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ORIGINAL RESEARCH

Impact of PNPLA3 p.I148M and Hepatic Steatosis on Long-Term Outcomes for Hepatocellular Carcinoma and HBsAg Seroclearance in Chronic Hepatitis B

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Background: Coexistence of hepatitis B and nonalcoholic fatty liver disease is common; however, little is known about the impact of hepatic steatosis and its major genetic determinants on the natural history of HBV infection. We aimed to study the effects of hepatic steatosis and PNPLA3 variant p.I148M on the risk of hepatocellular carcinoma (HCC) and the lifetime probability of HBsAg seroclearance, which is associated with functional remission and improved long-term outcome of HBV infection.

Methods: We conducted a cohort study of 2385 male, HBsAg-positive Taiwanese civil servants recruited in 1989–1992, and followed up until 2019. Cox regression with competing-risk models was used to estimate sub-distribution hazard ratios (sHRs) and 95% confidence intervals (CIs).

Results: Of 2385 participants, 628 experienced HBsAg seroclearance and 217 developed HCC. Hepatic steatosis, excess body-mass index, and the PNPLA3-148M variant were significantly associated with higher HBsAg seroclearance rate. However, multivariate analyses accounting for HBsAg seroclearance and various HCC risk factors showed that, while steatosis was associated with decreased HCC risk (sHR [95% CI]: 0.49 [0.36–0.66]), carriage of the PNPLA3-148M variant allele (vs II homozygotes: 1.64 [1.20–2.25] for MI heterozygotes; 1.83 [1.20–2.78] for MM homozygotes) and obesity (1.51 [1.07–2.13]) were associated with increased risk. The inverse hepatic steatosis-HCC association persisted after additional adjustment for other viral factors or using different follow-up time cut-offs to account for reverse causality. Moreover, the PNPLA3 MM genotype was positively associated with elevations of ALT and AST and liver cirrhosis, while hepatic steatosis was positively associated with ALT but inversely associated with AST and liver cirrhosis.

Conclusion: Hepatic steatosis and PNPLA3-148M variant appeared to have distinct impacts on the development of HBV-related progressive liver disease and HCC. PNPLA3 p.I148M, but not a diagnosis of hepatic steatosis, can help to identify HBV carriers with high-risk fatty liver disease in the progression to HCC.

Keywords: chronic hepatitis B, HBsAg seroclearance, hepatic steatosis, hepatocellular carcinoma, liver cirrhosis, PNPLA3

Introduction

Chronic hepatitis B virus (HBV) infection is a main cause of hepatocellular carcinoma (HCC) and liver-related death worldwide.[1] In addition, with the rising prevalence of obesity, nonalcoholic fatty liver disease (NAFLD) has been associated with increasing HCC burden in Western Countries. This disease begins with simple steatosis, a condition of excess fat in the liver, which is generally considered benign but can progress to nonalcoholic steatohepatitis (NASH) and advanced fibrosis/cirrhosis.[1] Although concomitance of chronic hepatitis B (CHB) and NAFLD is currently common, particularly in Asia-Pacific,[2] the impact of hepatic steatosis on liver-related outcomes in CHB remains controversial.

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Our earlier studies conducted with the same cohort of HBV carriers in the present work observed that hepatic steatosis on ultrasound was associated with a reduced risk of subsequent HCC,[3] although obesity and high burden of metabolic risk factors emerge as important risk factors.[3,4] Furthermore, in a cross-sectional analysis, obesity with moderate-severe hepatic steatosis was shown to be associated with increased chance of HBV surface antigen (HBsAg) seroclearance,[5] which generally confers a favorable outcome in CHB. This result is in line with the observation that steatotic CHB patients had significantly lower HBV viral load compared with nonsteatotic CHB patients, suggesting the potential effect of hepatic steatosis on suppressing viral replication.[2] So far, liver-biopsy cohort studies investigating the relationship between hepatic steatosis and HCC in CHB patients have reported contrasting results, but these studies were generally limited by small sample size and/or short-term follow-up.[6–8]

NAFLD is a complex trait associated with metabolic disorders and genetic susceptibility,[9] which frequently overlaps with hepatic steatosis and may contribute to liver-related outcomes. The patatin-like phospholipase domain-containing protein 3 (PNPLA3) isoleucine-to-methionine substitution at residue 148 (p.I148M) is a common nonsynonymous variant associated with the genetic predisposition to hepatic fat accumulation. In a series of genome-wide association studies, this PNPLA3 variant has emerged as the key genetic determinant of NAFLD.[10] The PNPLA3 homozygous variant genotype MM (vs II) has been associated with a 2- to 4-fold higher risk for HCC and liver cirrhosis,[10–12] as well as a hazard ratio of 18.2 for liver disease mortality in a recent US population-based study where number of HCC deaths is small.[13] However, almost all the previously reported positive associations were found among viral hepatitis negative Caucasians. The PNPLA3 148M-variant allele is particularly prevalent in East Asia, where CHB is hyperendemic. Little is known about the influence of this polymorphism on the development of CHB-related HCC.[11,14]

This study is an extended follow-up of our initial study[3] assessing hepatic steatosis for HCC risk. In the present work, we further dissected the role of hepatic steatosis in determining the seroclearance of HBsAg and HCC risk. Moreover, we investigated the impact of the PNPLA3 variant p.I148M on CHB progression, with an attempt to clarify whether steatosis has a causal role in CHB-related liver outcomes. The large-scale longitudinal cohort study and the long follow-up time allowed us to investigate liver-related outcomes across the spectrum of CHB and assess differences in associations between hepatic steatosis and HCC by length of follow-up time, which could help define the influence of premalignant conditions or establish causality by ruling out reverse causation.

Subjects and Methods

The Cohort

The cohort includes 2903 male, HBsAg-positive civil servants aged ≥30 years at baseline who were recruited from the Government Employees' Central Clinics during routine free physical examination between 1989 and 1992. The general design of the cohort study has been described elsewhere.[3,4] Participants completed a baseline examination, including an in-person questionnaire interview, blood pressure and anthropometric measurements, and urine routine and blood testing. All participants were followed through linking personal identification number to National Health Insurance profiles, including National Cancer Registry, National Death Certification, and National Registry for Catastrophic Illness Patient Database. The study was approved by the National Taiwan University ethics committee and all participants provided informed consent.

Participants were scheduled to undergo periodic follow-up examinations, comprising questionnaire interview concerning changes in lifestyle and medical history, imaging diagnosis, anthropometric assessment, urine strips testing and blood biochemistry. They were invited to take part in follow-up examinations every year for the first 15 years and at 2 to 3 years interval thereafter. Routine use of ultrasonography was performed since 1993 to detect hepatic steatosis, liver cirrhosis, and HCC. The widespread adoption of transient elastography devices for non-invasive assessment of liver fibrosis via liver stiffness measurement (LSM) since recently.[15] Since November 2016, FibroScan (Echosens, Paris, France) examination was also performed with both M and XL probes. Reimbursement of antiviral therapy under the National Health Insurance program for CHB began on October 2003. We expanded our follow-up interview questionnaire in 2002 to include questions about any antiviral therapy the participant might have received. The cumulative proportion of subjects who returned for examinations after 2001 and had a history of antiviral therapy was 8.2%. Among

the treated subjects, 8.3% received interferon-α and 91.7% received nucleos(t)ide analogues (mainly lamivudine and entecavir).

In this report, we focused on the relationship between hepatic steatosis, HBsAg seroclearance, and HCC. Among the 2903 enrolled participants, we excluded those who had missing data on HBsAg seroclearance (n=352), those who did not undergo initial ultrasonography between 1993 and 1996 (n=123), and those who had lost HBsAg before initial ultrasonography (n=43), resulting in a final sample consisting of 2385 subjects (Figure 1).

Main Outcomes

The events of interest during follow-up were defined as follows: HBsAg seroclearance, loss of HBsAg detectability; elevated ALT (AST), ALT (AST)≥40 U/L; liver cirrhosis, detection from imaging diagnosis (including liver imaging of coarse and nodular echotexture with ultrasound or LSM >12 kPa with FibroScan); HCC, detection through computerized linkage with national registry for cancer and catastrophic illness databases available from National Health Insurance profiles.

Case-Cohort Sample

A case-cohort sample based on a published database[16,17] was used to investigate the impact of major viral factors on the associations of hepatic steatosis and PNPLA3 p.I148M with HCC. This study was originally designed to assess long-term tracking of viral load and its effect on HCC risk, with 1143 HBV carriers (HCC cases: n=112; random subcohort: n=1084) sampled from the entire cohort in 2004. We included all of the HBV carriers (n=961, including 113 HCC cases that occurred by December 31, 2019 and a random subcohort of 919 subjects) who were eligible for inclusion in the present work. In analysis of HBsAg seroclearance rate, only the subcohort subjects were included as the case-cohort study design is not population-based because of over-sampling of cases outside the subcohort[18] (Figure 1).

Laboratory Assays

At recruitment, serum HBsAg was tested by a radioimmunoassay, antibodies against hepatitis C virus (anti-HCV) by an enzyme immunoassay, and α-fetoprotein by an enzyme-linked immunosorbent assay, using commercial reagents (Abbott Laboratories, North Chicago, IL). Testing for HBsAg in follow-up samples was performed by Abbott Architect HBsAg Qualitative assay. Serum antibody to HBsAg (anti-HBs) was measured in subjects with HBsAg seroclearance using quantitative analysis with Abbott Architect assay (concentration values ≥10 mIU/mL were considered anti-HBs positive). The PNPLA3 rs738409 polymorphism was genotyped using the TaqMan assay and QuantStudio 7 Flex Real-Time PCR System (Applied Biosystems, Foster City, CA). In the case-cohort sample, HBeAg (Roche Diagnostics, Indianapolis, IN) was tested by electrochemiluminescence immunoassay. Plasma HBV DNA levels, HBV genotype, and basal core promoter (BCP) 1762/1764 mutations were determined using polymerase chain reaction-based methods as described previously.[16,17]

Statistical Analysis

In the calculation of HBsAg seroclearance rate, the first instance in which a participant tested negative for HBsAg was defined as the date of seroclearance. Follow-up time was from the date of enrollment until the date of HBsAg seroclearance, death, or last follow-up visit, whichever came first. In the calculation of HCC incidence, follow-up time was from the date of enrollment to the time of diagnosis of HCC, death, or the last date of linked data available from National Health Insurance profiles, ie, December 31, 2019. Considering a long duration of follow-up, competing risk analysis was conducted. When studying the time until HBsAg seroclearance occurred, death and HCC occurrence were considered as competing risks. For HCC, we considered death as a competing event. Cumulative incidence function curve adjusted for competing risks with Gray's test were used for survival analysis. Univariate and multivariate competitive risk Cox regression models were used to estimate the sub-distribution hazard ratio (sHR) and its 95% confidence interval (CI). We analyzed the case-cohort component of the study using weighted Cox regression models with the Barlow weighting approach to investigate the association of NAFLD-related factors and HCC after adjustment for various viral markers. Based on longitudinal follow-up examination data collected by 2019, the generalized linear

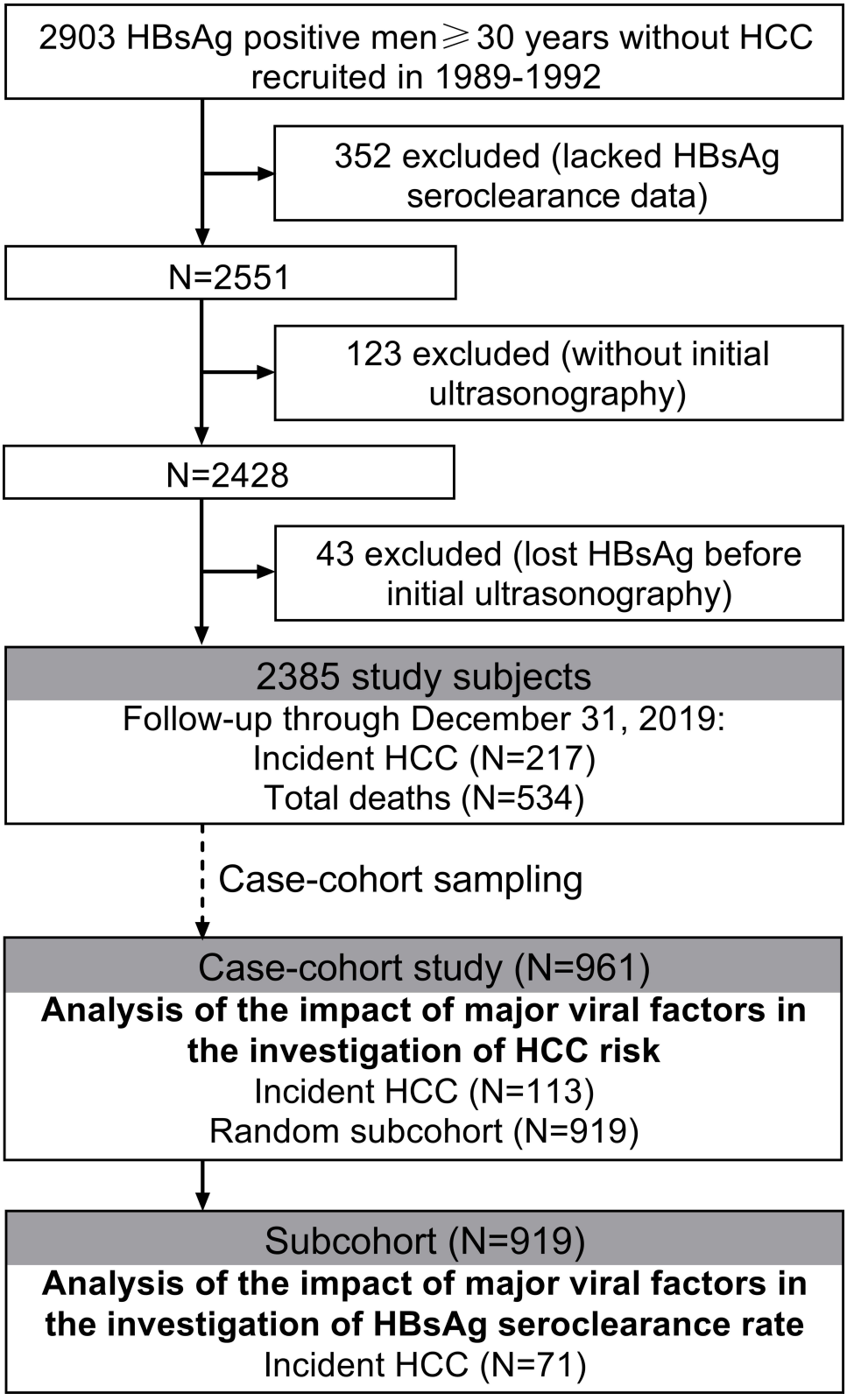


Figure 1 Derivation of the study population and case-cohort sample.

mixed model with the SAS GLIMMIX procedure was used to calculate the odds ratios (ORs) for episodes of liver enzyme elevation and liver cirrhosis detected by imaging modalities. All reported p values are two-sided. Statistical analyses were performed using SAS 9.4 software (SAS Institute, Carry, NC).

Results

Baseline Characteristics

Among study subjects, the median age was 43.2 years, median body-mass index (BMI) was 23.7 kg/m^2, 30.4% were obese (defined as BMI ≥25 kg/m^2 in Asians), and 2.1% had diabetes. Hepatic steatosis was detected in 45.8% of subjects and only 4.8% had liver cirrhosis diagnosed by ultrasonography. For the PNPLA3 p.I148M (rs738409 C>G) polymorphism, prevalence of II, MI, and MM genotype were 39.5%, 46.3%, and 14.2%, respectively (Table 1). The genotypic distribution of the PNPLA3 polymorphism was in Hardy–Weinberg equilibrium (p=0.6420). Status of anti-HCV was determined in 2309 (96.8%) participants at baseline, and the positive rate was 5.9%.

Table 1 Baseline Characteristics of Study Subjects and Main Outcomes

Variables	Entire Cohort (N=2385) No. of Participants (%)	Case-Cohort Study (N=961) No. of Participants (%)
Age (years)		
30–39	829 (34.8)	315 (32.8)
40–49	894 (37.5)	376 (39.1)
50–59	400 (16.8)	162 (16.9)
≥60	262 (11.0)	108 (11.2)
ALT≥ 40 U/L[a]	214 (9.0)	84 (8.8)
History of liver disease	169 (7.1)	70 (7.3)
First-degree family history of HCC[a]	156 (6.5)	72 (7.5)
Obesity (BMI ≥25 kg/m^2)	724 (30.4)	292 (30.4)
Diabetes mellitus	51 (2.1)	23 (2.4)
PNPLA3[a]		
II	940 (39.5)	381 (39.8)
MI	1103 (46.3)	442 (46.2)
MM	337 (14.2)	134 (14.0)
Cigarette smoking	730 (30.6)	297 (30.9)
Alcohol consumption	473 (19.8)	205 (21.3)
Heavy alcohol consumption (≥210 gram of ethanol per week)[a]	121 (5.1)	55 (5.8)
Ultrasonography		
Hepatic steatosis	1092 (45.8)	419 (43.6)
Liver cirrhosis	114 (4.8)	57 (5.9)
Viral markers		
HBeAg positive[a]		97 (10.3)
HBV DNA ≥ 4 log copies/mL		484 (50.4)
HBV genotype[a]		
B or B+C		790 (83.2)
C		159 (16.8)
BCP double mutations[a]		311 (33.5)
Follow-up		
HBsAg seroclearance	628 (26.3)	254 (26.4)
Incident HCC	217 (9.1)	113 (11.8)

Notes: [a]Data not available for all participants. Missing data in the entire cohort: ALT (n=6), HCC family history (n=1), PNPLA3 genotype (n=5), and amount of alcohol consumed (n=17); missing data in the case-cohort sample: ALT (n=3), PNPLA3 genotype (n=4), amount of alcohol consumed (n=10), HBeAg (n=23), HBV genotype (n=12), and BCP double mutations (n=34).

Abbreviations: ALT, alanine aminotransferase; BCP, basal core promoter; HCC, hepatocellular carcinoma.

Hepatic Steatosis, PNPLA3 p.I148M, and HBsAg Seroclearance

After 35603.1 person-years of follow-up, 628 individuals achieved HBsAg seroclearance (corresponding to a 1.76% annual seroclearance rate); their median (IQR) age at seroclearance was 58.8 (51.5–64.2) years; with 242 (38.5% of 628) having anti-HBs levels ≥10 mIU/mL at the time of seroclearance. In the multivariate Cox regression model adjusted for age and other HCC risk factors, a diagnosis of hepatic steatosis at baseline (p=0.0052) and the number of the PNPLA3-148M variant allele (p for trend=0.0316) were independently associated with higher rate of HBsAg loss in the presence of competing risks. Excess BMI was also an independent predictor of increased HBsAg seroclearance rate (Table 2).

Additive joint effect between BMI, PNPLA3 p.I148M, and steatosis was then examined, with the three factors combined into a prognostic score ranging from 0 to 5 (points: BMI [normal weight/underweight=0, overweight=1, obesity=2]; PNPLA3 [II=0, MI=1, MM=2]; steatosis [No=0, yes=1]). Excess BMI and PNPLA3 p.I148M were linked to hepatic steatosis (Supplementary Table 1) but an additive effect among the three factors was observed, showing the 20-year competing risk-adjusted cumulative incidence of HBsAg seroclearance increasing from 21.9% for score 0 to >35% for score ≥4 (sHR increased up to 2.2; p for trend<0.0001). In the subcohort (n=919) of the case-cohort sample, this association did not materially change after further adjustment for HBeAg, viral load, HBV genotype, and BCP double mutations at baseline (Figure 2).

HCC Risk

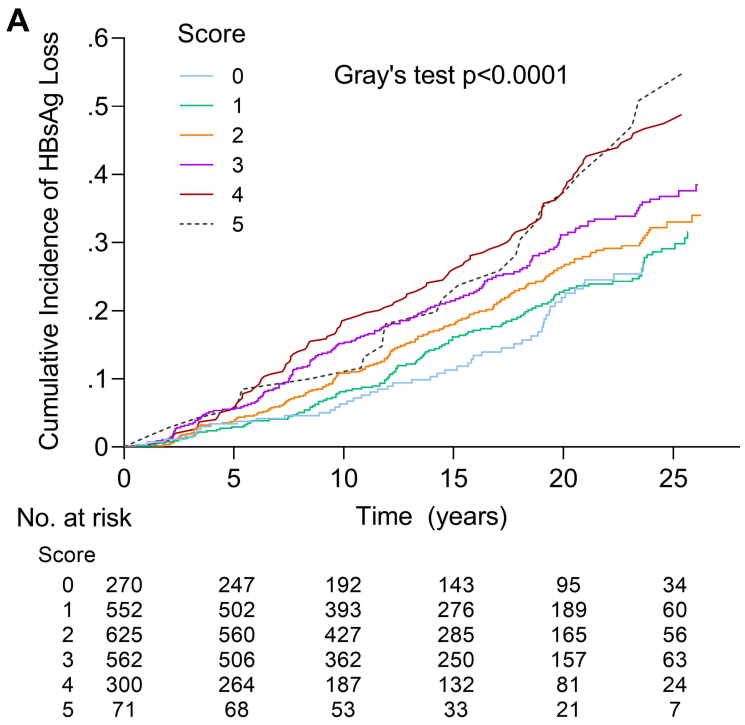
During a median (IQR) follow-up of 28.1 (27.5–28.8) years, 217 developed HCC, with an incidence rate of 352.73 per 100,000 person-years. HBsAg seroclearance (sHR=0.33; 95% CI=0.21–0.50) was strongly associated with a reduced risk

Table 2 Baseline Factors Associated with HBsAg Seroclearance

Variable	Entire Cohort (N=2385)									Subcohort (N=919)	
	Total No.	No. of HBsAg Seroclearance	Cumulative Incidence, %		Univariate Analysis[a]			Multivariate Analysis[b]		Multivariate Analysis[c]	
			10-y	20-y	sHR	95% CI	p-value	sHR	95% CI	sHR	95% CI
Hepatic steatosis											
No	1293	300	10.0	23.6	1.0			1.0		1.0	
Yes	1092	328	13.7	33.2	1.43	(1.22–1.67)	<0.0001	1.27	(1.07–1.50)	1.35	(1.03–1.78)
BMI (kg/m^2)											
Normal weight/ underweight (<23)	932	192	8.3	22.2	1.0			1.0		1.0	
Overweight (23–24.9)	729	214	13.1	31.5	1.50	(1.23–1.82)	<0.0001	1.47	(1.20–1.80)	1.55	(1.10–2.19)
Obesity (≥25)	724	222	14.7	31.3	1.58	(1.30–1.92)	<0.0001	1.55	(1.26–1.91)	1.51	(1.09–2.09)
PNPLA3[d]											
II	940	221	9.2	25.7	1.0			1.0[e]		1.0[f]	
MI	1103	310	13.2	29.5	1.19	(1.01–1.42)	0.0429	1.19	(1.00–1.41)	1.35	(1.00–1.81)
MM	337	95	13.4	28.5	1.25	(0.98–1.59)	0.0688	1.25	(0.98–1.59)	1.42	(0.95–2.13)
HBeAg											
Negative										1.0	
Positive										0.42	(0.21–0.85)
HBV DNA (log copies/mL) (continuous)										0.85	(0.79–0.90)
HBV genotype											
B or B+C										1.0	
C										2.09	(1.49–2.93)
BCP double mutations											
No										1.0	
Yes										1.27	(0.96–1.67)

Notes: [a]Adjusted for age at baseline. [b]Adjusted for age (continuous), ALT (continuous), history of liver disease, diabetes, cigarette smoking (non-smokers, ex-smokers, current smokers), alcohol consumption, and variables listed in the table [c]Adjusted for age (continuous), ALT (continuous), and variables listed in the table [d]5 subjects with missing data on PNPLA3 genotype. [e]p-value for trend=0.0316. [f]p-value for trend=0.0376.

Abbreviations: sHR, sub-distribution hazard ratio; BCP, basal core promoter.



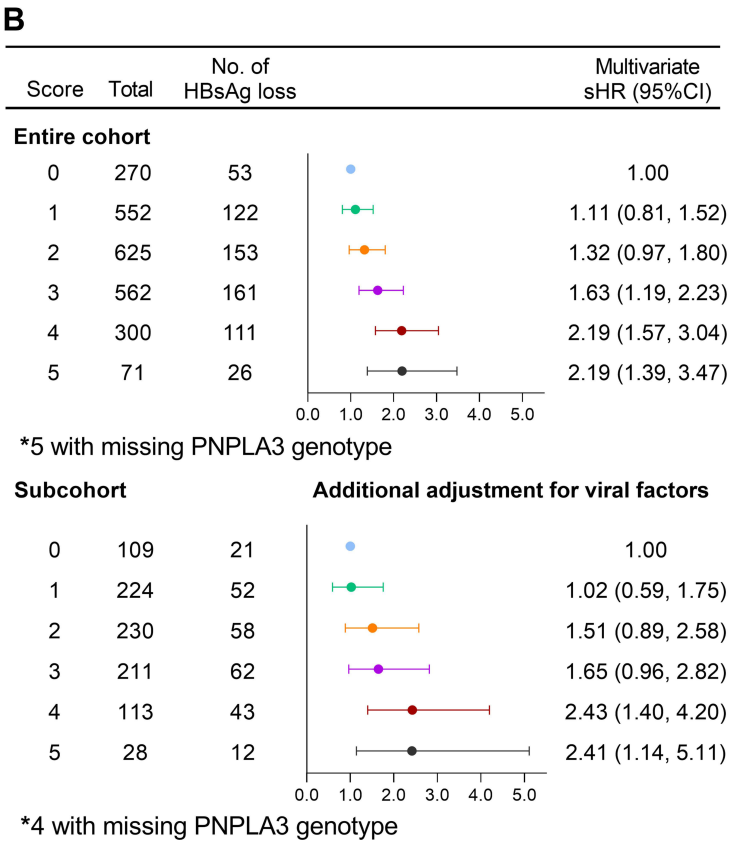


Figure 2 Additive joint effect between excess BMI, PNPLA3 148M-variant, and hepatic steatosis on the probability of HBsAg seroclearance. (**A**) Cumulative incidence function curve of HBsAg seroclearance according to a prognostic score ranging from 0 to 5 (points: BMI [normal weight/underweight=0, overweight=1, obesity=2]; PNPLA3 [II=0, MI=1, MM=2]; steatosis [No=0, yes=1]). (**B**) Sub-distribution hazard ratios (sHRs) with 95% CIs estimated from competitive risk Cox model. Solid circles and lines indicated sHRs and 95% CIs. sHRs derived from the entire cohort were adjusted for age, ALT, history of liver disease, diabetes, cigarette smoking, and alcohol consumption. sHRs derived from the subcohort were further adjusted for HBeAg, viral load, HBV genotype (C vs non-C), and BCP double mutations.

for HCC compared with persistently positive HBsAg. It also is notable that, even excess BMI and PNPLA3 p.I148M were associated with higher probability of HBsAg seroclearance, the HCC risk significantly increased for obesity (vs normal weight/underweight: sHR [95% CI]= 1.51 [1.07–2.13]) and carriage of the PNPLA3-148M variant allele (vs II homozygotes: sHR [95% CI]= 1.64 [1.20–2.25] for MI heterozygotes and 1.83 [1.20–2.78] for MM homozygotes; p for trend=0.0006). On the contrary, hepatic steatosis at baseline (sHR= 0.49, 95% CI=0.36–0.66) was inversely associated with HCC risk even after accounting for HBsAg seroclearance. Similar results were observed after additional adjustment for other viral factors in the case-cohort analysis (Table 3).

As hepatic fat accumulation may decrease with the progression of fibrosis,[19] to reduce potential influence of reverse causation on risk estimates, we repeated the analysis after excluding 114 subjects detected with liver cirrhosis on initial ultrasonography. The association between steatosis and HCC was attenuated (sHR= 0.65; 95% CI=0.46–0.92), but remained statistically significant. The sHRs for steatosis associated with HCC ranged from 0.64 to 0.67 (all p<0.04) after further exclusion of incident HCC cases over different follow-up times in the range of 3–12 years (Table 4).

Risk for Progressive Liver Disease

Of the 2385 subjects, 2304 (96.6%) had ≥2 follow-up imaging examinations (median= 7, IQR= 5–9), in which 50.6% had ALT ≥40 U/L and 11.3% had liver cirrhosis detected by ultrasonography or FibroScan (>12 kPa) in at least one follow-up visit. Multivariate analyses were performed on long-term dynamics of liver enzyme/imaging measurements with change in hepatic steatosis and diabetes status during follow-up treated as time-varying

Table 3 Multivariate-Adjusted sHRs of HCC Derived from the Entire Cohort and the Case-Cohort Analysis

Variable	Entire Cohort (N=2385)				Case-Cohort Analysis (N=961)		
	No. of HCC	sHR[a]	95% CI	p-value	HR[b]	95% CI	p-value
HBsAg seroclearance							
No	192	1.0			1.0		
Yes	25	0.33	(0.21–0.50)	<0.0001	0.44	(0.23–0.82)	0.0107
Baseline hepatic steatosis							
No	149	1.0			1.0		
Yes	68	0.49	(0.36–0.66)	<0.0001	0.53	(0.33–0.86)	0.0096
Baseline BMI (kg/m^2)							
Normal weight/underweight (<23)	79	1.0					
Overweight (23–24.9)	69	1.34	(0.95–1.88)	0.0948	1.0[c]		
Obesity (≥25)	69	1.51	(1.07–2.13)	0.0193	1.85	(1.18–2.89)	0.0072
PNPLA3							
II	65	1.0[d]			1.0[e]		
MI	115	1.64	(1.20–2.25)	0.0019	1.61	(1.00–2.60)	0.0500
MM	37	1.83	(1.20–2.78)	0.0052	2.22	(1.13–4.35)	0.0206
Baseline diabetes							
No	207	1.0			1.0		
Yes	10	1.43	(0.73–2.80)	0.3000	3.71	(1.50–9.19)	0.0046
Baseline HBV DNA (log copies/mL) (continuous)					1.37	(1.23–1.52)	<0.0001
Baseline HBV genotype							
B or B+C					1.0		
C					3.16	(2.01–4.96)	<0.0001
Baseline BCP double mutations							
No					1.0		
Yes					2.32	(1.45–3.70)	0.0004

Notes: [a]Adjusted for age (continuous), ALT (≥40 vs <40 U/L), history of liver disease, first-degree family history of HCC, cigarette smoking (non-smokers, ex-smokers, current smokers), and alcohol consumption at baseline, and variables listed in the table [b]Derived from a weighted Cox regression model adjusted for age (continuous), ALT (≥40 vs <40 U/L), history of liver disease, and first-degree family history of HCC at baseline, and variables listed in the table [c]BMI<25 kg/m^2 was used as reference group. [d]p-value for trend=0.0006. [e]p-value for trend=0.0090.

Abbreviations: sHR, sub-distribution hazard ratio; BCP, basal core promoter.

Table 4 Associations of Hepatic Steatosis at Baseline and PNPLA3 p.I148M Variation with Risk of HCC by Follow-Up Time[a]

Exclusion criteria	Remaining Participants Included in Analysis		Hepatic Steatosis		PNPLA3 p.I148M			
					MI vs II		MM vs II	
	No. of HCC	Person-years	sHR	95% CI	sHR	95% CI	sHR	95% CI
Excluding 114 subjects detected with liver cirrhosis at baseline from analysis	152[b]	59700.3	0.65	(0.46–0.92)	1.64	(1.13–2.37)	1.70	(1.03–2.81)
Excluding 114 subjects detected with liver cirrhosis at baseline plus HCC cases who occurred within following time period from analysis								
<3 years	150	59696.4	0.67	(0.47–0.95)	1.65	(1.13–2.39)	1.72	(1.04–2.86)
<6 years	140	59653.7	0.64	(0.45–0.91)	1.76	(1.19–2.60)	1.95	(1.16–3.27)
<9 years	124	59530.2	0.64	(0.43–0.94)	1.70	(1.12–2.58)	2.00	(1.16–3.45)
<12 years	105	59328.0	0.64	(0.42–0.98)	1.97	(1.24–3.11)	2.14	(1.17–3.91)

Notes: [a]Hepatic steatosis and PNPLA3 genotype were mutually adjusted, and HBsAg seroclearance and baseline age (continuous), ALT (≥40 vs <40 U/L), history of liver disease, first-degree family history of HCC, BMI (<23, 23–24.9, ≥25 kg/m^2), diabetes status, cigarette smoking (non-smokers, ex-smokers, current smokers), and alcohol consumption were also included as covariates in the multivariate analyses. [b]65 HCC cases detected with liver cirrhosis at baseline were excluded.
Abbreviation: sHR, sub-distribution hazard ratio.

variables. HBsAg seroclearance was observed to be strongly associated with reduced risks for ALT and AST elevations and liver cirrhosis. Obesity and the PNPLA3 MM genotype showed a positive association with liver cirrhosis and elevations of ALT and AST after adjustment for HBsAg seroclearance. Although hepatic steatosis was positively associated with elevated ALT, the OR was small. Furthermore, it was significantly associated with lower risks for elevated AST and liver cirrhosis (Table 5).

Table 5 Longitudinal Cohort Analysis for Factors Associated with Episodes of Liver Enzyme Elevation and Liver Cirrhosis Detected by Imaging Modalities[a, b]

Variable	Elevated ALT (≥40 U/L)		Elevated AST (≥40 U/L)		Liver Cirrhosis	
	OR	95% CI	OR	95% CI	OR	95% CI
HBsAg seroclearance						
No	1.0		1.0		1.0	
Yes	0.49	(0.42–0.59)	0.43	(0.35–0.54)	0.39	(0.26–0.58)
Baseline BMI (kg/m^2)						
Normal weight/underweight (<23)	1.0		1.0		1.0	
Overweight (23–24.9)	1.05	(0.88–1.26)	1.04	(0.84–1.28)	1.91	(1.32–2.75)
Obesity (≥25)	1.35	(1.13–1.61)	1.24	(1.01–1.54)	2.35	(1.62–3.40)
PNPLA3						
II	1.0		1.0		1.0	
MI	1.22	(1.04–1.43)	1.14	(0.94–1.37)	1.16	(0.84–1.61)
MM	1.56	(1.26–1.94)	1.48	(1.14–1.91)	1.64	(1.04–2.59)
Time-varying covariate[c]						
Hepatic steatosis						
No	1.0		1.0		1.0	
Yes	1.27	(1.13–1.43)	0.76	(0.65–0.89)	0.16	(0.12–0.23)
Diabetes						
No	1.0		1.0		1.0	
Yes	0.73	(0.60–0.90)	1.19	(0.89–1.60)	1.68	(1.13–2.47)

Notes: [a]Of the 2304 subjects with ≥2 follow-up imaging examinations, 6 lacked AST data. [b]ORs were adjusted for age and ALT (continuous) at baseline, number of visits, as well as variables listed in the table [c]Status at each visit during follow-up were included as a time-varying covariate in the generalized linear mixed model.
Abbreviation: OR, odds ratio.

Discussion

In this study, we found that a diagnosis of hepatic steatosis and its related factors including excess BMI and the PNPLA3-148M allele were associated with higher HBsAg seroclearance rate, and the analysis of their combined effect, summarized as a prognostic score, showed a highly significant increasing trend in the lifetime probability of HBsAg seroclearance with increase in the score. However, hepatic steatosis and the two steatosis-related factors had distinct roles in CHB progression after accounting for HBsAg seroclearance. Obesity and the PNPLA3 variant were observed to consistently increase the occurrence of elevated ALT and AST levels, liver cirrhosis, and HCC. In contrast, hepatic steatosis was associated with reduced risks for elevated AST, liver cirrhosis, and HCC, while had a small positive effect on ALT elevation.

Earlier cross-sectional studies had reported an inverse association between hepatic steatosis and HBV viral load.[2] Furthermore, hepatic steatosis combined with obesity was associated with increased chance of HBsAg seroclearance.[5] In a recent mouse model of HBV persistent replication, high-fat diet-induced hepatic steatosis was demonstrated to inhibit HBV-related antigens expression and viral replication.[20] Thus, our findings showing that hepatic steatosis was associated with increased HBsAg seroclearance rate are compelling and align with those from previous epidemiology studies and animal model. However, despite the extensive multivariate adjustment for viral and metabolic risk factors used to account for confounding in this study, unmeasured residual confounding is always a serious issue of concern in observational studies, which is an inherent methodological limitation.

Consistent with numerous other studies,[10,21] we observed a stepwise increase in the prevalence of hepatic steatosis with the number of the PNPLA3-148M allele. In contrast to a diagnosis of hepatic steatosis and metabolic risk factors, PNPLA3 p.I148M is less likely to be affected by confounders and robustly associates with lifelong exposure to steatosis. We also demonstrated the PNPLA3 p.I148M variant to be associated with increased HBsAg seroclearance rate. Moreover, the combined effect of steatosis, excess BMI, and PNPLA3 p.I148M appeared to be an important long-term predictor of HBsAg seroclearance independent of major viral factors. The quantity of hepatic fat varies widely among individuals with hepatic steatosis. In addition to the allele-dose effect of the PNPLA3-148M variant on hepatic fat content,[21] the accumulation of hepatic fat was also associated with unit increase in BMI.[22] Thus, it is reasonable to believe that the interaction on the additive scale between a diagnosis of hepatic steatosis, excess BMI, and PNPLA3-p.I148M variant mirrors the dose-response relationship between quantity of hepatic fat and suppression of viral replication, supporting the active role of hepatic fat in inducing viral clearance.

Interestingly, despite demonstrating beneficial effects of steatosis, excess BMI, and the PNPLA3-148M variant on the seroclearance of HBsAg, we found that the two steatosis-related factors were associated with increased risks for progressive liver disease and HCC, but steatosis revealed the opposite effect. Indeed, steatosis remained inversely associated with the risk of HCC, even when HBsAg seroclearance and other viral factors were accounted for by using multivariate analysis. It has been proposed that hepatic fat loss may accompany advanced fibrosis/cirrhosis in NASH.[19] This potentially leads to an inverse causal relationship between hepatic fat and HCC. With an attempt to clarify the causal nature of the inverse steatosis-HCC association, we re-analyzed the data after excluding subjects with cirrhosis detected at baseline and those developing HCC within the first 3–12 years of follow-up, who were presumed to represent those with undiagnosed advanced hepatic disease at baseline. However, since this inverse association remained significant and appeared consistent over the 3–12 years after baseline, it is likely that a premalignant condition altering liver fat does not fully explain the inverse association.

Although hepatic steatosis, characterized by hepatocyte accumulation of triglycerides, is a hallmark of NAFLD, the impact of simple steatosis on the development of NASH and NASH-related HCC has been debated.[23–26] Accumulating evidence suggests that simple steatosis does not need for NAFLD progression and it may not even be correlated with accumulation of toxic lipids.[26,27] In addition, simple accumulation of triglycerides, which are considered as an inert lipid species, could be hepatoprotective in obese, insulin-resistant individuals.[28] There are little data on the association between hepatic steatosis and long-term liver-related outcomes in CHB. The inverse association between steatosis and HCC observed in the present work is consistent with our previous study based on 134 HCC incidents ascertained during the first 15 years of follow-up.[3] Two prospective studies of CHB patients with biopsy-proven hepatic steatosis have also

investigated the possible association after 7–10 years of follow-up, but they are limited by the noticeably small number of HCC events and the results have been inconsistent.[6,8] Moreover, these two studies were based on a CHB population undergoing histologic assessment, which could induce a bias towards more severe CHB patients.

We found the PNPLA3-148M variant as a modifier for disease outcome across a wide spectrum of CHB encompassing ALT/AST elevation, liver cirrhosis, and HCC, in addition to confirming the well-known association of PNPLA3-148M variant with hepatic steatosis.[10,21] Importantly, we reported for the first time in a prospective manner the link between PNPLA3-148M variant and increased HCC incidence in a cohort of chronic HBV carriers. Furthermore, the PNPLA3 variant was associated with various disease states, independently of hepatic steatosis. In Caucasians, bearing the PNPLA3-148M variant has been associated not only with greater risk of hepatic steatosis but also with progressive NASH, advanced fibrosis/cirrhosis, and HCC.[10–13,29,30] However, few data are available in Asians and no positive association was detected for the PNPLA3 variant with HCC in CHB possibly due to sample size limitations.[11,31,32] The mechanisms underlying the association between the PNPLA3-148M variant and HCC remain unclear. However, besides its long-known role in accumulation of hepatic fat, recent in vitro studies suggest that PNPLA3 p.I148M may be involved in hepatic stellate cells activation and fibrogenesis.[33,34] Of note, in a recent liver-biopsy cohort study of CHB, it was also observed that NASH, particularly in the presence of advanced fibrosis, but not steatosis, was significantly associated with higher risk for a composite endpoint of end-stage liver disease.[8]

The strengths of our study include the large population-based cohort sample of chronic HBV carriers, and the simultaneous assessment of hepatic steatosis, PNPLA3 p.I148M, and metabolic risk factors. Due to the longitudinal cohort study design over a period of 30 years, we can look at the entire spectrum of disease progression and natural history of CHB, and examine potential impact of reverse causality in the relationship between hepatic steatosis and HCC using different cut-offs of follow-up time. Because the incidence of HBsAg seroclearance is approximately 1% per year,[35] the long-term data enabled assessment of lifetime risk statistic for the occurrence of HBsAg seroclearance. A limitation of this work is that we only studied men because HCC predominantly affects males with incidence two to four times more common in men than in women.[1] Another limitation is the use of a qualitative ultrasound approach to detect steatosis, although it provides fairly good accuracy to detect moderate-to-severe degree of hepatic steatosis.[36]

Conclusions

Hepatic steatosis and the PNPLA3-148M variant have distinct impacts on the development of progressive hepatic disease and HCC in CHB; however, they are both related to increased probability of HBsAg seroclearance. Our data suggest that hepatic steatosis is rather benign, perhaps has a favorable effect on progressive hepatic disease in the context of CHB, but the PNPLA3-148M variant predisposes chronic HBV carriers to HCC independent of hepatic steatosis. The results, combined with our previous studies,[3,4] suggest that intervention on obesity/metabolic risk factors and genetic predisposition instead of reducing hepatic fat in CHB is important for reduction of HCC development.

Abbreviations

ALT, alanine aminotransferase; AST, aspartate aminotransferase; BCP, basal core promoter; BMI, body-mass index; CHB, chronic hepatitis B; CI, confidence interval; HBeAg, hepatitis B e antigen; HBsAg, hepatitis B surface antigen; HBV, hepatitis B virus; HCC, hepatocellular carcinoma; IQR, interquartile range; sHR, sub-distribution hazard ratio; IQR, interquartile range; NAFLD, nonalcoholic fatty liver disease; NASH, nonalcoholic steatohepatitis; OR, odds ratio; PNPLA3, patatin-like phospholipase domain-containing protein 3.

Ethical Approval and Informed Consent

The study was conducted according to the guidelines of the Declaration of Helsinki. The study was approved by the National Taiwan University ethics committee, and informed consent was achieved from each participant.

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Author Contributions

All authors made a significant contribution to the work reported, whether that is in the conception, study design, execution, acquisition of data, analysis and interpretation, or in all these areas; took part in drafting, revising or critically reviewing the article; gave final approval of the version to be published; have agreed on the journal to which the article has been submitted; and agree to be accountable for all aspects of the work.

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Disclosure

No potential conflicts of interest for this work were disclosed by the authors.

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