Original Paper

Genetic Factors Involved in the Development and Progression of Nonalcoholic Fatty Liver Disease

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ABSTRACT: Purpose. The aim of our study was to identify the possible involvement of adiponutrine polymorphysm and of human leukocyte antigens (HLA) in the development of non-alcoholic fatty liver disease (NAFLD). Material and Methods. We included in this study a total of 138 subjects with non-invasive diagnosis of non-alcoholic hepatic steatosis. The patatin-like phospholipase domain containing protein 3 (PNPLA3) rs738409 (adiponutrine) polymorphism was genotyped by allelic discrimination TaqMan PCR assay (5' nuclease assay), using predesigned TaqMan SNP Genotyping Assays. Class I and II HLA antigens were determined by the polymerase chain reaction sequence specific oligonucleotide method (ADN-PCR-SSO). The results were compared with the same data from the control group subjects. Results. For PNPLA 3 polymorphism we found [CC] genotype in 82 subjects (59,42%), [GC] genotype in 45 (32,61%) and [GG] genotype in 11 subjects (7,97%). The frequency of minor [G] risk allele was 0.25. We found class I and II HLA antigens HLA A24, HLA B15, HLA DR15, HLA DR16, HLA DQ3 and HLA DQ5 more frequent in subjects with hepatic steatosis without any other risk factor and HLA-A2, HLA-32, HLA B18, HLA B49 and HLA B53 in patients with obesity or metabolic syndrome. Conclusions. Our results are consistent with the literature and show an association of PNPLA3 rs738409 polymorphism with hepatic steatosis. Regarding histocompatibility antigens, we studied for the first time in our country the relationship between HLA and non-alcoholic fatty liver disease.

***KEYWORDS**: Non-alcoholic fatty liver disease, PNPLA3 rs738409 polymorphism, histocompatibility antigens*

Introduction

Non-alcoholic fatty liver disease is considered nowadays a major public health problem worldwide [1,2]. Obesity, type II diabetes and dyslipidemia, in the absence of alcohol consumption, are the most important risk factors for non-alcoholic fatty liver disease [3]. The natural course of NAFLD is variable and is possible influenced by genetic factors. Polymorphisms in specific genes and histocompatibility complex genes have been studied in patients with NAFLD [4].

Genome-wide association studies identified single-nucleotide polymorphism (SNPs) that are associated with hepatic steatosis or elevated liver enzymes [5,6,7,8]. The interaction of genetic and environmental factors play an important role in the determination of the phenotype of the disease and its progression [9]. Consistent with these data, a common variant in patatin-like phospholipase domain containing protein 3 (PNPLA3) (adiponutrin) gene has been associated with hepatic triglyceride content and with the development of hepatic steatosis. Gene rs738409 polymorphism is associated with an increased risk for fatty liver, with the minor allele [G] as steatosis risk allele It is located on 22 chromosome and encodes a protein linked to the main hydrolase of adipose tissue-triglyceridlipase. As a result of the substitution of isoleucine with methionine at 148 position, patients carrying the risk variant will have increased hepatic fat content [9,10]. Some studies suggest that this genetic variant may confer increased susceptibility to hepatic injury and thus one of the individuals will develop steatohepatitis, fibrosis and liver cirrhosis [11].

The human leukocyte antigen is another genetic marker that may be implicated in the pathogenesis of nonalcoholic fatty liver disease. Although less studied than gene polymorphysm, HLA may contribute to disease susceptibility.

Given these data, the aim of our study was to identify the relationship between heapatic steatosis, PNPLA3 polymorphism, HLA typing

and the already known risk factors for non-alcoholic fatty liver disease.

Material and Methods

The study was conducted by the Research Center of Gastroenterology and Hepatology Craiova and included 138 subjects with non-alcoholic fatty liver disease and 125 age and sex matched healthy controls. For all patients, clinical and biological parameters were determined, including weight, height, waist and hip circumferences, liver enzymes, γ-glutamyl-tranpeptidase (GGT), platelet count, total cholesterol, HDL cholesterol, triglycerides, glycemia, insulinemia. Metabolic syndrome was defined according to IDF (International Diabetes Federation) criteria [12] and for insulin resistance we used HOMA-IR index.

Hepatic steatosis was assessed using non-invasive methods, abdominal ultrasonography and scoring systems as we already shown in a previous publication [13].

The genotyping assays were performed at Molecular and Cellular Biology Department, University of Medicine and Pharmacy of Craiova, using predesigned TaqMan SNP Genotyping Assays. Genomic DNA was extracted from peripheral blood, collected in an EDTA tube, using automatic extractor Maxwell® and 16 Blood DNA Purification Kit (Promega). The PNPLA3 rs738409 polymorphism was genotyped by Real-Time PCR using allelic discrimination TaqMan assay.

For 46 patients HLA typing was performed at the National Institute of Hematology and Blood Transfusion Bucharest, for A, B, C, D and DR antigens. The results was compared with 300 retrospective control subjects. The DNA from peripheral blood was extracted using ARROW BLOOD DNA kit and class I and IIHLA typing was performed by molecular biology technique-SSO-HISTO SPOT.

Statistical analysis was performed. The differences between continuous variables were expressed as mean±SD. A chi-square test was performed for the comparation between subjects and controls. For the groups with a lower number of subjects we used p Fisher test. A probability value<0.05 was considered significant.

The study was aproved by the Ethics Committee of University of Medicine and Pharmacy of Craiova and a written informed consent has been signed by all the participating subjects.

Results

From the 138 patients with abdominal ultrasoundshowing hepatic steatosis (39 men and 99 women, mean age 49±13 years), 107 (77.53%) were obese, 120 patients (86.95%) had metabolic syndrome, 53 (38.4%) were diabetic and 81 patients (58.69%) had elevated liver enzymes. The clinical and biological parameters of the patients are shown in Table 1.

Table 1. Clinical and biological parameters

Parameter	Stetosis I degree (n=28)	Steatosis II degree (n=57)	Steatosis IIIdegree (n=53)	p
BMI (kg/m^2)	31,98±7,42	31,98±7,28	38,21±7,26	<0.0001
Waist Circumference (cm)	103,03±13,14	109,68±13,75	117,59±14,21	<0.0001
Cholesterol (mg/dl)	185,4±44,27	201,38±44,92	194,46±44,72	0,154
Triglyceride (mg/dl)	120,87±71,64	156,81±76,49	139,71±76,34	0,130
AST (UI/L)	21,89±5,52	29,7±23,54	42,3±23,71	<0,0001
ALT (UI/L)	27,39±18,73	37,77±25,32	49,64±25,33	<0,0001
Cholesterol (mg/dl)	185,4±44,27	201,38±44,92	194,46±44,72	0,154
Triglyceride (mg/dl)	120,87±71,64	156,81±76,49	139,71±76,34	0,130
AST (UI/L)	21,89±5,52	29,7±23,54	42,3±23,71	<0,0001
ALT (UI/L)	27,39±18,73	37,77±25,32	49,64±25,33	<0,0001
GGT	25,25±35,53	40,36±46,52	54,45±46,64	0,009
HOMA-IR	2,9±1,91	4,16±2,55	5,65±3,11	<0,0001
FLI	65,66±24,6	74,77±24,2	91,73±23,83	<0,0001
NAFLD-LFS	0,07±1,55	1,28±2,11	2,70±2,30	<0,0001

Legend: BMI-Body mass index, FLI- fatty liver index, NAFLD-LFS- Non-alcoholic fatty liver disease-liver fat score

There were a significant correlation between the degree of hepatic steatosis, determined by ultrasonography, and the degree of obesity, the waist circumference, the ALT serum level and the insulin resistance index, but there was no correlation with cholesterol and triglycerides levels.

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The genotype frequencies for PNPLA3 rs738409 polymorphism in the study group was [CC](59,42%)>[CG](32,41%)>[GG](7,97%).
For the control group, genotype frequency was similar to that reported in the database *Entrez* SNP: [CC]>[CG]>[GG]. The [CG] genotype carriers had a 1.7 times higher risk for developing hepatic steatosis, compared with the [CC] genotype (p=0.046).

The PNPLA3 polymorphism was associated with an increased risk of hepatic steatosis in patients with BMI<30kg/m2, compared with the control population, when the risk allele [G] carriers were compared with the [C] allele carriers (p=0.038). By comparing the subgroup with steatosis without obesity with the subgroup with steatosis and BMI≥30kg/m2, we have noticed that the [G] allele carriers compared to the [CC] homozigotes in the dominant model, have a 2.5 times higher risk for developing hepatic steatosis (p=0.025). [G] risk allele was significantly associated with the risk of hepatic steatosis in patients without metabolic syndrome (p=0,005) and without insulino-resistance (p=0,033). (Table 2)

Table 2. PNPLA3 genotype frequency in the group with steatosis without metabolic syndrome

PNPLA3 rs738409 Polymorphism	Steatosis without metabolic syndrome n=18	Control n=125	OR(95%CI)	p
CC	7 (38,89%)	87 (69,60%)		
CG	7 (38,89%)	27 (21,60%)	3,222 [1,038-10,006]	0,046
GG	4 (22,22%)	11 (8,80%)	4,519 [1,138-17,953]	0,043
C	21 (58,33%)	201 (80,4%)	2,930 [1,408-6,095]	0,005
G	15 (41,67%)	49 (19,6%)		

The study group for HLA typing included 5 men and 31 women, aged between 24 and 69 years. 67.39% of the subjects were obese, 16 patients (34.78%) had the diagnostic criteria for metabolic syndrome and 8 patients (17.39%) were with diabetes. All the patients had ultrasound aspect of steatosis.

When we compared the antigen frequencies of the NAFLD subjects with the control group, HLA A2 (62,23%), A32 (13,34%), A24 (31,12%) şi A31 (11,12%), HLA B15 (13,34%), HLA B18 (26,67%), HLA DR15 (11,12%), HLA-DR16 (26,67%), HLA-DR8 (11,12%) were expressedmore significantly in the subjects group than control group.

The frequency of HLA A2, A31 and A32 was higher in patients with steatosis and metabolic syndrome compared with the patients without metabolic syndrome, whereas HLA A24 was found more frequently in patients with steatosis without metabolic syndrome.

A rarely antigen, HLA B53, was significantly present in the study group by comparing with controlers (6.67% vs. 0.08%, p<0.0001). All the patients with HLA B53 associated hepatic steatosis and metabolic syndrome, with a significant difference from the control group.

For HLA DR15 and DR16 there was a highly significant difference for the subgroup with steatosis and metabolic syndrome by comparing with the control group. For HLA DR16, the difference from the control population, remained highly significant in the absence of metabolic syndrome. (Table 3)

Table 3. p Fisher for HLA A, HLA B, HLA DR antigens

	Studied group vs Control group	Steatosis-SM vs Steatosis + SM	Steatosis-SM vs Lot martor	Steatosis + SM vs Control group
HLA A2	0,032	0,169	0,190	0,028
HLA A24	0,020	0,051	0,006	0,157
HLA A31	0,035	0,355	0,112	0,095
HLA A32	0,015	-	-	0,001
HLA B8	0,216	0,319	0,275	0,255
HLA B15	<0,0001	0,351	0,0061	0,0002
HLA B18	0,042	0,127	0,252	0,021
HLA B53	<0,0001	-	-	<0,0001
HLA DR15	0,102	0,0004	0,165	0,001
HLA DR16	0,255	<0,0001	<0,01	<0,01
HLA DQ5	<0,01	0,238	<0,01	<0,01

Discussions

In this study we evaluated the frequency of PNPLA3 rs738409 polymorphism in a group of subjects with non-alcoholic fatty liver disease. In the study group, [CG] genotype carriers had a 1.7 times higher risk to develop steatosis versus [CC] genotype carriers OR1,768 (95% CI, 1.006 to 3.110) (p=0.046). Furthermore, the [G] allele carriers had an increased risk of steatosis independent of obesity (p=0.038), of insulinresistance (p=0.033) and in the absence of metabolic syndrome (p=0.005).

Current evidences show that PNPLA3 polymorphysm don't affect insulin resistance, but rather sensitizes liver to the environmental stress factors [5]. In the study group, between the carriers of the risk allele [G] and homozygous [CC], there were significant differences related to the presence of obesity and metabolic syndrome. Thus, the [G] ([GG]+[CG]) allele carriers had a significantly lower BMI and the metabolic syndrome occurs less frequently than homozygotes [CC] (p=0.031, p=0.057). These aspects, as well as the lack of correlation with insulin resistance evaluated by HOMA-IR index (p=0.444), demonstrate that in the presence of the rs738409 polymorphism the development of hepatic steatosis occurs independently of insulin, and of the known risk factors. These results and the lack of correlation between the three genotypes and the serum levels of cholesterol and triglycerides are in accordance with the study by Romeo et al. 2011 [14].

Despite the international studies, there are a few studies published in Romania regarding the involvment of gene polymorphism and other genetic factors in NAFLD. In this study we attempt for the first time in our country to find a relationship between HLA and the susceptibility to NAFLD. In the study group we found two combinations of genes that appear to influence the disease: HLA A24, HLA B15, HLA DR15, DR16 HLA DQ5 HLA DQ3 in patients without other risk factors, and HLA-A2, HLA-32, HLA-B18, HLA-B49, and HLA B53 in patients with obesity or metabolic syndrome. There are very few studies at present. In a Turkish study population [15], HLA DQ5 was met with a greater frequency in patients with NAFLD, consistent with our findings. HLA B65 antigen, reported by the same study as being involved in the development of hepatic steatosis, was not found in any patients in our study group, nor in the control group. This fact may be explained by ethnic differences between the two populations.

Our study has some limits linked to the small number of patients who achieved HLA typing. It is possible that a greater number of subjects can concluded whether the histocompatibility antigens have an important role in determining susceptibility to NAFLD.

Conclusions

In conclusion genetic factors as gene polymorphism and histocompatibility antigens may confer susceptibility to non-alcoholic fatty liver disease and indicates new pathogenic ways for development of the disease. PNPLA3 polymorphism as well as the presence of some HLA (A24, B15, DR16, DQ5) could explain the disease in patients without any other risk factors. We need further studies for understanding better the involvement of genetic factors in development and progression of nonalcoholic fatty liver disease.

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