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Association of liver fibrosis with extrahepatic cancer in steatotic liver disease patients with PNPLA3 I148M GG genotype

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Abstract

The impacts of patatin-like phospholipase domain-containing protein 3 (*PNPLA3*) I148M-rs738409, methylenetetrahydrofolate reductase (*MTHFR*) Ala222Val-rs1801133, and aldehyde dehydrogenase 2 (*ALDH2*) Glu504Lys-rs671 on the outcomes of Taiwanese patients with steatotic liver disease (SLD) have remained elusive. An 8-year prospective cohort study of patients with ($n=546$) and without ($n=580$) SLD (controls) was undertaken in a Taiwanese tertiary care center. The 546 SLD patients comprised 306 (56.0%) men and 240 (44.0%) women with mean ages of 53.3 and 56.4 years, respectively. Compared with the controls, SLD patients had an increased frequency of the *PNPLA3* I148M-rs738409 GG genotype (25.5 vs. 5.9%, $p=0.001$). Among the SLD patients, 236 (43.1%) suffered cardiovascular events, 52 (9.5%) showed extrahepatic cancers, 13 (2.38%) experienced hepatic events, including hepatocellular carcinoma ($n=3$, 0.5%) and liver cirrhosis ($n=8$, 1.47%), and none died. The Fibrosis-4 (FIB-4) scores were associated with extrahepatic cancer (hazard ratio [HR] 1.325; 95% confidence interval [CI], 1.038–1.691) and cirrhosis development (HR 1.532; 95% CI, 1.055–2.224), and the *PNPLA3* I148M-rs738409 G allele ($\beta=0.158$, 95% CI, 0.054–0.325) was associated with the FIB-4 score. Stratified analyses showed that the impact of the FIB-4 score on extrahepatic cancer development was evident only in SLD patients with the *PNPLA3* I148M-rs738409 GG genotype (HR 1.543; 95% CI, 1.195–1.993) and not in patients with the GC or CC genotype. Moreover, the *ALDH2* Glu504Lys-rs671 G allele had a dose-dependent effect on alcoholism, and the *MTHFR* and *ALDH2* genotypes were not significantly associated with SLD patient outcomes. In conclusion, special vigilance should be exercised for emerging extrahepatic cancer in SLD patients with the *PNPLA3* I148M-rs738409 GG genotype and high FIB-4 scores.

KEYWORDS
ALDH2, cancer, fatty liver, FIB-4, MTHFR, PNPLA3

Abbreviations: ALD, alcoholic liver disease; ALDH2, aldehyde dehydrogenase 2; ALT, alanine aminotransferase; AST, aspartate aminotransferase; BMI, body mass index; CI, confidence interval; FIB-4, fibrosis-4; HCC, hepatocellular carcinoma; HDL-C, high-density lipoprotein-cholesterol; HOMA-IR, homeostasis model assessment–estimated insulin resistance; HR, hazard ratio; MAFLD, metabolic-associated fatty liver disease; MASLD, metabolic dysfunction-associated steatotic liver disease; MetALD, xxxx; MTHFR, methylenetetrahydrofolate reductase; NAFLD, Nonalcoholic fatty liver disease; PNPLA3, patatin-like phospholipase domain-containing protein 3; SLD, steatotic liver disease; SNP, single nucleotide polymorphism; STAT3, signal transducer and activator of transcription 3; TG, triglyceride.

1 | INTRODUCTION

Because of sedentary lifestyles and globalization of the Western diet,[1] the prevalence of fatty liver, defined as triglyceride fat accumulating in >5% of liver cells,[2] is increasing. SLD[3] is an overarching term that now encompasses the various etiologies of fatty liver, including MASLD, ALD, and MetALD. NAFLD indicates the presence of fatty liver in a nonalcoholic context.[2] The names chosen to replace NAFLD are MASLD[3] and MAFLD.[4] MASLD is defined by the presence of fatty liver in addition to at least one of five cardiometabolic risk factors, namely, overweight/obesity, presence of glucose intolerance/type 2 diabetes mellitus, and hypertension or dyslipidemia.[3] In addition to MASLD, MetALD describes patients with MASLD who consume higher amounts of alcohol per week.[3] The precise definition of MAFLD is evidence of SLD in addition to one of the following three criteria: overweight/obesity, presence of type 2 diabetes mellitus, or evidence of metabolic dysregulation.[4] Because MASLD or MAFLD is no longer diagnosed simply by exclusion of the alcoholic nature and is based on the presence of metabolic dysfunction, it is now possible to diagnose its coexistence with other liver diseases such as ALD.[4] The main causes of death in patients with NAFLD, MASLD, or MAFLD have been identified and include cardiovascular events, extrahepatic cancer, and hepatic complications such as liver cirrhosis and HCC.[5] However, the etiopathogenesis of SLD remains multifactorial,[6] and the most important factors include genetic predisposition, de novo lipogenesis, and adipose tissue dysfunction.[7] Among the reported genetic factors increasing susceptibility to risks of SLD, the *PNPLA3* I148M variant-rs738409 has large effects, with approximately twofold increased odds of NAFLD and threefold increased odds of nonalcoholic steatohepatitis and HCC per G allele.[8] The PNPLA3 protein has lipase activity toward triglycerides in hepatocytes and retinyl esters in hepatic stellate cells. *PNPLA3* displays hydrolase activity toward triglycerides and retinyl esters and has potential transacetylase activity to incorporate polyunsaturated fatty acids into phospholipids.[9] The I148M substitution leads to a loss of function and thus promotes triglyceride accumulation in hepatocytes.[10] The common missense sequence variant of *MTHFR*, C677T, favors the development of hyperhomocysteinemia and diminished DNA methylation.[11] Although a meta-analysis showed that the T/T genotype of the MTHFR C677T polymorphism is associated with susceptibility to NAFLD,[12] a large European cohort study has disputed the association between the *MTHFR* C677T genotype and the risk of NAFLD.[11] Aldehyde dehydrogenase 2 is a mitochondrial enzyme that detoxifies acetaldehyde and endogenous lipid aldehydes[13] and plays a key role in protecting the liver. A Japanese cohort study reported that the *ALDH2* Glu504Lys-rs671 A allele might be a risk factor for NAFLD.[14] The estimated local prevalence of SLD is reported to be as high as 66.5% in Taiwan,[15] an Asian country where chronic viral hepatitis B and C are endemic.[16] In Taiwan, the variant *PNPLA3* rs738409 genotypes have been shown to increase the risk of NAFLD in normoglycemic adults[17] and correlate with the histologic severity of NAFLD.[18] However, the interactive impacts of *PNPLA3*, *MTHFR*, and *ALDH2* on the phenotypes and outcomes of Taiwanese patients with SLD have remained unidentified. Accordingly, we sought to elucidate these topics by conducting a prospective SLD cohort study analyzing the baseline demographic, metabolic and genetic profiles and their correlation with outcomes, including cardiovascular, oncogenic and hepatic events, in these patients.

2 | MATERIALS AND METHODS

2.1 | Patients

The study group consisted of subjects aged ≥18 years with SLD, defined by the presence of fatty liver documented by a liver sonography, a FibroScan (Echosens, Paris, France) or a liver biopsy, as demonstrated by liver sonography with moderate or severe fatty liver according to the intensity, reflection level of echogenicity (namely, brightness) from the hepatic parenchyma with liver-to-kidney contrast, signs of far attenuation by echo penetration into the deep portion of the liver, and obscure changes in the vessel and gallbladder walls[19] as well as a controlled attenuation parameter ≥222 dB/m[20,21] upon FibroScan or a liver fat (triglyceride) content >5% in liver biopsy samples. FibroScan was carried out using an M or XL probe (for patients with BMI ≥ 30 kg/m^2). All the enrolled subjects were given questionnaires that included questions about the frequency of any consumption of alcohol, the amount of alcohol usually consumed per week (specified as number of glasses or volumes of beer, wine, and liquor) and the frequency of binge drinking.[22] The level of alcohol intake was then quantified as 10–60 g/week.[3] Long-term alcohol consumption was defined as >40 g alcohol consumption daily for women or >60 g alcohol consumption daily for men for ≥6 months.[23] The controls were identified by the absence of fatty liver. Subjects with HIV, hepatitis C virus, or hepatitis B virus infection, hemochromatosis, primary biliary cholangitis, primary sclerosing cholangitis, autoimmune hepatitis, or malignancy and were recipients of solid organ transplants were excluded.

2.2 | Methods

The experimental group was composed of 546 SLD patients; the control group was composed of 580 subjects without SLD. Both the experimental and control patients were recruited consecutively at a tertiary referral center between January 2015 and January 2023. For all included patients, several baseline factors were evaluated: sex, age, BMI, smoking, alcoholism, betel nut chewing, AST, ALT, presence of hepatic steatosis or cirrhosis, total cholesterol, TGs, HDL-C, HOMA-IR (fasting insulin [μU/mL] × fasting glucose [mmol/L]/22.5) index, FIB-4 (age [years] × aspartate transaminase [U/L]/[platelets (10^9/L) × (√[ALT (U/L)]) score, and *PNPLA3* I148M-rs738409, *MTHFR* C677T Ala222Val-rs1801133, and *ALDH2* Glu504Lys-rs671 SNP genotypes. The primer

sequences for the *PNPLA3* I148M-rs738409,[24] *MTHFR* C677T Ala222Val-rs1801133,[25] and *ALDH2* Glu504Lys-rs671[26] SNP genotypes are shown in Table S1. For DNA quantification, the DNA optical density was assayed by UV absorption analysis. The positive controls were adopted using the patients' DNA with positive PCR bands that had been sequenced to confirm the correct sequences, and the blank samples were used as negative controls. A total of 163 patients had diabetes, and HOMA-IRs were not measured in the patients with diabetes who received treatment with insulin ($n=3$) or insulin secretagogues such as sulfonylurea ($n=33$). The patients with SLD were followed and surveyed for incident cardiovascular, oncogenic, and hepatic events every 3–6 months. All enrolled patients were systematically surveyed at every follow-up to obtain their health information. Cardiovascular events were defined as ischemic heart disease, coronary revascularization, stroke, heart failure, cardiac arrest, and cardiovascular death identified using the International Classification of Diseases, Ninth Revision, Clinical Modification (ICD-9, CM) based on patient reports and confirmed by a review of medical records/registries. Extrahepatic cancers were diagnosed based on pathology and confirmed by specialists for each primary cancer, and the diagnosis and stage of each cancer were registered to the National Cancer Registration. Hepatic events comprised liver fibrosis, cirrhosis, and HCC. The diagnoses of liver cirrhosis and HCC were defined as described elsewhere.[27]

2.3 | Statistics

All statistical analyses were undertaken using SPSS version 21.0 (SPSS Inc.). Multivariate linear regression models were used to assess the relationship between dependent and independent factors by adjusting for all independent variables with a p value <0.05 in univariate analyses. Kaplan–Meier or univariate Cox regression analyses were used to assess the relationship among various variables and patient events. Multivariate Cox regression models were used to assess the relationship between various dependent and independent variables by adjusting for all the independent variables with a p value <0.05 in the univariate analyses. Genotype association tests were carried out using logistic regression analyses with additive, dominant, or recessive association models.[28] Statistical significance was defined at the 5% level based on two-tailed tests of the null hypothesis.

3 | RESULTS

3.1 | Baseline characteristics

In 8 years, a total of 546 patients with SLD were enrolled (Figure 1). Of 546 patients, 306 (56.0%) were male, and 240 (44.0%) were female, with mean ages of 53.3 and 56.4 years, respectively. A total of 580 controls were enrolled, comprising 330 (56.8%) male patients and 250 (43.1%) female patients, with mean ages of 51.8 and 53.2 years, respectively. Compared with controls, patients with SLD had increased rates of alcoholism and smoking, increased levels of AST, ALT, TGs and HOMA-IR, and reduced levels of HDL-C (Table 1).

3.2 | Frequency of *PNPLA3*, *MTHFR*, and *ALDH2* SNP genotypes

In patients with SLD, the major genotype for all three investigated SNPs is heterozygous, namely, *PNPLA3* I148M-rs738409 CG, *MTHFR* C677T Ala222Val-s1801133 CT, and *ALDH2* Glu504Lys-rs671 AT genotypes. Compared with controls, patients with SLD had a significant increase in the rate of the *PNPLA3* I148M-rs738409 GG genotype (25.5 vs. 5.9%, $p=0.001$), a marginally significant increase in the rate of the *MTHFR* C677T Ala222Val-rs1801133 TT genotype, a marginally significant decrease in the rate of the *MTHFR* C677T Ala222Val-s1801133 CC genotype, and a marginally significant increase in the rate of the *ALDH2* Glu504Lys-rs671 GG genotype (Table 2).

3.3 | Outcomes of patients with and without SLD

Of 580 controls, 152 (26.2%) had cardiovascular events; 27 (4.7%) had extrahepatic cancers, including lung ($n=14$, 2.4%) and genitourinary tract cancers ($n=13$, 2.2%), 2 (0.34%) had liver cirrhosis, and none developed HCC. None of the investigated factors were associated with the cumulative incidences of cardiovascular events or cancers, whereas the baseline HOMA-IR was associated with the development of liver cirrhosis (adjusted HR 5.489; 95% CI of HR, 1.198–25.144; $p=0.028$).

Of 546 patients with SLD, 236 (43.1%) suffered cardiovascular events. A total of 52 (9.5%) developed extrahepatic cancers, including breast cancers ($n=10$, 1.7%), genitourinary tract tumors ($n=8$, 1.4%), uterus/cervix/ovary cancers ($n=8$, 1.4%), lung cancers ($n=8$, 1.4%), head and neck cancers ($n=7$, 1.2%), colorectal cancers ($n=4$, 0.7%), skin cancers ($n=4$, 0.7%), lymphoma ($n=1$, 0.2%), pancreas cancer ($n=1$, 0.2%), thymus cancer ($n=1$, 0.2%), brain cancer ($n=1$, 0.2%), and gastric cancer ($n=1$, 0.2%). A total of 13 (2.38%) patients suffered hepatic events, including HCC ($n=3$, 0.5%) and liver cirrhosis ($n=8$, 1.47%). Compared with controls, patients with SLD had an increased rate of cardiovascular events ($p=0.021$) but similar rates of extrahepatic cancers ($p=0.203$), cirrhosis ($p=0.058$), and HCC ($p=0.809$). No mortality was observed in the whole cohort during the 8-year observation period. The univariate and multivariate analyses confirmed that age was an independent risk factor for cardiovascular events (Table 3 shows the additive model; the dominant and recessive models are shown in Tables S2 and S3) and that the FIB-4 score was a predictive factor for extrahepatic cancer (Table 4 shows the additive model; the dominant and recessive models are shown in Tables S4 and S5) and liver cirrhosis development (Table 5 shows

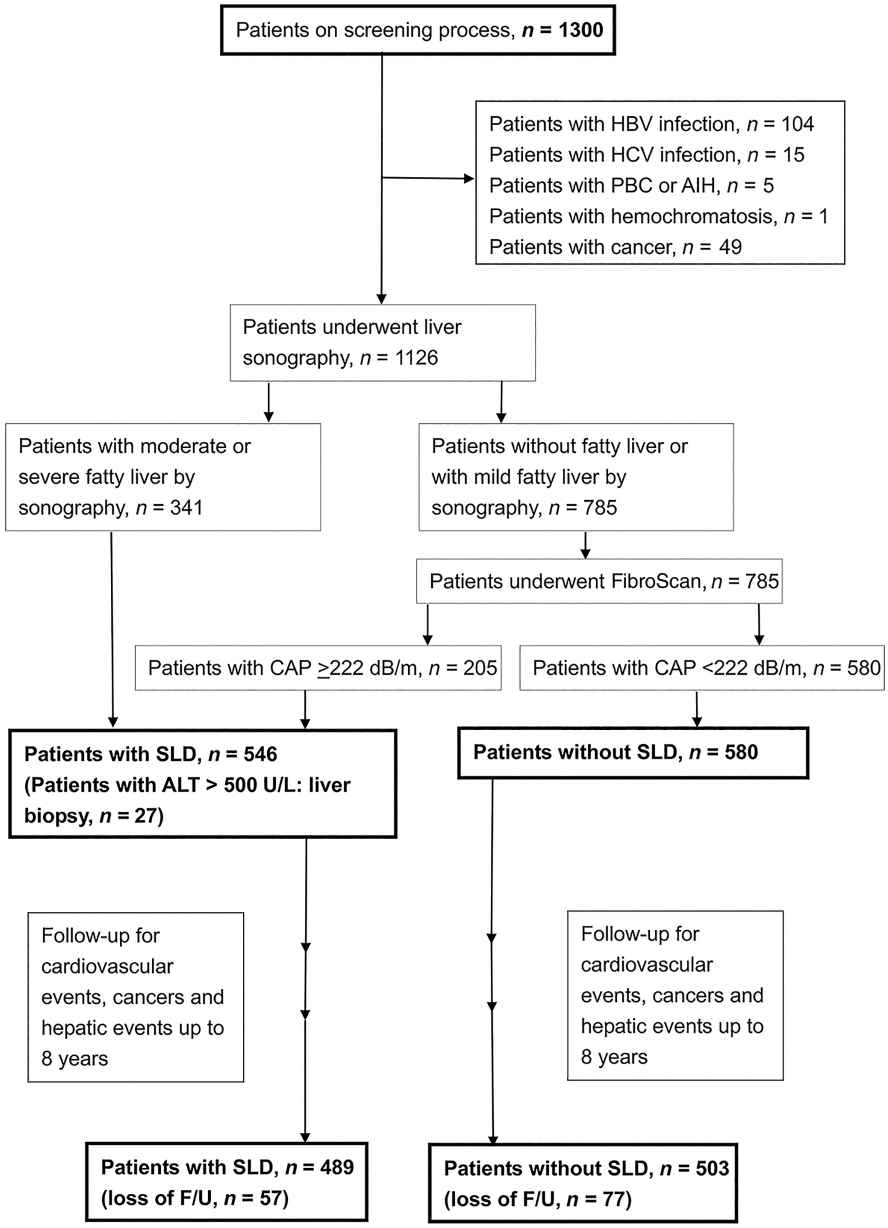

FIGURE 1 Flowchart of patient enrollment. AIH, autoimmune hepatitis; ALT, alanine transaminase; CAP, controlled attenuation parameter; F/U, follow-up; HBV, hepatitis B virus; HCV, hepatitis C virus; PBC, primary biliary cholangitis; SLD, steatotic liver disease.

the additive model; the dominant and recessive models are shown in Tables S6 and S7). No independent predictive factors were identified for HCC development.

3.4 | Independent factors associated with FIB-4 scores in patients with SLD

Because the FIB-4 score was a factor for extrahepatic cancer and cirrhosis development in SLD patients, we surveyed the crucial factors associated with FIB-4 scores. Age and the *PNPLA3* I148M-rs738409 G allele were independently associated with FIB-4 scores (Table 6 shows the additive model; the dominant and recessive models are shown in Tables S8 and S9). We stratified the patients with fatty liver by the *PNPLA3* I148M-rs738409 genotype to assess the impact of FIB-4 levels on extrahepatic cancer development. Interestingly, the impact of FIB-4 on extrahepatic cancer development was evident only in patients with the *PNPLA3* I148M-rs738409 GG genotype (HR 1.543; 95% CI HR, 1.195–1.993) but not in patients with the *PNPLA3* I148M-rs738409 GC genotype (95% CI HR, 0.493–2.106) or CC genotype (95% CI HR, 0.153–3.286).

3.5 | Dose effect of the *PNPLA3* I148M-rs738409 G allele on FIB-4 scores

We stratified the SLD patients by the *PNPLA3* I148M-rs738409 G allele to view the impact of the *PNPLA3* I148M-rs738409 G allele on FIB-4 scores. As shown in Figure S1A, there was a dose effect of the *PNPLA3* I148M-rs738409 G allele on FIB-4 scores. Namely, the FIB-4 scores of SLD patients with the GG genotype > patients with the CG genotype > patients with the CC genotype.

TABLE 1 Comparisons of baseline characteristics between patients with (W) and without (W/O) SLD

	Fatty liver (W) 546	Fatty liver (W/O) 580	*p* value
Male gender, *n* (%)	306 (56)	330 (56.8)	0.638
Age (year)	54.7 ± 11.8	52.9 ± 12.4	0.147
BMI (kg/m^2)	27.38 ± 4.1	25.3 ± 4.7	0.185
Alcohol, *n* (%)	73 (14.6%)	17 (2.9%)	0.046
Smoking, *n* (%)	97 (19.4%)	35 (6.0%)	0.042
Betel nut, *n* (%)	31 (6.3%)	10 (1.7%)	0.049
AST (U/L)	32.6 ± 20.5	25.8 ± 18.4	0.029
ALT (U/L)	47.5 ± 41.8	33 ± 35.6	0.021
Total cholesterol (mg/dL)	194.89 ± 37.4	190.1 ± 33.5	0.399
HDL-C (mg/dL)	49.3 ± 11.9	60.1 ± 15.8	<0.01
TG (mg/dL)	159.1 ± 98.6	100.1 ± 67.8	<0.01
HOMA-IR	2.58 ± 1.56	1.32 ± 0.79	<0.01
FIB-4	1.18 ± 0.93	1.13 ± 0.83	0.958

Abbreviations: ALT, alanine aminotransferase; AST, aspartate aminotransferase; BMI, body mass index; FIB-4, Fibrosis-4; HDL-C, high-density lipoprotein-cholesterol; HOMA-IR, homeostasis model assessment-estimated insulin resistance; TG, triglycerides.

3.6 | Dose effect of the *ALDH2* Glu504Lys-rs671 G allele on alcohol drinking

In SLD patients, although there was no association with any cardiovascular events, extrahepatic cancers, or hepatic events, the *ALDH2* Glu504Lys-rs671 genotype was associated with the number of patients with long-term alcohol consumption ($p = 0.003$). As shown in Figure S1B, there was a dose effect of the *ALDH2* Glu504Lys-rs671 G allele on long-term alcohol consumption. Namely, the numbers of patients with long-term alcohol consumption were as follows: patients with the *ALDH2* Glu504Lys-rs671 GG genotype > patients with the *ALDH2* Glu504Lys-rs671 GA genotype > patients with the ALDH2 Glu504Lys-rs671 AA genotype.

3.7 | Negligible effects of *MTHFR* alleles on outcomes in SLD patients

The current study did not show any association between *MTHFR* alleles and any of the investigated outcomes in SLD patients.

4 | DISCUSSION

The most compelling results are as follows. (1) Compared with controls, patients with SLD had increased rates of the *PNPLA3* I148M-rs738409 GG genotype and cardiovascular events. (2) Among the controls, the baseline HOMA-IR was associated

TABLE 2 Various genotypes of enrolled patients with (W) and without (W/O) SLD

PNPLA3 I148M-rs738409				*MTHFR* C677T Ala222Val-s1801133				*ALDH2* Glu504Lys-rs671			
Genotype	Fatty liver (W) *n* (%)	Fatty liver (W/O) *n* (%)	*p* value	Genotype	Fatty liver (W) *n* (%)	Fatty liver (W/O) *n* (%)	*p* value	Genotype	Fatty liver (W) *n* (%)	Fatty liver (W/O) *n* (%)	*p* value
CC	143 (26.2)	262 (45.1)	0.073	CC	273 (50.0)	373 (64.3)	0.051	AA	44 (8.1)	69 (11.9)	0.266
CG	264 (48.3)	284 (49.0)	0.245	CT	235 (43.0)	207 (35.7)	0.129	AG	256 (46.9)	290 (50.0)	0.189
GG	139 (25.5)	34 (5.9)	0.001	TT	38 (7.0)	0 (0)	0.061	GG	246 (45.1)	221 (38.1)	0.076

Abbreviations: ALDH2, aldehyde dehydrogenase 2; MTHFR, methylenetetrahydrofolate reductase; PNPLA3, patatin-like phospholipase domain-containing protein 3.

TABLE 3 Cox regression for cardiovascular events in patients with SLD

	Univariate		Multivariate	
Variable	**Crude HR (95% CI HR)**	***p* values**	**Adjusted HR (95% CI HR)**	***p* value**
Sex (male)	0.898 (0.696–1.16)	0.412	–	–
Age (year)	1.034 (1.022–1.046)	<0.001	1.037 (1.022–1.051)	<0.001
BMI (kg/m^2)	1.025 (0.995–1.056)	0.103	–	–
Alcohol (yes)	0.937 (0.637–1.378)	0.741	–	–
Smoking (yes)	1.034 (0.74–1.443)	0.846	–	–
Betel nut (yes)	1.05 (0.611–1.806)	0.859	–	–
AST (U/L)	1.002 (0.996–1.008)	0.487	–	–
ALT (U/L)	1 (0.997–1.003)	0.91	–	–
Total cholesterol (mg/dL)	0.994 (0.99–0.999)	0.008	0.996 (0.99–1.002)	0.082
HDL-C (mg/dL)	0.995 (0.982–1.009)	0.501	–	–
TG (mg/dL)	1 (0.999–1.002)	0.583	–	–
HOMA-IR	1.039 (0.915–1.18)	0.556	–	–
FIB-4	1.109 (0.944–1.303)	0.209	–	–
PNPLA3 I148M-rs738409 CC, CG, GG genotype (0, 1, 2)	1.029 (0.861–1.23)	0.753	–	–
MTHFR C677T Ala222Val-s1801133 CC, CT, TT genotype (0, 1, 2)	0.942 (0.763–1.164)	0.582	–	–
ALDH2 Glu504Lys-rs671 AA, AG, GG genotype (0, 1, 2)	1.044 (0.849–1.284)	0.684	–	–

Abbreviations: ALDH2, aldehyde dehydrogenase 2; ALT, alanine aminotransferase; AST, aspartate aminotransferase; BMI, body mass index; CI, confidence interval; FIB-4, Fibrosis-4; HDL-C, high-density lipoprotein-cholesterol; HOMA-IR, homeostasis model assessment-estimated insulin resistance; HR, hazard ratio; MTHFR, methylenetetrahydrofolate reductase; PNPLA3, patatin-like phospholipase domain-containing protein 3; TG, triglycerides.

TABLE 4 Cox regression for oncogenic events in patients with SLD

	Univariate		Multivariate	
Variables	**Crude HR (95% CI HR)**	***p* value**	**Adjusted HR (95% CI HR)**	***p* value**
Sex	0.568 (0.332–0.97)	0.038	0.542 (0.242–1.213)	0.136
Age (year)	1.034 (1.01–1.059)	0.006	1 (0.965–1.036)	0.982
BMI (kg/m^2)	0.916 (0.85–0.987)	0.021	0.974 (0.883–1.075)	0.604
Alcohol (yes)	0.891 (0.402–1.973)	0.776	–	–
Smoking (yes)	1.315 (0.706–2.451)	0.388	–	–
Betel nut (yes)	0.924 (0.288–2.964)	0.894	–	–
AST (U/L)	1.004 (0.992–1.015)	0.523	–	–
ALT (U/L)	1 (0.994–1.006)	0.936	–	–
Total cholesterol (mg/dL)	0.997 (0.989–1.005)	0.507	–	–
HDL-C (mg/dL)	1.006 (0.982–1.031)	0.622	–	–
TG (mg/dL)	0.998 (0.994–1.002)	0.294	–	–
HOMA-IR	0.837 (0.614–1.142)	0.262	–	–
FIB-4	1.312 (1.081–1.593)	0.006	1.325 (1.038–1.691)	0.024
PNPLA3 I148M-rs738409 CC, CG, GG genotype (0, 1, 2)	0.682 (0.468 – 0.995)	0.047	0.638 (0.366–1.11)	0.112
MTHFR C677T Ala222Val-s1801133 CC, CT, TT genotype (0, 1, 2)	1.203 (0.795 – 1.819)	0.382		
ALDH2 Glu504Lys-rs671 AA, AG, GG genotype (0, 1, 2)	0.958 (0.627 – 1.462)	0.841		

Abbreviations: ALDH2, aldehyde dehydrogenase 2; ALT, alanine aminotransferase; AST, aspartate aminotransferase; BMI, body mass index; CI, confidence interval; FIB-4, Fibrosis-4; HDL-C, high-density lipoprotein-cholesterol; HOMA-IR, homeostasis model assessment-estimated insulin resistance; HR, hazard ratio; MTHFR, methylenetetrahydrofolate reductase; PNPLA3, patatin-like phospholipase domain-containing protein 3; TG, triglycerides.

TABLE 5 Cox regression for liver cirrhosis in patients with SLD

	Univariate		Multivariate	
Variable	Crude HR (95% CI HR)	*p* value	Adjusted HR (95% CI HR)	*p* value
Sex	0.216 (0.024–1.94)	0.171	–	–
Age (year)	1.106 (1.02–1.199)	0.014	1.165 (0.96–1.414)	0.123
BMI (kg/m^2)	1.124 (0.95–1.33)	0.175	–	–
Alcohol (yes)	1.54 (0.172–13.79)	0.699	–	–
Smoking (yes)	1.236 (0.137–11.165)	0.85	–	–
Betel nut (yes)	4.326 (0.479–39.037)	0.192	–	–
AST (U/L)	1.029 (1.01–1.049)	0.003	–	–
ALT (U/L)	1.009 (0.995–1.022)	0.214	–	–
Total cholesterol (mg/dL)	1.009 (0.98–1.038)	0.555	–	–
HDL-C (mg/dL)	1.047 (0.972–1.127)	0.225	–	–
TG (mg/dL)	1.028 (0.682–1.551)	0.894	–	–
HOMA-IR	0.487 (0.048–4.973)	0.544	–	–
FIB-4	1.915 (1.451–2.527)	<0.01	1.902 (1.292–2.8)	0.001
PNPLA3 I148M-rs738409 CC, CG, GG genotype (0, 1, 2)	7.728 (1.001–59.63)	0.05	–	–
MTHFR C677T Ala222Val-s1801133 CC, CT, TT genotype (0, 1, 2)	1.724 (0.48–6.199)	0.404	–	–
ALDH2 Glu504Lys-rs671 AA, AG, GG genotype (0, 1, 2)	4.518 (0.545–37.55)	0.162	–	–

Abbreviations: ALDH2, aldehyde dehydrogenase 2; ALT, alanine aminotransferase; AST, aspartate aminotransferase; BMI, body mass index; CI, confidence interval; FIB-4, Fibrosis-4; HDL-C, high-density lipoprotein-cholesterol; HOMA-IR, homeostasis model assessment-estimated insulin resistance; HR, hazard ratio; MTHFR, methylenetetrahydrofolate reductase; PNPLA3, patatin-like phospholipase domain-containing protein 3; TG, triglycerides (AST was not lised as a factor in multivariate analysis due to high VIF with FIB-4).

TABLE 6 Linear regression for Fibrosis-4 (FIB-4) score in patients with fatty liver

	Univariable		Multivariable	
Variables	ß (95% CI ß)	*p* value	ß (95% CI ß)	*p* value
Sex	−0.082 (−0.386–0.08)	0.197	–	–
Age (year)	0.443 (0.026–0.043)	<0.001	0.438 (0.024–0.041)	<0.010
BMI (kg/m^2)	−0.112 (−0.053–0.003)	0.079	–	–
Alcohol (yes)	0.038 (−0.277–0.501)	0.57	–	–
Smoking (yes)	0.025 (−0.271–0.399)	0.708	–	–
Betel nut (yes)	−0.229 (−0.569–0.451)	0.819	–	–
Total cholesterol (mg/dL)	−0.11 (−0.006–0.001)	0.109	–	–
HDL-C (mg/dL)	0.008 (−0.011–0.012)	0.904	–	–
TG (mg/dL)	−0.056 (−0.002–0.001)	0.415	–	–
HOMA-IR	−0.057 (−0.166–0.053)	0.307	–	–
PNPLA3 I148M-rs738409 CC, CG, GG genotype (0, 1, 2)	0.18 (0.065–0.366)	0.005	0.158 (0.054–0.325)	0.006
MTHFR C677T Ala222Val-s1801133 CC, CT, TT genotype (0, 1, 2)	−0.02 (−0.221–0.161)	0.758	–	–
ALDH2 Glu504Lys-rs671 AA, AG, GG genotype (0, 1, 2)	0.109 (−0.024–0.321)	0.091	–	–

Abbreviations: ALDH2, aldehyde dehydrogenase 2; BMI, body mass index; CI, confidence interval; HDL-C, high-density lipoprotein-cholesterol; HOMA-IR, homeostasis model assessment-estimated insulin resistance; MTHFR, methylenetetrahydrofolate reductase; PNPLA3, patatin-like phospholipase domain-containing protein 3; TG, triglycerides.

with liver cirrhosis development; among the SLD patients, the baseline age was associated with cardiovascular events, and the FIB-4 scores were associated with extrahepatic cancer and liver cirrhosis development. (3) There was a dose effect of the *PNPLA3* I148M-rs738409 G allele on FIB-4 scores. (4) Among patients with SLD, the association between FIB-4 and extrahepatic cancer development was evident only in those with a *PNPLA3* I148M-rs738409 GG genotype. (5) There is a dose effect of the *ALDH2* Glu504Lys-rs671 G allele on long-term alcohol consumption.

In agreement with the fact that the *PNPLA3* I148M-rs738409 GG genotype is the genetic variant most robustly associated with NAFLD,[29] even after adjustment for alcohol consumption,[30] our SLD patients had a higher rate of the *PNPLA3* I148M-rs738409 GG genotype than controls. In addition, the distribution of *PNPLA3* genotypes in our patients with SLD (CC, 26.2%; CG, 45.1%; and GG, 25.5%) was close to that in a study of patients with histologically confirmed NAFLD (CC, 28%; CG, 46%; and GG, 25%).[31] Analogous to dysregulated adipokine secretion from inflamed and insulin-resistant adipose tissues, fatty liver may show differential expression and secrete hepatokines into the circulation,[32] and both adipokines and hepatokines are strongly associated with cardiometabolic risk in patients with NAFLD.[33] Thus, NAFLD is a systemic metabolic disorder that drives the progression of vascular disease,[34] supporting the observation that SLD patients had a higher rate of cardiovascular events than controls. That age was associated with cardiovascular events in SLD patients was in line with the fact that age >55 years predicts extrahepatic events in NAFLD patients.[35] Additionally, the finding that the *PNPLA3* I148M-rs738409 G allele is associated with the severity of fibrosis in patients with NAFLD[36] supports the concept of a dose-dependent association between the *PNPLA3* I148M-rs738409 G allele and FIB-4 scores. Consistent with the finding that HOMA-IR was a predictor of advanced liver fibrosis in nondiabetic patients with NAFLD,[37] patients with chronic hepatitis B[38] or patients with chronic hepatitis C,[39] the baseline HOMA-IR was associated with cirrhosis development in the controls. All the above-mentioned prior findings strongly support the reliability of the results from the current study.

There is a robust association between the *PNPLA3* I148M-rs738409 GG genotype and HCC in patients with SLD.[40] Intriguingly, the current study did not show any association between the *PNPLA3* I148M-rs738409 GG genotype and HCC development. Only three HCCs occurred in 8 years, which might blunt the association. In line with prior reports that hepatic fibrosis is associated with outcomes in patients with NAFLD,[5] extrahepatic cancer is the main long-term outcome for NAFLD,[5] and the overall risk of non-HCC malignancies is more than twofold greater for patients with cirrhosis than for the general population,[41] we observed that baseline FIB-4 scores were independently associated with extrahepatic cancer development in our SLD patients. Whether the *PNPLA3* genotype is associated with extrahepatic cancers has remained controversial.[42,43] We made the novel and noteworthy finding that the association between FIB-4 scores and extrahepatic cancer development was evident only in SLD patients carrying a *PNPLA3* I148M-rs738409 GG genotype. A simulated analysis of a cDNA dataset showed that *PNPLA3* was considerably related to cancer proliferation.[44] In addition, metabolic reprogramming is one of the most important hallmarks of malignant tumors.[45] *PNPLA3* encodes a lipid droplet-associated, carbohydrate-regulated lipogenic and/or lipolytic enzyme,[46] and the 148 M variant of *PNPLA3* extensively alters lipid metabolism. Specifically, the 148 M variant remodels liver TGs in a polyunsaturated direction,[47] reduces the lipidation of very-low-density lipoprotein,[48] and disturbs vitamin A metabolism.[49] Moreover, overexpression of the *PNPLA3* 148 M variant was associated with a shift to anaerobic metabolism and mitochondrial dysfunction[50] and increased ceramides with resultant STAT3 phosphorylation,[51] which elicits immunosuppression in the tumor microenvironment.[52] Altogether, the augmented cancer cell proliferation, lipid and vitamin A metabolic alterations, mitochondrial dysfunction, and STAT3 pathway activation driven by *PNPLA3* I148M overexpression may synergistically accelerate hepatic fibrosis-promoted extrahepatic cancer development in SLD patients carrying a *PNPLA3* I148M-rs738409 GG genotype.

It has been suggested that the increased mortality risk in patients with two 148 M alleles is greatest beginning in the second decade of follow-up.[53] The current study did not show any mortality, and 8 years might be too short to view the impact of the *PNPLA3* I148M-rs738409 GG genotype on mortality.

Whether the *MTHFR* C677T T/T genotype is[12] or is not[11] associated with NAFLD remains controversial, which might explain why the difference in the *MTHFR* C677T Ala222Val-rs1801133 TT genotype rate was only borderline in our SLD patients and controls. Consistent with the phenomenon that among Chinese individuals carrying the *ALDH2* Glu504Lys-rs671 AA, AG, and GG genotypes, the proportions of current drinkers were 1%, 16%, and 45%, respectively,[54] there was a dose effect of the *ALDH2* Glu504Lys-rs671 G allele on the number of patients with long-term alcohol consumption. Although the longitudinal risk of NAFLD was reported to be higher in *ALDH2* Glu504Lys-rs671 A allele carriers than in noncarriers,[14] we did not find any difference in *ALDH2* Glu504Lys-rs671 A allele rates between SLD patients and controls. Moreover, neither the *MTHFR* nor *ALDH2* genotype was found to be associated with outcomes in SLD patients. Future studies using large sample sizes might be needed to investigate the precise impacts of *MTHFR* and *ALDH2* genotypes on fatty liver risk and associated outcomes in the Taiwanese population.

The current study has some limitations. First, the results were obtained from a small database subset and are not supported by a priori hypotheses and functional data. Second, in addition to *PNPLA3*, some genes involved in lipid biology, including transmembrane 6

superfamily member 2, glucokinase regulatory protein, membrane bound O-acyltransferase domain containing 7, and hydroxysteroid 17-beta dehydrogenase 13,[9] were not investigated in the current study. Third, the precise mechanism of the increased risk of extrahepatic cancer in fatty liver patients with the *PNPLA3* GG genotype and high FIB-4 scores was not elucidated. Future studies with large-scale independent cohorts involving sophisticated molecular investigations and the aforementioned genetic surveys are needed to elucidate the fundamental mechanisms underlying the findings described in the current study.

Taken together, the data of the current study showed that compared with controls, patients with SLD had an increased frequency of the *PNPLA3* I148M-rs738409 GG genotype. Among the SLD patients, baseline FIB-4 scores were associated with extrahepatic cancer and cirrhosis development, and the *PNPLA3* I148M-rs738409 genotype was associated with FIB-4 scores. Special vigilance should be exercised for extrahepatic cancer development in SLD patients with the *PNPLA3* I148M-rs738409 GG genotype and high baseline FIB-4 scores.

AUTHOR CONTRIBUTIONS

Jennifer Tai: Formal analysis; writing – original draft. **Chao-Wei Hsu:** Methodology; writing – original draft. **Wei-Ting Chen:** Resources; writing – original draft. **Sien-Sing Yang:** Conceptualization; writing – original draft. **Cheng-Hsun Chiu:** Writing – original draft. **Rong-Nan Chien:** Resources; writing – original draft. **Ming-Ling Chang:** Conceptualization; data curation; formal analysis; funding acquisition; supervision; writing – original draft; writing – review and editing.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

ETHICS STATEMENT

Approval of the research protocol by an institutional review board: Chang Gung Memorial Hospital. institutional review board (IRB No. 104-7005B; 202000132B0; 202002302B0).

Informed consent: Obtained in writing from each patient, and the study protocol conformed to the ethical guidelines of the 1975 Declaration of Helsinki and was approved by the Chang Gung Memorial Hospital institutional review board.

Registry and the registration no. of the study/trial: N/A.

Animal studies: N/A.

REPORTING CHECKLIST

The authors have completed the Strengthening the Reporting of Observational Studies in Epidemiology (STROBE) reporting checklist.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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