Published in final edited form as:

Mol Carcinog. 2013 November ; 52(0): . doi:10.1002/mc.22057.

Genetic Variation in the *PNPLA3* Gene and Hepatocellular Carcinoma in USA: Risk and Prognosis Prediction

Manal M. Hassan^{1,*}, Ahmed Kaseb¹, Carol J. Etzel², Hashem El-Serag³, Margaret R. Spitz⁴, Ping Chang¹, Katherine S. Hale⁵, Mei Liu², Asif Rashid⁶, Mohamed Shama¹, James L. Abbruzzese¹, Evelyne M. Loyer⁷, Harmeet Kaur⁷, Hesham M. Hassabo¹, Jean-Nicolas Vauthey⁸, Curtis J. Wray⁹, Basmah S. Hassan¹, Yehuda Z. Patt¹⁰, Ernest Hawk², Khalid M. Soliman¹, and Donghui Li¹

¹Division of Cancer Medicine, Department of Gastrointestinal Medical Oncology, The University of Texas M.D. Anderson Cancer Center, Houston, Texas

²Division of Cancer Prevention and Population Science, Department of Epidemiology, The University of Texas M.D. Anderson Cancer Center, Houston, Texas

³Department of Gastroenterology and Hepatology, Baylor College of Medicine, Houston, Texas

⁴Department of Molecular and Cellular Biology, Baylor College of Medicine, Houston, Texas

⁵Department of Systems Biology, The University of Texas M.D. Anderson Cancer Center, Houston, Texas

⁶Division of Pathology/Lab Medicine, Department of Pathology, The University of Texas M.D. Anderson Cancer Center, Houston, Texas

⁷Division of Diagnostic Imaging, Department of Diagnostic Radiology, The University of Texas M.D. Anderson Cancer Center, Houston, Texas

⁸Division of Surgery, Department of Surgical Oncology, The University of Texas M.D. Anderson Cancer Center, Houston, Texas

⁹Department Surgery/Lyndon B. Johnson, The University of Texas Health Science Center at Houston Medical School, Houston, Texas

¹⁰Department of GI Oncology, University of New Mexico Cancer Center, Albuquerque, New Mexico

Abstract

Nonalcoholic fatty liver disease (NAFLD) is an emerging epidemic with high prevalence in Western countries. Genome-wide association studies had reported that a variation in the patatin-like phospholipase domain containing 3 (*PNPLA3*) gene is associated with high susceptibility to NAFLD. However, the relationship between this variation and hepatocellular carcinoma (HCC) has not been well established. We investigated the impact of *PNPLA3* genetic variation (rs738409: C>G) on HCC risk and prognosis in the United States by conducting a case-control study that included 257 newly diagnosed and pathologically confirmed Caucasian patients with HCC (cases) and 494 healthy controls. Multivariate logistics and Cox regression models were used to control for the confounding effects of HCC risk and prognostic factors. We observed higher risk of HCC for subjects with a homozygous GG genotype than for those with CC or CG

genotypes, the adjusted odds ratio (OR) was 3.21 (95% confidence interval [CI], 1.68–6.41). We observed risk modification among individuals with diabetes mellitus (OR = 19.11; 95% CI, 5.13–71.20). The *PNPLA3* GG genotype was significantly associated with underlying cirrhosis in HCC patients (OR = 2.48; 95% CI, 1.05–5.87). Moreover, GG allele represents an independent risk factor for death. The adjusted hazard ratio of the GG genotype was 2.11 (95% CI, 1.26–3.52) compared with CC and CG genotypes. *PNPLA3* genetic variation (rs738409: C>G) may determine individual susceptibility to HCC development and poor prognosis. Further experimental investigations are necessary for thorough assessment of the hepatocarcinogenic role of *PNPLA3*.

Keywords

molecular epidemiology; genetic susceptibility; case-control; single nucleotide polymorphism

INTRODUCTION

Despite the multi-etiological nature of hepatocellular carcinoma (HCC), it is difficult to pinpoint the specific underlying factors that have contributed to the increased incidence of HCC in the United States, especially in young people [1]. Concurrent with the increase in HCC incidence, the prevalence of fatty liver or hepatic steatosis has increased and is currently considered a growing global health problem. An estimated 20–40% of the adult population in Western countries have fatty liver [2]. Interestingly, each of the major risk factors of HCC such as hepatitis C virus (HCV), hepatitis B virus (HBV), alcohol consumption, prior history of obesity, and diabetes mellitus has been significantly associated with a wide spectrum of hepatobiliary diseases [3–5].

Nonalcoholic fatty liver disease (NAFLD) is a general term for multiple liver disorders. The initial condition is simple hepatocyte fatty infiltration (steatosis) defined by deposition of lipid droplets (triglycerides) in liver cell [6]. Steatosis is often reversible condition but it can progress to a second disorder named nonalcoholic steatohepatitis (NASH) with underlying hepatic inflammation and or collagen deposition (fibrosis). Approximately 10–29% of patients with NASH may progress to nonreversible end stage liver disorder (cirrhosis) within 10 yrs [7] and will be at high risk for HCC development [8]. The Serum level of alanine aminotransferase (ALT) is elevated in patients with liver injury and NAFLD than in individuals with normal liver [9].

Various genetic and environmental mediators are involved in the progression of liver diseases [10] and may explain the observed variations in individuals' susceptibility for HCC development among high risk populations.

Recently, a genome-wide association study reported that a variation in the patatin-like phospholipase domain containing 3 (*PNPLA3*) gene is associated with high susceptibility to NAFLD [11]. The I148M allele (rs738409: C>G) of the *PNPLA3* gene represents a cytosine to guanine substitution, resulting in an isoleucine to methionine switch at codon 148. Individuals who were homozygous for the G allele had a higher hepatic triglyceride level [11] and an elevated serum level of ALT [12]. Moreover, *PNPLA3* (rs738409: C>G) may influence fibrosis severity in patients with fatty liver [13–16]. Thus, we hypothesized that *PNPLA3* genetic variation (rs738409: C>G) may determine individual susceptibility to HCC and may correlate with underlying cirrhosis and poor prognosis of American men and women with HCC.

SUBJECTS AND METHODS

Population and Study Design

Case patients were prospectively recruited from the population of patients with newly diagnosed HCC who were evaluated and treated at the M. D. Anderson Cancer Center. The inclusion criteria were as follows: pathologically confirmed diagnosis of HCC and U.S. residency. The exclusion criteria were the presence of other types of primary liver cancer and concurrent or past history of cancer at another organ site.

From January 2000 through December 2009, 724 patients with suspected HCC were identified, 553 of whom were eligible for this study. We enrolled 446 eligible patients with HCC (participation rate = 80.7%); 107 were not recruited due to patients refusal, patients sickness, or inadequate time to complete the interview; 75 were Caucasians (70%). Among the total of 446 eligible HCC patients 313 cases were Caucasians (70.2%); 257 (82.1%) had sufficient and high-quality DNA samples for genotyping and were included in this investigation.

The healthy control subjects were genetically unrelated family members (spouses or in-laws) of patients at MD Anderson who had cancers other than liver, gastrointestinal, lung, or head and neck cancer. The eligibility criteria for controls were the same as for cases, except for having a cancer diagnosis. Control subjects were recruited from the Institution's Central Diagnostic Radiology Clinics, where all cancer patients and their companions are sent for the initial cancer diagnosis or post-treatment follow-up examination. Research data coordinator of the study followed a detailed protocol to identify candidates of control subjects according to the eligibility criteria of control selection.

A short self-administered structured questionnaire was used to determine the perception of the healthy individuals to participate in medical research and to identify potential controls from the healthy individuals on the basis of the eligibility criteria. The questionnaire requested information about age, sex, race, educational level, place of residency, personal history of cancer, relationship to the cancer patient, and the patient's type of cancer. We estimated that 83.6% of the eligible candidates for control selection agreed to participate in the study and did not differ significantly from eligible candidates who refused to participate.

Using the same short self-administered structured questionnaire, we sought to confirm the control subjects' reasons for coming to the hospital with cancer patients and whether these reasons could have been related to the risk factors of HCC. We found that the underlying causes for the controls' companionship were care and altruism. Moreover, all spouses of patients with other cancers who served as control subjects reported that they would have chosen to be referred to M.D. Anderson if they had been diagnosed with cancer during the same time period because they tended to share the same family physician, had the same health insurance coverage, and lived in the same geographic location. All of the above-mentioned results indicated that the patients and controls had the same catchments, which further supported the idea that the control subjects were representative of the M.D. Anderson population from which HCC patients were selected. A total of 494 Caucasian healthy control subjects who were frequency-matched to the HCC cases by gender and age (± 5 yrs) were recruited and included in this investigation.

HCC cases and controls were recruited during the same time frame and were personally interviewed for 25–30 min. To control for the confounding impact of the major risk factors of HCC, the interviewers used a structured and validated questionnaire to collect information of demographic variables and HCC risk factors including cigarette smoking, alcohol consumption, chronic medical conditions, and family history of cancers.

We recently revised the study questionnaire to collect information about BMI at different ages (20, 20, 30, 40, and 50 yrs) prior to HCC development. Total of 155 Caucasian HCC cases and 307 Caucasian control subjects had been interviewed for obesity history using the revised questionnaire. Detailed methods of cases and controls ascertainment were previously described in Refs. [17–21].

Several clinical variables were considered in determining survival outcome and were retrieved from HCC patients' medical records: alpha-fetoprotein levels (ng/ml) at the time of diagnosis; tumor size (50% vs. >50%) of the total liver size; tumor nodularity (uninodular vs. multinodular); radiological and pathological evidence of cirrhosis, fibrosis, and steatosis; presence or absence of portal thrombosis; presence or absence of macrovascular or microvascular invasion; extrahepatic metastasis; lymph node involvement; tumor differentiation (well differentiated, moderately differentiated, poorly differentiated); Child-Pugh classification (A–C); Barcelona Clinic Liver Cancer (BCLC) stage (A–D); Eastern Cooperative Oncology Group (ECOG) performance status; and the type of first treatment initiated at time of diagnosis [no therapy, chemotherapy with or without others modalities, surgical resection/transplantation with or without other modalities, and transcatheter arterial chemoembolization (TAC)].

Other modalities included percutaneous ethanol injection, radiofrequency thermal ablation, or immune therapy.

The current investigation is part of an ongoing hospital-based case–control study, which was approved by the Institutional Review Board (IRB) at The University of Texas MD Anderson Cancer Center. Written informed consent for participation was obtained from each study participant. Under IRB approval we retrieved the medical records of the eligible and non-recruited HCC patients. There were no significant differences in demographic, epidemiologic, and clinical factors, among the eligible and the included Caucasian patients with DNA samples ($N = 257$) as compared to eligible Caucasian patients without DNA samples ($n = 56$) or as compared to Caucasian patients who were eligible but not enrolled in the study ($n = 75$).

Laboratory Methods

Blood samples were collected in heparinized vacutainers (BD Biosciences, Franklin Lakes, NJ). DNA was extracted from mononuclear cells by using a FlexiGene DNA kit (Cat# 51206, Qiagen, Valencia, CA) and the Maxwell 16 automated system with Cell DNA Purification Kit (Cat # AS1020, Promega, Madison, WI). DNA concentration was determined by Nano-Drop ND1000 and diluted DNA (10 ng/μl) stored at 4°C for immediate use. Separated plasma samples were tested for HBV and HCV markers. HCV antibodies (anti-HCV), hepatitis B surface antigen (HBsAg), and antibodies to hepatitis B core antigen (anti-HBc) were detected by use of a third-generation enzyme-linked immunosorbent assay (ELISA; Abbott Laboratories, North Chicago, IL). Positive results prompted repeated confirmatory ELISA.

Sequenom polymerase chain reaction (PCR) and single nucleotide polymorphism (SNP) primers were designed with use of Sequenom's Assay Design software, limiting the number of targets to 20 per multiplex reaction. Multiplex PCR reactions were amplified by using Qiagen Hotstar Taq polymerase; the PCR products were cleaned by using EXO-SAP (Sequenom); and the primer extension reactions were run by using iPLEX Gold (Sequenom). The samples were spotted onto Spectrochip II matrix chips and then run in the MassARRAY. All samples were run in duplicate. Sequenom Typer 3 software and in-house software were used to interpret the mass spectra. Moreover, Genotyping confirmation in all

cases and controls was performed by using TaqMan allelic discrimination assays (Applied Biosystems, Foster City, CA).

Sample Size Consideration and Statistical Methods

We estimated the sample size based on probability of $\alpha = 0.05$ and $\beta = 0.1$ and assuming that the prevalence of the risk allele (G) in the control group was 23% [11], and estimated odds ratio (OR) was 2. Approximately 2 to 1 control–case ratio was chosen to increase the power of the study. According to the above parameters the estimated sample size had enough power to assess the risk of the *PNPLA3* genetic variation (rs738409: C>G) on HCC development.

Univariate analyses were conducted with χ^2 or Fisher exact tests for categorical variables and the Kruskal–Wallis test for continuous variables. All clinical, genetic, and epidemiological data were merged and analyzed with use of STATA software (STATA Corp, College Station, TX). For controls, deviations of the genotype frequencies from those expected under Hardy–Weinberg equilibrium were assessed by χ^2 tests (1 df), and genotype frequencies in cases and controls were compared by using χ^2 tests (2 df).

We performed multivariable logistic regression to estimate the adjusted odds ratio (AOR) and 95% confidence intervals (CI) using the maximum likelihood estimate. All AORs were adjusted for age, gender, educational level, cigarette smoking, alcohol consumption, diabetes mellitus, family history of cancer, and HBV/HCV infection. The final model was determined on the basis of backward stepwise selection procedure. In addition, we used multivariable logistic regression models to investigate possible interactions on an additive scale of *PNPLA3* genotypes with HCV/HBV infection, diabetes mellitus, and alcohol consumption.

Overall survival (OS) was defined as the time between diagnosis and death (as a result of all causes) or end of follow-up (censored observations). Median survival was estimated by using the Kaplan–Meier product-limit method, and significant differences between the survival times were determined by using the log-rank test. To identify independent prognostic factors for OS, hazard ratios (HR) and 95% CIs were calculated by using Cox proportional hazard models with a backward stepwise selection procedure, considering the clinical covariates of HCC without multicollinearity between the clinical features of HCC.

RESULTS

Participants' Characteristics

Table 1 shows the demographic characteristics of the 257 Caucasian cases with HCC and the 494 Caucasian healthy controls. Gender distribution was comparable between HCC cases and controls. Overall, cases were slightly older than controls but frequency matched by ± 5 yrs; the mean age $\pm$ standard error was 62.39 ± 0.78 yrs for HCC cases and 60.35 ± 0.51 yrs for controls. Higher education (college degree) was more frequent among controls (46.4%) than among HCC cases (33.1%; $P = 0.001$). Cases and controls had a similar distribution of geographical region (U.S. state of residency) with the majority of study participants (70.7%) from Texas and adjacent states (i.e., Louisiana, Arkansas, New Mexico, and Oklahoma). The observed HCC risk factors including chronic infection with HCV/HBV, alcohol consumption, cigarette smoking, prior history of diabetes mellitus, and first-degree family history of liver cancer were consistent with those of our previous reports addressing the potential association between environmental risk factors and HCC [17–21].

PNPLA3 Genotypes and HCC

The distribution of *PNPLA3* genotypes (CC, GC, and GG) was significantly different between HCC cases (50.6%, 36.6%, and 12.8%) and controls (55.5%, 39.9%, and 4.7%), respectively, $P < 0.001$ (Table 2). The control genotype distribution was consistent with the Hardy–Weinberg equilibrium in all ($P = 0.1$), in men ($P = 0.2$), and in women ($P = 0.3$). Although we found deviations from the expected proportions of homozygote and heterozygote classes in HCC case population ($P = 0.02$), this supports the association between HCC and G allele of *PNPLA3* gene.

Multivariable logistic regression analyses indicated that having the homozygous GG variant of *PNPLA3* afforded a threefold increased risk for HCC compared with participants with the homozygous or heterozygous C allele (CC/CG), irrespective of the potential confounding effects of HCC risk factors. A significant risk association was observed for both men and women (Table 2).

An interaction of the GG genotype and diabetes (risk modification) was observed on the additive scale. The AOR (95% CI) was 19.11 (5.13–71.20) for diabetic patients with GG genotype as compared to non-diabetic individuals who are homozygous or heterozygous for the C allele, P for the interaction = .04 (Table 3). No risk modification was observed by hepatitis virus infection or by alcohol consumption.

Association Between PNPLA3 GG Genotype and Underlying Cirrhosis in HCC Patients

At the time of HCC diagnosis, a total of 156 HCC cases (60.7%) had pathological ($n = 98$) and/or radiological ($n = 58$) evidence of cirrhosis ($n = 145$), as well as fibrosis with or without steatosis ($n = 11$). High prevalence of underlying cirrhosis was observed in HCC cases with the GG *PNPLA3* genetic variation (72.7%) compared with that in HCC cases with the CC (53.1%) or CG genotype (67%; $P = 0.03$). The final logistic stepwise regression model for underlying cirrhosis indicated that the GG genotype of *PNPLA3* was an independent and significant predictor for cirrhosis development before HCC diagnosis. The estimated AOR (95% CI) was 2.48 (1.05–5.87; Table 4). Other significant predictors including: HCV, HBV, and diabetes mellitus. The estimated AORs were 5.77 (2.93–11.36); 5.26 (1.07–25.77); and 3.87 (1.76–8.55) for HBV, HCV, and diabetes, respectively (Table 4).

Association Between PNPLA3 GG Genotype and Overall Survival

The survival duration for HCC cases with the GG genotype was shorter than that of HCC cases with the CC or CG genotypes. The estimated median survival was 16.8 months (95% CI, 9.9–23.7 months) for HCC cases with the GG genotype and 25.9 months (95% CI, 21.5–30.3 months) for HCC cases with the CC/CG genotypes ($P = 0.1$).

In an analysis restricted to HCC patients with advanced stages, poor median survival was observed in patients with the GG genotypes [5.17 months (95% CI, 1.66–8.68)] compared with that in patients with the CC and GC genotypes [13.53 months (95% CI, 10.73–16.34); $P = .005$].

Cox proportional hazard models, which included predictors of prognosis, revealed that the GG allele was an independent risk factor for mortality (Table 5). Compared with homozygous CC allele or heterozygous CG allele carriers, the adjusted HR for OS for cases with the GG genotype was more than two times higher (HR = 2.04; 95% CI, 1.22–3.41; $P = 0.007$). In addition, male gender and poor tumor differentiation were associated with a higher risk of death after adjustment for BCLC staging, ECOG performance status, and

treatment exposure. Treated patients experienced better survival than non-treated patients, with a range of 54–89% improvement in OS by different types of treatment (Table 5).

DISCUSSION

Despite the overwhelming impact of environmental factors on HCC development, this large case–control study revealed an independent effect of GG *PNPLA3* genetic variation (rs738409: C>G) on HCC development in American men and women.

The current analysis was performed in American Caucasians, in whom the prevalence of the risk allele (G) was 24.6%, similar to that presented by GWAS (23%) [11].

Two recent genomic-wide association studies reported non-synonymous sequence variation in the *PNPLA3* gene in association with NAFLD and with ALT concentration [11,12]. Results from both studies have been replicated and indicated a significant risk of the GG genotype on the development of NAFLD and NASH [13–16,22–24]. Moreover, the GG genotype influenced the severity of fibrosis and enhanced the risk of cirrhosis development in patients with NAFLD. This may explain our observation of the association between GG genotype of *PNPLA3* and underlying cirrhosis in HCC patients. We estimated that 60.7% of our HCC patients had underlying cirrhosis, which was supported by population [25] and clinical studies in USA [26–28].

Our results is supported by two Italian studies indicating that patients with cirrhosis complicated by HCC were more likely to be GG homozygous than were the remaining cirrhotic patients [14,24].

PNPLA3 mRNA is expressed in the liver and the adipose tissue [29], encodes 481- amino acids protein of unclear physiological and biological function. Nevertheless, *PNPLA3* exhibits strong activity in lipolysis and triglyceride hydrolysis in vitro [30]. Moreover, expression of *PNPLA3*-I148M, but not the wild type *PNPLA3*, promotes triglyceride accumulation by impairing the triglyceride hydrolysis in cultured hepatocyte and in mice liver [31].

The carcinogenic role of *PNPLA3* in HCC development has not been examined. However, the genetic susceptibility of *PNPLA3* for HCC development may be considered as a natural extension for the previously reported association between NAFLD and *PNPLA3* genetic variation (rs738409: C>G). Similarly, fatty liver disease is a common condition in patients with other HCC risk factors including HCV infection [3], diabetes mellitus, obesity [4], and alcohol drinking [5]. Both alcoholic and non-alcoholic fatty liver diseases have a similar pathological feature [32,33].

Once steatosis has developed in genetically susceptible patients, lipid peroxidation, inflammation, and generated free oxygen radicals may play a central role in NASH development, during which the initiation phase of the HCC mechanism takes place. The balance between apoptotic and anti-apoptotic factors and the disturbance in the growth factors may facilitate oval cell proliferation and the promotion phase of hepatocarcinogenesis [34].

Hyperinsulinemia and insulin resistance is another pathway involved in hepatocarcinogenesis in patients with high susceptibility for fatty liver diseases, particularly in patients with obesity and diabetes-induced NAFLD [35,36]. This may explain our observation of synergistic interaction between the GG genotype of *PNPLA3* (rs738409: C>G) and diabetes mellitus on the risk of HCC development.

PNPLA3 expression is positively correlated with body mass index (BMI) and carbohydrate intake [29,37]. Because obesity is a risk factor of type 2 diabetes and NAFLD, one may argue that obesity may confound the independent effect of GG genotype of *PNPLA3* (rs738409) or its interaction with diabetes mellitus. However, the statistical adjustment for the confounding effect of obesity in subset of the study population with available obesity information (155 cases and 307 controls) or the restricted analysis to those without diabetes did not meaningfully change the observed significant association between the *PNPLA3* GG genotype (rs738409) and HCC risk or poor prognosis.

Since the effect of the *PNPLA3* genetic variation (rs738409: C>G) on HCC risk is possibly mediated through the effect of NAFLD, fatty liver is considered an intermediate factor and should not be treated as a confounding factor [38]. In fact, adjusting for the effect of the intermediate factors may bias the results toward the null. Nevertheless, our estimated ORs were adjusted for the major etiological factors of HCC as surrogate variables of NAFLD due to the presence of fatty liver in conjunction with these etiological factors of HCC. Large follow-up studies of patients with fatty liver may be warranted to understand how *PNPLA3* GG genotype (rs738409) modulates the risk of NASH to develop HCC with and without underlying cirrhosis.

The association between the *PNPLA3* (rs738409) polymorphism and HCC extended to the poor survival outcome of HCC patients with the GG genotype of *PNPLA3* (rs738409) compared with patients with the CC or CG genotypes. The prognostic role of the GG genotype in HCC survival was independent of the major survival predictive factors. Although there is no specific explanation for such observation, it is possible that patients with the GG genotype of *PNPLA3* (rs738409) have high susceptibility for lymph node involvement, vascular invasion, poor tumor differentiation, or other clinical parameters associated with HCC prognosis. Given the complexity of the natural history of HCC, we hypothesized that manifestations of underlying cirrhosis including ascites, bleeding, and low platelet count are a huge burden to HCC clinical management, often forcing oncologists to reduce the optimal dose of aggressive chemotherapeutic agents or prohibit surgical resection. This may ultimately affect patients' response to the therapy and limits patients' options for effective treatment, leading to diminished survival.

Most HCC patients received several types of systemic therapy, correlation between *PNPLA3* genotypes and response to each type of treatment will be very difficult to assess independently from others due to overlapping between used regimens.

It has been reported that the highest frequency of the G allele was observed among Hispanics [11]. In this case-control study, only a small number of Hispanic patients with HCC were recruited, which precluded us from determining the impact of *PNPLA3* GG genotype (rs738409) on HCC development and HCC prognosis among Hispanics. Further studies are warranted to identify common genetic predisposition factors that may contribute to susceptibility to HCC development in minorities, in whom the incidence of HCC and the prevalence of metabolic syndrome are higher than in Caucasians. Should our findings be confirmed, it may help to develop novel strategies in identifying high-risk individuals for HCC especially among diabetic patients and in future personalized cancer treatment.

Acknowledgments

This research is supported by National Institutes of Health (NIH grants) RO3 ES11481 (to M.H.), CA106458 (to M.H.), and Texas Tobacco Settlement (to M.H.).

Abbreviations

HCC	hepatocellular carcinoma
HCV	hepatitis C virus
HBV	hepatitis B virus
NAFLD	nonalcoholic fatty liver disease
NASH	nonalcoholic steatohepatitis
OS	overall survival
AOR	adjusted odds ratio

References

1. Altekruse SF, McGlynn KA, Reichman ME. Hepatocellular carcinoma incidence, mortality, and survival trends in the United States from 1975 to 2005. *J Clin Oncol*. 2009; 27:1485–1491. [PubMed: 19224838]
2. Browning JD, Szczepaniak LS, Dobbins R, et al. Prevalence of hepatic steatosis in an urban population in the United States: Impact of ethnicity. *Hepatology*. 2004; 40:1387–1395. [PubMed: 15565570]
3. Czaja AJ, Carpenter HA, Santrach PJ, Moore SB. Host- and disease-specific factors affecting steatosis in chronic hepatitis C. *J Hepatol*. 1998; 29:198–206. [PubMed: 9722200]
4. Tolman KG, Fonseca V, Tan MH, Dalpiaz A. Narrative review: Hepatobiliary disease in type 2 diabetes mellitus. *Ann Intern Med*. 2004; 141:946–956. [PubMed: 15611492]
5. Bellentani S, Saccoccio G, Costa G, et al. Drinking habits as cofactors of risk for alcohol induced liver damage. The Dionysos Study Group. *Gut*. 1997; 41:845–850. [PubMed: 9462221]
6. Cohen JC, Horton JD, Hobbs HH. Human fatty liver disease: Old questions and new insights. *Science*. 2011; 332:1519–1523. [PubMed: 21700865]
7. Argo CK, Caldwell SH. Epidemiology and natural history of non-alcoholic steatohepatitis. *Clin Liver Dis*. 2009; 13:511–531. [PubMed: 19818302]
8. Starley BQ, Calcagno CJ, Harrison SA. Nonalcoholic fatty liver disease and hepatocellular carcinoma: A weighty connection. *Hepatology*. 2010; 51:1820–1832. [PubMed: 20432259]
9. Kishino T, Ohnishi H, Ohtsuka K, et al. Low concentrations of serum n-3 polyunsaturated fatty acids in non-alcoholic fatty liver disease patients with liver injury. *Clin Chem Lab Med*. 2011; 49:159–162. [PubMed: 21034259]
10. Browning JD, Horton JD. Molecular mediators of hepatic steatosis and liver injury. *J Clin Invest*. 2004; 114:147–152. [PubMed: 15254578]
11. Romeo S, Kozlitina J, Xing C, et al. Genetic variation in PNPLA3 confers susceptibility to nonalcoholic fatty liver disease. *Nat Genet*. 2008; 40:1461–1465. [PubMed: 18820647]
12. Yuan X, Waterworth D, Perry JR, et al. Population-based genome-wide association studies reveal six loci influencing plasma levels of liver enzymes. *Am J Hum Genet*. 2008; 83:520–528. [PubMed: 18940312]
13. Valenti L, Al-Serri A, Daly AK, et al. Homozygosity for the patatin-like phospholipase-3/adiponutrin I148M polymorphism influences liver fibrosis in patients with nonalcoholic fatty liver disease. *Hepatology*. 2010; 51:1209–1217. [PubMed: 20373368]
14. Valenti L, Rumi M, Galmozzi E, et al. Patatin-like phospholipase domain-containing 3 I148M polymorphism, steatosis, and liver damage in chronic hepatitis C. *Hepatology*. 2011; 53:791–799. [PubMed: 21319195]
15. Rotman Y, Koh C, Zmuda JM, Kleiner DE, Liang TJ. The association of genetic variability in patatin-like phospholipase domain-containing protein 3 (PNPLA3) with histological severity of nonalcoholic fatty liver disease. *Hepatology*. 2010; 52:894–903. [PubMed: 20684021]

16. Sookoian S, Castano GO, Burgueno AL, Gianotti TF, Rosselli MS, Pirola CJ. A nonsynonymous gene variant in the adiponutrin gene is associated with nonalcoholic fatty liver disease severity. *J Lipid Res.* 2009; 50:2111–2116. [PubMed: 19738004]
17. Hassan MM, Hwang LY, Hatten CJ, et al. Risk factors for hepatocellular carcinoma: Synergism of alcohol with viral hepatitis and diabetes mellitus. *Hepatology.* 2002; 36:1206–1213. [PubMed: 12395331]
18. Hassan MM, Spitz MR, Thomas MB, et al. Effect of different types of smoking and synergism with hepatitis C virus on risk of hepatocellular carcinoma in American men and women: Case–control study. *Int J Cancer.* 2008; 123:1883–1891. [PubMed: 18688864]
19. Hassan MM, Spitz MR, Thomas MB, et al. The association of family history of liver cancer with hepatocellular carcinoma: A case–control study in the United States. *J Hepatol.* 2009; 50:334–341. [PubMed: 19070394]
20. Hassan MM, Kaseb A, Li D, et al. Association between hypothyroidism and hepatocellular carcinoma: A case–control study in the United States. *Hepatology.* 2009; 49:1563–1570. [PubMed: 19399911]
21. Hassan MM, Curley SA, Li D, et al. Association of diabetes duration and diabetes treatment with the risk of hepatocellular carcinoma. *Cancer.* 2010; 116:1938–1946. [PubMed: 20166205]
22. Kotronen A, Johansson LE, Johansson LM, et al. A common variant in PNPLA3, which encodes adiponutrin, is associated with liver fat content in humans. *Diabetologia.* 2009; 52:1056–1060. [PubMed: 19224197]
23. Hotta K, Yoneda M, Hyogo H, et al. Association of the rs738409 polymorphism in PNPLA3 with liver damage and the development of nonalcoholic fatty liver disease. *BMC Med Genet.* 2010; 11:172. [PubMed: 21176169]
24. Falletti E, Fabris C, Cmet S, et al. PNPLA3 rs738409C/G polymorphism in cirrhosis: Relationship with the aetiology of liver disease and hepatocellular carcinoma occurrence. *Liver Int.* 2011; 31:1137–1143. [PubMed: 21745286]
25. El-Serag HB, Siegel AB, Davila JA, et al. Treatment and outcomes of treating of hepatocellular carcinoma among Medicare recipients in the United States: A population-based study. *J Hepatol.* 2006; 44:158–166. [PubMed: 16290309]
26. Patt YZ, Hassan MM, Lozano RD, et al. Phase II trial of systemic continuous fluorouracil and subcutaneous recombinant interferon Alfa-2b for treatment of hepatocellular carcinoma. *J Clin Oncol.* 2003; 21:421–427. [PubMed: 12560429]
27. Patt YZ, Hassan MM, Lozano RD, et al. Thalidomide in the treatment of patients with hepatocellular carcinoma: A phase II trial. *Cancer.* 2005; 103:749–755. [PubMed: 15660400]
28. Patt YZ, Hassan MM, Aguayo A, et al. Oral capecitabine for the treatment of hepatocellular carcinoma, cholangiocarcinoma, and gallbladder carcinoma. *Cancer.* 2004; 101:578–586. [PubMed: 15274071]
29. Romeo S, Huang-Doran I, Baroni MG, Kotronen A. Unravelling the pathogenesis of fatty liver disease: Patatin-like phospholipase domain-containing 3 protein. *Curr Opin Lipidol.* 2010; 21:247–252. [PubMed: 20480550]
30. Lake AC, Sun Y, Li JL, et al. Expression, regulation, and triglyceride hydrolase activity of Adiponutrin family members. *J Lipid Res.* 2005; 46:2477–2487. [PubMed: 16150821]
31. He S, McPhaul C, Li JZ, et al. A sequence variation (I148M) in PNPLA3 associated with nonalcoholic fatty liver disease disrupts triglyceride hydrolysis. *J Biol Chem.* 2010; 285:6706–6715. [PubMed: 20034933]
32. Reddy JK, Rao MS. Lipid metabolism and liver inflammation. II. Fatty liver disease and fatty acid oxidation. *Am J Physiol Gastrointest Liver Physiol.* 2006; 290:G852–G858. [PubMed: 16603729]
33. Teli MR, Day CP, Burt AD, Bennett MK, James OF. Determinants of progression to cirrhosis or fibrosis in pure alcoholic fatty liver. *Lancet.* 1995; 346:987–990. [PubMed: 7475591]
34. Xu L, Han C, Lim K, Wu T. Cross-talk between peroxisome proliferator-activated receptor delta and cytosolic phospholipase A(2)alpha/cyclooxygenase-2/prostaglandin E(2) signaling pathways in human hepatocellular carcinoma cells. *Cancer Res.* 2006; 66:11859–11868. [PubMed: 17178883]

35. Bugianesi E, McCullough AJ, Marchesini G. Insulin resistance: A metabolic pathway to chronic liver disease. *Hepatology*. 2005; 42:987–1000. [PubMed: 16250043]
36. Groop PH, Forsblom C, Thomas MC. Mechanisms of disease: Pathway-selective insulin resistance and microvascular complications of diabetes. *Nat Clin Pract Endocrinol Metab*. 2005; 1:100–110. [PubMed: 16929378]
37. Davis JN, Le KA, Walker RW, et al. Increased hepatic fat in overweight Hispanic youth influenced by interaction between genetic variation in PNPLA3 and high dietary carbohydrate and sugar consumption. *Am J Clin Nutr*. 2010; 92:1522–1527. [PubMed: 20962157]
38. Greenland S, Robins JM. Confounding and misclassification. *Am J Epidemiol*. 1985; 122:495–506. [PubMed: 4025298]

Table 1

Demographic and Epidemiological Characteristics of the Study Population

Demographic and risk factors	HCC patients		Controls		P-value
	N = 257	%	N = 494	%	
Sex					0.5
Male	176	68.5	336	68	
Female	81	31.5	158	32	
Age (yrs old)					0.039
40	13	5.1	20	4	
41–50	28	10.9	84	17	
51–59	67	26.1	149	30.2	
60	149	58	241	48.8	
Mean age ($\pm$ SE)	62.39 $\pm$ 0.78		60.35 $\pm$ 0.51		0.02
Education level					<0.001
<College degree	172	66.9	265	53.6	
College degree	85	33.1	229	46.4	
State of residency					0.1
TX, LA, AK, NM, OK ^a	174	67.7	357	72.3	
Other states	83	32.3	137	27.7	
Hepatitis virus infection					<0.001
HCV–, HBsAg–, Anti-Hbc–	157	61.1	474	96	
HCV+, HBsAg–, Anti-Hbc+	21	8.2	2	0.4	
HCV+, HBsAg–, Anti-Hbc–	54	21	2	0.4	
HCV–, HBsAg+, Anti-Hbc+	9	3.5	2	0.4	
HCV–, HBsAg–, Anti-Hbc+	13	5.1	14	2.8	
HCV+, HBsAg+, Anti-Hbc+	3	1.2	0	0	
Alcohol consumption					<0.001
No	79	30.7	194	39.3	
60 ml ethanol/d	119	46.3	267	54	
>60 ml ethanol/d	59	23	33	6.7	
Cigarette smoking					<0.001

Demographic and risk factors	HCC patients		Controls		P-value
	N = 257	%	N = 494	%	
No	88	34.2	244	49.4	<.001
20 pack yrs	49	19.1	109	22.1	
>20 pack yrs	120	46.7	141	28.5	
Prior history of diabetes					
No	180	70	444	89.9	0.010
1 yr	9	3.5	9	1.8	
>1 yr	68	26.5	41	8.3	
First degree history of liver cancer					
No	249	96.9	491	99.4	0.010
Yes	8	3.1	3	0.6	

^aStates of Texas, Louisiana, Arkansas, New Mexico, and Oklahoma.

Table 2

PNPLA3 Genotypes and the Independent Risk of HCC Development

<i>PNPLA3</i> genotypes (rs738409)	Total population ^a cases/controls		Men ^b cases/controls		Women ^b cases/controls	
	257/494	AOR (95% CI)	176/336	AOR (95% CI)	81/158	AOR (95% CI)
CC	130/274	1 (reference)	85/186	1 (reference)	45/88	1 (reference)
GC	94/197	1.05 (0.71–1.55)	67/134	0.76 (.45–1.26)	27/63	1.30 (0.68–2.48)
GG	33/23	3.28 (1.68–6.42)	24/16	2.87 (1.19–6.91)	9/7	4.44 (1.38–14.33)
CC	130/274	1 (reference)	85/186	1 (reference)	45/88	1 (reference)
GG GC	127/220	1.28 (0.89–1.84)	91/150	1.58 (0.98–2.57)	36/70	1 (0.55–1.81)
CC GC	224/471	1 (reference)	152/320	1 (reference)	72/151	1 (reference)
GG	33/23	3.21 (1.68–6.41)	24/16	3.34 (1.44–7.72)	9/7	3.77 (1.26–11.33)

^aOdds ratios is adjusted for confounding of age, sex, educational level, HCV, HBV, alcohol drinking, cigarette smoking, diabetes mellitus, and family history of cancer.

^bOdds ratios is adjusted for confounding of age, educational level, HCV, HBV, alcohol drinking, cigarette smoking, diabetes mellitus, and family history of cancer.

Table 3Risk Modification of *PNPLA3* Genotypes by HCC Established Risk Factors

Risk variables		Cases/controls	AOR (95% CI)	
HCV/HBV	Genotypes	257/494	Model (1) ^a	P-value
Negative	CC/GC	133/452	1 (reference)	
Positive	CC/GC	91/19	17.64 (9.71–29.82)	<0.001
Negative	GG	24/22	3.41 (1.75–6.63)	<0.001
Positive	GG	9/1	23.03 (2.71–195.82)	0.004
Risk variables		Cases/controls	AOR (95% CI)	
Alcohol	Genotypes	257/494	Model (2) ^b	P-value
No	CC/GC	67/185	1 (reference)	
Yes	CC/GC	157/286	1.60 (1.04–2.46)	0.03
No	GG	12/9	5.18 (1.91–14.05)	0.001
Yes	GG	21/14	3.52 (1.49–8.32)	0.004
Risk variables		Cases/controls	AOR (95% CI)	
Diabetes	Genotypes	257/494	Model (3) ^c	P-value
No	CC/GC	162/424	1 (reference)	
Yes	CC/GC	62/47	3.93 (2.40–6.46)	<0.001
No	GG	18/20	2.65 (1.24–5.69)	0.01
Yes	GG	15/3	19.11 (5.13–71.20)	<0.001

^a Adjusted odds ratio for age, sex, education, diabetes, alcohol drinking, cigarette smoking, and family history of cancer.^b Adjusted odds ratio for age, sex, education, diabetes, HCV, HBV, cigarette smoking, and family history of cancer.^c Adjusted odds ratio for age, sex, education, HCV, HBV, alcohol drinking, cigarette smoking, and family history of cancer.

Table 4**Predictors of Underlying Cirrhosis in Patients With HCC**

Risk factors	No cirrhosis, N = 101 (%)	Cirrhosis, N = 156 (%)	AOR (95% CI)	P-value
Chronic HCV infection (anti-HCV+)				
No	87 (86.1)	92 (59)	1 (reference)	
Yes	14 (13.9)	64 (41)	5.77 (2.93–11.36)	0.000
Chronic HBV infection (HBsAg+)				
No	99 (98)	146 (93.6)	1 (reference)	
Yes	2 (2)	10 (6.4)	5.26 (1.07–25.77)	0.041
Prior history of diabetes mellitus				
Non-diabetic or diabetics for ≤ 5 yrs	91 (90.1)	123 (78.9)	1 (reference)	
>5 yrs with diabetes	10 (9.9)	33 (21.1)	3.87 (1.76–8.55)	0.001
<i>PNPLA3</i> genotyping ^a				
CC/CG	92 (91.1)	132 (84.6)	1 (reference)	
GG	9 (8.9)	24 (15.4)	2.48 (1.05–5.87) ^a	0.039

^a Adjusted odds ratio of *PNPLA3* GG genotype after controlling for age, sex, HCV, HBV, diabetes mellitus, cigarette smoking, and heavy alcohol consumption using stepwise backward logistic regression.

Table 5

Prognostic Factors of Overall HCC Survival

Prognostic variables	Variable label	N	Multivariable HR (95% CI)	P-value
Sex	Female	81	1 (reference)	
	Male	176	1.54 (1.04–2.28)	0.03
HCV	Negative	179	1 (reference)	
	Positive	78	1.34 (0.95–1.90)	0.099
Tumor differentiation ^a	Well	88	1 (reference)	
	Moderate	84	1.50 (1.04–2.69)	0.033
	Poor	46	1.77 (1.17–2.69)	0.007
	0, A, B	83	1 (reference)	
BCLC staging system	C, D	174	2.43 (1.19–4.98)	0.004
ECOC	0, 1	217	1 (reference)	
	2	27	2.56 (1.52–4.35)	<0.0001
	3	13	4.77 (2.39–11.54)	<0.0001
Treatment exposure	No treatment	31	1 (reference)	
	Chemotherapy	88	0.48 (0.29–0.82)	0.007
	Chemotherapy and others	35	0.40 (0.21–0.76)	0.005
	Surgical resection/liver transplant	32	0.21 (0.09–0.48)	<0.0001
	Surgery and others	41	0.26 (0.13–0.49)	<0.000
	TAC ^c	30	0.37 (0.18–0.77)	0.008
	CC/CG	224	1 (reference)	
<i>PNPLA3</i> genotypes (rs7384409) ^b	GG	33	2.11 (1.26–3.52) ^b	0.004

^aMissing information of pathological tumor differentiation for 39 HCC patients.

^bHazard ratio of *PNPLA3* GG genotype is adjusted for age, sex, HCV, HBV, diabetes mellitus, alcohol consumption, tumor differentiation, prior treatment, baseline ECOC performance status, baseline BCLC staging system, and presence of cirrhosis using stepwise backward Cox regression.

^cTranscatheter arterial chemoembolization.