
ORIGINAL PAPER

ASSOCIATION OF A313G POLYMORPHISM OF GSTP1 GENE WITH BIOCHEMICAL BLOOD PARAMETERS IN PATIENTS WITH NONALCOHOLIC FATTY LIVER DISEASE

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SUMMARY

Background: One of the key gene involved in chronic diffuse liver diseases pathogenesis, including nonalcoholic fatty liver disease are genes encoding the synthesis of glutathione-S-transferases. The objective of the study was to investigate a possible link between A313G polymorphism of the GSTP1 gene and biochemical blood parameters in nonalcoholic fatty liver disease patients.

Methods: Gene polymorphism of GSTP1 (A313G) in 64 non-alcoholic fatty liver disease patients and 20 healthy individuals (control group) was studied. The range of investigated biochemical blood parameters included: total bilirubin and its fractions, cholesterol, triglycerides, uric acid, total protein and albumin, urea, creatinine, plasma enzyme activity.

Results: G-allele significantly more frequently by 2.47 times (OR = 2.47, CI = 1.01-6.03, $p < 0.05$) occurred in observed patients compared with healthy individuals. Total bilirubin level in blood of patients with GG-genotype was by 19.7% ($p = 0.03$) and by 36.9% ($p = 0.04$) higher compared with AA-genotype and AG-genotype carriers respectively. Presence of G-allele was associated with increased alanine aminotransferase activity, which was significantly higher in observed patients AG- and GG-genotypes carriers compared to patients with-AA genotype at 60.7% ($p = 0.03$) and 51.0% ($p = 0.04$) respectively.

Conclusions: Frequency of G-allele of GSTP1 gene was in 2.47 times (OR = 2.47, CI = 1.01-6.03, $p < 0.05$) higher in nonalcoholic fatty liver disease patients compared with healthy individuals in Ukrainian population. Presence of G-allele was associated with increased concentration of total bilirubin and higher activity of cytolytic syndrome in observed patients.

Abbreviations: CDLD – chronic diffuse liver diseases, NAFLD – nonalcoholic fatty liver disease, GST – glutathione-S-transferase, AST – aspartate aminotransferase, ALT – alanine aminotransferase, LDH – lactate dehydrogenase, GGT – gamma-glutamyl transferase, AP – alkaline phosphatase

RÉSUMÉ

Association entre le polymorphisme A313G du gène GSTP1 et les paramètres biochimiques sanguins chez les patients avec stéatose hépatique non alcoolique

L'introduction: L'un des principaux gènes impliqués dans la pathogenèse des maladies chroniques du foie, y compris la stéatose non alcoolique hépatique sont des gènes codant la synthèse de glutathion-S-transférases. L'objectif de notre étude est d'enquêter sur un lien possible entre polymorphisme A313G du gène GSTP1 et les paramètres biochimiques sanguins chez les patients avec de la possédant la stéatose non alcoolique hépatique. **Matériaux et Méthodes:** On a étudié le Polymorphisme du gène de la GSTP1 (A313G) chez les 64 patients malades de stéatose non alcoolique hépatique et de 20 individus sains (groupe de contrôle). La gamme de paramètres biochimiques sanguins concernés inclus: bilirubine totale et ses fractions, cholestérol, triglycérides, acide urique, protéines totales et de l'albumine, de l'urée, la créatinine, l'activité enzymatique du plasma.

Résultats: G-allèle de gène GSTP1 a été observée 2,47 fois plus fréquemment (OR = 2,47, IC = 1,01-6,03, $p < 0,05$) chez les patients observés par rapport aux individus sains. Le niveau de bilirubine totale dans le sang des patients de génotype GG-est à 19,7% ($p = 0,03$) et de 36,9% ($p = 0,04$) plus élevé par rapport à un génotype AA-AG-porteuses et génotypiques respectivement. La présence de G-allèle a été associée à une augmentation de l'activité d'alanine aminotransférase, ce qui était significativement plus élevé chez les patients observés et AG-GG-porteuses génotypes par rapport aux patients avec le génotype AA à 60,7% ($p = 0,03$) et 51,0 % ($p = 0,04$), respectivement.

Conclusions: Fréquence de G-allèle du gène GSTP1 était 2,47 fois (OR = 2,47, IC = 1,01-6,03, $p < 0,05$) plus élevée chez les patients avec de la stéatose non alcoolique hépatique par rapport aux individus sains. Présence de G-allèle a été associée à une augmentation de concentration de la bilirubine totale et

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Key words: nonalcoholic fatty liver disease, glutathione-S-transferase gene, polymorphism

l'activité plus élevée du syndrome cytolitique chez les patients observés.

Mots clés: la stéatose non alcoolique hépatique, des gènes glutathion-S-transférase, polymorphisme

INTRODUCTION

One of the key genes involved in chronic diffuse liver diseases (CDLD) pathogenesis, including nonalcoholic fatty liver disease (NAFLD) are genes encoding the synthesis of glutathione-S-transferase (GST), – second phase enzyme detoxification systems that protect from endogenous oxidative stress and exogenous toxins, by catalysing of glutathione sulfuric groups conjugation and decontaminating of lipid and DNA oxidation products [8, 10]. The group of GST enzymes consists of cytosolic, mitochondrial and microsomal fractions. Eight classes of soluble cytoplasmic enzyme isoforms of GST are known [3]. In turn, there are three GST genes, each of them is responsible for the synthesis of different enzyme isoforms [11]. Single (A to G) substitution at nucleotide position 313 in GSTP1 gene, changing an isoleucine to a valine amino acid, is present homozygously in 4 to 12% of the population, causes to the reduced enzymatic GST activity and plays an important role in different diseases development [5]. K. Wu et al. investigated that GSTP1 313 G/G polymorphism is a strong predisposing risk factor for bladder cancer [12]. L. Qin et al., found that the genes GSTM3 and GSTP1 promoter methylation, which causes dysfunction of intracellular antioxidant defense system, more often occurs in patients with acute and chronic liver failure due to hepatitis B virus, compared with patients with compensated viral hepatitis. Determination of methylated promoters of GSTP1 and GSTM3 genes can serve as a prognostic factor in the development of acute and chronic liver failure in these patients [9]. I.A. Goncharova et al. showed that patients with liver cirrhosis AA genotype carriers has 2.5 times higher survival rate compared with the patients with the GG and AG genotypes of GSTP1 gene [4]. Despite of intensive investigations of the role of GSTP1 gene polymorphism (A313G) in the pathogenesis of different forms of CDLD, its association with appearance and severity of NAFLD should be clarified.

The objective of the study was to investigate a possible link between A313G polymorphism of the GSTP1 gene and biochemical blood parameters in NAFLD patients.

MATERIAL AND METHODS

A313G polymorphism of GSTP1 gene was studied in 64 patients with NAFLD and 20 healthy individuals (control group). Blood samples were obtained in the morning before taking meal from antecubital vein in the first day of hospitalization until the appointment of treatment. 5% solution of disodium salt of ethylene diamine tetraacetate was used as an anticoagulant. The study protocol was in accordance with the revised Helsinki Declaration (2008) and was approved by

the local medical ethics committee. Written informed consent was obtained from all the participants.

All of the patients and healthy volunteers underwent general complex clinical, laboratory and instrumental diagnostic investigations. Biochemical studies were performed on the blood biochemical analyzer "Accent-200" ("Cormay SA", Poland). The range of indicators of biochemical blood analysis included: total bilirubin and its fractions, cholesterol, triglycerides, uric acid, 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)).

Investigation of A313G polymorphism of GSTP1 gene was performed in the state institution "Reference Center for Molecular Diagnostics of the Ministry of Public Health of Ukraine" (Kyiv, Ukraine). Genomic DNA for molecular genetic studies was isolated from peripheral blood using commercial test kits "innuPREP Blood DNA Mini Kit" (Analytik Jena, Germany) using centrifugal filters. To determine the polymorphic variants of GSTP1 (A313G) rs 1695 modified protocols of oligonucleotide primers were used [2] using the method of polymerase chain reaction and subsequent analysis of restriction fragment length polymorphism. Investigation of gene domain was amplified using specific primers («Metabion», Germany), mentioned in [table 1](#).

The products of amplified DNA fragments of GSTP1 gene underwent hydrolytic cleavage using restriction endonucleases and BstUI Alw261 (BsmAI) («Thermo Scientific», USA). Amplified fragments were analyzed by 2% agarose gel, and restriction fragment GSTP1 (A313G) – 3% agarose gel (agarose company «Cleaver Scientific», UK), with the addition of ethidium bromide, molecular weight marker GeneRuler 50 bp DNA Ladder («Thermo Scientific», USA) and subsequent visualization by the computer program Vitran. [fig. 1](#) shows electroforegram of GSTP1 (A313G) gene restricts.

Hydrolytic cleavage of normal allele by restryctase was performed by one restriction site appearance 5'...GTCTCN1↓...3', resulting molecular weight fragments formation 328 and 104 b.p. (AA-genotype). When nucleotides adenine replace to guanine second restriction site appeared, resulting molecular weight fragments formation 222 and 104 b.p. (GG-genotype)

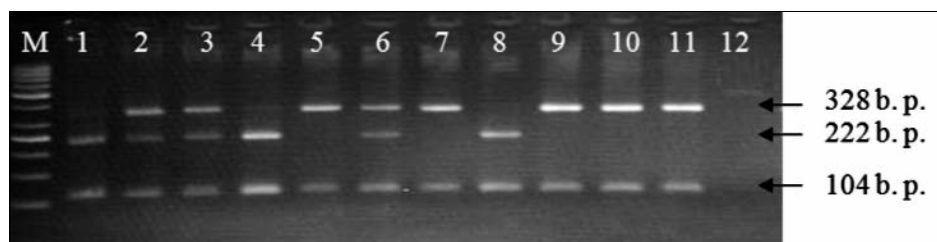
Examples 1, 4, 8 – GG-genotype, examples 2, 3, 6 – AG-genotype, examples 5, 7, 9-11 –AA-genotype, example 12 – negative control, M – molecular weight marker.

The quality of DNA isolation and conditions for multiplexed PCR setting was controlled by fragment of the albumin gene amplification with a molecular weight of 350 b.p. In case of separate fragment absence in the sample during the multiplexed polymerase chain reaction electrophoretic division, results for this sample were not considered.

Table 1- Primer sequences, restriction enzyme and allele calling for GSTP1 SNPs

Gene (polymorphism)	Primer sequences (5'-3')	Allele calling (size of fragments, b.p.)
GSTP1 (A313G)	GTAGTTTGGCCCAAGGTCAAG - forward AGCCACCTGAGGGGTAAG - reverse	432 b.p.
Internal control of amplification	GCCCTCTGCTAACAAAGTCCTAC GCCCTAAAAAGAAAATCGCCAATC	350 b.p.

Figure 1 - Electrophoregram of GSTP1 gene amplified fragment distribution



Statistical processing of the data was performed by the computer program PAST Version 2.05 [6]. To determine the type of data distribution, comparing the arithmetic mean, median and mode, and Wilcoxon-Shapiro test were used. To determine the statistical differences between two independent groups Mann-Whitney test was applied. Hardy-Weinberg equilibrium was calculated by a chi-square test. Odds-ratio was determined. P values < 0,05 were considered statistically significant.

RESULTS AND DISCUSSION

A313G polymorphism of GSTP1 gene in 64 patients with NAFLD and 20 healthy individuals (control group) was studied. The distribution of genotypes of GSTP1 gene polymorphism in NAFLD patients is presented in table 2. Among these patients AA genotype was diagnosed in 30 persons (46.9%), AG - 24 (37.5%), GG - 10 (15.6%); A-allele of GSTP1 gene was observed in 84 cases (65.6%) among 128 selected alleles, G-allele - in 44 cases (34.4%) respectively.

In the group of healthy volunteers homozygous carriers of Ala-allele were found 14 (70.0%), 5 persons (25.0%) from this group were heterozygotes, 1 person (5.0%) - homozygous carriers of G-allele. A-allele of GSTP1 gene was observed in 33 cases (82.5%) among 40 selected alleles, G-allele - in 7 cases (17.5%) respectively. Similar distribution of the GSTP1

polymorphic variants among healthy people of Bashkir population was obtained [1]. By OR calculation we found that G-allele significantly more frequently in 2.47 times (OR=2,47, CI=1.01-6.03, $p < 0.05$) occurred in NAFLD patients compared with healthy individuals. Our data are consonant with the results of M. Hashemi et al., which shows that G-allele of GSTP1 gene is a risk factor for NAFLD formation in the Iranian population [7].

Association of biochemical blood parameters in NAFLD patients with GSTP1 gene polymorphic variants are shown in table 3. The analysis of possible differences in parameters of synthesizing, detoxification, excretory liver functions and activity of cytolytic and cholestatic syndromes between different polymorphic variants of A313G polymorphism GSTP1 gene was performed. Total bilirubin level in blood of patients with GG-genotype was by 19.7% ($p=0.03$) and 36.9% ($p = 0.04$) higher compared with AA-genotype and AG-genotype carriers respectively. Presence of G-allele was also associated with increased ALT activity, which was significantly higher in NAFLD patients AG- and GG-genotypes carriers compared to patients with AA-genotype at 60.7% ($p = 0.03$) and 51.0% ($p = 0.04$) respectively. Moreover homozygotes by G-allele showed a tendency to increased AST and GGT activities, but this was not confirmed statistically. We have also detected some peculiarities in the carbohydrates metabolism in NAFLD patients depending on polymorphic variants of GSTP1 gene. In particular, glucose

Table 2 - Distribution of GSTP1 gene polymorphism (A313G) in patients with nonalcoholic fatty liver disease and healthy individuals

GSTP1 gene genotype	Patients with NAFLD (n = 64)		Healthy volunteers (n = 20)	
	Absolute quantity, n	Percentage	Absolute quantity, n	Percentage
AA	30	46.9%	14	70.0%
AG	24	37.5%	5	25.0%
GG	10	15.6%	1	5.0%

Table 3 - Biochemical blood parameters in patients with nonalcoholic fatty liver disease according to A313G polymorphism of GSTP1 gene

Plasma level	Healthy volunteers, n = 20	Patients with NAFLD, n = 64		
		AA-genotype carriers, n = 30	AG-genotype carriers, n = 24	GG-genotype carriers, n = 10
Glucose, mmol/L (N= 3.9-6.0 mmol/L)	4.8 ± 0.19	7.7 ± 0.68 p1 = 0.00001	5.5 ± 0.21 p1 = 0.02, p2 = 0.008	6.6 ± 0.66 p1 = 0.004, p3 = 0.05
Total bilirubin, mkmol/L (N=5.0-20.5)	10.3 ± 0.76	12.7 ± 1.12	11.1 ± 1.32	15.2 ± 1.36 p1 = 0.007 p2 = 0.03, p3 = 0.04
Direct bilirubin, mkmol/L (N=0.5-5.0)	2.6 ± 0.29	2.7 ± 0.32	2.8 ± 0.36	3.3 ± 0.46
Cholesterol, mmol/L (N=3.1-5.2)	4.72 ± 0.24	5.6 ± 0.20 p1 = 0.03	5.3 ± 0.28	5.5 ± 0.39 p1 = 0.03
Triglycerides, mmol/L (N=0.4-1.8)	1.26 ± 0.13	2.0 ± 0.19 p1 = 0.005	2.0 ± 0.22 p1 = 0.004	2.1 ± 0.38 p1 = 0.01
Uric acid, mkmol/L (N=200-450)	258.7 ± 13.81	347.4 ± 24.96 p1 = 0.005	352.2 ± 20.67 p1 = 0.004	342.6 ± 29.28 p1 = 0.01
Albumin, g/L (N=35-50)	46.3 ± 0.53	44.1 ± 0.62 p1 = 0.02	44.5 ± 0.98	44.1 ± 0.97
Total protein, g/L (N=65-85)	70.8 ± 0.93	72.2 ± 1.04	70.9 ± 1.43	72.3 ± 1.41
Urea, mmol/L (N=2.4-8.3)	4.3 ± 0.39	5.9 ± 0.57 p1 = 0.04	4.4 ± 0.34	6.5 ± 1.05 p1 = 0.03
Creatinine, mkmol/L (N=40-110)	80.6 ± 2.99	92.4 ± 5.28	84.1 ± 2.64	84.6 ± 3.21
Aspartate aminotransferase, units of action/L (N < 37)	24.9 ± 2.50	28.1 ± 2.79	27.0 ± 2.78	33.7 ± 8.15 p1 = 0.04
Alanine aminotransferase, units of action/l (N < 32)	22.1 ± 3.01	24.7 ± 3.13	39.7 ± 4.72 p1 = 0.03 p2 = 0.03	37.3 ± 7.74 p1 = 0.03 p3 = 0.04
Lactate dehydrogenase, units of action/L (N=210-420)	385.1 ± 21.15	486.2 ± 22.17 p1 = 0.005	488.6 ± 32.70 p1 = 0.02	494.5 ± 35.62 p1 = 0.03
Alkaline phosphatase, of action/L (N=42-141)	84.7 ± 5.40	90.1 ± 4.55	88.2 ± 7.43	80.5 ± 2.28
Gamma-glutamyl transferase, units of action/L (N=10-50)	25.3 ± 2.85	42.6 ± 4.58 p1 = 0.004	37.9 ± 4.55 p1 = 0.03	62.7 ± 6.03 p1 = 0.01

p1 - significance of differences compared with the indicators in the group of healthy people; p2 - significance of differences compared with rates in NAFLD patients AA-genotype carriers, p3 - significance of differences compared with rates in NAFLD patients AG-genotype carriers.

plasma level was the lowest in heterozygotes and the highest in homozygotes by A-allele. Homozygotes by G-allele showed intermediate concentration of glucose in blood. The mechanisms of revealed dependence remain unclear, thus requiring more investigations to explain this phenomenon.

CONCLUSIONS

Frequency of G-allele of GSTP1 gene was by 2.47 times (OR = 2.47, CI = 1.01-6.03, $p < 0.05$) higher in NAFLD patients compared with healthy individuals in Ukrainian population. Presence of G-allele was associated with increased concentration of total bilirubin and higher activity of cytolytic syndrome in NAFLD patients.

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