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Research of *PNPLA3* I148M Gene Polymorphism in Patients with Non-Alcoholic Fatty Liver Disease, with Liver Cirrhosis and with Hepatocellular Carcinoma

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Aim: to determine the frequency of *PNPLA3* rs738409 C>G gene polymorphism, leading to *p*.I148M substitution, in patients with non-alcoholic fatty liver disease (NAFLD), and to reveal the association between polymorphism and probable NAFLD outcomes: liver cirrhosis (LC) and hepatocellular carcinoma (HCC).
Materials and methods. The study was conducted according to the "case-control" design, three main groups were formed: a group with NAFLD (n = 46), a group with LC (n = 61), a group with HCC (n = 50), as well as a control group (n = 70), for all groups we performed genotyping of the rs738409 polymorphism of the *PNPLA3* gene. The relationship between the occurrence of different genotype variants and the diagnosis of patients was evaluated, the odds ratio (OR) of progression of NAFLD and the reliability of intergroup differences were determined.
Results. NAFLD patients with *PNPLA3* I148M polymorphism have a significantly higher chance of developing LC and HCC. The odds ratio for the GG genotype was 7.94 (95 % CI: 2.19–28.84; p = 0.030) for LC and 6.51 (95 % CI: 1.15–4.08; p = 0.039) — for HCC with concomitant LC. The presence of the minor G allele also increases the likelihood of transition from NAFLD to LC (OR = 2.38; 95 % CI: 1.41–4.02; p = 0.010) and HCC in the presence of cirrhosis (OR = 2.17; 95 % CI: 1.15–4.08; p = 0.039). Differences in the frequency of *PNPLA3* polymorphism between the NAFLD and HCC groups were not significant. Additional risk factors for HCC associated with NAFLD are overweight (OR = 5.14; 95 % CI: 1.94–13.67; p < 0.001), arterial hypertension (OR = 8.49; 95 % CI: 3.05–23,62; p < 0.001) and diabetes mellitus (OR = 8.57; 95 % CI: 1.03–71.48; p = 0.032).
Conclusion. The frequency of single nucleotide polymorphism *PNPLA3* significantly differs in patients with NAFLD, cirrhosis and HCC compared with the control group of healthy volunteers. The *PNPLA3* I148M polymorphism increases the incidence of NAFLD progression to cirrhosis and HCC, but only with concomitant cirrhosis.
Keywords: *PNPLA3*, NAFLD, liver cirrhosis, hepatocellular cancer
Conflict of interest: the authors declare that there is no conflict of interest.

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Исследование полиморфизма гена *PNPLA3* I148M у пациентов с неалкогольной жировой болезнью печени, циррозом печени и гепатоцеллюлярным раком

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Цель исследования: определение частоты полиморфизма rs738409 C>G в гене *PNPLA3*, приводящего к аминокислотной замене *p*.I148M, у пациентов с неалкогольной жировой болезнью печени (НАЖБП) и выявление ассоциации данного полиморфизма с вероятными исходами НАЖБП — циррозом печени (ЦП) и гепатоцеллюлярной карциномой (ГЦР).

Материалы и методы. Исследование проведено по дизайну «случай-контроль». Были сформированы три основные группы: группа с НАЖБП (n = 46), группа с ЦП в исходе НАЖБП (n = 61), группа с ГЦР на фоне НАЖБП (n = 50), а также контрольная группа (n = 70), для которой выполнялось генотипирование полиморфизма rs738409 в гене *PNPLA3*. Оценивалась связь между частотой различных вариантов генотипа rs738409 C>G и диагнозом пациентов, рассчитано отношение шансов прогрессирования НАЖБП до ЦП и ГЦР, достоверность межгрупповых различий.
Результаты. Пациенты с НАЖБП с полиморфизмом *PNPLA3* I148M имеют статистически значимо больший шанс развития ЦП и ГЦР. Отношение шансов (ОШ) при генотипе GG составило 7,94 (95 % ДИ: 2,19–28,84; p = 0,030) для ЦП и 6,51 (95 % ДИ: 1,15–4,08; p = 0,039) для ГЦР на фоне ЦП. Наличие минорного аллеля G также увеличивает вероятность перехода НАЖБП в ЦП (ОШ = 2,38; 95 % ДИ: 1,41–4,02; p = 0,010) и ГЦР на фоне ЦП (ОШ = 2,17; 95 % ДИ: 1,15–4,08; p = 0,039). Отличия частоты полиморфизма *PNPLA3* между группами НАЖБП и ГЦР были недостоверны. Дополнительными факторами риска ГЦР на фоне НАЖБП являются избыточная масса тела (ОШ = 5,14; 95 % ДИ: 1,94–13,67; p < 0,001), артериальная гипертензия (ОШ = 8,49; 95 % ДИ: 3,05–23,62; p < 0,001) и сахарный диабет (ОШ = 8,57; 95 % ДИ: 1,03–71,48; p = 0,032).
Выводы. Частота однонуклеотидного полиморфизма *PNPLA3* значимо различается у пациентов с НАЖБП, ЦП и ГЦР по сравнению с контрольной группой здоровых добровольцев. Полиморфизм *PNPLA3* I148M повышает частоту прогрессирования НАЖБП до ЦП и ГЦР, но только на фоне ЦП.
Ключевые слова: ген *PNPLA3*, неалкогольная жировая болезнь печени, цирроз, гепатоцеллюлярный рак
Конфликт интересов: авторы заявляют об отсутствии конфликта интересов.

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Introduction

Non-alcoholic fatty liver disease (NAFLD) is the leading chronic liver disease in the world, which can be defined as a hepatic manifestation of a metabolic syndrome characterized by steatosis, which in a number of patients can progress and lead to the development of steatohepatitis, fibrosis, and then liver cirrhosis (LC) or hepatocellular carcinoma (HCC) [1, 2]. Currently, there are no reliable predictors of NAFLD progression, so it is necessary to pay attention to the search for new potential molecular genetic markers [3].

Researchers associate the development of NAFLD with a number of single-nucleotide polymorphisms, in particular, the connection of the polymorphism of the *PNPLA3* gene (patatin like phospholipase domain containing 3) p.I148M (rs738409 C>G) with the development of steatosis was revealed [4]. The more frequent development of HCC may also indicate a probable connection between the polymorphism of the *PNPLA3* gene and HCC secondary to NAFLD [5]. Particular attention is paid to the carrier of the minor G allele as a risk factor of the development of aggressive forms of NAFLD [6].

According to Russian researchers, the presence of a minor allele of the *PNPLA3* gene in patients with NAFLD is associated with the severity of steatosis and cytolytic syndrome [7, 8]. The rs738409 C>G polymorphism in the *PNPLA3* gene can be considered as a marker of the formation and progression of fibrosis in patients with NAFLD being carriers of the G allele [9], as well as an increased risk of liver cirrhosis [10].

Currently, the feasibility of screening in a group of patients with NAFLD and GG variant rs738409 of the *PNPLA3* gene is being studied. Experts believe that additional data are needed to include active monitoring of such patients in clinical recommendations [11].

The aim of the study was to determine the frequency of *PNPLA3* gene polymorphism in patients with NAFLD, and to identify the association of this polymorphism with probable outcomes of NAFLD: liver cirrhosis (LC) and hepatocellular carcinoma (HCC).

Materials and methods

The selection of patients with NAFLD and LC (group 1 and group 2, respectively) was carried out in the Regional Hepatological Center on the basis of the Sverdlovsk Regional Clinical Hospital No. 1. The diagnosis of HCC secondary to NAFLD was established and morphologically confirmed in patients of the Sverdlovsk Regional Oncological Dispensary (group 3). In all patients included in the observation, the etiological connection of the development of liver pathology with factors such as viral hepatitis, alcohol abuse, taking hepatotoxic drugs or other toxic compounds, cholangitis, hereditary metabolic diseases was excluded; at the same time, steatosis could act as a background or independent disease. When taking medical history, data on concomitant cardiovascular and endocrine pathology, as well as the body mass index (BMI) of patients were taken into account.

To assess the intergroup differences, the "case-control" study design was chosen. Monitoring

of patients with laboratory tests was carried out from January to August 2022. The control group consisted of 70 healthy volunteers — 43 (61 %) women and 27 (39 %) men, without liver pathology and without the above-mentioned risk factors of liver lesions at the age of 30 to 69 years old (mean age — 40 years old).

The genotyping of the rs738409 polymorphism in the *PNPLA3* gene, which leads to the amino acid substitution of p.I148M, was carried out on the basis of the Laboratory of Molecular Biology, Immunophenotyping and Pathomorphology of the Regional Children's Clinical Hospital of Yekaterinburg. For genotyping, genomic DNA isolated from 300 ul of peripheral blood collected in a test tube with EDTA using a DNA-Extran kit (ZAO "Syntol", Russia) was used according to the manufacturer's instructions. The concentration of the isolated DNA was measured on a Qubit 4 fluorimeter (Thermo Fisher Scientific, USA) using the Qubit dsDNA BR Assay Kit (Thermo Fisher Scientific, USA). DNA was diluted to the concentration of 20 ng/mL and 5 mL of solution was introduced into the PCR reaction, which corresponded to 100 ng of DNA. Genotyping by allelic discrimination was performed on the Rotor Gene Q 5 plex device (Qiagen, Germany) using fluorescent probes and primers synthesized by ZAO "DNA-Synthesis" (Russia), the sequences of probes and primers were published earlier [12]. 100 ng of the DNA under study, 900 nmol of each of the primers, 300 nmol of fluorescently labeled hydrolyzable probes, 2.5 μL of 10 mM deoxynucleotide triphosphates, 2.5 units of SynTaq DNA polymerase (ZAO "Syntol", Russia) were added to the PCR reaction mixture. The amplification conditions were as follows: 95 °C for 5 min, then 40 cycles: 95 °C for 10 s, 55 °C for 30 s and 72 °C for 30 s. The results of real-time PCR were evaluated as follows: in the presence of a fluorescent signal only via the FAM channel, a wild-type homozygote (CC genotype) was detected, in the presence of a fluorescent signal only via the HEX channel — a homozygote for the pathogenic variant (GG genotype), with simultaneous detection of fluorescent signals via two FAM and HEX channels — heterozygote (CG genotype). Negative control (TE buffer used for DNA dissolution) was used in all setups. No fluorescent signals were recorded in the negative control.

Statistical processing of study results was carried out in software products Statistica 10 (StatSoft Inc., USA), Microsoft Excel (Microsoft Corp., USA) with the calculation of statistical averages (mean and median values, standard sampling error, standard deviation, etc.). The relationship between the frequency of various variants of the *PNPLA3* gene genotype (in particular, the frequency of the minor G allele) and the diagnosis (NAFLD, LC, HCC) was assessed based on the results of constructing conjugacy tables and subsequent determination of the odds ratio (OR) within the 95 % confidence interval (95 % CI). The statistical significance of the intergroup differences was assessed based on the results of the calculation of the non-parametric Mann — Whitney test for unconjugated samples.

Results

Group 1 with NAFLD included 46 patients 17 (37 %) men and 29 (63 %) women), mean age — 43.0 ± 9.3 years old. Overweight was found in 7 patients (15.2 %), arterial hypertension (AH) — in 6 (13.0 %), coronary heart disease (CHD) — in 6 (13.0 %), diabetes mellitus (DM) — in 1 (2.2 %). Group 2 with LC included 61 patients (19 (31 %) men and 42 (69 %) women), mean age — 52.0 ± 9.0 years old. In this group, there were more patients with overweight (n = 30; 49.2 %) and concomitant diseases in the form of hypertension (n = 29; 47.5 %), coronary heart disease (n = 13; 21.3 %) and diabetes (n = 15; 24.6 %). Group 3 with HCC was represented by 50 patients (33 (66 %) men and 17 (34 %) women) with a slightly higher average age — 61.0 ± 6.3 years old, and similar concomitant diseases: 24 patients (48.0 %) — with overweight, 28 patients (56.0 %) — with hypertension, 13 patients (26.0 %) — with coronary artery disease and 8 patients (16.0 %) — with diabetes. Differences in the frequency of overweight, hypertension, diabetes were statistically significant between patients with LC and NAFLD, between patients with HCC and NAFLD, and insignificant between patients with HCC and LC (Table 1).

Unfortunately, it was not possible to evaluate the effect of *PNPLA3* polymorphism on the progression of NAFLD to LC and HCC in isolation in patients with additional risk factors due to the insufficient number of such patients. The main analysis revealed significant differences in the frequency of single-nucleotide polymorphism rs738409 in the *PNPLA3* gene between the study groups and the control group (Fig. 1).

CG and GG genotypes were significantly more common in the follow-up groups of patients with NAFLD, LC and HCC (58.7 %, 62.3 % and 64.0 %, respectively) compared with the control group (45.7 %). The frequency of GG homozygotes is especially high in the LC group (26.2 % compared to 4.3 % in the control group).

The minor G allele was determined significantly more often in the NAFLD, LC and HCC groups compared to the control (Fig. 2).

There are also more pronounced differences in the frequency of the minor G allele in the LC group (44.3 % compared with 25.0 % in the control group).

The assessment of the reliability of differences in the frequency of a particular variant of the *PNPLA3*

Table 1. Pairwise comparison of the incidence of overweight, arterial hypertension, coronary heart disease and diabetes mellitus in the study groups according to odds ratios with a 95 % confidence interval, taking into account a two-sided *p*-test for the significance of differences

Таблица 1. Попарное сравнение частоты избыточной массы тела, артериальной гипертензии, ишемической болезни сердца и сахарного диабета в исследуемых группах по показателям отношения шансов при 95%-ном доверительном интервале с учетом двухстороннего *p*-критерия значимости различий

Parameter Параметр	LC — NAFLD ЦП — НАЖБП	HCC — NAFLD ГЦР — НАЖБП	HCC — LC ГЦР — ЦП
Overweight Избыточная масса тела	5.392 (2.089–13.918) $p < 0.001$	5.143 (1.935–13.666) $p < 0.001$	0.954 (0.451–2.016) $p = 1.000$
AH / АГ	6.042 (2.235–16.331) $p < 0.001$	8.485 (3.048–23.619) $p < 0.001$	1.404 (0.663–2.977) $p = 0.446$
CHD / ИБС	1.806 (0.629–5.183) $p = 0.315$	2.342 (0.807–6.799) $p = 0.131$	1.297 (0.538–3.128) $p = 0.654$
DM / СД	14.674 (1.860–115.774) $p = 0.001$	8.571 (1.028–71.481) $p = 0.032$	0.584 (0.225–1.517) $p = 0.348$

Note: AH — arterial hypertension; CHD — coronary heart disease; DM — diabetes mellitus; NAFLD — non-alcoholic fatty liver disease; LC — liver cirrhosis; HCC — hepatocellular carcinoma.

Примечание: АГ — артериальная гипертензия; ИБС — ишемическая болезнь сердца; СД — сахарный диабет; НАЖБП — неалкогольная жировая болезнь печени; ЦП — цирроз печени; ГЦР — гепатоцеллюлярная карцинома.

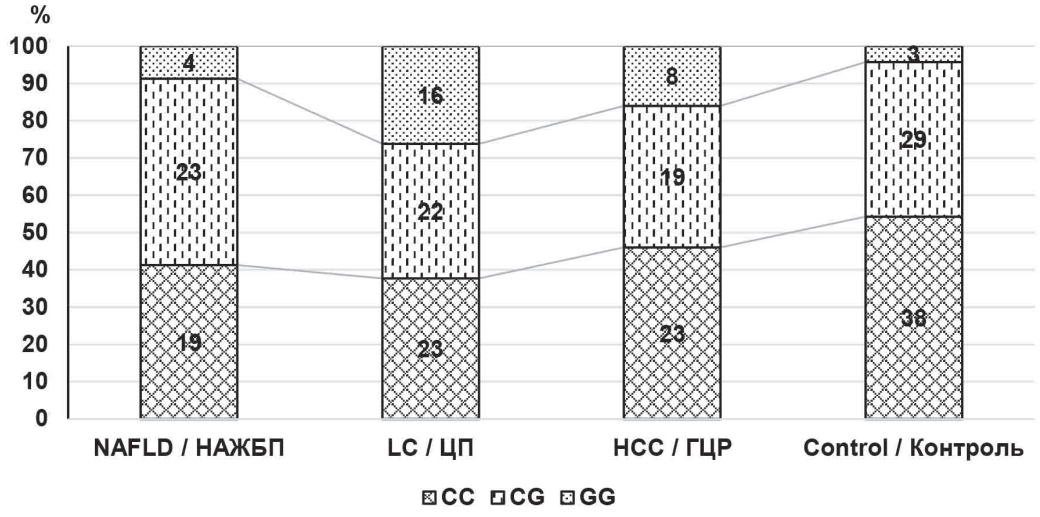

Figure 1. Frequency of various *PNPLA3* genotypes occurrence (NAFLD — non-alcoholic fatty liver disease; LC — liver cirrhosis; HCC — hepatocellular carcinoma)

Рисунок 1. Частота различных генотипов *PNPLA3* (НАЖБП — неалкогольная жировая болезнь печени; ЦП — цирроз печени; ГЦР — гепатоцеллюлярная карцинома)

gene in patients with NAFLD and the calculation of OR enable to estimate the probability of progression of NAFLD to LC and/or to HCC in patients with the polymorphism under study. Indeed, more extensive and long-term studies are needed to form reasonable conclusions about the role of *PNPLA3* polymorphism in the development of aggressive forms of NAFLD, however, the calculated OR indicators can be fully applied to the population under consideration (Table 2).

In the course of the study, we found that patients with a minor G allele had a statistically significantly higher chance of LC development (OR = 2.38; 95 % CI: 1.41–4.02; $p = 0.010$), and an insignificant chance of HCC development (OR = 1.62; 95 % CI: 0.92–2.83; $p = 0.217$). However, if we take patients with HCC secondary to LC, the differences become significant (OR = 2.17; 95 % CI: 1.15–4.08; $p = 0.039$). There are also significant differences in the higher frequency of the GG genotype in comparison with the control group in patients with LC ($p = 0.030$), HCC ($p = 0.029$) and HCC with LC ($p = 0.005$).

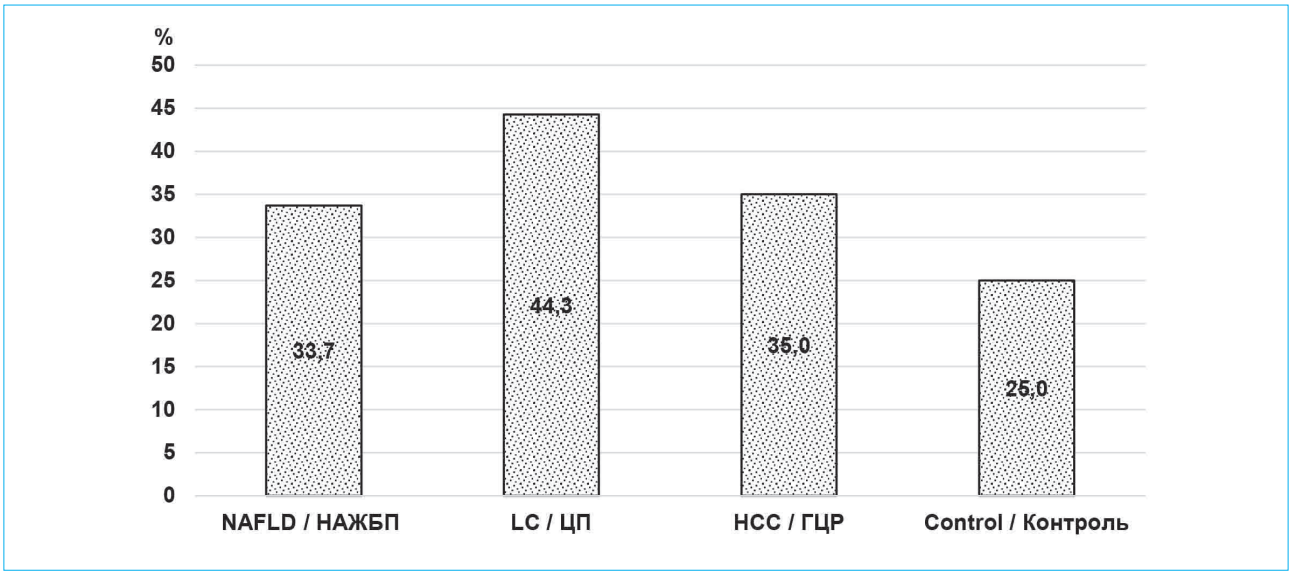

Figure 2. Frequency of the minor G allele occurrence (NAFLD — non-alcoholic fatty liver disease; LC — liver cirrhosis; HCC — hepatocellular carcinoma)

Рисунок 2. Частота минорного аллеля G (НАЖБП — неалкогольная жировая болезнь печени; ЦП — цирроз печени; ГЦР — гепатоцеллюлярная карцинома)

Table 2. Pairwise comparison of the main groups with the control group (n = 70) according to odds ratios with a 95 % confidence interval, taking into account a two-sided p-test for the significance of differences

Таблица 2. Попарное сравнение основных групп с контрольной группой (n = 70) по показателям отношения шансов при 95%-ном доверительном интервале с учетом двухстороннего p-критерия значимости различий

Genotype Генотип	NAFLD / НАЖБП, n = 46	LC / ЦП, n = 61	HCC / ГЦР, n = 50	ГЦР и ЦП / HCC and LC, n = 31
CC	0.59 (0.28–1.26) p = 0.240	0.51 (0.253–1.03) p = 0.102	0.72 (0.35–1.49) p = 0.443	0.53 (0.23–1.26) p = 0.216
CG	1.41 (0.67–2.99) p = 0.439	0.80 (0.39–1.62) p = 0.598	0.87 (0.41–1.82) p = 0.752	0.89 (0.38–2.12) p = 0.829
GG	2.13 (0.45–9.98) p = 0.691	7.94 (2.19–28.84) p = 0.030	4.25 (1.07–16.94) p = 0.029	6.51 (1.56–27.24) p = 0.005
G allele Аллель G	1.53 (0.86–2.72) p = 0.190	2.38 (1.41–4.02) p = 0.010	1.62 (0.92–2.83) p = 0.217	2.17 (1.15–4.08) p = 0.039

Note: NAFLD — non-alcoholic fatty liver disease; LC — liver cirrhosis; HCC — hepatocellular carcinoma

Примечание: НАЖБП — неалкогольная жировая болезнь печени; ЦП — цирроз печени; ГЦР — гепатоцеллюлярная карцинома.

Discussion

The *PNPLA3* p.I148M polymorphism increases the frequency of LC and HCC development secondary to NAFLD, and differences are observed both when compared with the control group and when comparing the main groups with each other. The frequency of the minor G allele in the HCC group was lower than in patients from the LC group (35.0 and 44.3 %, respectively), however, when analyzing the subgroup of patients in whom HCC occurs secondary to LC, we observe identical frequency indicators in this subgroup (41.9 %) and in the main LC group (44.3 %). At the same time, in the HCC subgroup without LC, the minor G allele is observed only in 23.7 % of patients, which is comparable with the control group (25.0 %). This can be explained by the fact that HCC develops in patients with NAFLD through the LC stage.

Our results of estimating the frequency of single-nucleotide polymorphism of *PNPLA3* and minor G allele in patients with liver pathology

Table 3. PNPLA3 polymorphism
Таблица 3. Полиморфизм *PNPLA3*

Group of patients Группа пациентов	Location Локация	Authors / Авторы	Number of observations Количество наблюдений	*PNPLA3* polymorphism (OR; 95% CI) Полиморфизм *PNPLA3* (ОШ; 95 % ДИ)	Frequency of genotype occurrence, % Частота выявления генотипа, %		
					CC	CG	GG
Fibrosis / LC as a result of NAFLD Фиброз / ЦП в результате НАЖБП	Europe Европа	Burlone M.E. et al. [13]	60	3.21 (1.56–8.65)	46	32	22
		Krawczyk M. et al. [12]	899	1.47 (1.00–2.15)	54	39	7
	USA США	Guichelaar M.M.J. et al. [14]	72	2.00 (0.71–5.80)	50	39	11
		Rotman Y. et al. [15]	894	1.20 (0.97–1.48)	26	45	29
	Japan Япония	Hotta K. et al. [16]	253	1.31 (0.95–1.79)	18	42	40
	Russia Россия	The author's research Авторское исследование	61	2.38 (1.41–4.02)	38	36	26
HCC / ГЦР	Europe Европа	Falleti E. et al. [17]	91	1.22 (0.90–1.65)	35	45	20
		Guyot E. et al. [18]	159	1.06 (0.82–1.38)	47	39	14
		Liu Y.-L. et al. [19]	33	2.25 (1.05–4.82)	42	44	14
	Japan Япония	Takeuchi Y. et al. [20]	50	1.27 (0.51–3.17)	7	39	53
	Russia Россия	The author's research Авторское исследование	50	1.62 (0.92–2.83)	46	38	16

Note: NAFLD — non-alcoholic fatty liver disease; LC — liver cirrhosis; HCC — hepatocellular carcinoma.

Примечание: НАЖБП — неалкогольная жировая болезнь печени; ЦП — цирроз печени; ГЦР — гепатоцеллюлярная карцинома.

compared with healthy volunteers are comparable with the literature data (Table 3).

Small volume of samples of patients with HCC, and especially patients with HCC of non-viral and non-alcoholic etiology, in comparison with samples of patients with LC as a result of NAFLD, is worth noting. Significant differences in the frequency of *PNPLA3* polymorphism inpatients with LC secondary to NAFLD were noted both in our study and in European studies. However, no statistical significance was obtained in HCC, which caused us to select a separate group of HCC secondary to LC and NAFLD.

Overweight is also considered as a predictor of the progression of NAFLD and the development of LC and HCC in patients being carriers of *PNPLA3* 148M alleles. In the study by M.A. Burza et al. [21], OR was 5.9 (95 % CI: 1.5–23.8; $p = 0.013$). In our study, the chance of LC as a result of NAFLD was 5.4 times higher, and the chance of HCC was 5.1 times higher in overweight patients. The data obtained by us also indicate an increase in the frequency of LC and HCC development secondary to NAFLD in patients with hypertension and diabetes. Answering the question whether screening should be carried out among patients with NAFLD, M. Reig et al., in their review of research data around the world, draw the following conclusions [22]: the probability of HCC development within 10 years in patients with LC secondary to NAFLD ranges from 6.7 to 15 %, while without cirrhosis — 23 cases per 100,000 patients per year. Screening is most appropriate in patients with NAFLD and LC, or without cirrhosis, but in patients older than 55 years, with increased alanine aminotransferase and/or with diabetes.

The observations above lead us to the hypothesis that it is inappropriate to consider the rs738409 polymorphism in the *PNPLA3* gene as a significant risk factor of HCC development. It is likely that patients with at least one minor allele G rs738409 should be included in screening programs for the detection of HCC after the development of cirrhosis or in the presence of additional risk factors. However, to confirm the hypotheses put forward, it is necessary to include patients with polymorphism in long-term prospective studies.

Conclusion

The frequency of single-nucleotide polymorphism significantly differs in patients with NAFLD, LC and HCC compared with the control group with a significant predominance of GG homozygotes in the LC group (26.2 % compared with 4.3 % in the control group).

The results of the study of the frequency of various rs738409 genotypes in the *PNPLA3* gene in patients with NAFLD, LC and HCC indicate a higher chance of NAFLD progression to LC with the subsequent development of HCC in carriers of minor G alleles. Additional risk factors are overweight, hypertension and diabetes.

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