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Association between single nucleotide polymorphisms in *PNPLA3*, *TM6SF2* and *MBOAT7* genes and markers of cancer aggressiveness in a Sri Lankan NASH-related HCC cohort

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Abstract

Background Single nucleotide polymorphisms (SNPs) in patatin-like phospholipase domain-containing protein 3 (*PNPLA3*), transmembrane 6 superfamily member 2 (*TM6SF2*) and membrane bound O-acyltransferase domain containing 7 (*MBOAT7*) genes were reported to be strongly associated with non-alcoholic fatty liver disease (NAFLD) pathogenicity among different populations. We investigated whether these SNPs are associated with prognostic factors and genetic biomarkers of non-alcoholic steatohepatitis (NASH)-related hepatocellular carcinoma (HCC) in the Sri Lankan context.

Methods We conducted an exploratory study to evaluate the prevalence of five SNPs (*PNPLA3* rs738409, *PNPLA3* rs2281135, *PNPLA3* rs2294918, *TM6SF2* rs58542926 and *MBOAT7* rs641738) as genetic risk factors for NASH-HCC pathogenicity. We genotyped 48 NASH-HCC patient samples collected at a clinical setting using a minisequencing method. Impact of each SNP with tumor prognostic factors such as nodularity, tumor size and AFP (alpha-feto protein) level was analyzed using chi square test. We also analyzed the expression of micro RNA-122 (miR-122) in serum and leukocyte telomere length via quantitative real-time PCR. Associations between each SNP with micro RNA-122 (miR-122) expression level and leukocyte telomere length of NASH-HCC patients were analyzed using one-way analysis of variance (ANOVA) test and independent t test. Relationships among tested SNPs and some well-established HCC risk factors such as age, BMI, gender, diabetes status and the cirrhotic stage were also analyzed using chi square test, independent t-test and One-way ANOVA test.

Results Our analyses demonstrated significant associations between *PNPLA3* rs2281135 variant and tumor nodularity. Also, *PNPLA3* rs2281135 and *PNPLA3* rs2294918 variants were significantly associated with miR-122 expression levels of NASH-HCC patients. Further, age and body mass index (BMI) were significantly associated with *PNPLA3* rs2281135 variant in our study cohort.

Conclusion We found that in the Sri Lankan NASH-related HCC cohort, some *PNPLA3* variants (rs2281135 and rs2294918) correlate with tumor nodularity, higher miR-122 expression, and distinct demographic features such

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as age and BMI. Our work highlights the role of specific SNPs in tumor aggressiveness, contributing to the precision screening for HCC in NASH patients.

Keywords Non-alcoholic steatohepatitis, Hepatocellular carcinoma, Prognosis, Single nucleotide polymorphisms, *PNPLA3*

Background

Hepatocellular carcinoma (HCC) is becoming a challenging global health concern, ranking as the sixth most common cancer worldwide. In 2020, more than 900,000 new cases were reported globally and it was estimated that in 2025, more than a million new cases will be reported annually [1]. Non-alcoholic fatty liver disease (NAFLD) is becoming the leading cause for liver disease and HCC globally. Environmental risk factors on genetic predisposition may play a critical role in developing HCC related to NAFLD. In the first ever genome-wide association study done on NAFLD pathogenicity, Romeo et al. 2008 identified a prominent single nucleotide polymorphism (SNP) (rs738409) on the gene encoding patatin-like phospholipase domain-containing protein 3 (PNPLA3) that was significantly associated with the hepatic fat/triglyceride content and susceptibility to NAFLD [2]. PNPLA3 is a hepatic triglyceride hydrolase [3]. Subsequent genetic studies have demonstrated that rs738409 is associated with various features of NAFLD including hepatic steatosis [4–6] and NAFLD severity [4]. The *PNPLA3* rs738409 variant G allele was reported to be more prevalent in non-cirrhotic NAFLD patients compared to cirrhotic patients [7]. Further, *PNPLA3* rs738409 variant has shown a significant association with non-cirrhotic HCC development [8]. *PNPLA3* rs738409 was reported to confer a loss-of-function, reducing nearly 80% of the triglyceride hydrolase activity [9] causing steatosis and hepatic injury [3]. In contrast, a recent report suggests that hepatic steatosis is promoted by the gain-of-function nature of *PNPLA3* rs738409 variant [10]. Another polymorphism in the *PNPLA3* gene (rs2281135) was reported to be significantly associated with NAFLD and NAFLD severity [11, 12]. A rare variant of *PNPLA3* (rs2294918) promotes hepatic steatosis in the development of non-alcoholic steatohepatitis (NASH) [8]. An exome-wide association study showed a variant (rs58542926) in the transmembrane 6 superfamily member 2 (*TM6SF2)* gene to be associated with higher liver fat levels [13] where several successive studies showed its significant association with NAFLD risk [14, 15] and NAFLD-related HCC risk [14, 16]. In addition, membrane bound O-acyltransferase domain containing 7 (*MBOAT7*) rs641738 showed associations with non-cirrhotic NAFLD-related HCC [8] and NAFLD severity [17].

Tumor size is considered as an independent prognostic factor for HCC [18, 19] whereas larger tumors predicting a poor prognosis [20]. Tumor size of HCC is reported to be significantly associated with genetic aberrations [21]. Also, larger tumors exhibit enhanced aggressiveness, stimulating invasive behavior [22]. In addition, tumor nodularity serves as an important prognostic factor for HCC [23] that can influence the patient outcomes. The elevation of (Alpha fetoprotein) AFP as a tumor marker is associated with poorer survival outcomes of HCC, indicating its role as a significant prognostic feature [24]. Liver injury induced by these reported genetic variants might significantly impact on serum micro RNA-122 (miR-122) levels and peripheral blood leukocyte telomere length. miR-122 is the predominant hepatic miRNA [25]. Oxidative stress and inflammation caused by liver damage in NAFLD may release miR-122 from hepatocytes showing elevated serum levels [26]. Also, chronic inflammation can induce oxidative stress, thus damaging the telomeres [27]. Previous studies have reported significant correlations between the formation of HCC and peripheral blood telomere length alterations [28, 29]. Therefore, miR-122 and the peripheral blood leukocyte telomere length are potential biomarkers in NASH-related HCC.

A Sri Lankan community-based prevalence study has shown that nearly one-third of the Sri Lankan adults in an urban population have NAFLD [30]. Previous data in Sri Lanka has shown association of *PNPLA3* rs738409 with NAFLD susceptibility [31]. The presence of such a large at-risk population for HCC, urges the need for better surveillance in detecting HCC at an early stage. Abdominal ultrasound is an effective HCC surveillance strategy, but still has questionable benefits in cost-effectiveness when used in a large population [32]. In order to overcome this, many attempts are made to individualize screening based on risk stratification [33, 34]. Genetic variant analysis is an essential component that increase the validity of precision screening. However, there is hardly any data from the Asian continent regarding genetic predisposition and the importance on clinical prognostic factors of NAFLD-related HCC. To the best of our knowledge, our study represents the first investigation to explore the interplay of genetic biomarkers and tumor aggressiveness factors in NASH-related HCC in the Sri Lankan context,

and potentially be the first study in the broader Southeast Asian region. Our research provides valuable insight into the genetic and clinical factors influencing the disease progression and prognosis in terms of NASH-related HCC addressing a significant knowledge gap in the current literature. Therefore, this study aims to determine the genetic background of a Sri Lankan NASH-related HCC cohort in terms of the prevalence data of five previously identified SNPs (*PNPLA3* rs738409, *PNPLA3* rs2281135, *PNPLA3* rs2294918, *TM6SF2* rs58542926 and *MBOAT7* rs641738) that are associated with NAFLD pathogenicity. Further the analysis seeks to address the impact of these SNPs on selected tumor prognostic factors (tumor size, nodularity and AFP level) and HCC biomarkers such as miR-122 and leukocyte telomere length.

Methods

Subjects

The study included patients diagnosed with NASH-related HCC ($N=48$), referred to North Colombo Center for Liver Diseases, Ragama, Sri Lanka. Median age of the sample cohort was 63 years (range 19–69). HCC was diagnosed based on the Asian Pacific association for the study of the liver (APASL) diagnostic criteria [35]. All participants gave written informed consent and the study was approved by the ethics review committee of the Faculty of Medicine, University of Kelaniya, Ragama, Sri Lanka (P/230/11/2019). Subjects with a history of unsafe alcohol consumption, infective hepatitis or a family history of other metabolic diseases except NAFLD were excluded. Information on socio-demographical data, clinical and biochemical characteristics were obtained using a structured questionnaire which was developed for the study (supplementary file) and clinical records. Baseline characteristics of NASH-related HCC patients is summarized in Table 1. Design of the study is represented in Fig. 1.

Sample collection

Peripheral venous blood samples (10 mL) were collected from the patients attending the North Colombo Center for Liver Diseases, Ragama, Sri Lanka. A 5 mL aliquot was immediately transferred to ethylenediaminetetraacetic acid (EDTA) coated tube and gently inverted several times to separate the plasma. Remainder (5 mL) was transferred to a plain tube to separate the serum. The blood samples were transported to the laboratory in ice, and they were centrifuged at 900 relative centrifugal force (RCF) for 7 min at 4 °C. Separated blood cells were stored at -20 °C until used for genomic DNA extraction. Separated serum samples were stored at -80 °C until miRNA extraction.

Table 1 Socio-demographic data, anthropometric data, co-morbidities, characteristics of tumor, and biochemical investigations of the NASH-HCC cohort ($n=48$)

Variables	Mean (± SD)/ Median (IQR) or N (%)
Socio-Demographic Data	
Age	
Mean (± SD), years	59.69 (± 10.533)
Median (IQR), years	63 (19–69)
Gender	
Male	40 (83.3)
Female	8 (16.67)
Marital status	
Yes	44 (91.67)
No	4 (8.33)
Ethnicity	
Sinhalese	47 (97.91)
Muslims	1 (2.1)
Anthropometric Data	
Weight	
Mean (± SD), kg	67.29 (± 14.314)
Median (IQR), kg	65 (28–102)
Height	
Mean (± SD), cm	159.42 (± 8.082)
Median (IQR), cm	159.50 (138–180)
BMI[1]	
Mean (± SD), kg/m^2	26.44 (± 5.050)
Median (IQR), kg/m^2	26.02 (13–42)
Co-morbidities	
Diabetes mellitus	29 (60.42)
Liver Cirrhosis	39 (81.25)
Child-Pugh[2] class A	30/39
Child–Pugh class B	8/39
Child–Pugh class C	1/39
Coronary heart disease	13(27.1)
Retinopathy	17(35.42)
Renal complications[3]	9 (18.75)
Neuropathy	17 (35.42)
Respiratory disorders[4]	12 (25)
Peripheral vascular disorder	23 (47.9)
Family history of liver diseases	
Yes	9 (18.75)
No	39 (81.25)
The characteristics of tumor and biochemical investigations	
Tumor size	
Mean (± SD), cm	5.32 (± 3.84)
Median (IQR), cm	4.25 (2.65 to 7.00)
Tumor type	
Single	35 (73)
Multiple	10 (20.8)
Diffuse type	3 (6.25)

Table 1 (continued)

Variables	Mean (± SD)/ Median (IQR) or N (%)
Vascular invasion	
Yes	4 (8.33)
No	44 (91.67)
Alpha-Fetoprotein (AFP), ng/mL	
Mean (± SD)	122.68 (± 244.38)
Median (IQR)	5.93 (2.92 to 62.50)
Aspartate aminotransferase (AST), IU/L	
Mean (± SD)	66.83 (± 32.87)
Median (IQR)	64.00 (38 to 87)
Alanine aminotransferase (ALT), IU/L	
Mean (± SD)	55.31 (± 28.33)
Median (IQR)	51.00 (36.11 to 61.75)
Serum Alkaline Phosphatase (ALP), IU/L	
Mean (± SD)	165.22 (± 97.58)
Median (IQR)	135.00 (83.50 to 211.50)
Gamma Glutamyltransferase (GGT), IU/L	
Mean (± SD)	155.85 (± 153.38)
Median (IQR)	99.00 (63.00 to 188.00)

SD Standard deviation

IQR Inter quartile range

[1] Body Mass Index: Asia–pacific cut-off points were used as; underweight (< 18.5 kg/m2), normal weight (18.5–22.9 kg/m2), overweight (23–24.9 kg/m2) and obese (≥ 25 kg/m2)

[2] Child-pugh classification; class A: least severe disease; class B: moderately severe disease; class C: most severe disease [36]

[3] Chronic kidney diseases

[4] Asthma, COPD (Chronic obstructive pulmonary disease)

SNP genotyping

DNA was extracted from whole blood obtained from each study participant using commercially available reagents (Qiagen, Cat. No: 51104, CA, USA). Prevalence of five SNPs- *PNPLA3* rs738409, *PNPLA3* rs2281135, *PNPLA3* rs2294918, *TM6SF2* rs58542926 and *MBOAT7* rs641738 were evaluated. SNPs were detected using polymerase chain reaction (PCR). The amplification was conducted as follows: initial denaturation at 95°C for 2 min; 30 cycles of denaturation at 95°C for 1 min, annealing for 1 min (rs738409 and rs2281135 at 58°C, rs2294918 and rs641738 at 55°C, rs58542926 at 57°C) and extension at 72°C for 1 min, with a final extension at 72°C for 5 min. SNP analysis was performed using the SNapshot™ Multiplex kit (Applied Biosystems). SNaPshot products (0.5 µL) were mixed with HiDi™ formamide (9 µL) and GeneScan-120 LIZ (0.5 µL) size standard (Applied Biosystems) to detect SNPs. Data generation was carried out after capillary electrophoresis on ABI 3500dx genetic analyzer (Applied biosystems) and analyzed with Gene Mapper™ software version 3.7 (Applied Biosystems). To confirm genotyping results, representative samples with different genotypes were subjected to Sanger sequencing and further validated by clinico-histopathological diagnosis to avoid false positives.

Real-time quantitative PCR to determine the leukocyte telomere length

Average leukocyte telomere length was measured using real-time quantitative PCR technique described by Cawthon [37]. QuantiNova® SYBR® Green PCR kit (QIAGEN, Hilden, Germany) was used to perform the qPCR analysis using genomic DNA extracted from blood samples (patients & controls) as templates.

Real-time quantitative-reverse transcription (RT)-PCR to detect cell free miR-122

RT-PCR technique was used to detect and quantify serum miR-122 levels. miR-122 was extracted from serum samples using a column-based method (miRNeasy Serum/Plasma kit: Qiagen, USA) and detection and quantification was done using a miR-122 specific real time RT-PCR detection kit (Cohesion Biosciences, UK).

Sample size calculation

Given that this study focuses on the genetic analysis of NASH-related HCC patients, the determination of the sample size was influenced by both statistical considerations and the practical constraints associated with recruiting individuals with this rare condition in Sri Lanka. A total of 48 patients were included in the study, a number dictated primarily by the limited availability of diagnosed cases and the logistical challenges inherent to genetic studies of rare diseases. To ensure the validity of this sample size, a post hoc power analysis was conducted, confirming that with a significance level (α) of 0.05, the study achieves 80% power ($1-\beta=0.80$) to detect a minimum effect size (Δ) of 1.5. This calculation was based on previously reported moderate effect sizes for key genetic variants, including *PNPLA3* and *TM6SF2*, both of which have been implicated in the progression of NASH to HCC in various populations [38, 39].

The assumed standard deviation (σ) of 1.2 was derived from prior studies investigating the metabolic impact of genetic variants in liver disease, particularly in relation to insulin resistance, hepatic lipid accumulation, and fibrotic progression [40]. While an expanded sample size would enhance statistical power and allow for finer stratification in subgroup analyses, the rarity of NASH-related HCC cases, coupled with the unique genetic composition of the Sri Lankan population, necessitates the utilization of available data to extract meaningful insights. The study, therefore, adopts a targeted approach to examining the genetic underpinnings of NASH-related HCC in

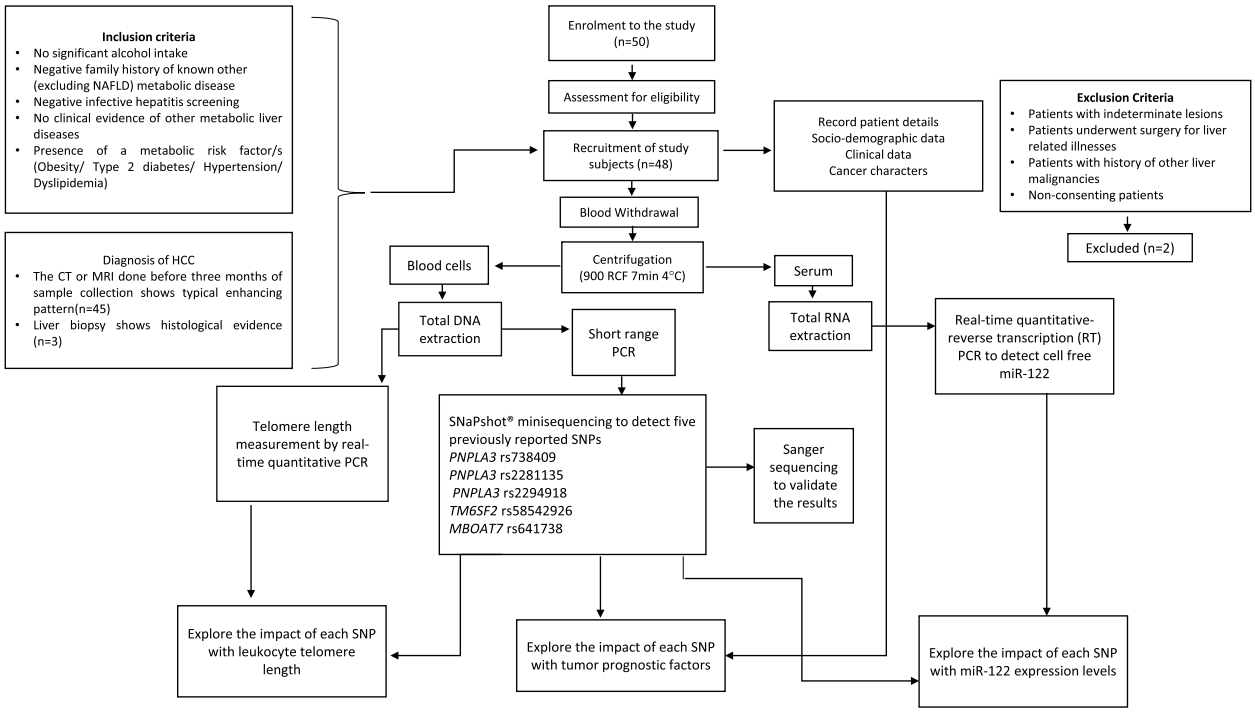

Fig. 1 Flowchart summarizing the study design

this cohort, acknowledging the limitations while striving to contribute novel findings relevant to disease pathogenesis and potential biomarkers in this population.

Statistical analysis

Genotypic data of selected SNPs were analyzed as categorical variables (homozygous wild type, heterozygous, and homozygous mutant). The Chi-square test was utilized to evaluate associations between categorical variables, such as nodularity, total tumor diameter, and alpha-fetoprotein (AFP) levels in NASH-related HCC patients and the tested SNPs.

For continuous variables, one-way analysis of variance (ANOVA) and independent t-tests were performed to assess relationships between the tested SNPs and HCC biomarkers, specifically miR-122 expression levels and leukocyte telomere length. The selected sample provided sufficient statistical power for detecting significant associations. Prior to these analyses, assumptions of normality and homogeneity of variance were examined. When ANOVA results were significant, post hoc tests were conducted to identify specific group differences. Associations between other risk factors of NASH-related HCC patients and the SNPs were similarly analyzed using Chi-square tests, independent t-tests, and one-way ANOVA.

All statistical analyses were performed using IBM® SPSS Statistics software, version 26.0 (IBM Corp., Armonk, NY, USA). Statistical significance was set at p-values < 0.05.

Results

Genotype distribution of *PNPLA3* rs738409, *PNPLA3* rs2281135, *PNPLA3* rs2294918, *TM6SF2* rs58542926 and *MBOAT7* rs641738 observed in this study cohort is shown in Fig. 2. Homozygous wild-type was the commonest for *TM6SF2* rs58542926 (genotype frequencies: CC: 68.75%, CT: 27.0%, TT: 4.2%), homozygous mutant was the commonest genotype for *PNPLA3* rs2294918 (genotype frequencies: AA:10.42%, AG:25.0%, GG:64.6%) while heterozygous genotype was the commonest for *PNPLA3* rs738409 (genotype frequencies: CC: 20.8%, CG:79.2%), *PNPLA3* rs2281135 (genotype frequencies GG:23.0%, GA:73.0%, AA:4.2%) and *MBOAT7* rs641738 (genotype frequencies: CC: 14.6%, CT:52.08%, TT:33.33%).

Associations between SNPs and prognosis factors for NASH-HCC

Total tumor diameter, number of tumor nodules and AFP level were evaluated as poor prognostic factors. No association was observed among the tested SNPs with the tumor size and AFP levels of NASH-related HCC

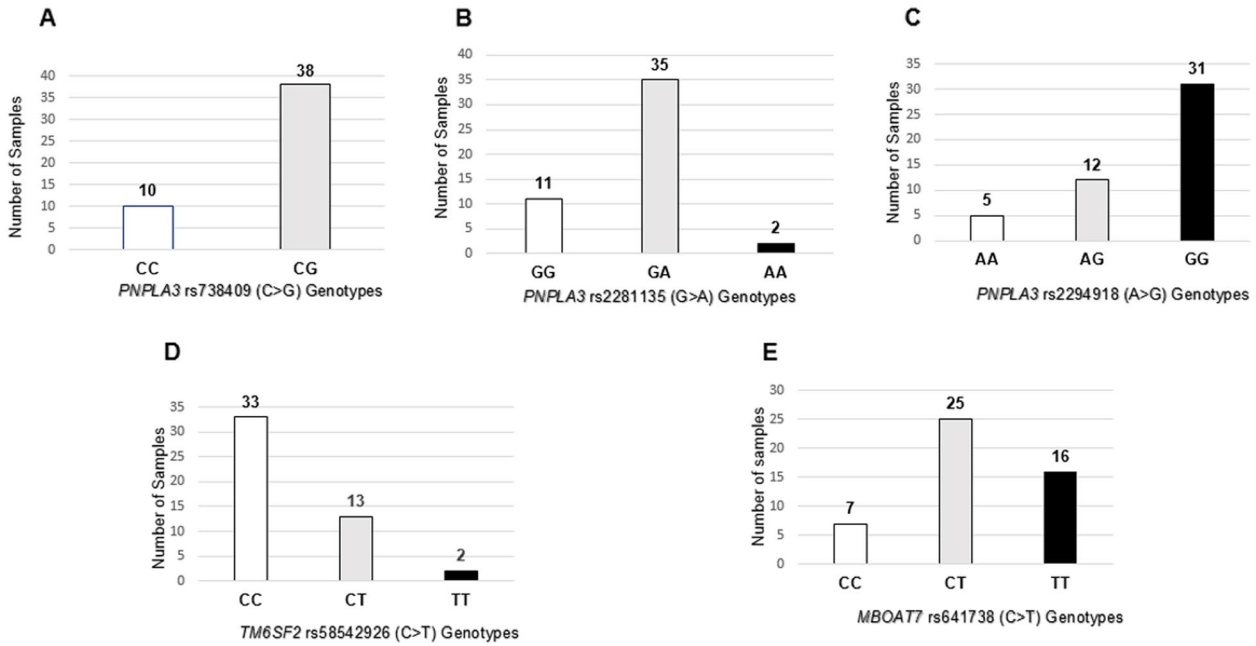

Fig. 2 Genotype distribution of the Sri Lankan NASH-HCC cohort. **A** *PNPLA3* rs738409 (CC: 20.8%, CG:79.2) **B** *PNPLA3* rs2281135 (GG: 23.0%, GA:73.0%, AA: 4.2%) **C** *PNPLA3* rs2294918 (AA:10.42%, AG:25.0%, GG: 64.6%) **D** *TM6SF2* rs58542926 (CC: 68.75%, CT: 27.0%, TT: 4.2%) and **E** *MBOAT7* rs641738 (CC: 14.6%, CT: 52.08%, TT: 33.33%) polymorphisms (n = 48). *PNPLA3*: Patatin-like phospholipase domain-containing protein 3. *TM6SF2*: Transmembrane 6 superfamily member 2. *MBOAT7*: Membrane bound O-acyltransferase domain containing 7. (A) Adenine, (C) Cytosine, (G) Guanine, (T) Thymine

patients. *PNPLA3* rs2281135 (p = 0.038) had significant associations with HCC tumor nodularity (Table 2).

Associations between SNPs and predictive biomarkers for NASH-HCC

Leukocyte telomere length of the NASH-related HCC patients did not show any statistical significant association with the tested SNPs. *PNPLA3* rs2281135 ($p = 0.001$) and *PNPLA3* rs2294918 ($p = 0.019$) had significant associations with miR-122 expression levels of the NASH-HCC patients (Table 3). Also, we found that miR-122 levels are significantly higher in larger tumors (Smaller Vs. Larger 5.1 ± 7.47 Vs. 832.69 ± 1388.62; $t_{20} = -3.033$; $p < 0.001$).

Associations between SNPs and risk factors for NASH-HCC

None of the SNPs show association with the tested risk factors, except *PNPLA3* rs2281135 that showed statistically significant associations with age ($p = 0.000$) and BMI ($p = 0.050$) of the NASH-HCC cohort (Supplementary Material 2: Table 4).

Discussion

In this study, we report the prevalence data of *PNPLA3* rs738409, *PNPLA3* rs2281135, *PNPLA3* rs2294918, *TM6SF2* rs58542926 and *MBOAT7* rs641738 in the Sri Lankan NASH-related HCC cohort. The most common genotypes observed were, homozygous wild type for *TM6SF2* rs58542926, homozygous mutant for *PNPLA3* rs2294918, and heterozygous for *PNPLA3* rs738409, *PNPLA3* rs2281135, and *MBOAT7* rs641738. Further, the chi-square test showed no association between the five selected SNPs with tumor size, and AFP levels while, *PNPLA3* rs2281135 variant had a statistically significant association with tumor-nodularity of NASH-related HCC patients. Also, there were no statistically significant telomere length differences between risk genotypes and their wild-type genotypes for the NASH-related HCC cohort. *PNPLA3* rs2281135 AA (homozygous mutant) and *PNPLA3* rs2294918 AA (homozygous wild type) genotypes showed a statistically significant increase in the miR-122 expression levels in NASH-related HCC patients.

To control for potential confounding factors in our analysis, we employed multivariable regression models, with tumor size, nodularity, and AFP levels selected as dependent variables. Independent variables included age, gender, BMI, diabetes status, smoking history, and undiagnosed alcohol consumption. Although some variables were not statistically significant during the model-building process, they were retained based on their clinical relevance and support from the literature. Specifically, undiagnosed alcohol consumption was accounted for

Table 2 Associations of SNPs with prognosis factors for NASH-HCC

SNP	Nodularity[4]			Significance[5]
	Single (N%)	Multiple (N%)		
PNPLA3[1] rs738409				$\chi^2 = 1.867$
CC	(25.7)	(7.7)		df = 1
CG	(74.3)	(92.3)		$p = 0.172$
PNPLA3[1] rs228135				
GG	(65.7)	(69.2)		$\chi^2 = 6.563$
GA	(0)	(15.4)		df = 2
AA	(34.3)	(15.4)		$p =$ **0.038**
PNPLA3[1]rs2294918				
AA	(11.4)	(7.7)		$\chi^2 = 1.738$
AG	(20)	(38.5)		df = 2
GG	(68.6)	(53.8)		$p = 0.419$
TM6SF2[2]				
rs58542926				
CC	(71.4)	(61.5)		$\chi^2 = 0.756$
CT	(25.7)	(30.8)		df = 2
TT	(2.9)	(7.7)		$p = 0.685$
MBOAT7[3]				
rs641738				
CC	(8.6)	(30.8)		$\chi^2 = 3.886$
CT	(54.3)	(46.2)		df = 2
TT	(37.1)	(23.1)		$p = 0.143$
	Tumor size[6]			
	Large HCC > 10 cm (N%)		Small HCC < 10 cm (N%)	
PNPLA3[1] rs738409				$\chi^2 = 0.002$
CC	(20)		(20.9)	df = 1
CG	(80)		(79.1)	$p = 0.961$
PNPLA3[1]rs2281135				
GG	(20)		(23.3)	$\chi^2 = 3.507$
GA	(60)		(74.4)	df = 2
AA	(20)		(2.3)	$p = 0.173$
PNPLA3[1]rs2294918				
AA	(20)		(9.3)	$\chi^2 = 1.517$
AG	(40)		(23.3)	df = 2
GG	(40)		(67.4)	$p = 0.468$
TM6SF2[2]				
rs58542926				
CC	(60)		(69.8)	$\chi^2 = 0.639$
CT	(40)		(25.6)	df = 2
TT	(0)		(4.7)	$p = 0.727$
MBOAT7[3] rs641738				
CC	(0)		(37.2)	$\chi^2 = 2.808$
CT	(80)		(48.8)	df = 2
TT	(20)		(14)	$p = 0.246$
	AFP level[7]			
	High secretion > 20 ng/mL (N%)		Low secretion < 20 ng/mL (N%)	
PNPLA3[1] rs738409				$\chi^2 = 0.257$
CC	(18.2)		(26.7)	df = 1
CG	(81.8)		(73.3)	$p = 0.612$

Table 2 (continued)

PNPLA3[1] rs2281135			
GG	(18.2)	(26.7)	$\chi^2 = 2.101$
GA	(81.8)	(60)	df = 2
AA	(0)	(13.3)	$p = 0.350$
PNPLA3[1] rs2294918			
AA	(9.1)	(13.3)	$\chi^2 = 0.257$
AG	(9.1)	(13.3)	df = 2
GG	(81.8)	(73.3)	$p = 0.879$
TM6SF2[2] rs58542926			
CC	(54.5)	(73.3)	$\chi^2 = 1.022$
CT	(36.4)	(20)	df = 2
TT	(9.1)	(6.7)	$p = 0.6$
MBOAT7[3] rs641738			
CC	(23.1)	(20)	$\chi^2 = 1.282$
CT	(57.7)	(53.3)	df = 2
TT	(19.2)	(26.7)	$p = 0.527$

(A) Adenine, (C) Cytosine, (G) Guanine, (T) Thymine

HCC Hepatocellular carcinoma

AFP Alpha fetoprotein

[1] Patatin-like phospholipase domain-containing protein 3

[2] Transmembrane 6 superfamily member 2

[3] Membrane bound O-acyltransferase domain containing 7

[4] Single nodularity: Presence of one nodule in the liver; multiple nodularity: Presence of two or more nodules in the liver

[5] Chi square test

[6] HCC size categorizations were done according to [41]

[7] AFP cut-off values were selected based on [42]

thorough review of medical records and self-reported data during patient interviews. This approach ensured that the models accounted for factors known to influence the dependent variables, even if their statistical significance was limited. By adjusting for these covariates, we aimed to minimize bias and provide a robust analysis. While cirrhosis is a key determinant of HCC progression, our cohort size limited stratified analyses. Additionally, fibrosis progression in NAFLD is a continuous spectrum rather than a binary classification. Future studies with larger sample sizes will aim to stratify patients accordingly to clarify these associations.

This investigation represents the first study conducted on the genetic background of NASH-related HCC patient cohort in the Sri Lankan context addressing a critical gap in literature. Our study cohort can be considered as a homogenous group as majority of the study subjects (47 out of 48) were Sinhalese. Therefore, strong associations observed between the tested SNPs (especially *PNPLA3* rs2281135) and tumor aggressiveness might be a unique feature to Sinhalese ethnicity. However, the overrepresentation of the Sinhalese individuals in our cohort limits generalizability to other ethnic groups. While this ensures a homogenous genetic background, replication in more diverse populations is essential to establish broader applicability.Previous data from a follow-up study done by Seko et al. has shown that the *PNPLA3* rs738409 G allele is significantly associated with developing HCC in NAFLD patients [43]. Furthermore, it was significantly high in NAFLD-related HCC compared to patients with viral etiology. The same observation was reported by Uyema et al. on NAFLD-related HCC subjects with diabetes [44]. All these East Asian cohort studies on NAFLD-related HCC have shown that the *PNPLA3* rs738409 G allele is a significant genetic factor for the disease onset and progression, comparable with what we have observed in our cohort. Similar observations were made in Western populations for *PNPLA3* rs738409 [45, 46]. Also, *TM6SF2* rs58542926 [47] and *MBOAT7* rs6417387 [8, 34, 48] have indicated significant contributions to the NAFLD-related HCC etiology.

Furthermore, Longo et al. and Donati et al. have investigated the synergetic effect of *PNPLA3* rs738409, *TM6SF2* rs58542926, and *MBOAT7* rs641738 variants on NAFLD-related HCC and found that the additive effect of either two [8] or three [47] of the variants can increase the burden towards HCC as shown in our results as well.

Table 3 Association of telomere length and miR-122 expression levels of NASH-HCC patients with SNPs studied

SNP	Telomere length		miR-122 expression level	
	Mean (± SD)	Significance	Mean (± SD)	Significance
PNPLA3[1] rs738409				
CC	19.18(± 40.74)	$t^4_{23} = 0.533$	12.56(± 23.24)	$t^4_{23} = -0.519$
CG	13.46(± 14.42)	$p = 0.026$	132.60(± 557.79)	$p = 0.284$
PNPLA3[1] rs2281135				
GG	17.98(± 37.32)	$F^5 = 2.316$	10.84(± 21.70)[a]	$F^6 = 10.146$
GA	9.86 (± 10.52)	$p = 0.122$	4.65(± 8.00)[a]	$p =$ **0.001**
AA	43.60(± 7.14)		1222.31(± 1716.12)[b]	
PNPLA3[1] rs2294918				
AA	14.17(± 21.11)	$F^5 = 0.043$	813.0(± 1405.38)[b]	$F^6 = 4.770$
AG	12.58(± 13.43)	$p = 0.958$	3.47(± 3.62)[a]	P = **0.019**
GG	15.81(± 26.26)		8.44(± 15.81)[a]	
TM6SF2[2] rs58542926				
CC	20.77(± 26.48)	$F^5 = 1.627$	155.54(± 608.08)	$F^5 = 0.236$
CT	4.58(± 3.81)	$p = 0.219$	13.06(± 21.25)	$p = 0.792$
TT	1.89		1.66	
MBOAT7[3] rs641738				
CC	13.93(± 17.18)	$F^5 = 0.113$	1.8 (± 1.4)	$F^5 = 0.314$
CT	14.31(± 26.10)	$p = 0.893$	168.56(± 627.39)	$p = 0.734$
TT	22.37(± 21.45)		7.84(± 9.91)	

miR = Miro RNA

A) Adenine, (C) Cytosine, (G) Guanine, (T) Thymine

[1] Patatin-like phospholipase domain-containing protein 3

[2] Transmembrane 6 superfamily member

[3] Membrane bound O-acyltransferase domain containing 7

[4] Independent t-test

[5] One-way ANOVA test

[6] One-way ANOVA test: Post hoc test

[a b] Mean values are similar

NAFLD is a disease that exposes a large cross-section of a population to its complications. However, only a small proportion of patients would progress further into developing complications. Thus, extensive population screening has shown to be a failed strategy [49]. In order to overcome this, risk stratification and screening of high-risk groups are becoming the trend for the future [50]. However, risk factors for risk stratification are rapidly evolving with the emergence of new data.

In precision screening, the patients are selected based on the risks and the tool for screening is individualized. Combining genetic risk with conventional risk factors could give a greater validity for precision screening in NALD-related HCC. This risk prediction can facilitate the early detection of high-risk individuals and develop personalized health measures to diminish disease onset and progression. The clinical implication of such genetic discoveries is still evolving but calculating polygenic risk scores (PRS) is currently practiced to determine the total burden of risk alleles of an individual for clinical utility. PRS is calculated as a weighted sum of the number of risk alleles and prioritizes them in preventable clinical actions [51].

HCC is known to be an aggressive tumor [19]. About 70%-85% of patients present at an advanced or unresectable stage [52]. NASH-related HCC is known to present at an advanced stage with a poor prognosis [53]. Tumor size [19, 54, 55] nodularity [54, 56] and AFP level [57] are considered as poor prognostic factors for HCC at diagnosis. Valenti et al. reported that the *PNPLA3* rs738409 G allele was significantly associated with multiple HCC nodules at presentation in liver disease patients with alcoholic and non-alcoholic etiologies in an Italian population [58]. Although we did not achieve significant associations between the tested SNPs and the tumor size, *PNPLA3* rs738409 was previously reported to be directly associated with tumor size in HCC [59]. AFP is reported to be an important predictive biomarker for hepatocarcinogenesis [60]. Significant correlations between serum AFP levels and *PNPLA3* rs738409 in HCC patients were previously reported, with GG genotype having higher levels of AFP [61, 62]. In our cohort, *PNPLA3* rs2281135 variant had a significant association with HCC nodularity indicating a relationship between *PNPLA3* variations and tumor aggressiveness that can be a unique genetic feature in the Sri Lankan context with respect to the Sinhalese ethnicity. Our study revealed that the mean levels of miR-122 were significantly higher in larger tumors ($p = 0.000$) compatible with the published evidence. Delik et al. 2020 studied the distribution of miR-122 levels according to *PNPLA3* rs738409 genotypes and observed a significantly higher miR-122 expression levels associated with risk genotypes [62]. In our sample, homozygous mutant genotype (AA) of *PNPLA3* rs2281135 variant correlate with significantly higher miR-122 expression level ($p = 0.001$). Taken together, our work highlights previously unreported correlations among *PNPLA3* rs2281135 with tumor nodularity and higher miR-122 expression level, that might potentially be unique to the Sri Lankan NASH-related HCC patient cohort. MiR-122 is the most abundant miRNA present in the liver [63]. The *PNPLA3* rs2281135 variant may contribute to tumor nodularity and miR-122 elevation through its role in hepatic lipid metabolism. Altered lipid accumulation could promote hepatocyte stress and inflammation, leading to increased miR-122 release [64]. Additionally, *PNPLA3* variants have been implicated in fibrogenesis, which may influence tumor aggressiveness [4]. We further analyzed any possible association between *PNPLA3*, *TM6SF2* and *MBOAT7* variants and well-established HCC risk factors. Among the tested variants *PNPLA3* rs2281135 was significantly associated with age and BMI ($p = 0.000$ and

$p=0.050$ respectively) of NASH-related HCC patients. The observed associations between *PNPLA3* rs2281135 and age/BMI could reflect survivorship bias or confounding by comorbidities. Since HCC is a late-stage disease, genetic predisposition may interact with lifestyle and metabolic factors over time. We recommend longitudinal studies to assess causality. Mean age in carriers of both the risk alleles (AA) was nearly 30 years younger than heterozygous (GA) and non-carriers of the risk allele (GG). Although we observed an unusual mean age gap among individuals bearing different genotypes for *PNPLA3* rs2281135, probably due to the small sample size, our findings suggest a role for genetic data in distinguishing HCC risk in younger age groups, reflecting the evidence reported by Walker et al., 2020 [65] previously. Similarly, AA homozygotes for *PNPLA3* rs2281135 variant had a significantly lower mean BMI compared to GA and GG genotypes. This observation indicates that mutant homozygotes for *PNPLA3* variants might actually be leaner, demonstrating such common anthropometric characters associated with hepatocarcinogenesis are not necessarily seen at presentation. Although we did not see any statistically significant association between risk variants and gender of the patients, men with HCC had a greater proportion of any of the tested risk alleles than females, reflecting the predominancy of HCC among males than females [66]. Overall, our findings show that some SNPs in the *PNPLA3* gene are significantly associated with an increased risk for tumor aggressiveness and certain demographic features (age and BMI) in NASH-related HCC patients, warranting these genetic data to be incorporated into practical risk stratification models that could enhance better clinical decision-making. The study was powered primarily to detect associations for *PNPLA3* SNPs, given their well-documented impact on NAFLD and HCC. The lack of significance for *TM6SF2* and *MBOAT7* SNPs may be due to the relatively small sample size, which limited our ability to detect associations with moderate effect sizes for these variants. Future studies with larger cohorts will be necessary to confirm their roles. Also, we acknowledge that multiple comparisons increase the risk of Type I errors. However, given the exploratory nature of our study, we opted not to apply strict Bonferroni corrections, as they may overly reduce statistical power. Instead, we have interpreted significant p-values with caution and encourage further validation in larger cohorts.

SNPs, as the most common form of genetic variation might be affecting the NASH-related HCC as genetic heterogeneity can directly influence the genetic susceptibility towards carcinogenesis. These genetic data can significantly contribute for predictive testing for liver carcinogenesis in clinical utility. We acknowledge that small sample size due to resource constrains and financial difficulties is a limitation of the study. Yet, our data provide valuable information regarding the genetic background of the NASH-related HCC patients in Sri Lanka addressing an unexplored geographic context. However, we expect to project future cohort studies on NASH-related HCC in Sri Lanka with larger sample sizes to generalize our study findings.

Conclusion

We found that in the Sri Lankan NASH-related HCC cohort, particular *PNPLA3* variants rs2281135 and rs2294918 are correlating with tumor nodularity, higher miR-122 expression, and distinct demographic features such as age and BMI. *PNPLA3* variations seem to have an impact on tumor aggressiveness. In order to generalize our findings, a larger ethnically diverse cohort need to be studied. Furthermore, predictive testing using the genetic markers identified is warranted to analyse the potential of identifying NASH patients progressing to HCC in due course.

Abbreviations

HCC	Hepatocellular carcinoma
NAFLD	Non-alcoholic fatty liver disease
SNP	Single nucleotide polymorphism
PNPLA3	Patatin-like phospholipase domain-containing protein 3
NASH	Non-alcoholic steatohepatitis
TM6SF2	Transmembrane 6 superfamily member 2
MBOAT7	Membrane bound O-acyltransferase domain containing 7
AFP	Alpha fetoprotein
miR-122	Micro RNA-122
BMI	Body mass index
APASL	Asian Pacific association for the study of the liver
SD	Standard deviation
IQR	Inter quartile range
COPD	Chronic obstructive pulmonary disease
PCR	Polymerase chain reaction
RT	Reverse transcription
ANOVA	Analysis of variance
PRS	Polygenic risk scores

Supplementary Information

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Supplementary Material 1.

Supplementary Material 2: Table 4. Association of the risk factors of NASH HCC patients with SNPs.

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Authors' contributions
SMS, ASH, RCS, KHT, MAN and SDS contributed in the study designing. RCS and MAN provided patient samples and clinical information. VA and SMS contributed in statistical analysis and data interpretation. SMS wrote the manuscript. ASH, RCS, KHT, MAN and SDS reviewed the manuscript providing critical comments and approved the final manuscript.

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Data availability
Data is provided within the manuscript.

Declarations

Ethics approval and consent to participate
This study was approved by the ethics review committee of the Faculty of Medicine, University of Kelaniya, Ragama, Sri Lanka (P/230/11/2019). All methods were performed in accordance with the relevant guidelines and regulations of declaration of Helsinki. Participation of potential subjects in the research was absolutely voluntary and written informed consent was obtained from all the volunteered study participants.

Consent for publication
Not applicable.

Competing interests
The authors declare no competing interests.

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