideration that in 99 patients of the main group genetic testing of polymorphic variants of *eNOS* (T-768C)*, PNPLA3* (C10109G)*, CD14* (C-159T) genes were analyzed, differences in the duration of alcohol abuse were analyzed depending on the genetic characteristics of the examined patients (Table 3).

At first glance, the shortest experience of alcohol abuse in the main group prior to the development of clinical manifestations of ALD was found for patients with the -768CS genotype for the *eNOS* gene, which seemed quite logical, since in the presence of this polymorphic variant, the detoxification function of the liver is reduced and the circulatory processes in the liver slow down but listed in Table. 3 the average duration of abuse did not differ significantly depending on the polymorphism of the *eNOS gene.* The shorter was the experience of alcohol abuse in the presence of genotypes 10109CC for the genome PNPLA3 and -159TT for the CD14 gene, but also without significant difference. Probably this is due not only to the genetic features of patients but also to the amount of alcohol consumed during the period of alcohol abuse per day.

Table 4 shows the results of the calculation of the average daily dose of alcohol intake, depending on the genetic characteristics of patients on ALD. As it turned out, carriers of the -768CS and -768TC genotypes for the eNOS gene consumed larger daily doses of alcoholic beverages prior to the ALD onset but these differences were not significant.

TABLE 4. *Indicators of daily alcohol abuse prior to the detection of ALD in patients depending on the polymorphism of eNOS, PNPLA3, CD14 genes*

Gene (polymorphism)	Genotype	Daily consumption, ml (M±m)	Significance of differences (p)
eNOS (T-768C)	-768TT	273.68±67.52	for all variants (p>0,05)
	-768TC	286.73±75.54	
	-768CC	300.83±70.71	
PNPLA3 (C10109G)	10109CC	290.00±77.59	for all variants (p>0,05)
	10109CG	279,55±62,98	
	10109GG	274.07±68.46	
CD14 (C-159T)	-159CC	270.27±68.17	for all variants (p>0,05)
	-159CT	290.00±81.02	
	-159TT	293.18±58.34	

TABLE 3. *Indicators of alcohol abuse duration prior to the detection of ALD in patients depending on the polymorphism of eNOS, PNPLA3, CD14 genes*

Gene (polymorphism)	Genotype	Abuse duration, years (M±m)	Significance of differences (p)
eNOS (T-768C)	-768TT	9.37±2.44	for all variants (p>0,05)
	-768TC	9.27±2.33	
	-768CC	8.83±1.53	
PNPLA3 (C10109G)	10109CC	8.94±2.20	for all variants (p>0,05)
	10109CG	9.86±1.96	
	10109GG	9.33±2.62	
CD14 (C-159T)	-159CC	9.92±2.70	for all variants (p>0,05)
	-159CT	8.90±1.91	
	-159TT	8.77±1.93	

Table 4 shows that similar features were established for patients with C-159T polymorphism for the CD14 gene. In the presence of the minor allele -159T (in a heterozygous or homozygous state), patients consumed a larger daily dose of alcohol prior to the ALD onset. In contrast, patients with the minor allele 10109G (10109CG and 10109GG genotypes), on the contrary, consumed a lower daily dose of alcohol than patients with a genotype 10109CC, but the indicated daily alcohol consumption in patients with the listed genotypes did not have meaningful differences.

In the examined patients with ALD, the frequency of genotype propagation for the *e-NOS* (T-786C) genome did not differ significantly from the frequencies identified in the surveyed individuals of the comparison group (Table 5).

Significant differences were established in the frequency of distribution of these genotypes between subgroups of patients with ALD, between patients with AH and ALC (Table 5), which consisted of decrease in the frequency of distribution of -786 TC and -786 CC genotypes at liver cirrhosis compared with their distribution in the presence of hepatitis, and accordingly, an increase in the distribution of the -786 TT genotype (χ^2 = 7.02; p <0.01) among ALC patients. The frequency of the genotype -786 TS spreading was significantly increased among patients with AH compared with ALC patients (χ^2 = 5.58; p <0.05). It is worth emphasizing that the given frequencies of genotypes on the investigated genome did not differ from the frequency of their distribution in the group of comparison persons (Table 5). Thus, we established the association between the -786 TT genotype on the *e-NOS* gene and the development of ALC, as well as between -786 TC as a genotype for the *e-NOS* gene and the development of AH at prolonged alcohol abuse have been established. The absence of a specific one-nucleotide substitution in the *e-NOS* gene resulted according to our study in the development of a heavier liver injury, taking into account the same duration of alcohol abuse.

Endothelial dysfunction due to genetic polymorphism is an important factor in the increased risk of liver damage. Polymorphic variants of the *e-NOS* gene affect the level of nitric oxide (or NO) production. According to research data, NO deficiency due to reduced gene expression is observed in people with T-786C single-nucleotide substitution. Due to the lack of NO in the liver, lipids accumulate, blood flow slows, and irreversible dystrophic structural changes occur (8). The consumption of ethanol suppresses gene expression and reduces NO production. For these molecular changes, a correlation was established with the degree of ethanol damage of the liver (9). Violation of regulating the inflammatory response and lipid metabolism due to long-time intake of ethanol is associated with the development of AH. Liver inflammation is the result of excessive production of cytokines at a long-term alcohol load (10). In hepatic sinusoids and hepatocytes at liver damage by any toxin varying degrees of inflammation and fibrosis, activation and migration of stem cells are observed (11). The listed processes just determine what morphological changes and, accordingly, the clinical symptoms will prevail in ALD. For the *e-NOS* gene, in some studies, association with non-alcoholic fatty liver disease (NAFLD) and insulin resistance were shown that, in turn, was due to the prevalence of dystrophy or steatohepatosis (12). But similar studies have not been performed in patients with ALD.

Genome-wide studies have found that the PNPLA3 gene, which is released in the liver, affects the overall lipid content of the liver and its function by participating in the metabolism of the liver lipoproteins (13). The most studied polymorphism of the gene based on the analysis of research sources was C10109G (rs738409). Some authors found the effect of the polymorphism of the C10109G, the G-allele of the gene on increasing the accumulation of lipids in the liver, an increased risk and severity of NAFLD and the faster development of cirrhosis (14), and was also recognized as the newest marker in the risk assessment of ALD (15) and progressive steatosis and fibrosis in the case of chronic virus hepatitis C (16). In the presence of the G-allele in patients, the hydrolysis of triglycerides decreases or does not occur, which

TABLE 5. *Distribution of polymorphous variants of the eNOS (T-786C) gene in the examined patients with ALD*

Gene / Polymorphism	Genotypes	Clinically healthy individuals (n=21)		Patients with AH (n=37)		Patients with ALC (n=62)		General group of patients with ALD (n=99)	
		n	%	n	%	n	%	n	%
eNOS (T-786C)	TT	6	28.57	8	21.6	30	48.4	38	38.4
	TC	13	61.90	24	64.9	25	40.3	49	49.5
	CC	2	9.52	5	13.5	7	11.3	12	12.1

TABLE 6. *Distribution of polymorphous variants of the PNPLA3 (C10109G) gene in the examined patients with ALD*

Gene / Polymorphism	Geno types	Clinically healthy individuals (n=21)		Pa ts with AH (n=37)		Pa ts with ALC (n=62)		General group of pa ts with ALD (n=99)	
		n	%	n	%	n	%	n	%
PNPLA3 (C10109G)	CC	13	61.90	20	54.0	30	48.4	50	50.5
	CG	4	19.05	8	21.6	14	22.6	22	22.2
	GG	4	19.05	9	24.4	18	29.0	27	27.3

are accumulated in the liver tissue as a result. It has been shown that steatoshepatosis and hepatic fibrosis never develop in PNPLA3 knockout mice, and in conditions of gene hyperexpression, the synthesis of triglycerides and their accumulation in the carriers of the G-allele increases in connection with the genetically determined lower hydrolytic activity of the adiponutrin enzyme (17). The presence or appearance of obesity also affects the growth of the accumulation of triglycerides in carriers of the G-allele (18). Some authors point out that liver damage at non-alcoholic fatty dystrophy and ALD is diverse in nature, they differ in the phenotype of the disease and, accordingly, varying degrees of its progression (19).

In the references mentioned above, the polymorphism of the PNPLA3 gene (C10109G) was considered as a promising prognostic marker of progressive liver disease, but with the same duration of consumption of identical doses of alcoholic beverages in two groups of patients with ALD (Table 6), we did not find any significant differences, as well as in the comparison group.

Consequently, we have not identified the association of the investigated polymorphism of the *PNPLA3 (C10109G)* gene with ALD and its phenotypic manifestations (AH or ALC).

The absence of the expected association with the risk of ALD development and the severity of the course indicates the need for further analysis in the context of the search for gene modifiers and the study of other polymorphic variants of the PNPLA3 (C10109G) gene. Donati B. et al. proved that another common polymorphic variant of this rs2294918 gene with the replacement of G> A (E434K protein variant) did not affect the enzymatic activity at all, had a relationship with the gene expression level and the amount of protein (20). Thus, the polymorphism of the locus rs2294918 of the PNPLA3 gene modifies the adverse effect of the rs738409 variant by reducing the synthesis of the mutant protein, which has a co-dominant negative effect on the increased accumulation of triglycerides in the liver (hep28370 Donati). The evaluation of the risk of liver damage including the development of ALD taking into account the results obtained by Donati B. et al. should be performed with molecular genetic studies of two loci of the PNPLA3 gene (rs738409 and rs2294918). Separate studies have also shown that the effect of PNPLA3 (C10109G) polymorphism is modified by overweight, physical activity and prolonged sedentary way of life or sedentary behavior (21).

Table 7 presents the results of the molecular genetic analysis of the *CD14* (C-159T) polymorphism in the examined patients with ALD and individuals in the comparison group. There were no significant differences in the frequency of genotype distribution in the presented groups and subgroups.

Clinical and experimental studies have shown that endotoxins in the intestine are important pathogenetic factors for the progression of ALD and faster ALC development. The leading role in the progression of endotoxic injury is believed to be due to an increase of the *CD14* gene expression due to the presence of the (C-159T) polymorphism gene in the promoter location. The functional effect of this polymorphism on gene expression accompanies liver damage and a course of the disease, as well as changes in serum transaminase parameters, so some authors suggested using the results of genetic testing as an additional (surrogate) biological marker (22).

TABLE 7. *Distribution of polymorphous variants of the CD 14 (C-159T) in the examined patients with ALD*

Gene / Polymorphism	Geno types	Clinically healthy individuals (n=21)		Pa ts with AH (n=37)		Pa ts with ALC (n=62)		General group of pa ts with ALD (n=99)	
		n	%	n	%	n	%	n	%
CD 14 (C-159T)	CC	5	23.81	12	32.4	25	40.3	37	37.4
	CT	11	52.38	18	48.6	22	35.5	40	40.4
	TT	5	23.81	7	18.9	15	24.2	22	22.2

CONCLUSIONS

Association between the -786 TT genotype on the *e-NOS* gene and the development of alcoholic cirrhosis of the liver, as well as between -786 TC as a genotype for the *e-NOS* gene and the development of alcoholic hepatitis at prolonged alcohol abuse have been established. The absence of a specific one-nucleotide substitution in the *e-NOS* gene results in the development of a heavier liver injury, despite the same duration of alcohol abuse. Polymorphic variants of *PNPLA3 (C10109G), CD14 (C-159T)* genes are not additional risk factors for alcoholic liver disease in the examined patients.

Conflict of interest: none declared
Financial support: none declared

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