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Research article

ON ASSESSING RISKS OF DEVELOPING AND PROGRESSING NON-ALCOHOLIC FATTY LIVER DISEASE USING TNF-A, IL6, AND VEGF FACTORS AND POLYMORPHISMS OF THEIR GENES

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Our research aim was to develop a system for calculating risks of development and progression of non-alcoholic fatty liver disease (NAFLD). The system would be based on interleukin-6 (IL-6), tumor necrosis factor-alpha (TNF-α), vascular endothelial growth factor (VEGF) and TNF-α gene polymorphism in the region -308G/A (rs1800629), IL-6 in the region –174G/C (rs1800795), and VEGFA in the region –634G/C (rs2010963).

We examined 52 patients with NAFLD and 65 healthy donors. The examination involved estimating levels of cytokines TNF-α, IL-6 and VEGF in blood serum. We also studied the polymorphism of the TNF-α genes in the -308G/A region, IL-6 in the -174G/C region, and VEGFA in the -634G/C region.

Women aged from 32 to 54 years prevailed among patients with NAFLD (67 %). We established in this research that concentrations of the pro-inflammatory cytokines TNF-α, IL-6 and the level of VEGF in the blood serum were significantly higher in patients with NAFLD than in the reference group ($p = 0.03$; $p = 0.00003$ and $p = 0.001$ accordingly). This confirms an occurring inflammatory syndrome and endothelial dysfunction that are typical for this pathology. Patients with NAFLD tended to have the AA genotype of the TNF-α -308G/A gene (rs1800629) significantly more frequently than healthy donors ($p = 0.04$). Homozygote CC and allele C of the VEGFA gene (G-634C) in the position rs2010963 were significantly more often detected in the test group (patients with NAFLD) than in the reference one ($p = 0.02$ and $p = 0.01$ respectively). We didn't detect any statistically significant differences in the IL-6 gene polymorphism in the -174G/C (rs1800795) region in the analyzed groups. TNF-α -308G/A gene polymorphism correlated with activating production of TNF-α and IL-6 cytokines ($Ki = 0.588$; $p = 0.043$ and $Ki = 0.597$; $p = 0.04$, respectively), which can lead to developing immune-inflammatory syndrome in its carriers. When determining genetic profiles, we established that 51 % donors had low risks of NAFLD development whereas the risk was high for 75 % of patients with the disease.

The risk of developing NASP is associated with carrying the AA genotype of the TNF-α -308G/A gene and the CC genotype of the VEGFA -634G/C gene. Assessment of a genetic profile using these markers provides an opportunity to assess risks of developing NAFLD in healthy people and to predict its progression in patients with the disease.

Key words: *non-alcoholic fatty liver disease, cytokines, tumor necrosis factor alpha, interleukin-6, vascular endothelial growth factor, gene polymorphism.*

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Non-alcoholic fatty liver disease (NAFLD) holds the first rank place among liver pathologies [1]. The All-Russian study DIREG 2 established than more than a half adult population in the country had NAFLD and the disease was diagnosed in 80 % of them. According to some researchers, NAFLD is associated with metabolic syndrome in 95 % cases, most frequently as fatty hepatosis [2]. Some patients also suffer from developing inflammation in the liver as a complication of the disease (non-alcoholic steatohepatitis). Chronic inflammation leads to developing fibrosis and cirrhosis and makes for occurrence of liver cancer as well. All this calls for more profound examination of how NAFLD develops in order to determine risk factors causing the disease, including genetic ones.

According to some data, NAFLD is more frequently detected among women aged 40–50 years and men to women ratio is 1:3, though the disease can occur in any age group [3][1]. All-Russian study DIREG 1 with 30,754 people participating in it produced the following results: women with NAFLD accounted for 56 %. According to this study there were several prevailing risk factors that caused NAFLD including dyslipidemia (type II as per Fredrikson classification) detected in 75.9 % patients; hypertension, 69.9 %; and hypercholesterolemia, 68.8 % [4]. Some other studies discovered such risk factors causing NAFLD as male sex, age from 30 to 59, overweight and obesity (body mass index being higher than 25 kg/m^2), hyperglycemia, hypertriglyceridemia, and hypercholesterolemia [5].

There is no unified and well-explored mechanism of NAFLD development. The disease has multifactorial pathogenesis which includes the following processes: the main component is resistance to insulin and changes in the hormonal profile regarding hormones that regulate lipid metabolism, occurring hyperinsulinemia, activation of lipolysis in fat tissue, an increase in contents of free fatty acids, activated gluconeogenesis in the liver resulting in hyperglycemia, increased production of very low density lipoproteins and decreasing seizure of triglycerides with developing dyslipidemia, pro-inflammatory cytokines and free radicals becoming more active with developing inflammation in the liver. Ultimately all this leads to steatosis progressing into steatohepatitis and liver fibrosis which can later turn into cirrhosis [6].

Inflammation is a component in complicated pathogenesis of NAFLD development and progress. Its basic mediators are cytokines, the key ones being interleukin-6 (IL6) and tumor necrosis factor alpha (TNF-α). Apart from stimulating inflammation, these cytokines regulate apoptosis and necrosis of hepatocytes, exacerbate resistance to insulin and induce fibrosis as well [7].

According to several research works, TNF-α correlates with a degree of liver fat dystrophy and activates adhesive properties of endothelial cells [8–10]. Several authors established higher IL6 contents in blood of patients with NAFLD [11–13]. IL6 concentration in the liver correlated directly with inflammation and fibrosis intensity and contents of this cytokine in blood, especially when NAFLD was progressing [14].

Vascular endothelial growth factor (VEGF) activates production of collagen by astrocytes in the liver and neoangiogenesis mechanisms in case of inflammation. There were several examinations on rats with NAFLD and metabolic syndrome which established that endothelial dysfunction occurs prior to inflammation and developing fibrosis in the liver [15–18].

Recently, a lot of attention has been given to hereditary predisposition to NAFLD. A significant aspect here is polymorphism of genes

[1] Nealkogol'naya zhirovaya bolezn' pecheni: metodicheskie rekomendatsii [Non-alcoholic fatty liver disease: methodical guidelines]. In: G.I., Storozhakov ed. Moscow, N.I. Pirogov's Russian National Research Medical University Publ., 2015, 42 p.

that regulate immune inflammatory processes [19]. Cytokine genes are highly polymorphic. Nevertheless, there are still rather few studies focusing on examining an association between polymorphism of TNF-α and IL6 genes and developing NAFLD and they tend to produce somewhat controversial results. It can probably be due to certain peculiarities related to how frequencies of alleles and genotypes are distributed in a population; there could be differences explained by a region, race, or a different methodical approach.

For example, a ratio of allele frequencies (A to G) as per -308G>A polymorphic marker of TNF gene amounts to 1.2–7 % among people living in the Asian-Pacific region; about 3.3 %, among healthy people in China; and it varies from 12 to 24 % in some other populations. AA genotype as per this gene doesn't occur in people living in the Asian-Pacific region whereas it is usually detected in 1.2–7.9 % cases among people from other populations [20].

There were some research works focusing on determining a contribution made by mutations in the promoter part of IL6 gene to developing liver pathologies; however, they produced rather controversial results. According to data available in literature, frequency of allele C as per -174G>C marker of IL6 gene was significantly higher among Europeans suffering from non-alcoholic steatohepatitis and hepatocellular carcinoma than among healthy people [21, 22]. It was also discovered that having allele C as per -174G>C polymorphism of IL6 gene was associated with developing non-alcoholic steatohepatitis in the Russian population [23]. However, another research didn't establish any correlation between this polymorphism and developing pathology in the liver [24].

Our research aim was to develop a system for calculating risks of developing and progressing non-alcoholic fatty liver disease (NAFLD) based on interleukin-6 (IL6), tumor necrosis factor alpha (TNF-α), vascular endothelial growth factor (VEGF), and polymorphism of TNF-α gene in -308G/A (rs1800629) region, IL6 gene in -174G/C (rs1800795) region, and VEGFA gene in -634G/C (rs2010963) region.

Materials and methods. We examined 52 patients with non-alcoholic fatty liver disease (NAFLD) in its clinical form; 35 of them were women (67 %) and 17 were men (33 %). Patients' average age amounted to 43.0 ± 11.1 years. Liver steatosis was established by an ultrasound examination. We excluded patients with alcoholic or drug-induced fatty liver disease confirmed by data taken from their clinical case histories; we also excluded patients with non-alcoholic steatohepatitis that was diagnosed by estimating transaminase levels. The analyzed samplings were similarly susceptible to factors that could cause NAFLD development. Prevalence of women in our random sampling made up of people with NAFLD living in Perm region is comparable with data obtained in much larger All-Russian studies [3, 4][1].

Our reference group consisted of 65 practically healthy people that were comparable with the test group as per sex and age; they didn't have either liver pathology or any other pathology associated with metabolic syndrome. All participants were provided with the comprehensive information about the study and they all gave their voluntary informed written consent to take part in it.

We determined contents of TNF-α, IL6 and VEGF cytokines in blood serum of 15 practically healthy people and 40 patients with NAFLD by ELISA tests using "Stat-Fax-2100" microplate reader (USA) and reagent sets produced by "Vector-Best" LLC (Novosibirsk).

We examined polymorphism of TNF-α gene in -308G/A region, IL6 gene in -174G/C region, and VEGFA gene in -634G/C region in 52 patients with NAFLD and 65 healthy donors using "CFX-96" real-time PCR detection system (Bio-Rad Laboratories, Inc., USA) and "SNP-Screen" allele-specific PCR (Syntol LLC, Moscow).

To determine risks of NAFLD development and progression, we estimated genetic profiles of

patients and donors depending on frequencies of genotypes and alleles of examined gene polymorphisms according to a score estimate scale developed specifically for this study.

Score estimates:

0 means that a patient is homozygous as per protective alleles regarding all three polymorphisms;

1 means a patient is heterozygous as per one of two genes;

2 means a patient is heterozygous as per two genes;

3 means a participant has two risk alleles as per one gene and is homozygous as per protective alleles in another gene;

4 means that a participant has both risk alleles as per one gene but he or she is heterozygous as per another gene;

5 means a participant is homozygous as per risk alleles of TNF-α (AA) / VEGFA (CC) regarding both genes.

According to this scale, when healthy donors scored 0–1, risk of developing NAFLD was low; 2–3 scores, moderate; 4–5 scores, high. When patients with NAFLD scored 0–1, it meant a risk of the disease progression was low; 2–3 scores, moderate; 4–5 scores, high.

All the data were statistically analyzed using Statistica 7.0 software package. Quantitative parameters were given as median and interquartile range (Q1–Q3). Significance of difference between two independent groups was estimated using Mann – Whitney test. We applied Spearman's correlation coefficient (r) with determining the significance level to estimate correlations. We applied χ^2 technique to describe frequency ratios for genotypes and alleles of the examined gene polymorphisms. Contingency tables and Pearson contingency coefficients $(Ki)^2$ were used to establish any dependence between examined qualitative attributes. Differences between the samplings were considered to be authentic at $p < 0.05$.

Results and discussion. We established that patients with NAFLD had significantly higher concentrations of TNF-α and IL6 and VEGF level in their blood serum than their counterparts from the reference group ($p = 0.03$, $p = 0.00003$, and $p = 0.001$, respectively) (Table 1).

Higher contents of the examined pro-inflammatory cytokines in blood of patients with NAFLD mean that inflammation is already developing and it was also mentioned as a typical NAFLD sign by some other authors [8, 11–13]. TNF-α authentically correlated with IL6 concentration ($r = 0.54$; $p = 0.0001$).

Growing VEGF concentration in blood of patients with NAFLD can indicate that endothelial dysfunction is developing against this pathology. Our data are well in line with results produced by several other studies that confirmed occurring endothelial dysfunction against liver steatosis by not only biochemical but also functional techniques [25, 26].

Table 1

Concentrations of TNF-α, IL6 and VEGF cytokines in the reference group and in patients with NAFLD (*Me*, 25 and 75 percentiles)

Parameter	Reference group ($n = 15$)	Patients with NAFLD ($n = 40$)	p
TNF-α, pg/ml	0 (0; 0.02)	1.1 (0; 3.15)	0.03*
IL6, pg/ml	0 (0; 0)	0.9 (0; 2.2)	0.0003*
VEGF, pg/ml	86.65 (10.7; 132.4)	184.6 (94.7; 291.6)	0.001*

Note: p is significance of differences, * means differences are statistically significant.

[2] Shelud'ko V.S., Podluzhnaya M.Ya. Teoreticheskie osnovy meditsinskoi statistiki: metodicheskie rekomendatsii [Theoretical grounds of medical statistics: methodical guidelines]. Perm, 2001, 36 p. (in Russian).

Table 2

Frequency of allele types of TNF-α gene in -308G/A (rs1800629) region, IL6 gene in -174G/C (rs1800795) region, and VEGFA gene in -634G/C (rs2010963) region in patients with NAFLD and healthy donors

Genotype / gene alleles		Donors (n = 65) % ± m	NAFLD (n = 52) % ± m	*OR*	p
TNF-α -308G/A	GG, %	21.54 ± 5.1	13.46 ± 4.73	0.57	0.25
	GA, %	78.46 ± 5.1	78.85 ± 5.66	1.02	0.96
	AA, %	0 ± 0	7.69 ± 3.69	1.76	0.04*
Alleles	G-allele, %	58.46 ± 4.32	52.88 ± 4.89	0.72	0.58
	A-allele, %	39.23 ± 4.28	47.12 ± 4.89	1.38	0.58
IL6 -174G/C	GG, %	32.31 ± 5.8	28.85 ± 6.28	0.85	0.69
	GC, %	52.31 ± 6.2	51.92 ± 6.93	0.98	0.97
	CC, %	15.38 ± 4.47	19.23 ± 5.47	1.31	0.59
Alleles	G-allele, %	58.46 ± 4.32	54.81 ± 4.88	0.86	0.58
	C-allele, %	41.54 ± 4.32	45.19 ± 4.88	1.16	0.58
VEGFA -634G/C	GG, %	35.38 ± 5.93	19.23 ± 5.47	0.43	0.04*
	GC, %	53.85 ± 6.18	51.92 ± 6.93	0.93	0.84
	CC, %	10.77 ± 3.85	28.85 ± 6.28	3.36	0.02*
Alleles	G-allele, %	62.31 ± 4.25	45.19 ± 4.88	0.50	0.01*
	C-allele, %	37.69 ± 4.25	54.81 ± 4.88	2.00	0.01*

Note: *OR* is odds ratio, p is significance of differences, * means differences are statistically significant.

52 patients with NAFLD and 65 healthy donors had practically the same frequency of GG genotype of TNF-α gene, 13.46 % and 21.54 % respectively (χ^2 = 3.23; p = 0.25; *OR* = 0.57) and GA genotype, 78.85 % and 78.46 % respectively (χ^2 = 1.28; p = 0.96; *OR* = 1.02), of TNF-α gene polymorphism in rs1800629 region. We didn't detect any significant differences regarding distribution of alleles G and A of TNF-α gene in -308G/A region between the examined groups (p = 0.58 and p = 0.58, respectively) (Table 2).

However, AA genotype of TNF-α gene in -308G/A region was detected significantly more frequently in patients with NAFLD than among healthy people (7.69 % and 0 % respectively; χ^2 = 6.05; p = 0.04; *OR* = 1.76) and similar results were also discovered by other researchers among people living in the Asian-Pacific region [20]. Probably, carrying AA genotype of TNF-α gene (rs1800629) plays a certain role in hereditary predisposition to NAFLD.

We didn't detect any significant differences as per IL6 gene polymorphism in -174G/C (rs1800795) region. GC genotype prevailed in both groups and was detected in 52.31 % and 51.92 % respectively (χ^2 = 0.36; p = 0.97; *OR* = 0.98). Alleles G and C also occurred with the same frequency (p = 0.58 and p = 0.50 respectively) (Table 3). Other researchers also pointed out that there was no correlation between this polymorphism and developing NAFLD [24].

When examining allele variants of VEGFA (G-634C) gene in rs2010963 region, we established GC genotype in 35.38 % of healthy people (χ^2 = 7.71; p = 0.04). However, CC homozygote was detected among patients with NAFLD significantly more frequently since it was highly probable in 28.85 % cases (*OR* = 3.36) whereas it was detected only in 10.77 % of healthy people (χ^2 = 6.18; p = 0.02). Allele C of VEGFA gene in -634G/C region was detected in 54.81 % cases among patients with NAFLD and this was significantly higher than among healthy

Table 3

Genetic profiles of healthy donors and patients with NAFLD

Groups / scores	0	1	2	3	4	5
Donors, % (*n*)	9 % (6)	42 % (27)	35 % (23)	3 % (2)	11 % (7)	–
NAFLD, % (*n*)	2 % (1)	23 % (12)	40 % (21)	4 % (2)	29 % (15)	2 % (1)

donors (χ^2 = 6.83; p = 0.01; OR = 2.00) (see Table 2).

We didn't detect any significant differences between men and women with NAFLD regarding frequencies of genes. Since the examined genes are located in autosomes (and not in the sex ones) and are inherited regardless of a sex, we assume that sex as a factor doesn't produce any significant effects on risks of developing NAFLD.

Therefore, we can assume allele C in the locus of VEGFA (G-634C) gene to be a predictor of developing NAFLD. We showed its significance in the process of viral liver diseases becoming chronic in our previous works [27].

Consequently, a risk of developing NAFLD is associated with carrying AA genotype of TNF-α -308G/A gene and CC genotype of VEGFA -634G/C gene.

When estimating dependences with contingency tables, we discovered a correlation between polymorphism of TNF-α in -308G/A region and activated production of TNF-α and IL6 cytokines in patients with NAFLD (Ki = 0.558; p = 0.043 and Ki = 0.597; p = 0.042, respectively). This can result in progressing immune-inflammatory syndrome in its carriers.

To determine risks of NAFLD development and progressing, we estimated genetic profiles of healthy donors and patients with the disease depending on frequencies of genotypes and alleles of TNF-α gene in -308G/A region and VEGFA gene in -634G/C region. Score estimates were given using our own scale. According to this scale, if healthy donors scored 0–1, then a risk of developing NAFLD was low; 2–3 scores, a risk was moderate; 4–5 scores meant a high risk. When patients with NAFLD scored 0–1, a risk that the disease would progress further was low; 2–3 scores meant a moderate risk; 4–5 scores meant a risk was high.

More than a half donors (51 %) had low risks of developing NAFLD (0–1 scores); 38 %, moderate (2–3 scores); 11 %, high (4 scores) (Table 3).

25 % of patients had low risks that NAFLD would progress further (0–1 scores); almost half of them (44 %) had moderate risks (2–3 scores), and one third (31 %) had high risks (4–5 scores) accordingly to the scale.

Conclusion. Women aged from 32 to 54 years prevail among patients with NAFLD (67 %). Patients with NAFLD have high concentrations of pro-inflammatory cytokines TNF-A and IL6, as well as elevated concentrations of VEGF which means there is inflammation and endothelial dysfunction developing against the pathology.

Homozygote AA in -308G/A region of TNF-α gene was significantly more frequently detected among patients with NAFLD than among healthy donors.

Having examined combinations of allele variants in -634G/C VEGFA gene in rs2010963 region, we established that homozygote CC b allele C were detected significantly more frequently in patients with NAFLD than in practically healthy people.

We didn't detect any authentic differences with respect to genotypes and alleles of IL6 gene polymorphism in -174G/C (rs1800795) region.

Consequently, a risk of developing NAFLD is associated with carrying AA genotype of TNF-α-308G/A gene and CC genotype of VEGFA -634G/C gene, especially when it is combined with high contents of pro-inflammatory cytokines.

The suggested approaches which involve analyzing genetic profiles as per TNF-α gene in -308G/A region and VEGFA gene in -634G/C region provide an opportunity to perform early non-invasive diagnostics and to determine whether there are any risks of developing NAFLD for healthy people (risks are considered to be high if the score estimate is 4–5) and risks of probable further NAFLD progressing for patients with the disease (score estimates equal to 4–5 men that risks of the disease progressing are high). These approaches can be applied in medical practice.

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