Title:
The impact of patatin-like phospholipase domain-containing protein 3 polymorphism on hepatocellular carcinoma prognosis

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Short title: PNPLA3 SNP and HCC

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ABSTRACT

Background and aim: The single nucleotide polymorphism (SNP) rs738409 in patatin-like phospholipase domain-containing protein 3 (PNPLA3) is associated with hepatic fat accumulation and disease progression in patients with non-alcoholic fatty liver disease and alcoholic liver disease (ALD). This study was conducted to determine whether PNPLA3 rs738409 SNPs affect development and prognosis of hepatocellular carcinoma (HCC) in patients with various liver diseases. **Methods:** We enrolled 638 consecutive Japanese patients newly diagnosed with HCC between 2001 and 2010: 72 patients with hepatitis B virus (HBV), 462 with hepatitis C virus (HCV), and 104 with non-B non-C (NBNC). **Results:** NBNC patients exhibited large tumors of advanced TNM stages at HCC diagnosis, and had significantly poorer prognosis than HBV or HCV patients ($P < 0.001$ and <0.001, respectively; log-rank test). The G/G genotype of PNPLA3 rs738409 SNP had significantly higher distribution in NBNC patients ($P < 0.001$) and was significantly associated with higher body mass index (BMI) and an increased aspartate aminotransferase to platelet ratio index. No significant differences were observed in survival with differences in PNPLA3 SNP genotypes among the patients, although ALD patients with the G/G genotype of PNPLA3 SNP and low BMI had significantly poorer survival than those with high BMI ($P = 0.028$). **Conclusions:** The G/G genotype of PNPLA3 rs738409 SNP was more frequently distributed, and associated with BMI and fibrosis among NBNC-HCC patients while not among HBV or HCV patients. These genotypes might affect on HCC prognosis in ALD patients, but not in HBV, HCV, or NAFLD patients.

List of abbreviations:

HCC, hepatocellular carcinoma; HCV, hepatitis C virus; HBV, hepatitis B virus; NBNC, non-B non-C; NAFLD, non-alcoholic fatty liver disease; PNPLA3, patatin-like phospholipase domain-containing protein 3; SNP, single nucleotide polymorphism; AFP, alpha-fetoprotein; DCP, des-gamma-carboxy prothrombin; BMI, body mass index; APRI, aspartate aminotransferase to platelet ratio index; ALD, alcoholic liver disease

INTRODUCTION

Hepatocellular carcinoma (HCC) is the fifth most common cancer and the third most common cause of cancer-related deaths worldwide (1-3). Various factors, including chronic viral infection, age, sex, alcoholic liver disease (ALD), and diabetes mellitus, are involved in HCC development and progression. Hepatitis C virus (HCV) and/or hepatitis B virus (HBV) infections are the major cause of HCC development in Japan (4-6). Recently, the incidence of HCC without HBV or HCV infection has increased, accounting for 12–16% of HCC patients in Japan (7, 8). Obesity is the leading cause of non-alcoholic fatty liver disease (NAFLD) (9) and represents the second major cause of cancer development in western countries and Japan (10, 11). Almost 25% of all HCC cases in western countries, and 8 to 15% in Japan are estimated to originate from NAFLD (12, 13). Because the presence of effective predictive biomarkers for non-B non-C (NBNC) HCC development in NAFLD patients have not been clarified, NBNC-HCC is often diagnosed in advanced stages with poor prognosis (14).

There is compelling evidence that host genetic polymorphisms are involved in HCC pathogenesis and may be useful as predictive biomarkers (15). The associations of functional genetic polymorphisms of interleukin 6 and tumor necrosis factor alpha with HCC development have been reported (16, 17). In the present study, we focused on the association of patatin-like phospholipase domain-containing protein 3 (PNPLA3) genetic polymorphisms with HCC prognosis. PNPLA3, also called as adiponutrin, is a member of the patatin-like phospholipase family and its activity is regulated by the same hormonal pathways that regulate fat deposition in the liver (18-20). Non-synonymous single nucleotide polymorphism (SNP) rs738409 C>G transversion (Ile148Met) in exon 3 of the gene encoding for PNPLA3 has been reported to be associated with NAFLD development (21). Moreover, the PNPLA3 rs738409 SNP is related to the degree of fibrosis observed in ALD and NAFLD (22-24); however, it is not clear whether this polymorphism affects HCC development and prognosis in NAFLD and ALD. The present study was conducted to determine whether rs738409 C>G non-synonymous PNPLA3 SNP is associated with HCC development and prognosis in HBV, HCV, or NBNC patients.

METHODS

Patients

The present study enrolled 638 consecutive Japanese patients newly diagnosed with HCC at the Okayama University Hospital between 2001 and 2010. Liver disease etiologies were as follows: 462 patients with HCV-related disease, 72 with HBV-related disease, and 104 with non-B non-C (NBNC) disease. NBNC etiologies included metabolic disorders such as NAFLD in 57 patients, ALD in 34 patients, and autoimmune liver diseases and cryptogenic liver injury in 13 patients. Heavy drinking behavior was defined as daily alcohol intake of ≥70 grams for ALD patients, and non-drinking behavior was defined as daily alcohol intake of <20 grams for NAFLD patients. Fifty NAFLD patients without HCC were also included in the study. The study was conducted in accordance with the Helsinki Declaration, and all protocols were approved by the ethics committees of the institutes. All patients provided informed consent before study enrollment.

HCC diagnosis and follow-up

In accordance with the clinical practice manual from the Japan Society of Hepatology (25, 26), HCC diagnosis was accomplished using imaging techniques, including ultrasonography, computed tomography, magnetic resonance imaging, hepatic angiography, and/or tumor biopsy, in combination with the detection of serum levels of alpha-fetoprotein (AFP), AFP-L3, and des-gamma-carboxy prothrombin (DCP). HCC staging conformed to the standards of general rules for clinical and pathological study of primary liver cancer by the liver cancer study group of Japan (27). The cancer was treated following suitable measures according to the treatment algorithm for HCC in the clinical practice manual from the Japan Society of Hepatology (18, 19). After curative treatment of primary HCC, all patients underwent liver function tests every 1–3 months and ultrasonography or dynamic study every 3–4 months. Serum levels of AFP, AFP-L3, and DCP were also determined every 1–3 months. HCC recurrence was diagnosed using the same criteria as those used for primary HCC diagnosis. HCC recurrence was treated following suitable measures.

SNP genotyping

Genomic DNA was extracted from whole blood samples using the QIAamp DNA Mini Kit according to the manufacturer’s protocol (Qiagen, Tokyo, Japan). PNPLA3 SNP was genotyped using the TaqMan predesigned SNP genotyping assays, as recommended by the manufacturer (Applied Biosystems, Tokyo, Japan).

Statistical analysis

Data are expressed as mean ± standard deviation or median (range). Patient laboratory data were compared among the groups using the Kruskal–Wallis test. Survival rates of patients were estimated using the Kaplan–Meier method and compared with the log-rank test. $P < 0.05$ was considered significant. Statistical analysis was performed using JMP software (SAS Institute, Cary, NC, USA).

RESULTS

Patient characteristic at diagnosis

HCC development occurred at a much younger age in HBV patients than in HCV or NBNC patients ($P < 0.001$, Kruskal–Wallis test, Table 1). Majority of NBNC and HBV patients were male ($P < 0.001$). Body mass index (BMI) of NBNC patients was significantly higher than that of HBV or HCV patients ($P = 0.001$). The NBNC patients also exhibited the highest incidence of diabetes mellitus (38.5%). The incidence of diabetes mellitus was much lower among HCV (18.8%) and HBV patients (4.0%, lowest, $P < 0.001$). In terms of drinking behavior, 32.7% of NBNC patients drank heavily, while only 5.5% of HBV patients and 11.0% of HCV patients ($P < 0.001$) were heavy drinkers. No significant differences were observed among the experimental groups regarding the ratio of patients with Child-Pugh A ($P = 0.44$). Significantly higher serum levels of aspartate aminotransferase and alanine aminotransferase as well as lower platelet counts were observed in HCV patients ($P < 0.001$, 0.003, and <0.001, respectively). Gamma-glutamyl transpeptidase was significantly higher in NBNC patients than in HBV or HCV patients ($P < 0.001$).

HCC diagnosis and prognosis

At diagnosis, NBNC patients exhibited a higher incidence of large HCC tumors of advanced TNM stages as well as higher serum DCP levels than HBV or HCV patients ($P < 0.001$, Table 1). After curative treatment of primary HCC, all patients underwent periodical liver function and imaging tests at follow-up. The mean follow-up period was approximately 3.0 years (range, 0.1–9.5 years). This period was defined as the time between diagnosis and death or the latest confirmation of survival. The accumulated observation time was 1914 person-years. The survival rate of NBNC patients was significantly lower than that of HBV or HCV patients ($P = 0.011$, log-rank test, Figure 1), with an estimated 68.7% survival at 3 years and 41.8% survival at 5 years. The survival of HCV, or NBNC patients was not dependent on TNM stage (Figure 2). The survival of HBV patients on TNM stage Ⅱ was significantly better than that of HCV or NBNC patients ($P = 0.044$).

Subgroup analysis based on PNPLA3 rs738409 SNP

The ratio of patients with the G/G genotype of PNPLA3 SNP was significantly greater among NBNC patients (46.2%) than among HBV or HCV patients (16.7% and 24.0%, respectively, $P < 0.001$, Table 1). Age and gender did not significantly correlate with PNPLA3 SNP genotype distribution among HBV, HCV, or NBNC patients. Among NBNC patients, patients with the G/G genotype of PNPLA3 SNP had significantly higher BMI than those with the C/C or C/G genotype ($P < 0.001$, Table 2), while no significant correlation was

observed between HBV or HCV patients regarding BMI and PNPLA3 SNP genotypes. As for laboratory data, NBNC patients with the G/G genotype exhibited slight tendency of smaller tumors (P = 0.047), and higher aspartate aminotransferase to platelet ratio index (APRI) (P = 0.054) than those with C/C or C/G genotype; however, no significant differences were observed in the TNM stage and serum levels of AFP and DCP. We also compared patient survival to the distribution of the different PNPLA3 SNP genotypes (Figure 3). No significant correlation was observed between survival and the genotypes among HBV, HCV or NBNC patients. For HBV patients, the viral status including HBe antigen, HBs antigen, and HBV DNA at diagnosis did not differ based on the different PNPLA3 SNP genotypes. We further investigated ALD or NAFLD patients among NBNC patients. The ratio of patients with the G/G genotype of PNPLA3 SNP was significantly greater among NAFLD patients (56.1%) than the other groups with ALD or other etiologies (35.3% and 30.8%, respectively, P = 0.046, Table 3). As presented in Figure 4, ALD patients with the G/G genotype of PNPLA3 SNP who had BMI of <25 kg/m^2 exhibited significantly lower survival rates than those who had BMI of ≥25 kg/m^2 (P = 0.028, log-rank test). A similar association was not observed for ALD patients with the C/C or C/G genotype or NAFLD patients. To investigate the association of PNPLA3 SNP with carcinogenesis, the NAFLD patients with HCC were compared to those without HCC in the prevalence of the G/G genotype of PNPLA3 SNP. The genotypes of PNPLA3 SNP in the NAFLD patients without HCC were C/C in 3 patients (6.0%), C/G in 22 patients (44.0%), and G/G in 25 patients (50.0%), which did not have significant difference from those in the NAFLD patients with HCC (P = 0.61).

DISCUSSION

HCC is commonly associated with HBV or HCV infections; however, the incidence of NBNC-HCC with NAFLD or ALD has increased significantly in recent years (12, 13). The incidence of NBNC-HCC in the present study support the trend that non-viral associated HCC is increasing as the ratio of NBNC-HCC has increased from 11.8% between 2001 and 2005 to 15.1% between 2006 and 2010. Several studies have attempted to elucidate useful predictors for NBNC-HCC; however, these studies were not completely successful. Recent genome-wide association studies revealed that PNPLA3 rs738409 SNPs are associated with hepatic steatosis and fibrosis (21, 28). PNPLA3 (rs738409 C>G) SNP can modulate hepatic inflammation and fibrosis progression (29). The G allele of PNPLA3 SNP is associated with increased steatosis (30-32) and severe fibrosis (22, 27, 33, 34 and 35). Liver cirrhosis and steatohepatitis are associated with carcinogenesis and increased susceptibility to HCC development; however, no published studies have focused on the role of PNPLA3 SNP genotypes in HCC development and prognosis. Therefore, we investigated PNPLA3 SNP genotypes in HCC patients to clarify the importance of this polymorphism as a predictor for HCC development and prognosis.

Our results of patient characteristic at HCC diagnosis revealed that NBNC-HCC patients exhibited a significantly higher incidence of the G/G genotype than HCV or HBV patients with a higher incidence of diabetes mellitus, obesity, and alcoholic liver injury, supporting the results of previous studies (36-38). Furthermore, subgroup analysis revealed that the NBNC-HCC patients with the G/G genotype had higher BMI and APRI, reflecting obesity and severe fibrosis, than those with the other genotypes, and that the patients with NAFLD had a higher frequency of the G/G genotype than those with ALD. In contrast, among HBV or HCV patients with HCC, the presence of obesity or severe hepatic fibrosis showed no significant correlation with the PNPLA3 SNP genotypes. Therefore, these results indicated that among the patients with HCC, the G/G genotype of PNPLA3 SNP is strongly associated with NAFLD with obesity and severe fibrosis, which is a similar situation in those without HCC in the previous studies.

Our results of HCC prognosis clearly showed that no significant association was observed between HCC prognosis and the PNPLA3 SNP genotypes in HCV or NAFLD patients. Interestingly, subgroup analysis of ALD patients indicated prognostic differences by the genotypes of PNPLA3 SNP; the ALD patients with the G/G genotype of PNPLA3 SNP and high BMI might maintain body weight against HCC progression and malnutrition, and resulted in better prognosis, by comparing to the ALD patients with low BMI, although the number of the ALD patients might be relatively small.

In conclusion, our results indicate that differences in the distribution of PNPLA3 SNP

genotypes have important and independent roles in the regulation of various liver disease etiologies and HCC prognosis. While the G/G genotype showed greater distribution in NBNC-HCC patients, only among ALD patients did the G/G genotypes but not the C/C and C/G genotype was associated with HCC prognosis. More research is necessary to further clarify the exact manner in which differences in expression of PNPLA3 SNP genotypes may regulate liver etiology as well as HCC development and prognosis. Longitudinal study of HCC development in patients without HCC is required.

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DISCLOSURE STATEMENT

The authors have no conflict of interest.

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FIGURE LEGENDS

Figure 1. The patient survival rates in the three groups

The patient survival rate was presumed for each group by the Kaplan–Meyer method. The survival of NBNC patients was significantly poorer than that of HBV or HCV patients (P = 0.011, log-rank test), and it was estimated as 68.7% at 3 years and 41.8% at 5 years.

Figure 2. The comparison of survival rates among the three groups in different TNM stages

By dividing the patients based on the TNM stage at diagnosis, the patient survival rate was compared among the three groups of HBV, HCV, and NBNC etiology for each TNM stage. No prognostic differences were observed among the three groups for TNM stage Ⅰ, Ⅲ, or Ⅳ (P = 0.13, 0.36, and 0.64, respectively, log-rank test). The survival of HBV patients on TNM stage Ⅱ was significantly better than that of HCV or NBNC patients (P = 0.044).

Figure 3. The comparison of survival rates based on the different PNPLA3 SNP genotypes in the three groups

The survival rates in relation to the different PNPLA3 SNP genotypes were compared for the three groups of HBV, HCV, and NBNC etiology. The PNPLA3 SNP genotype was not significantly related to survival rates among HBV, HCV, or NBNC patients.

Figure 4. The comparison of survival rates based on different BMI for NBNC patients with ALD or NAFLD

The impact of PNPLA3 SNP on survival was evaluated by BMI for NBNC patients with ALD or NAFLD. Among ALD patients with the G/G genotype, patients with BMI of <25 kg/m^2 had significantly poorer survival than those with BMI of ≥25 kg/m^2 (P = 0.028, log-rank test). ALD patients with the C/C or C/G genotype of PNPLA3 or NAFLD patients showed no prognostic difference in BMI.

Table 1: Patient characteristics

	HBV (n = 72)	HCV (n = 462)	NBNC (n = 104)	*P*
Age (years)	61 (38–87)[+]	75 (42–93)[+]	71 (27–91)[+]	<0.0001
Gender (female/male)	11/61	151/311	22/82	<0.0001
Diabetes mellitus (yes)	3	87	40	<0.0001
Heavy drinking behavior (yes)	4	51	34	<0.0001
Body mass index	23.2 ± 3.5[‡]	22.7 ± 3.1[‡]	24.6 ± 4.3[‡]	0.0001
PNPLA rs738409 (CC/CG/GG)	20/40/12	125/226/111	17/39/48	<0.0001
Child-Pugh score (A/B/C)	57/13/2	332/118/12	83/19/2	0.14
APRI	4.5 ± 3.8[‡]	7.0 ± 7.0[‡]	4.6 ± 4.6[‡]	<0.0001
TNM stage (1/2/3/4)	24/24/13/11	161/155/111/35	22/32/24/26	<0.0001
Tumor size (mm)	22 ± 3.6[‡]	20 ± 0.9[‡]	32 ± 4.0[‡]	<0.0001
Tumor number (solitary)	36	260	52	0.36
Laboratory data				
ALT (IU/l)	40 ± 23[‡]	57 ± 39[‡]	46 ± 44[‡]	0.003
γGTP (IU/l)	103 ± 129[‡]	85 ± 95[‡]	143 ± 146[‡]	<0.0001
Albumin (g/dl)	3.9 ± 0.6[‡]	3.6 ± 0.5[‡]	3.7 ± 0.5[‡]	<0.0001
Total bilirubin (mg/dl)	1.1 ± 0.7[‡]	1.0 ± 0.6[‡]	1.1 ± 0.7[‡]	0.15
Platelet count ($\times 10^4$/μl)	16.2 ± 8.7[‡]	12.4 ± 5.9[‡]	13.4 ± 6.2[‡]	<0.0001
AFP (ng/ml)	10 (0.9–67951)[+]	19 (0.5–85292)[+]	26 (2–455560)[+]	0.54
DCP (mAU/ml)	46 (2–410500)[+]	36 (10–708400)[+]	126 (11–1323600)[+]	<0.0001
Anti-HBc antibody (yes)		135	35	0.44

[+]: Median (range); [‡]: Mean ± standard deviation. TNM, Tumor–node–metastasis stage according to the Liver Cancer Study Group of Japan; APRI, aspartate aminotransferase to platelet ratio index; ALT, alanine aminotransferase; γGTP, γ−glutamyl transpeptidase; AFP, Alpha-fetoprotein; DCP, des-gamma-carboxy prothrombin.

Table 2: Characteristics of NBNC patients with PNPLA3 rs738409 polymorphisms

rs738409 SNP genotype	C/C or C/G (n = 56)	G/G (n = 48)	*P*
Age (years)	70 (27–91)[+]	72 (46–83)[+]	0.85
Gender (female/male)	10/46	12/36	0.26
Diabetes mellitus (yes)	20	20	0.34
Heavy drinking behavior (yes)	22	12	0.09
Body mass index	23.6 ± 3.2[‡]	26.3 ± 3.8[‡]	<0.0001
Child-Pugh score (A/B/C)	44/8/4	39/6/3	0.46
APRI	2.4 ± 2.2[‡]	3.6 ± 3.1[‡]	0.054
TNM stage (1/2/3/4)	13/15/9/19	7/14/14/13	0.21
Tumor size (mm)	38 (10–200)	28 (5–100)	0.047
Laboratory data			
ALT (IU/l)	38 ± 34[‡]	40 ± 22[‡]	0.66
γGTP (IU/l)	101 ± 95[‡]	85 ± 82[‡]	0.68
Albumin (g/dl)	3.7 ± 0.6[‡]	3.7 ± 0.5[‡]	0.92
Total bilirubin (mg/dl)	0.9 ± 0.5[‡]	1.0 ± 0.7[‡]	0.35
Platelet count ($\times 10^4$/μl)	16.1 ± 6.9[‡]	13.7 ± 6.7[‡]	0.08
AFP (ng/ml)	8.1 (0.9–67951)[+]	10.1 (2–37387)[+]	0.34
DCP (mAU/ml)	183.5 (16–472100)[+]	100 (11–1323600)[+]	0.33
Anti-HBc antibody (yes)	17	18	0.29

[+]: Median (range). [‡]: Mean ± standard deviation. TNM, Tumor–node–metastasis stage according to the Liver Cancer Study Group of Japan; APRI, aspartate aminotransferase to platelet ratio index; ALT, alanine aminotransferase; γGTP, γ−glutamyl transpeptidase; AFP, Alpha-fetoprotein; DCP, des-gamma-carboxy prothrombin.

Table 3: Frequency of PNPLA3 rs738409 polymorphisms in NBNC patients

rs738409 SNP genotype	C/C (n = 17)	C/G (n = 39)	G/G (n = 48)
NAFLD	5 (8.8%)	20 (35.1%)	32 (56.1%)
Alcoholic liver disease	7 (20.6%)	15 (44.1%)	12 (35.3%)
Disease for other etiologies	5 (38.4%)	4 (30.8%)	4 (30.8%)

PNPLA3, patatin-like phospholipase domain-containing protein 3; NBNC, non-B non-C; SNP, single nucleotide polymorphism; NAFLD, non-alcoholic fatty liver disease.