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Does Genetic Variation in *PNPLA3*, *TM6SF2* and *HSD17B13* have a Role in the Development or Prognosis of Hepatocellular Carcinoma in Turkish Patients with Hepatitis B?

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ABSTRACT

Background & Aims: Progression to hepatocellular carcinoma (HCC) is restricted by viral suppression in chronic hepatitis B (CHB); however, some patients still progress despite antiviral therapy. Presence of single nucleotide polymorphisms (SNPs) such as PNPLA3 rs738409 and TM6SF2 rs58542926 are associated with the development and progression of steatotic liver disease to HCC, whereas a splice variant in HSD17B13 rs72613567:TA has been shown to be protective. We investigated the role of these SNPs in the development or prognosis of HCC in pure CHB etiology, in the absence of hepatic steatosis, remains unknown.
Materials: We analysed *PNPLA3* rs738409, *TM6SF2* rs58542926, and *HSD17B13* rs72613567 SNPs in a prospectively recruited cohort (n=323) consisting of healthy controls, CHB and CHB-HCC patients without hepatic steatosis. SNPs were determined by PCR analysis and associations for the alleles and genotypes were investigated using adjusted-logistic regression analyses. The overall survival (OS) data were collected from CHB-HCC patients for survival analysis.
Results: The genotype and allelic distribution of *PNPLA3* rs738409, *TM6SF2* rs58542926, and *HSD17B13* rs72613567 were similar between healthy controls, CHB, and CHB-HCC groups. No genotype, allele or haplotype analysis was found to be associated with increased risk for CHB-HCC. Survival analysis revealed no genotype or allele to be associated with OS in patients with CHB-HCC.
Conclusions: We could not demonstrate any association of *PNPLA3* rs738409, *TM6SF2* rs58542926, and *HSD17B13* rs72613567 with the development or prognosis of CHB-HCC, supporting the initial hypothesis that they should be considered specific hotspots for liver diseases characterized with hepatic steatosis.

Key words: patatin – like phospholipase-3 – *PNPLA3* – transmembrane 6 superfamily member 2 – *TM6SF2* – 17 beta-hydoxysteroid dehydrogenase B13 – *HSD17B13* – hepatitis B – hepatocellular carcinoma – genetic risk score.

Abbreviations: ALD: alcoholic liver disease; CHB: chronic hepatitis B; GRS: genetic risk score; HCC: hepatocellular carcinoma; HCV: hepatitis C virus; HSD17B13: 17 beta-hydoxysteroid dehydrogenase B13; IQR: interquartile range; NAFLD: non-alcoholic fatty liver disease; OS: overall survival; PNPLA3: patatin-like phospholipase-3; SD: standard deviation; SNP: single nucleotide polymorphism; TM6SF2: transmembrane 6 superfamily member 2.

INTRODUCTION

Chronic hepatitis B (CHB) virus infection affects approximately 6% of the world population on average, including more than 296 million people being chronic carriers [1]. It is still the dominant etiology in cirrhosis and hepatocellular carcinoma (HCC) (>60%) worldwide, and the leading cause of death among viral hepatitis in Asian countries [2]. Among all CHB cases, approximately 20-30% would develop cirrhosis, and 5% further progress to HCC, and about 820,000 patients die from these complications per year [3]. Hepatitis B virus has carcinogenetic potential itself by several insertional mutagenetic routes, and up to 30% of CHB-related HCCs arise in the non-cirrhotic liver [4]. Current management of CHB with long-term nucleotide analogues treatment by inhibiting HBV replication had been shown to decrease the incidence of HCC. Nevertheless, despite on-therapy virological remission, the annual incidence of HCC ranges from 0.01% to 5.4% in CHB [5].

The large diversity in clinical outcome of HBV infection emphasize the importance of identifying the mechanisms

underlying the progression of CHB to HCC to prevent against CHB-related mortality. Although the environmental factors such as older age, male gender, alcohol abuse, and co-infection with other hepatitis virus are unveiled as risk factors of progressive HBV-induced liver disease, genetic factors may also influence progression in CHB [6]. In fact, recent genome-wide association studies (GWAS) revealed multiple candidate genes and genetic predispositions in the HLA haplotypes with risk-conferring or protective impact have also been proposed, that have been extensively investigated in the progression of CHB to HCC [7].

In the recent years, several single nucleotide polymorphsims (SNPs) such as patatin-like phospholipase-3 (*PNPLA3*) rs738409, transmembrane 6 superfamily member 2 (*TM6SF2*) rs58542926 and 17 beta-hydoxysteroid dehydrogenase B13 (*HSD17B13*) rs72613567 have been identified as risk factors for progression of various liver diseases including non-alcoholic fatty liver disease (NAFLD), alcoholic-liver disease (ALD) and hepatitis C virus (HCV) [8-20]. As these SNPs are located in the liver fat modulating genes, they were initially thought to be only influential on the occurrence and progression of liver diseases that cause liver steatosis. However, they have also been shown to have an association with a risk of cirrhosis and HCC in the general population, as well as other rare liver diseases including autoimmune hepatitis and hereditary hemochromatosis [21-24]. These findings raised the question whether this holds true taking into account carriage of the variants of *PNPLA3* rs738409, *TM6SF2* rs58542926 and *HSD17B13* rs72613567 for the progression of CHB disease in the absence of hepatic steatosis. To date, these SNPs have never been investigated in combination for patients with CHB by excluding those with hepatic steatosis that.

The aim of the present study was to evaluate whether the characteristic SNPs that have been established to influence the progression of various liver diseases characterized by hepatic steatosis (*PNPLA3* rs738409, *TM6SF2* rs58542926, and *HSD17B13* rs72613567), have an impact on the development of HCC in the CHB etiology, and to assess their potential influence on survival outcomes in the CHB-related HCC patients.

METHODS

Study Population

A total of 323 adult participants, including 148 healthy liver disease-free controls, 91 patients with CHB and 84 patients with CHB-related HCC were prospectively recruited at Marmara University, School of Medicine, Division of Gastroenterology and Hepatology from January 2019 to January 2022. The healthy controls were community residents entering the same hospital for routine check-up during the study period. The control group members had no evidence of HCC or other types of cancer, history of liver disease, radiologic evidence of hepatic steatosis, serological evidence of HBV or HCV, or any relation to the experimental group. All CHB patients were diagnosed with their biochemical and virological features as HBsAg positivity for more than six months, all were under the antiviral treatment and had undetectable HBV-DNA. The enrolled CHB-related HCC cases were all newly diagnosed using the guidelines proposed by the European Association for the Study of the Liver (EASL), under antiviral treatment with undetectable HBV-DNA but treatment-naïve for their cancer at the time of the enrolment [25]. All participants were Caucasians and over the age of 18.

For all three groups, those with radiological evidence of hepatic steatosis, significant alcohol use or concomitant other chronic liver disease was excluded from the study to prevent any interference and bias. Significant alcohol consumption was defined as >21 units per week for men and >14 units per week for women. The diagnosis of liver cirrhosis was confirmed by imaging evidence, laboratory results or histological findings via liver biopsy.

After signed written informed consent, whole-blood samples treated with ethylenediaminetetraacetate (EDTA) were taken for the genetic testing of 323 participants. Survival time was calculated from the date of HCC diagnosis until the date of exact notified death as retrieved from hospital records and/or national death notification system, or 1st of January 2022 if alive.

Genotyping of *PNPLA3* rs738409, *TM6SF2* rs58542926 and *HSD17B13* rs72613567 Polymorphisms

Genomic DNA was isolated from peripheral blood mononuclear cells using the "Pure Link Genomic DNA Purification Kit" (Invitrogen, California, USA) according to the manufacturer's instructions. Isolated DNA concentration was quantified using Qubit™ dsDNA BR Assay Kit (Thermo Fischer Scientific, USA) and 5ng/ul of DNA was used for all allelic discrimination of polymorphisms. DNA concentrations were measured using a Qubit flourometer. Genotyping of *HSD17B13* rs72613567 was performed using a custom TaqMan® SNP Genotyping Assay with primer and probes (FAM and VIC labelled) [19], whereas *PNPLA3* rs738409 and *TM6SF2* rs58542926 polymorphisms were genotyped by using rhAmp™ SNP Assays. All assays were performed in 96-well plates, including negative template controls. Allelic discrimination was performed on Light Cycler 480 Real-Time System (Roche, Germany) by measuring allele-specific fluorescence.

We calculated a previously proposed genetic risk score (GRS) using only these three SNPs for each participant, that has been shown to be associated with risk of cirrhosis and HCC in NAFLD etiology [22]. Accordingly, *PNPLA3* rs738409, *TM6SF2* rs58542926, and *HSD17B13* rs72613567 genotypes were coded as 0, 1, and 2 for non-carriers, heterozygous, and homozygous carriers of the risk-increasing allele, respectively. For *PNPLA3* and TM6SF2, the risk alleles were appointed as the minor alleles, while the major T allele was considered risk increasing because the minor TA allele of this variant associates with protection from chronic liver disease. For each participant, a combined GRS was calculated as the sum of these risk-increasing alleles (ranging from 0 to 6).

Statistical Analysis

All statistical analyses were conducted using the SPSS software version 24.0 (IBM, Armonk, NY, USA). The median and interquartile range (IQR) were used to display continuous skewed data, and mean ± standart deviation (SD) was used if normally distributed. For the comparison of continuous

demographic variables amongst three groups; one-way ANOVA test was performed if normally distributed, and the Kruskal-Wallis test was conducted if the data was not normally distributed with a Bonferroni-adjusted Mann-Whitney test for the post-hoc analysis when needed. The categorical parameters were compared using Chi-square test or Fisher's exact test. Using the Hardy Weinberg equilibrium theory, expected and actual genotype and allele frequencies were compared among cases and controls. Associations between control and case groups with the alleles and genotypes were investigated using logistic regression analyses, and adjustment for age and sex was utilized.

Overall survival (OS) time was estimated from the date of HCC diagnosis to the last known date of follow-up or death. The survival outcomes were compared using Kaplan-Meier curves with the log-rank test. A p-value ≤ 0.05 was considered statistically significant.

Ethical Statement

Participant recruitment was approved by the Committee for Ethics of Medical Experiments on Human Subjects, Faculty of Medicine in Marmara University (Protocol No: 09.2019.716, Approval date: 13.09.2019). All investigations were conducted in accordance with the guidelines of the 1975 Declaration of Helsinki.

RESULTS

General Characteristics

The general characteristics of the three groups are summarized in Table I. CHB-HCC patients were older and there were more males in comparison to CHB and healthy controls (both p values<0.001). CHB and CHB-HCC groups had no difference in baseline characteristics regarding comorbidities (diabetes and hypertension), body-mass index, cigarette use, Child-Pugh-Turcotte or MELD scores.

Table I. General characteristics of whole cohort

	Controls (n=148)	CHB (n=91)	CHB-HCC (n=84)	p-value
Age, mean ± SD	45 (18-62)	52 (30-77)	61 (30-78)	<0.001*
Gender, n (%)				<0.001*
Female	75 (50.7)	34 (37.4)	15 (17.9)	
Male	73 (49.3)	57 (62.6)	69 (82.1)	
Diabetes mellitus,, n (%)	-	15 (16.5)	17 (20.2)	0.561
Hypertension, n (%)	-	19 (20.9)	16 (19.0)	0.851
BMI, median (min- max)	-	28.4 (16.9-46.7)	27.7 (16.8-45.7)	0.255
Cigarette, n (%)	-	23 (25.3)	31 (37.3)	0.086
CPT score, median (min- max)	-	5 (5-12)	5 (5-9)	0.437
MELD score, median (min- max)	-	11 (6-28)	9 (7-20)	0.409
PNPLA3, n (%)				0.445
• CC	75 (50.7)	45 (49.5)	50 (59.5)	
• CG	57 (38.5)	38 (41.8)	30 (35.7)	
• GG	16 (10.8)	8 (8.8)	4 (4.8)	
o Minor Allele (G)	89 (30.1)	54 (29.7)	38 (22.6)	o 0.348
▫ MAF, %	0.108	0.087	0.047	
TM6SF2, n (%)				0.422
• CC	133 (89.9)	81 (89.0)	76 (90.5)	
• CT	15 (10.1)	9 (9.9)	6 (7.1)	
• TT	-	1 (1.1)	2 (2.4)	
o Minor Allele (T)	15 (5.1)	11 (6.0)	10 (6.0)	o 0.892
▫MAF, %	0	0.010	0.023	
HSD17B13, n (%)				
• T/T	96 (64.9)	65 (71.4)	63 (75.0)	0.401
• T/TA	46 (31.1)	25 (27.5)	20 (23.8)	
• TA/TA	6 (4.1)	1 (1.1)	1 (1.2)	
o Minor Allele (TA)	58 (19.6)	27 (14.8)	22 (13.1)	o 0.153
▫ MAF, %	0.040	0.010	0.011	
Genetic risk score, n (%)				0.550
• 1	1 (0.7)	1 (1.1)	2 (2.4)	
• 2	17 (11.5)	10 (11.0)	5 (6.0)	
• 3	35 (23.6)	30 (33.3)	25 (29.8)	
• 4	71 (48.0)	35 (38.5)	37 (44.0)	
• 5	22 (14.9)	15 (16.5)	15 (17.9)	
• 6	2 (1.4)	0 (0)	0 (0)	

BMI: body-mass index; CHB: chronic hepatitis B; CPT: Child-Pugh Turcotte; HCC: Hepatocellular carcinoma; HSD17B13: 17 beta-hydoxysteroid dehydrogenase B13; MAF: minor allele frequency; MELD: model of end-stage liver disease; PNPLA3: patatin-like phospholipase-3; TM6SF2: transmembrane 6 superfamily member 2

Genotype and Allele Frequencies of *PNPLA3* rs738409, *TM6SF2* rs58542926 and *HSD17B13* rs72613567

Of the 148 healthy controls, 75 (50.7%) had CC, 57 (38.5%) had CG, and 16 (10.8%) had GG genotype for *PNPLA3* rs738409, 133 (89.9%) had CC and 15 (10.1%) had CT genotype for *TM6SF2* rs58542926, 96 (64.9%) had T/T, 46 (31.1%) had T/TA and 6 (4.1%) had TA/TA genotype for *HSD17B13* rs72613567 SNP. Minor allele frequency was 0.108% for *PNPLA3* rs738409, 0% for *TM6SF2* rs58542926 and 0.040% for *HSD17B13* rs72613567 in healthy controls.

In CHB group, 45 (49.5%) had CC, 38 (41.8%) had CG, and 8 (8.8%) had GG genotype for *PNPLA3* rs738409, 81 (89.0%) had CC, 9 (9.9%) had CT and 1 (1.1%) had TT genotype for *TM6SF2* rs58542926, 65 (71.4%) had T/T, 25 (27.5%) had T/TA and 1 (1.1%) had TA/TA genotype for *HSD17B13* rs72613567 SNP. Minor allele frequency was 0.087% for *PNPLA3* rs738409, 0.010% for *TM6SF2* rs58542926 and 0.010% for *HSD17B13* rs72613567 in CHB.

Of the 84 patients in CHB-HCC group, 50 (59.5%) had CC, 30 (35.7%) had CG, and 4 (4.8%) had GG genotype for *PNPLA3* rs7384, 76 (90.5%) had CC, 5 (7.1%) had CT, and 2 (2.4%) had TT genotype for *TM6SF2* rs58542926, while 63 (75.0%) had T/T, 20 (23.8%) had T/TA, and 1 (1.2%) T/TA genotype for *HSD17B13* rs72613567 SNP. Minor allele frequency was 0.047% for *PNPLA3* rs738409, 0.023% for *TM6SF2* rs58542926 and 0.011% for *HSD17B13* rs72613567 in CHB-HCC group.

The distribution of genotype and allele frequencies of *PNPLA3* rs738409 SNPs, *TM6SF2* rs58542926, *HSD17B13* rs72613567 and the GRS showed no statistically significant difference among healthy controls, CHB, and CHB-HCC groups (Table I). The genotypic frequencies of the patients and controls were in Hardy–Weinberg equilibrium, suggesting that there was no population stratification and no sampling bias.

Association of Three SNPs with HCC Development in CHB Patients

To investigate the potential risk association of three SNPs with the presence of CHB and progression to HCC, we performed univariate and multivariate logistic regression analysis with each genotype and allele adjusting for age and sex. As a result, none of the *PNPLA3* rs738409, *TM6SF2* rs58542926, and *HSD17B13* rs72613567 genotypes or alleles was found to be associated with CHB and CHB-HCC (Table II). To prevent any bias of age and gender difference related bias among groups, we performed age and gender adjusted logistic regression analysis and again found no association of three SNPs with HCC risk. The haplotypes of *PNPLA3* rs738409, *TM6SF2* rs58542926, and *HSD17B13* rs72613567 were also compared, and none was found to be associated with increased risk for CHB or CHB-HCC (Table III).

Table II. Logistic regression analysis to reveal association of genotypes and alleles with CHB and CHB-HCC

Genotype	uOR (95% CI)	p-value	aOR (95% CI)	p-value
Healthy controls (n=148) vs CHB (n=91)				
***PNPLA3* rs738409**				
- CC		0.821		0.565
- CG	1.111 (0.640-1.930)	0.708	1.216 (0.662-2.237)	0.528
- GG	0.833 (0.330-2.103)	0.699	0.699 (0.246-1.982)	0.500
- Minor allele (G)	0.981 (0.655-1.469)	0.927	0.963 (0.616-1.506)	0.869
***TM6SF2* rs58542926**				
- CC		0.999		0.995
- CT	0.985 (0.412-2.355)	0.973	0.952 (0.365-2.486)	0.921
- TT	2.653E+9 (0.000-)	1.000	380481177	1.000
- Minor allele (T)	2.305 (0.541-2.684)	0.648	1.031 (0.418-2.543)	0.947
***HSD17B13* rs72613567**				
- T/T		0.357		0.291
- T/TA	0.803 (0.449-1.433)	0.458	1.055 (0.552-2.015)	0.871
- TA/TA	0.246 (0.029-2.093)	0.199	0.162 (0.016-1.626)	0.122
- Minor allele (TA)	0.715 (0.434-1.178)	0.188	0.795 (0.458-1.379)	0.414
CHB (n=91) vs CHB-HCC (n=84)				
***PNPLA3* rs738409**				
- CC		0.329		0.224
- CG	0.711 (0.380-1.328)	0.284	0.615 (0.313-1.206)	0.157
- GG	0.450 (0.127-1.596)	0.216	0.412 (0.106-1.602)	0.201
- Minor allele (G)	0.693 (0.428-1.121)	0.135	0.634 (0.379-1.060)	0.082
***TM6SF2* rs58542926**				
- CC		0.674		0.488
- CT	0.711 (0.241-2.091)	0.535	0.685 (0.215-2.179)	0.522
- TT	2.132 (0.189-23.990)	0.540	4.343 (0.241-78.342)	0.320
- Minor allele (T)	0.984 (0.407-2.380)	0.971	1.106 (0.424-2.888)	0.836
***HSD17B13* rs72613567**				
- T/T		0.858		0.814
- T/TA	0.825 (0.417-1.633)	0.582	0.885 (0.426-1.837)	0.743
- TA/TA	1.032 (0.063-16.855)	0.983	0.439 (0.026-7.430)	0.568
- Minor allele (TA)	0.865 (0.472-1.587)	0.639	0.850 (0.445-1.625)	0.623

aOR: adjusted Odds-ratio (all are adjusted for age and sex); HSD17B13: 17 beta-hydoxysteroid dehydrogenase B13; PNPLA3: patatin-like phospholipase-3; TM6SF2: transmembrane 6 superfamily member 2; uOR: unadjusted odds-ratio.

Table III. Logistic regression analysis to reveal association of haplotypes with CHB and CHB-HCC

Healthy controls vs CHB						
	PNPLA3	*TM6SF2*	*HSD17B13*	**Freq**	**OR (95% CI)**	**p-value**
1	C	C	T	0.5356	1.000	-
2	G	C	T	0.2454	1.040 (0.630-1.720)	0.870
3	G	C	TA	0.1326	0.820 (0.410-1.650)	0.580
4	C	T	TA	0.0863	0.760 (0.340-1.720)	0.520
CHB vs CHB-HCC						
	PNPLA3	*TM6SF2*	*HSD17B13*	Freq	OR (95% CI)	p-value
1	C	C	T	0.5866	1.000	-
2	G	C	T	0.2192	0.620 (0.340-1.130)	0.120
3	G	C	TA	0.1152	0.790 (0.340-1.820)	0.580
4	C	T	TA	0.079	0.950 (0.420-2.180)	0.910

CHB: Chronic hepatitis B; CI: confidence interval; Freq: frequency; HCC: hepatocellular carcinoma; HSD17B13: 17 beta-hydoxysteroid dehydrogenase B13; OR: odds-ratio; PNPLA3: patatin-like phospholipase-3; TM6SF2: transmembrane 6 superfamily member 2.

Survival Analysis of Patients with CHB-related HCC

Tumoral characteristics and applied treatment modalities in patients with HCC are presented in Table IV. The median OS time of patients with CHB-HCC was 29.7 (23.6-35.9) months. We analysed the OS for each genotype and allele, as *PNPLA3* genotypes (CC: 29.7 [19.7-39.7], CG: 38.3 [15.6-61.0], GG: 10.2 [19.7-39.7] months; p=0.461), *TM6SF2* genotypes (CC: 31.0 [24.1-37.9], CT: 10.9 [7.4-14.3], TT: 24.3 months; p=0.520), *HSD17B13* genotypes (TT: 29.7±4.7, TTA: 10.3±12.9 months; p=0.221), *PNPLA3* alleles (C: 31.0 [25.2-36.8], G: 29.5[21.0-38.1] months; p=0.945), *TM6SF2* alleles (C: 29.7 [24.2-35.2], T: 24.3 [4.1-44.5] months; p=0.229), *HSD17B13* alleles (T: 29.7 [24.9-34.5], TA: 31.0 [4.1-57.9] months; p=0.633), and found no association with survival outcomes in the CHB-HCC group (Fig. 1). For the survival analysis in HCC patients, we performed an adjusted Cox-regression analysis to preclude the impacts of age, gender and tumor stage on survival outcomes. However, the results did not change and we could demonstrate that regardless of the differences in age, sex and tumor stage, the three SNPs are not associated with the survival outcomes of CHB-HCC patients (Table V).

We also compared the survival outcomes for each GRS, and found no significant difference among median OS with increasing GRS (GRS 1: 8.1 months, GRS 2: 26.2 [9.1-43.3] months, GRS 3: 22.8 [8.2-37.3] months, GRS 4: 42.9 [20.0-65.8] months, GRS 5-6: 10.3 [6.7-13.9] months, p=0.296). The Kaplan-Meier survival analysis of patients with CHB-HCC with regard to GRS is presented in Fig. 2.

Table IV. Clinical characteristics of CHB-HCC patients

	CHB-HCC (n=84)
BCLC, n (%)	
- 0	6 (7.1)
- A	41 (48.8)
- B	20 (23.8)
- C	16 (19.0)
- D	1 (1.2)
ECOG PS, n (%)	
- 0	55 (65.5)
- 1	13 (15.5)
- 2	5 (6.0)
AFP, median (min- max)	9.45 (1.09-230140.00)
Portal vein thrombosis, n (%)	14 (16.7)
Lymph node involvement, n (%)	8 (9.5)
Vascular invasion, n (%)	8 (9.5)
Metastasis, n (%)	5 (6.0)
Treatment modality, n (%)	
- Surgical resection	11 (13.1)
- OLT	1 (1.2)
- RFA	19 (22.6)
- TACE	30 (35.7)
- Sorafenib	6 (7.1)
- TARE	5 (6.0)
- TACE + RFA	1 (1.2)
- BSC	11 (13.1)
Survival, median (min- max), months	29.7 (23.6-35.9)

AFP: Alfa fetoprotein; BCLC: Barcelona Clinic Liver Cancer; BSC: Best supportive care; ECOG PS: Eastern Cooperative Oncology Group Performance Score; OLT: orthotropic liver transplantation; RFA: radiofrequency ablation; TACE: transarteriel chemoembolization; TARE: transarteriel radioembolization.

DISCUSSION

Among fatty liver associated genetic polymorphisms, *PNPLA3* rs738409, *TM6SF2* rs58542926, and *HSD17B13* rs72613567 are regarded as the most important three SNPs that have been universally associated with the development of chronic liver disease and fibrosis, and progression to HCC. In the recent years, the association of these three genetic variants with the progression of fibrosis and development of HCC in ALD and HCV etiology have also been demonstrated. Just like in NAFLD and ALD, chronic HCV disease can also cause hepatic steatosis especially in the presence of genotype 3 infection [26-28]. Different than HCV, HBV infection is not a familiar cause of hepatic steatosis [29]. From that point of view, chronic HCV can theoretically share some common genetic hotspots with steatotic liver disease, while they are not expected to have a role in the disease course of CHB.

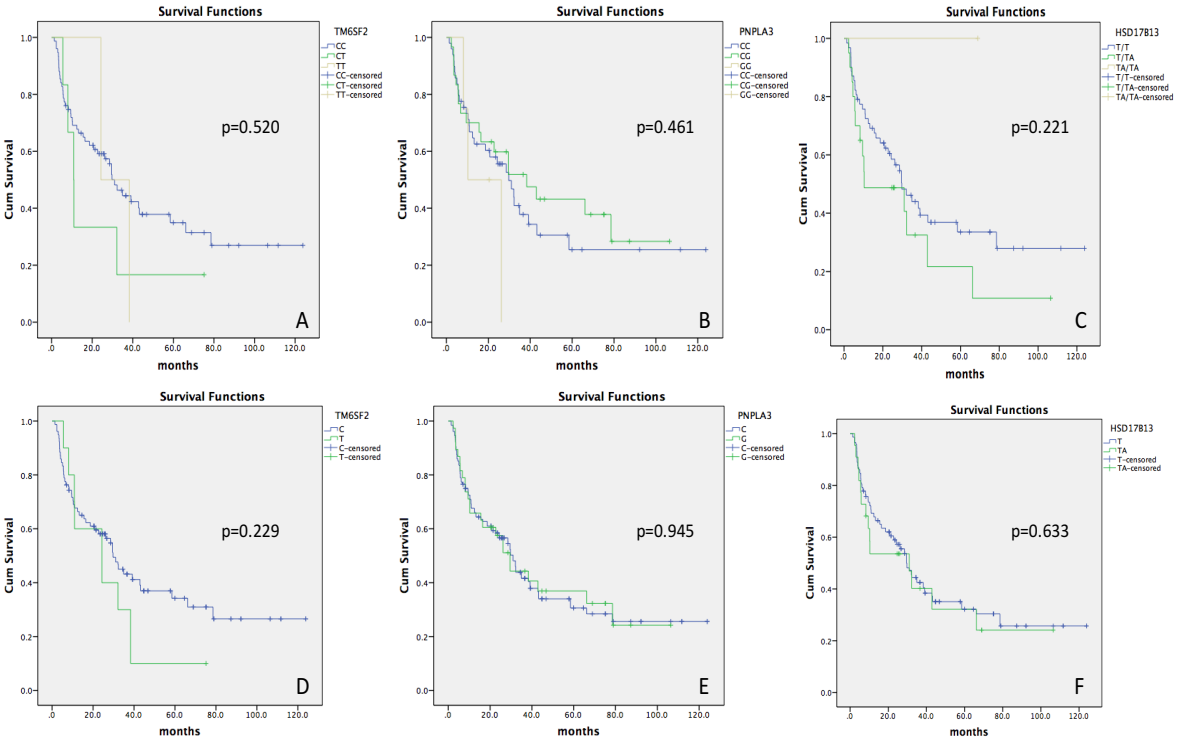

Fig. 1. Kaplan-Meier Survival Curves for each genotype and allele of three SNPs. A. *TM6SF2* genotypes (CC: 31.0 [24.1-37.9], CT: 10.9 [7.4-14.3] TT: 24.3 months; p=0.520); B. *PNPLA3* genotypes (CC: 29.7 [19.7-39.7], CG: 38.3 [15.6-61.0], GG: 10.2 [19.7-39.7] months; p=0.461); C. *HSD17B13* genotypes (TT: 29.7 [4.7, TTA: 10.3] 12.9 months; p=0.221)D. *TM6SF2* alleles (C: 29.7 [24.2-35.2], T: 24.3 [4.1-44.5] months; p=0.229); E. *PNPLA3* alleles (C: 31.0 [25.2-36.8], G: 29.5 [21.0-38.1] months; p=0.945); F. *HSD17B13* alleles (T: 29.7 [24.9-34.5], TA: 31.0 [4.1-57.9] months; p=0.633).

Despite the linkage of these aforementioned SNPs with liver diseases characterized by fatty liver in the literature, two recent studies reported the association of *PNPLA3* rs738409 GG genotype with poorer prognosis in autoimmune hepatitis, and the presence of *PNPLA3* rs738409, *TM6SF2* rs58542926 genetic variants with increased risk of cirrhosis in hereditary hemochromatosis [23, 24]. One possible explanation could be the lack of exclusion of those with hepatic steatosis in their study. On the basis of this conflict, we performed the present study to expose whether these 3 characteristic SNPs might have a role in disease course of pure HBV etiology and prognosis in CHB-HCC.

Table V. Cox-regression analysis to reveal association of three SNPs and the combined Genetic Risk Score with survival outcomes in CHB-HCC

Genotype	HR (95% CI)	p-value	aHR (95% CI)	p-value
***PNPLA3* rs738409**				
- CC		0.470		0.787
- CG	0.808 (0.448-1.459)	0.479	1.069 (0.549-2.082)	0.844
- GG	1.709 (0.514-5.680)	0.382	1.532 (0.453-5.186)	0.493
***TM6SF2* rs58542926**				
- CC		0.527		0.148
- CT	1.638 (0.647-4.146)	0.298	2.618 (0.970-7.065)	0.157
- TT	1.450 (0.350-6.010)	0.609	0.759 (0.150-3.825)	0.738
***HSD17B13* rs72613567**				
- T/T		0.427		0.670
- T/TA	1.509 (0.813-2.798)	0.192	1.335 (0.552-2.015)	0.372
-TA/TA	0.000 (0.01-2.093)	0.974	0.001 (0.001-6.524)	0.943
GRS				
- 1		0.315		0.410
- 2	0.577 (0.096-3.477)	0.548	0.659 (0.095-4.548)	0.672
- 3	0.670 (0.154-2.923)	0.594	0.877 (0.181-4.257)	0.871
- 4	0.426 (0.099-1.842)	0.253	0.468 (0.103-2.133)	0.327
- 5-6	0.931 (0.203-4.278)	0.927	0.800 (0.162-3.938	0.783

aHR: adjusted-Hazard ratio; GRS: genetic risk score; HSD17B13: 17 beta-hydoxysteroid dehydrogenase B13; PNPLA3: Patatin-like Phospholipase-3; TM6SF2: Transmembrane 6 Superfamily Member 2; HR: hazard ratio.

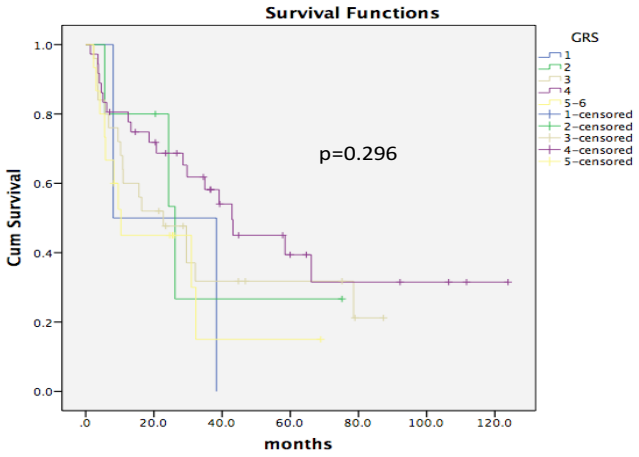

Fig. 2. Kaplan-Meier Survival Curves for the combined Genetic Risk Score (GRS).

The importance of CHB and fatty liver disease co-existence have been better understood in the last few years. Although fatty liver disease is a well-known risk factor for adverse outcomes such as cirrhosis and HCC, its interactions with HBV presence and clinical impacts seem complex. The presence of hepatic steatosis may suppress HBV viral activity, potentially leading to attenuated liver injury. To this end, the impact of co-existing fatty liver disease in previous studies targeting HBV etiology have been underestimated with regard to clinical and genetic features. In the present study, we demonstrated that three sequence variations with vigorous impact on the progression of hepatic steatosis are not associated with increased risk of HCC in pure HBV etiology. Thus, it would be convenient to say that our findings are generally in support with the initial hypothesis that these 3 previously defined fatty liver disease-associated SNPs are probably only linked to chronic liver diseases causing hepatic steatosis. In support of this concept, there are studies in the literature showing the relation of hepatic steatosis development in HBV-infected patients carrying the risk alleles of *PNPLA3* [30-32]. To prevent any bias or interference with fatty liver disease patients, we excluded all subjects with hepatic steatosis detected in radiological imaging in the present study.

The limited available evidence on the role of these genetic variants with the spectrum of CHB is generally in line with the results of our case-control study. In a multicentre retrospective case-control study, *PNPLA3* rs738409 and *TM6SF2* rs58542926 variants are identified as independent predisposing factors in patients with ALD, but not in subjects with CHB[33]. In that study, 87 CHB and 90 CHB-HCC patients were investigated but *HSD17B13* rs72613567 analysis was not included. Whether *HSD17B13* rs72613567:TA carries an increased risk for progression of CHB has been evaluated in a study investigating 94 CHB and 113 CHB-HCC patients, and the authors found no potential association neither in CHB nor in HCC development [19]. Case-control studies from Asia and a subsequently performed meta-analysis revealed that *PNPLA3* rs738409 might increase HCC risk in comparison to healthy subjects, but it had no influence on the development of HCC among HBV-infected subjects [34, 35]. Our study highlighted that *PNPLA3* rs738409, either alone or in combination with *HSD17B13* rs72613567 and *TM6SF2* rs58542926, is not associated with the risk of HCC in CHB individuals. We also tested a combined GRS developed for patients with NAFLD, using the three SNPs, which also did not show any prognostic impact in our CHB cohort. Additionally, we demonstrated that none of these variants were associated with the prognosis of patients with CHB-HCC. This finding is rather rational considering the multifactorial and heterogeneous behaviour of the HCC disease course, and an independent impact of a single genetic variant on HCC prognosis would have been surprising.

There were some similarities and discrepancies regarding the allocation of genotypes in our cohort compared to other studies. The distribution of *TM6SF2* genotypes among our patients with CHB was 89% CC, 9.9% CT, and 1.1% TT; results comparable with a previous report, whose analysis of 87 European patients with CHB revealed 90.8% for CC and 8.0% for CT+TT [33]. The *PNPLA3* genotype frequencies in our CHB (49.5% CC, 41.8% CG, and 8.8% GG) and CHB-HCC (59.5% CC, 35.7% CG, and 4.8% GG) groups were slightly different, but comparable, than the reported ratios in the previous European study (CHB: 60.9% CC, 29.9% CG, and 9.2% GG // CHB-HCC: 52.2% CC, 40% CG, and 7.7% GG) [33]. In a study analysing Asian patients, distribution of *PNPLA3* genotypes among 695 patients with CHB (37.3% CC, 49.7% CG, and 13.0% GG) and 786 patients with CHB-HCC (40.6% CC, 46.1% CG, and 13.2% GG) were found more similar with our patients [34]. The distribution of *HSD17B13* genotypes in our healthy controls (64.9% TT, 31.1% T/TA, and 4.1% TA/TA), patients with CHB (71.4% T/T, 27.5% T/TA, and 1.1% TA/TA) and CHB-HCC (T/T:75.0%, T/TA: 23.8%, TA/TA: 1.2%) were slightly different comparing to a previous European CHB cohort, showing 53% T/T, 47% T/TA+TA/TA in healthy controls, 55% T/T, 45% T/TA+TA/TA in CHB patients, and 59% T/T, 41% T/T+TA/TA in CHB-HCC patients [19]. The discrepancies in the distribution of genotypes in our study may result, at least in part, from the population representativeness of the selected sample or the genetic configuration. Either way, the results of our study are generally in harmony with previous reports, suggesting that the differences in the genetic configuration or a different sample selection probably would not have affected the results of our study.

There are a few limitations of the present study that must be taken into consideration when interpreting results. The major limitation of the present study was the small sample size. Due to the fact that this study was a hospital-based case-control study, and cases were selected from a single tertiary institution, it is possible that our relatively small sample was unrepresentative of CHB and CHB-HCC patients in the general population. However, the number of patients analysed was pretty close with the previous studies on this subject, and power calculation ensured the accuracy of our results. Moreover, our control subjects were also recruited from the same hospital and observed distributions of polymorphism genotype frequencies suggested no selection bias according to the Hardy–Weinberg equilibrium model. Secondly, addition of an intermediate cirrhotic control group could add value to our study. In that regard, our study requires further validation and subgroup analysis in a larger series. Considering the increasing incidence of fatty liver disease not only in general population but also in CHB patients, the present study focused on pure HBV etiology

excluding those with hepatic steatosis which prevented us from reaching a larger number of subjects.

CONCLUSIONS

Our case-control study highlighted that *PNPLA3* rs738409, *TM6SF2* rs58542926, and *HSD17B13* rs72613567 SNPs are not associated with susceptibility to HCC in HBV etiology. We also reported the negligible association of these three genetic variants with regard to the prognosis of those with CHB-associated HCC. To the best of our knowledge, this was the first report to analyse the association of these three SNPs in combination for HCC development in hepatitis B virus etiology and prognosis of CHB-related HCC.

Conflicts of interest: None to declare.

Authors' contribution: C.O.D. contributed significantly to the conception, design, data collection, analysis and drafting the article. F.E. contributed significantly to the conception, design, analysis, data interpretation and critically reviewed the manuscript. D.Y. helped in the data acquisition and laboratory analysis. O.C.O. and F.G. critically revised the manuscript and approved the final version of the manuscript. All authors approved the final version to be published and agreed to be accountable for all aspects of the work.

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