

ANALYSIS OF THE ASSOCIATION BETWEEN THE RS767455 T>C *TNFRSF1A* AND RS1061622 T>G *TNFRSF1B* POLYMORPHISMS AND NONALCOHOLIC STEATOHEPATITIS

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Poor diet, sedentary behavior and genetic background are major factors contributing to the etiology and pathogenesis of non-alcoholic fatty liver disease (NAFLD). It is hypothesized that polymorphisms of the *TNFR1* and *TNFR2* genes coding for the receptors that bind the proinflammatory cytokine tumor necrosis factor alpha (TNF α) can be implicated in the susceptibility to NAFLD, but not much data is available in the literature. In the present work we aimed to investigate a possible association between the rs767455 T>C *TNFRSF1A* and rs1061622 T>G *TNFRSF1B* polymorphisms and one of NAFLD forms, nonalcoholic steatohepatitis (NASH), and to assess their effect on blood biochemistry. Samples of DNA isolated from the venous blood of 151 healthy donors and 242 patients with NASH were genotyped using PCR-RFLP. TNF α concentrations were measured by ELISA. We have not found any association between the rs767455 T>C *TNFRSF1A* polymorphism and the development of NASH in the residents of Karelia. However, we have discovered an association between NASH and the T>G *TNFRSF1B* rs1061622 polymorphism. Carriers of the G allele have a higher risk of developing NASH (OR = 4.83; 95% CI: 2.72–8.57). The rs1061622 T>G genotype of the *TNFRSF1B* gene appears to have no effect on TNF α concentrations and the activity of alanine aminotransferase (ALT), aspartate aminotransferase (AST) and alkaline phosphatase (ALP). Our findings suggest a possible association between the rs1061622 T>G *TNFRSF1B* polymorphism and a risk of developing NASH in the residents of Karelia.

Keywords: non-alcoholic steatohepatitis, tumor necrosis factor alpha, tumor necrosis factor alpha receptors, mbTNFR1, sTNFR, *TNFRSF1A* gene, *TNFRSF1B* gene, gene polymorphism, alanine aminotransferase, aspartate aminotransferase

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АНАЛИЗ АССОЦИАЦИИ ПОЛИМОРФНЫХ ВАРИАНТОВ T>C RS767455 ГЕНА *TNFRSF1A* И T>G RS1061622 ГЕНА *TNFRSF1B* С РАЗВИТИЕМ НЕАЛКОГОЛЬНОГО СТЕАТОГЕПАТИТА

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В этиологии и патогенезе неалкогольной жировой болезни печени (НАЖБП) важны особенности питания, малоподвижный образ жизни и наследственность. Предполагают, что полиморфные варианты генов, кодирующих рецепторы к провоспалительному цитокину фактору некроза опухоли альфа (TNF α) (*TNFR1* и *TNFR2*), влияют на предрасположенность людей к развитию НАЖБП. Однако сведения о связи данного заболевания с носительством полиморфных вариантов генов *TNFR1* и *TNFR2* почти отсутствуют в литературе. Целью исследования было изучить связь полиморфных вариантов генов *TNFRSF1A* (T>C rs767455) и *TNFRSF1B* (T>G rs1061622) с развитием одной из форм НАЖБП — неалкогольного стеатогепатита (НАСГ) и их влияние на биохимические показатели крови. Методом ПЦР-ПДРФ генотипировали ДНК, выделенную из венозной крови 151 здорового донора и 242 пациентов с диагнозом НАСГ. Содержание TNF α оценивали с помощью иммуноферментного анализа (ИФА). По результатам, связь полиморфного маркера T>C rs767455 гена *TNFRSF1A* с развитием НАСГ у жителей Карелии отсутствует. Обнаружена ассоциация с НАСГ полиморфного варианта T>G rs1061622 гена *TNFRSF1B*. У носителей аллеля G повышен риск развития данного заболевания ОШ = 4,83 (95% ДИ: 2,72–8,57). Влияние генотипа по T>G rs1061622 маркеру гена *TNFRSF1B* на содержание TNF α и активность аланинаминотрансферазы (АлАТ), аспаратаминотрансферазы (АсАТ) и щелочной фосфатазы (ЩФ) не выявлено. Сделано заключение, что полиморфный вариант T>G rs1061622 гена *TNFRSF1B* может быть вовлечен в предрасположенность населения Карелии к НАСГ.

Ключевые слова: неалкогольный стеатогепатит, наследственность, фактор некроза опухоли альфа, рецепторы к фактору некроза опухоли альфа, mbTNFR1, sTNFR, ген *TNFRSF1A*, ген *TNFRSF1B*, полиморфизм генов, аланинаминотрансфераза, аспаратаминотрансфераза

Финансирование: финансирование осуществлялось из федерального бюджета на выполнение государственного задания КарНЦ РАН (тема № 0221-2017-0049) на оборудовании Центра коллективного пользования ФИЦ «Карельский научный центр Российской академии наук», при поддержке стипендии Президента РФ для молодых ученых и аспирантов, осуществляющих перспективные научные исследования и разработки по приоритетным направлениям модернизации российской экономики на 2015–2017 гг.; в рамках «Программы стратегического развития Петрозаводского государственного университета в 2013–2017 г.», (НИОКР № 115070110006, ИКРБС № 216022450003), проекта «Разработка технологии скрининговой диагностики неалкогольной жировой болезни печени у лиц с избыточным весом и метаболическим синдромом» № 9173ГУ/2015 от 15.12.2015 г. программы «У.М.Н.И.К.».

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Progression of nonalcoholic fatty liver disease (NAFLD) and more specifically one of its serious forms, nonalcoholic steatohepatitis (NASH), is accompanied by elevated concentrations of proinflammatory cytokines, such as tumor necrosis factor alpha (TNF α), in the blood plasma and liver [1, 2]. Abnormally high TNF α promotes hepatic inflammation, lipid deposition and peroxidation, stimulates activation of Kupffer cells and hepatocyte apoptosis, and leads to insulin resistance [3]. As plasma TNF α levels go back to normal, the liver function recovers [4, 5].

Proteins belonging to the TNF family exert their biological effects by interacting with TNFR superfamily receptors [6]. TNF α -binding receptors (mbTNFR) are represented by two types of transmembrane proteins: mbTNFR I and mbTNFR II. The intracellular region of mbTNFR I carries a death domain absent in mbTNFR II. Once activated, the death domain triggers either apoptosis or necroptosis [7]. Another type of TNF α receptors are soluble sTNFRs, a product of mbTNFR ectodomain shedding mediated by ADAM metalloproteinases [8]. sTNFRs bind to TNF α and act as mbTNFR antagonists preventing activation of TNF α -signaling pathways. Low concentrations of soluble TNF α receptors can be found in the blood serum and urine of healthy individuals. Patients with chronic viral hepatitis [9], cirrhosis [10], or NAFLD [11, 12] have elevated levels of circulating TNFR, which indicates inflammation and activation of T-cell immunity, in particular CD8⁺ T-cells that express metalloproteinase ADAM-17 [8]. It is hypothesized that both levels and ratio of soluble to membrane-bound TNF α receptors play a significant role not only in inducing hepatocyte death and damage to the liver, but also in the regeneration and homeostasis of this organ [13, 14, 15, 16]. It appears that the ratio of soluble to membrane-bound TNFR I and TNFR II largely determines the intensity of immune response and inflammatory reactions. It has been shown that mutations in the *TNFRSF1A* and *TNFRSF1B* genes affect sTNFR I and sTNFR II concentrations in the blood plasma and the number of mbTNFR I and mbTNFR II proteins on the surface of innate immunity cells [17]. Therefore, we can hypothesize that polymorphisms of genes coding for TNF α receptors may substantially contribute to the etiology and pathogenesis of liver diseases, including NAFLD. At present, attempts are made to establish an association between polymorphic variants of TNFR-encoding genes and NAFLD. The data is still scarce, describing mostly a link between *TNFRSF1A* or *TNFRSF1B* polymorphisms and biliary cirrhosis, alcoholic liver disease and hepatocellular carcinoma [18, 19, 20]. Associations between polymorphisms of TNF α receptor genes and NAFLD are hardly reported in the literature. That said, we decided to investigate how *TNFRSF1A* and *TNFRSF1B* polymorphisms contribute to the development of NAFLD in Karelian residents.

METHODS

Venous blood sample collection was aided by the Department of Propedeutics of Internal Diseases and Hygiene (Institute of Medicine, Petrozavodsk State University) and the Laboratory for Clinical Diagnostics of the Clinical Hospital at Petrozavodsk Station (Russian Railways JSC). The study recruited 110 male and 132 female patients with NASH (242 patients in total) and 151 healthy individuals (64 males and 87 females). The healthy donors also underwent a medical checkup by the doctors of the Clinical Hospital at Petrozavodsk Station (Russian Railways JSC). All participants were divided into 2 groups: healthy controls with no clinical symptoms of NAFLD (mean age of 48.04 \pm 2.26 years) and patients with NASH (mean age of 50.14 \pm 2.46 years). The age did not differ significantly

between the groups ($U = 132.5$; $p = 0.637$). The study included individuals of both sexes who gave informed consent to participate. Among other general criteria for inclusion were: Karelian residency, negative HBsAg and hepatitis C antibody tests (no chronic viral hepatitis), the absence of alcoholic, drug-induced or autoimmune liver diseases confirmed by medical history and clinical or laboratory tests. The main group included patients with a first-time diagnosis of mild to moderate NASH (prior to treatment). Exclusion criteria for both groups were: infectious or inflammatory diseases within a month before the study, pregnancy or lactation, smoking, diabetes mellitus, body mass index ≥ 30 kg/m², drug therapy, intake of hepatotropic drugs. The diagnosis was established based on standard clinical, laboratory, instrumental and histological tests. The following blood parameters were evaluated: ALT, AST, and ALP (measured on the RandomAccessF-15 analyzer by BioSystems, Spain). Ultrasound scans revealed enlarged liver and increased parenchymal echogenicity in all patients with NASH. In some cases, the diagnosis was confirmed by liver biopsy.

Prior to drug therapy, 10 ml of venous blood were collected into EDTA-containing vacuum test tubes, of which 250 μ L were used for DNA extraction. Some venous blood was used to obtain 200 μ L plasma samples for measuring TNF α concentrations. The remaining blood volume was used for biochemistry tests.

The study was approved by the Committee on Medical Ethics of Petrozavodsk State University and Ministry of Health and Social Development of the Republic of Karelia (Protocol 39 dated November 15, 2017).

TNF α concentrations were measured in randomly selected blood plasma samples by ELISA using the Human TNF α Platinum ELISA kit (eBioscience, Austria). In total, 30 plasma samples of healthy donors (mean age of 49.11 \pm 1.81 years) and 60 samples of patients with NASH (mean age of 49.95 \pm 2.74 years) were tested; male and female samples were equally represented. The age did not differ significantly between the groups ($U = 181.5$; $p = 0.535$). Optical density of the solution was measured on the microplate reader Sunrise (Tecan, Austria) at 450 nm wavelength and 620 nm reference wavelength.

DNA was extracted from the peripheral blood on microcolumns using the K-Sorb kit (Syntol, Russia). Quality and quantity of the obtained DNA were evaluated on the SmartSpec spectrophotometer (Bio-Rad, USA).

To amplify the region of the *TNFRSF1A* gene harboring position 339 (rs767455), the following primers were used: forward 5'agtggctgaggttagac3' and reverse 5'ctatgcccgagtctcaac3' described in [21]. To amplify the region of the *TNFRSF1B* gene harboring position 587 (rs1061622), the following primers were used: forward 5'gcacacatcgctactctc3' and reverse 5'aaggagtgaatgaatgagac3' described in [21]. Polymerase chain reaction (PCR) was carried out in the iCycler iQ5 (Bio-Rad, USA) using a reaction mix by Evrogen, Russia. PCR products containing rs767455 were incubated with 1 unit Bse I restriction endonuclease (SibEnzyme, Russia) for 3 hours at 65 $^{\circ}$ C. PCR products containing rs1061622 were incubated with 1 unit Fat I restriction endonuclease (SibEnzyme, Russia) for 1 hour at 55 $^{\circ}$ C. Then DNA fragments were separated in 1.5% agarose gel using the tris-acetate buffer.

The obtained data were processed in Statgraphics 2.1. Differences in allele and genotype frequencies between the two groups were assessed using the χ^2 test; differences in biochemical parameters were assessed using the nonparametric Mann-Whitney-Wilcoxon U test. The latter was employed because distribution in the groups was not normal.

To assess how different genotypes affected blood biochemistry, the Kruskal–Wallis test was used. To estimate the risk of developing NASH, we calculated the odds ratio (OR) and the 95% confidence interval (CI) [22]. Differences were considered significant at $p < 0.05$.

RESULTS

Figures 1 and 2 show electrophoresis of rs767455- and rs1061622- containing PCR products after restriction digest.

TNFRSF1A>C (rs767455) allele and genotype frequencies have been analyzed in patients with NASH and healthy controls.

The datasets were tested for deviations from the Hardy-Weinberg equilibrium. Both healthy controls and patients with NASH demonstrated deviations for allele and genotype frequencies ($\chi^2 = 8.25$ (df = 2, $p < 0.05$), $\chi^2 = 21.64$ (df = 2, $p < 0.05$), respectively).

Table 1 shows that frequencies of T>C (rs767455) alleles and genotypes did not differ between patients with NASH and healthy donors.

We have also analyzed the frequencies of *TNFRSF1B* 587T>G alleles and genotypes in patients with NASH and healthy controls.

The two studied groups did deviate from the Hardy-Weinberg equilibrium ($\chi^2 = 0.30$ (df = 2, $p > 0.05$), $\chi^2 = 4.16$ (df = 2, $p > 0.05$) for healthy donors and patients with NASH, respectively).

Table 2 shows that *TNFRSF1B* 587T>G allele and genotype frequencies differed between the healthy donors and patients with NASH. The G allele was far more frequent in patients with NASH than in healthy individuals. Carriers of the G allele are at a higher risk of developing NASH (OR = 4.83; 95% CI: 2.72–8.57).

We have also assessed the effect of the *TNFRSF1B* polymorphism (rs1061622) on liver function tests and plasma TNF α levels (Table 3). No significant differences were observed regarding the studied parameters between carriers of two different genotypes in the compared groups. The genotype did not have any effect on blood biochemistry both in patients with NASH and healthy controls ($p > 0.05$).

DISCUSSION

We have attempted to establish an association between two polymorphisms rs767455 and rs1061622 of genes *TNFRSF1A* and *TNFRSF1B*, respectively, and susceptibility to NASH. According to the literature, these polymorphisms are associated with a few inflammatory diseases and abnormal levels of TNF α in the blood plasma [23]. The rs767455 polymorphism of gene *TNFRSF1A* is a synonymous mutation at position 36 of exon 1. Synonymous mutations are known to disrupt mRNA splicing, alter mRNA structure and affect protein folding [24]. It has been shown that adenine to guanine substitution at position 36 of *TNFRSF1A* leads to a CCA to CCG codon change, disrupting translation [25]. In combination with other *TNFRSF1A* mutations (haplotype T-A-T at rs4149570-rs767455-rs1800692), it leads to the reduced abundance of exon 2-skipping products [26]. We have not established an association between the rs767455 polymorphism and susceptibility to NASH in the study participants. However, we have discovered an association between the G allele carriership (rs1061622, *TNFRSF1B*) and the risk of this disease.

The rs1061622 polymorphism of the *TNFRSF1B* gene is a thymine to guanine substitution at position 587 of exon 6 that leads to a methionine to arginine amino acid substitution

at position 196 of the protein's transmembrane domain, near the site of proteolytic cleavage by ADAM metalloproteases. This mutation affects ectodomain shedding (cleavage of the intracellular fragment of the transmembrane protein and its release into the extracellular matrix). Some researchers have shown that TT (Met196) genotype carriers have lower levels of sTNFRII than those with the Arg196 receptor variant [27]. Other authors report that carriers of TT+TG genotypes at this locus have higher levels of sTNFRII in the blood plasma than donors with the GG genotype [28].

Thus, the rs1061622 polymorphism can alter the ratio of membrane-bound to soluble TNFRII both in health and inflammation. Patients with liver diseases have elevated levels of sTNFRII and sTNFRII in the blood plasma and liver that positively correlate with disease severity [10, 12, 29, 30]. However, the role of increased ectodomain shedding of TNF α receptors in inflammation is not absolutely clear. Elevated concentrations of sTNFRII accompanied by reduced number of mbTNFRII on cell surface can trigger mbTNFRII-mediated signaling pathways leading to apoptosis [7]. Besides, soluble TNFR can act as physiological attenuators of TNF α activity, competing for the ligand with membrane-bound receptors. However, it appears that soluble receptors are capable of stabilizing and preserving circulating TNF α and thus act as its agonists [31].

The Met196 and Arg196 variants of TNFRII differ in their ability to mediate TNF signaling and trigger apoptosis or necroptosis. Epithelial HeLaS3 cells transfected with the pcDNA3.1 plasmid containing the Arg196 allele of *TNFRII* demonstrated reduced activity of the nuclear factor κ B and poor recruitment of TRAF2 upon stimulation with recombinant TNF α [32]. Subsequent activation of TNFRII signaling pathway in these cells induced apoptosis while in the cells transfected with the plasmid containing the wild type Met196, survival rates were better. Importantly, NASH is accompanied by the

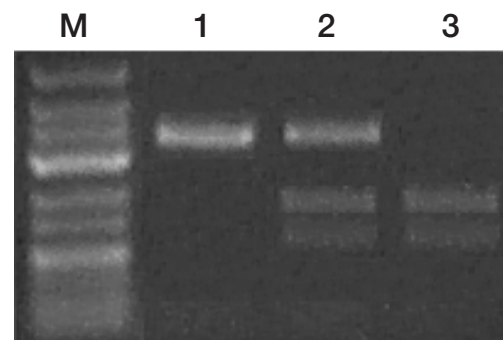


Fig. 1. Electrophoresis of rs767455-containing PCR products after restriction digest: M — Thermo Scientific GeneRuler Low range DNA Ladder, 1 — genotype CC (330 bp), 2 — genotype TC (330, 184 and 146 bp), 3 — genotype TT (184 and 146 bp)

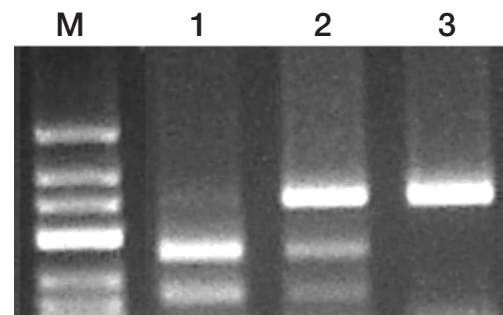


Fig. 2. Electrophoresis of rs1061622-containing PCR products after restriction digest: M — Thermo Scientific GeneRuler Low range DNA Ladder, 1 — genotype TT (235 and 144 bp), 2 — genotype TG (379, 235 and 144 bp), 3 — genotype GG (379 bp)

Table 1. Distribution of *TNFRSF1AT>C* (rs767455) alleles and genotypes in patients with NASH and healthy control

Alleles and genotypes	Controls (n = 131)	Patients with NASH (n = 242)	χ^2
T	119 (0.45)	229 (0.47)	0.24 (df = 1, p > 0.05)
C	143 (0.55)	255 (0.53)	
TT	35 (0.26)	72 (0.30)	0.61 (df = 2, p > 0.05)
TC	48 (0.37)	85 (0.35)	
CC	48 (0.37)	85 (0.35)	

Table 2. Distribution of *TNFRSF1BT>G* (rs1061622) alleles and genotypes in patients with NASH and healthy controls

Alleles and genotypes	Controls (n = 151)	Patients with NASH (n = 133)	χ^2
T	206 (0.68)	116 (0.44)	16.60 (df = 1, p < 0.05)
G	96 (0.32)	150 (0.56)	
TT	69 (0.46)	20 (0.15)	37.07 (df = 2, p < 0.01)
TG	68 (0.45)	77 (0.58)	
GG	14 (0.09)	36 (0.27)	

Table 3. Liver function parameters in healthy and diseased *TNFRSF1B587T>G* (rs1061622) carriers

Parameter	Controls		Patients with NASH	
	TT (n = 14)	TG + GG (n = 16)	TT (n = 20)	TG + GG (n = 40)
ALT, un/l	17.29 ± 2.05 (17.05)	18.31 ± 2.34 (18.27)	59.36 ± 8.53 (45.90)	65.56 ± 8.68 (48.30)
AST, un/l	24.36 ± 2.64 (19.50)	22.08 ± 2.72 (19.09)	51.22 ± 9.65 (41.05)	51.51 ± 6.27 (35.60)
ALP, un/l	118.42 ± 10.82 (117.46)	123.51 ± 17.14 (125.48)	218.00 ± 18.70 (210.00)	214.26 ± 11.28 (197.00)
TNF α , pg/ml	5.53 ± 1.38 (4.69)	4.82 ± 0.39 (5.08)	6.09 ± 0.43 (5.83)	6.36 ± 0.27 (6.27)

Note: data are represented as mean and error mean (M ± m). The median is shown in brackets.

death of hepatocytes [33]. We hypothesize that the rs1061622 polymorphism of the *TNFRSF1B* gene contributes to the development and progression of NASH through activation of signaling pathways that induce hepatic cell death.

The rs1061622 polymorphism affects the levels of proinflammatory cytokines, which provides another explanation of its involvement into the etiology and pathogenesis of NASH[34]. We have studied plasma concentrations of TNF α in healthy and diseased carriers of different alleles and genotypes to reveal no significant differences between the groups. However, we cannot claim the absence of any effect of the *TNFRSF1B* polymorphism rs1061622 on TNF α levels because of a small sample size (especially true for the controls) and

some other factors that may affect this parameter. Therefore, the role of this polymorphism in the development of NASH needs to be further investigated.

CONCLUSIONS

No association has been found between the rs767455 T>C *TNFRSF1A* polymorphism and the development of NASH in Karelian residents. We have however discovered an association between the rs1061622 T>G *TNFRSF1B* polymorphism and the disease. This polymorphic marker can be implicated in the genetic predisposition to NASH among the residents of Karelia.

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