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# Association of PNPLA3 SNP With the Development of HBV-related Hepatocellular Carcinoma

HIRAYUKI ENOMOTO, NOBUHIRO AIZAWA, NAOTO IKEDA, TOMOYUKI TAKASHIMA, YUKIHISA YURI, MAMIKO OKAMOTO, RYOTA YOSHIOKA, SHOKI KAWATA, KOHEI YOSHIHARA, SHOGO OTA, RYOTA NAKANO, HIDEYUKI SHIOMI, TAKASHI NISHIMURA and HIROKO IIJIMA

*Division of Hepatobiliary and Pancreatic Disease, Department of Internal Medicine, Hyogo Medical University, Nishinomiya, Japan*

**Abstract.** *Background/Aim: Concomitant nonalcoholic fatty liver disease (NAFLD)/hepatic steatosis (HS) is suggested to increase the risk of hepatocellular carcinoma (HCC) in hepatitis virus B (HBV)-infected patients. Patatin-like phospholipase domain-containing 3 (PNPLA3) rs738409 gene single-nucleotide polymorphism (SNP) is well-known to be associated with the development of NAFLD/HS; however, it is still unclear whether this SNP is related to the development of HCC in HBV-infected patients. Patients and Methods: We investigated a total of 202 HBV-infected patients who received percutaneous liver biopsy, and simultaneously assessed biopsy-proven HS, insulin resistance, and the PNPLA3 SNP status. We further investigated the relationships of these factors with the development of HCC in HBV-infected patients. Results: Most of the enrolled cases (196/202: 97.0%) were non-cirrhotic patients. One hundred seventy-three patients (85.6%) received antiviral therapy. A Kaplan-Meier analysis showed that the incidence of HCC development in patients with HS was higher than that in patients without HS (p<0.01). An increased homeostasis model assessment as an index of insulin resistance (HOMA-IR) value (≥1.6) was associated not only with the presence of HS (p<0.0001) but also with the development of HCC (p<0.01). The PNPLA3 rs738409 SNP was also associated with the presence of HS (p<0.01) and the development of HCC (p<0.05) in HBV-infected patients. Conclusion: In addition to HS and IR, PNPLA3 rs738409 SNP was suggested to be associated with the development of HCC in Japanese patients with HBV infection.*

*Correspondence to*: Hirayuki Enomoto, Division of Hepatobiliary and Pancreatic Disease, Department of Internal Medicine, Hyogo Medical University, Mukogawa-cho1-1, Nishinomiya, Hyogo 663-8501, Japan. Tel: +81 798456472, Fax: +81 798456474, e-mail: enomoto@hyo-med.ac.jp

*Key Words:* HBV, hepatocellular carcinoma, hepatic steatosis, HOMA-IR, PNPLA3.



Hepatitis B virus (HBV) infection is a worldwide health concern, especially in Asia and Africa (1-3). In light of remarkable advances in the treatment of viral hepatitis, including HBV and hepatitis C virus, the World Health Organization (WHO) launched a plan to eliminate hepatitis viruses by 2030 (3). On the other hand, because of recent changes in lifestyle, the clinical importance of non-viral chronic liver diseases, including nonalcoholic fatty liver disease (NAFLD)/hepatic steatosis (HS) has been increasing (4-9). Regarding the development of HS, metabolic disorders, particularly insulin resistance (IR), play an important role; however, genetic factors are also known to be related to the development of HS (10-12). Among various gene single-nucleotide polymorphisms (SNPs), patatin-like phospholipase domain-containing 3 (*PNPLA3*) rs738409 (C>G, I148M) is a well-known NAFLD/HS-related SNP (13). We investigated the clinical role of HS in HBV-infected patients and reported that PNPLA3 SNP was associated not only with the development of HS but also with the treatment efficacy of antiviral therapy for HBV-infected patients (14).

HBV-infection is a well-recognized risk factor for the development of hepatocellular carcinoma (HCC) (15-17), and concomitant HS is suggested to increase the risk of developing HCC in HBV-infected patients (18). *PNPLA3* SNP and IR have been suggested to play cooperative roles in the development of fatty liver disease (19, 20); however, it is still uncertain whether *PNPLA3* SNP is related to the development of HCC in HBV-infected patients (21-25). In addition, there are few studies in which biopsy-proven HS, IR and *PNPLA3* SNP were simultaneously evaluated in HBV-infected patients. In the present study, we simultaneously

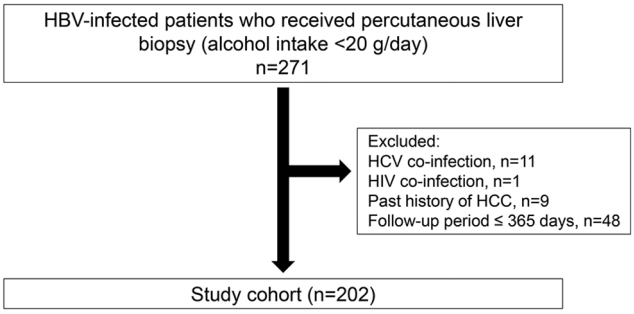


Figure 1. *Study patients. HBV: Hepatitis B virus; HCV: hepatitis C virus; HIV: human immunodeficiency virus; HCC: hepatocellular carcinoma.*

assessed these three factors (HS, IR and *PNPLA3* SNP) and investigated their associations with the development of HCC in HBV-infected patients.

## Patients and Methods

*Patients*. We retrospectively investigated chronic HBV-infected patients who received percutaneous liver biopsy in our department between August 2010 and March 2020, and who were followed up for more than one year after the biopsy. Patients who were positive for HBsAg for more than six months were defined as having chronic HBV infection. Patients with alcohol intake (≥20 g/day), past history of HCC, HIV co-infection, or HCV co-infection were excluded from the study (Figure 1). The current research was conducted under the ethical approval of our institutional review board (Nos. 1831 and Hi-92). Written informed consent was obtained from all of the enrolled patients before the liver biopsy for the use of their clinical data and genomic samples in research.

*Liver biopsy and laboratory data*. Ultrasound-guided percutaneous liver biopsy was conducted according to the standard methods (26, 27). The stages of liver fibrosis were determined by the METAVIR scoring system; namely, F0 stage (no fibrosis), F1 stage (portal fibrosis without septa), F2 stage (portal fibrosis with rare septa), F3 stage (numerous septa without cirrhosis), and F4 stage (cirrhosis) (28). The degree of HS was also histologically assessed, and the presence of HS was defined as HS ≥5% (29). The histological findings were evaluated by external expert pathologists who did not receive any clinical information (SMC Laboratories, Inc., Tokyo, Japan).

Regarding blood tests, general liver functional variables, including total bilirubin, aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), platelet count, prothrombin time (PT), and albumin were measured. To detect HBV infection, we tested for HBeAg positivity and measured the HBV-DNA titer. We also measured metabolic variables, including total cholesterol, triglyceride, glucose, and immunoreactive insulin (IRI). We calculated the homeostasis model assessment as an index of insulin resistance (HOMA-IR) according to the following formula: HOMA-IR=Fasting plasma glucose (mg/dl)×IRI (μU/ml)/405 (Japanese normal value <1.6) (30, 31). All blood samples were collected on the day of liver biopsy under fasting conditions.

Table I. *Basic clinical characteristics of the patients (N=202).*

| | |
|---|---|
| Age (years) | 45 (37-57) |
| Sex (Male/Female) | 107/95 |
| AST (U/l) | 24 (19-33.25) |
| ALT (U/l) | 23 (15.75-42.25) |
| γ-GTP (U/l) | 20 (15-32) |
| ALP (U/l) | 202 (166.75-240) |
| Total bilirubin (mg/dl) | 0.8 (0.6-1.1) |
| Albumin (g/dl) | 4.0 (3.8-4.2) |
| Platelet ($\times10^3$/mm$^3$) | 188 (158.5-230.25) |
| Prothrombin time (%) | 89.0 (83.3-96.625) |
| HBeAg (+/–) | 71/131 |
| HBV genotype (A/B/C/D/ND) | 8/22/163/2/7 |
| HBV-DNA (Log copies/ml) | 2.75 (LLOQ -5.4) |
| Antiviral therapy at the biopsy (+/–) | 95/107 |
| Antiviral therapy after the biopsy (+/–) | 173/29 |
| Liver fibrosis stage (F0-1/F2/F3/F4) | 119/47/30/6 |

AST: Aspartate aminotransferase; ALT: alanine aminotransferase; γ-GTP: γ-glutamyl transpeptidase; ALP: alkaline phosphatase; HBV: Hepatitis B virus; LLOQ: Lower limit of quantification; ND: not determined.

*Genotyping of PNPLA3 gene polymorphisms*. Genomic DNA was obtained from peripheral mononuclear cells and stored until use at –20˚C. The PNPLA3 SNP C>G (rs738409) was determined with a standard real-time PCR method (TaqMan® SNP Assays; Thermo Fisher Scientific Japan, Catalogue No. 4351379; Assay ID: C__7241_10) according to the manufacturer's instructions (14).

*Statistical analysis*. Quantitative variables are shown as the median [interquartile range (IQR)], and the statistical significance of differences between two groups was evaluated using the Mann-Whitney *U*-test. The chi-squared test or Fisher's exact test was used as appropriate to analyze the statistical significance of differences in frequency between groups. The frequency trends among three groups were determined using the Cochran-Armitage test. The incidence of HCC occurrence after liver biopsy was analyzed using the Kaplan-Meier method. *p*-Values of <0.05 were considered to be statistically significant.

## Results

*Basic characteristics of the HBV-infected patients*. Among HBV-infected patients who received a liver biopsy in our department, we analyzed the cases who met the criteria described in the *Patients and Methods* section. The basic clinical characteristics are shown in Table I. Among the total of 202 patients, 107 (53.0%) were men and 95 (47.0%) were women. The majority of the enrolled cases (163/202: 80.7%) was infected with HBV-genotype C. A total of 95 patients were treated with nucleotide/nucleoside analogues at the time of biopsy. One hundred seventy-three patients (85.6%) received antiviral therapy (treatments with interferon and/or nucleotide/nucleoside analogues); HBeAg-negative HBV-carriers with a low viral load (<2,000 IU/ml) did not receive

Table II. *Comparison of general variables in patients with and without hepatic steatosis.*

| | Hepatic steatosis | | |
|---|---|---|---|
| | Absent (N=152) | Present (N=50) | *p*-Value |
| Age (years) | 45 (37-57) | 54 (38-56.25) | NS |
| Sex (Male/Female) | 79/73 | 28/22 | NS |
| AST (U/l) | 23 (19-31.75) | 30 (22-38.5) | <0.05 |
| ALT (U/l) | 22 (14-32.75) | 36 (22-63) | <0.05 |
| γ-GTP (U/l) | 17 (14-26.5) | 30 (22-42) | <0.01 |
| ALP (U/l) | 191 (156.5-229) | 216 (187.25-256) | <0.05 |
| Total bilirubin (mg/dl) | 0.85 (0.6-1.1) | 0.8 (0.6-1.0) | NS |
| Albumin (g/dl) | 4.05 (3.7-4.3) | 4.0 (3.8-4.2) | NS |
| Platelet ($\times 10^3/mm^3$) | 186 (153-229.5) | 185 (169.75-240.25) | NS |
| Prothrombin time (%) | 89.7 (83-96) | 89.0 (84.375-95.1) | NS |
| HBeAg (+/–) | 53/99 | 18/32 | NS |
| HBV genotype (A/B/C/D/ND) | 7/14/125/2/4 | 1/8/38/0/3 | NS |
| HBV-DNA (Log copies/ml) | 2.35 (LLOQ-4.975) | 3.5 (LLOQ-6.55) | NS |
| Antiviral therapy at the biopsy (+/–) | 75/77 | 20/30 | NS |
| Antiviral therapy after the biopsy (+/–) | 132/20 | 41/9 | NS |
| Liver fibrosis stage (F0-1/F2/F3/F4) | 96/35/18/3 | 23/12/12/3 | NS |

AST: Aspartate aminotransferase; ALT: alanine aminotransferase; γ-GTP: γ-glutamyl transpeptidase; ALP: alkaline phosphatase; HBV: Hepatitis B virus; NS: Not significant; LLOQ: lower limit of quantification.

antiviral therapy. Most of the enrolled patients were non-cirrhotic (F0-F3 stages; 196/202: 97.0%), HCC developed in six patients during the follow-up period (mean observation period: 5.77 years).

*Association of hepatic steatosis with the development of HCC in HBV-infected patients*. The characteristics of patients with or without HS are shown in Table II. In our cohort, the distribution of the liver fibrosis stages in the two groups did not differ to a statistically different significant extent; however, the Kaplan-Meier method showed that the rate of HCC development in patients with HS was higher in comparison to patients without HS (Figure 2). When we performed a sub-analysis of cases that were followed for more than five years (N=123), the development of HCC within 5 years after liver biopsy was more frequently observed in patients with HS than in patients without HS (Figure 3).

Based on the association of HS with the development of HCC, we next investigated the association of HS-related factors with the development of HCC. In the present study, we focused on HOMA-IR and *PNPLA3* SNP, as a recent study suggested that *PNPLA3* SNP and HOMA-IR have different and important roles in the development of fatty liver disease (19, 20).

*Association of insulin resistance with the presence of hepatic steatosis and the development of HCC in HBV-infected patients*. We evaluated the relationship between HOMA-IR and the presence of histologically-proven HS (≥5%). The

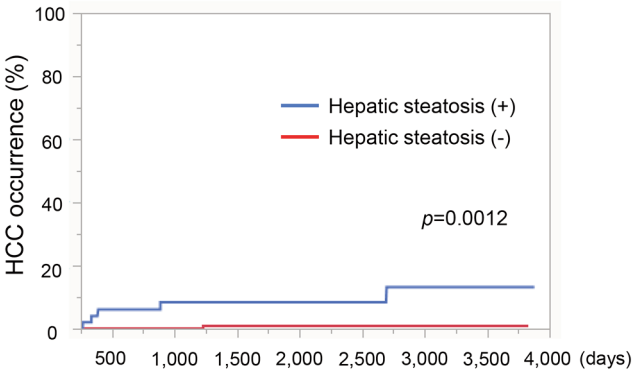


Figure 2. *The association of hepatic steatosis with the development of hepatocellular carcinoma (HCC) in hepatitis B virus-infected patients. Two hundred two HBV-infected patients who received a liver biopsy were analyzed. A Kaplan-Meier analysis showed that the rate of HCC development in patients with hepatic steatosis was significantly higher than that in patients without hepatic steatosis.*

HOMA-IR values were higher in patients with HS than in patients without HS (Table III). Patients with increased HOMA-IR values had a higher frequency of HS than patients without increased HOMA-IR values (Figure 4). In addition, the Kaplan-Meier analysis showed that high HOMA-IR was related to the development of HCC (Figure 5). When we performed a sub-analysis of cases that were followed for more than five years, high HOMA-IR values were associated with the development of HCC within 5 years after liver biopsy (Figure 6).

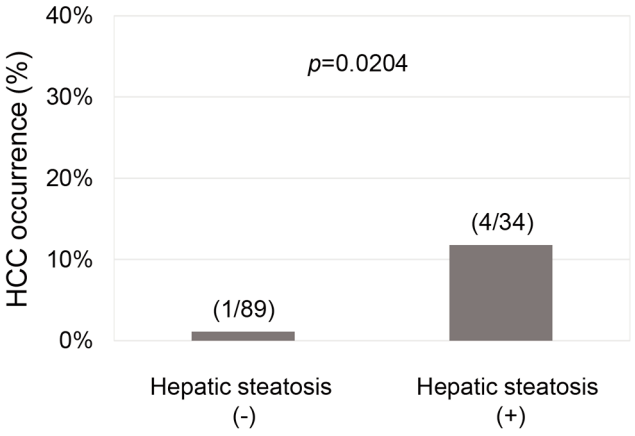


Figure 3. *The sub-analysis of the occurrence of hepatocellular carcinoma (HCC) in hepatitis B virus -infected patients with or without hepatic steatosis. We performed a sub-analysis of patients who were followed for more than five years (N=123) and found that the development of HCC within 5 years after liver biopsy more frequently occurred in patients with hepatic steatosis than in patients without hepatic steatosis.*

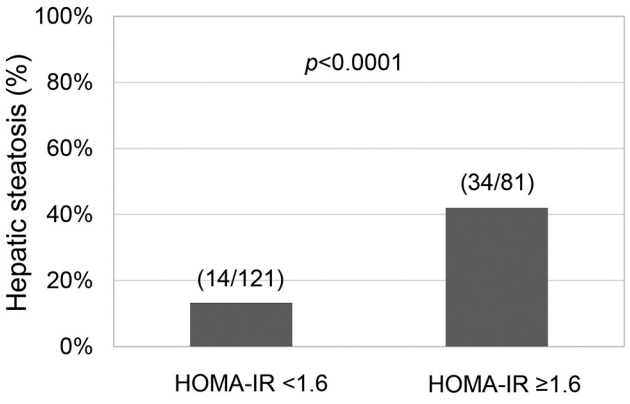


Figure 4. *The association of the homeostasis model assessment as an index of insulin resistance (HOMA-IR) value with the presence of hepatic steatosis in hepatitis B virus-infected patients. We evaluated the relationship between HOMA-IR and the presence of the histologically-proven hepatic steatosis. The frequency of hepatic steatosis in patients with an increased HOMA-IR (≥1.6) value was higher than that in patients with a normal HOMA-IR value (<1.6).*

*Association of PNPLA3 SNPs with the presence of hepatic steatosis and the development of HCC in HBV-infected patients*. We also evaluated whether *PNPLA3* SNP was related to the presence of histologically-evaluated HS. *PNPLA3* mutation was related to an increased frequency of HS (Figure 7). We further assessed the association of *PNPLA3* SNP with the development of HCC. The Kaplan-Meier method showed that *PNPLA3* SNPs were related to the development of HCC (Figure 8). Our sub-analysis

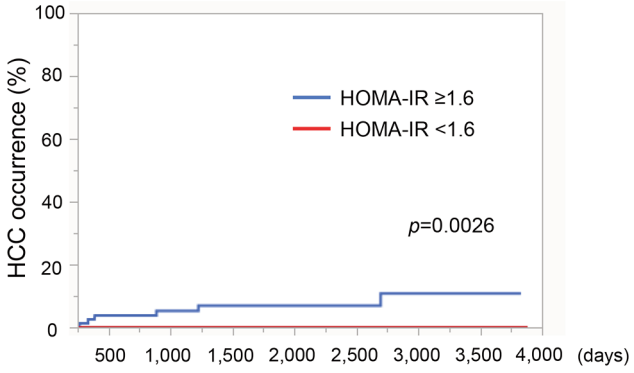


Figure 5. *The association of homeostasis model assessment as an index of insulin resistance (HOMA-IR) with the development of hepatocellular carcinoma (HCC) in hepatitis B virus-infected patients. The Kaplan-Meier analysis showed that the incidence of HCC in patients with an increased HOMA-IR (≥1.6) value was higher than that in patients with a normal HOMA-IR value (<1.6).*

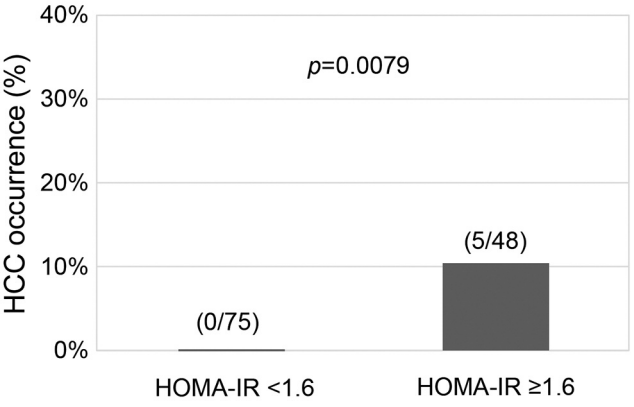


Figure 6. *The sub-analysis of the association of homeostasis model assessment as an index of insulin resistance (HOMA-IR) with the development of hepatocellular carcinoma (HCC) in hepatitis B virus-infected patients. We performed a sub-analysis of patients who were followed for more than five years (N=123) and found that high HOMA-IR values were associated with the development of HCC within 5 years after liver biopsy.*

revealed that *PNPLA3* SNP was associated with the development of HCC within 5 years after liver biopsy (Figure 9).

## Discussion

HBV-infection is an important cause of HCC (15-17). Because of recent changes in lifestyle, the prevalence of HS has been increasing, and concomitant HS and HBV-infection is suggested to increase the risk of developing HCC, although this conclusion may still be under debate (32). The development of HS is mainly influenced by lifestyle-related

Table III. *Comparison of non-viral variables in patients with or without hepatic steatosis.*

| | Hepatic steatosis | | |
|---|---|---|---|
| | Absent (N=152) | Present (N=50) | *p*-Value |
| BMI (kg/m$^2$) | 21.8 (20.1-23.5) | 24.8 (22.7-27.0) | <0.01 |
| Glucose (mg/dl) | 88 (83-92) | 90.5 (86.75-101.25) | <0.01 |
| Triglyceride(mg/dl) | 84.5 (66.25-104.5) | 120 (79.25-160.25) | <0.01 |
| Total cholesterol (mg/dl) | 173.5 (155.25-200.5) | 183 (162-201) | NS |
| IRI (μU/ml) | 6.01 (4.39-8.15) | 8.425 (6.83-11.25) | <0.01 |
| HOMA-IR | 1.31 (0.90-1.79) | 1.92 (1.49-2.63) | <0.01 |
| PNPLA3 rs738409 (CC/CG/GG) | 50/76/26 | 9/21/20 | <0.01 |

BMI: Body mass index; IRI: immunoreactive insulin; HOMA-IR: homeostasis model assessment-insulin resistance; PNPLA3: patatin-like phospholipase domain-containing 3.

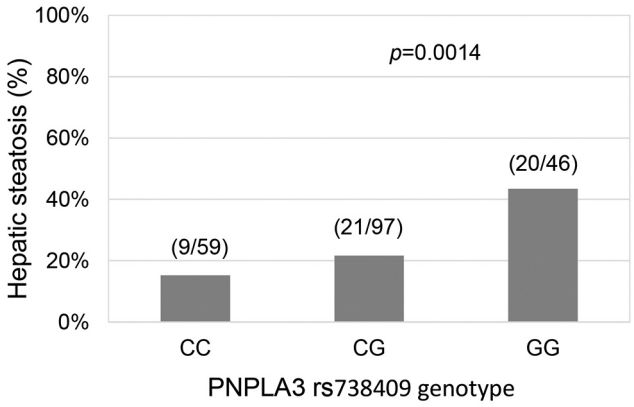


Figure 7. *The association of patatin-like phospholipase domain-containing 3 (PNPLA3) single-nucleotide polymorphism (SNP) with the presence of hepatic steatosis. We evaluated whether PNPLA3 SNP was related to the presence of histologically-proven hepatic steatosis. PNPLA3 mutation was associated with an increased frequency of hepatic steatosis.*

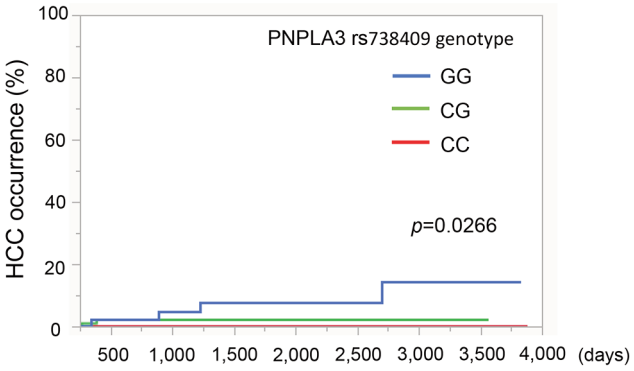


Figure 8. *Association of patatin-like phospholipase domain-containing 3 (PNPLA3) single-nucleotide polymorphism with the development of hepatocellular carcinoma (HCC) in hepatitis B virus-infected patients. The Kaplan-Meier analysis showed that PNPLA3 SNP was related to the development of HCC in HBV-infected patients.*

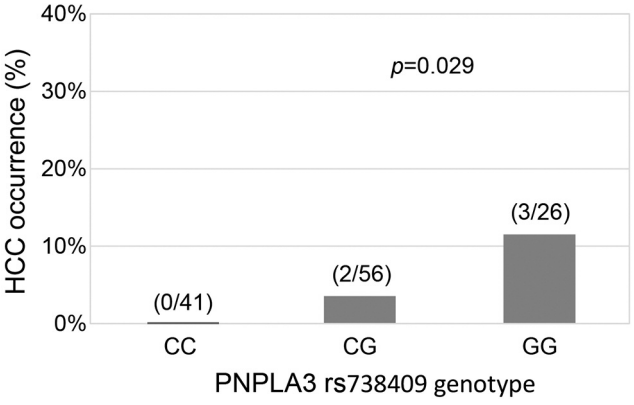


Figure 9. *Sub-analysis of the association of patatin-like phospholipase domain-containing 3 (PNPLA3) single-nucleotide polymorphism (SNP) with the development of hepatocellular carcinoma (HCC) in hepatitis B virus-infected patients. We performed a sub-analysis of patients who were followed for more than five years (N=123) and found that PNPLA3 SNP was associated with the development of HCC within 5 years after liver biopsy.*

factors, including IR; however, genetic factors are also known to be clinically relevant, and *PNPLA3* SNP is well-known to be a disease-sensitive gene, and is suggested to have an IR-independent clinical role in the development of NAFLD (19). In agreement with a previous report (18), the presence of HS was associated with the development of HCC in HBV-infected patients. In addition, our present results also suggest that abnormal HOMA-IR values and *PNPLA3* SNPs could relate to the presence of HS and development of HCC. The current study is unique in that histologically-proven HS, HOMA-IR, and *PNPLA3* SNP were simultaneously evaluated in more than 200 of HBV-infected patients.

In agreement with previous studies including ours (14, 33), *PNPLA3* mutation was related to an increased frequency of HS (Figure 7). However, the involvement of *PNPLA3*

SNPs in the occurrence of HCC in HBV-infected patients is controversial (21-25). In a recent case-control study (22), Wang *et al*. mentioned that *PLPLA3* SNP was unlikely to have a significant impact on the development of HCC in HBV-infected patients. Their analysis included all HBV-infected patients, regardless of their HBV infection status (*e.g*., HBeAg positivity or HBV-DNA titer). We have to note that our patients with a high HBV titer received antiviral treatments after liver biopsy, and our cohort included patients with a low viral titer during the observation period. HBV-infection itself is associated with a high risk of developing HCC, and our previous study suggested that HS or *PNPLA3* SNP would mainly have a clinical impact in cases in which the influence of HBV is low, such as HBeAg-negative cases (14, 34, 35). Thus, the cohort may have affected the results of the current research. We should also note that most of the enrolled cases are Japanese patients with HBV genotype C. Furthermore, results were obtained from a relatively long observation period (median: 5.77 years). These features of the current study may have also affected the results.

The present study was associated with some limitations. First, although we analyzed the prospectively collected cohort, this was a retrospective single-center study. Second, our cohort mainly included cases with mild to moderate fibrosis (stage F0-F2) and additionally, >85% of the patients received antiviral treatments after liver biopsy. Thus, our cohort was mainly composed of patients with low viral replication and without severe liver fibrosis, and the incidence of HCC development was low, despite the relatively long observation period. Finally, as mentioned above, most of the enrolled cases were Japanese patients with HBV genotype C.

In summary, in addition to HS and IR, *PNPLA3* rs738409 SNP was suggested to be associated with the development of HCC in HBV-infected Japanese patients. A future prospective study with a larger study population is warranted.

## Funding

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## Conflicts of Interest

The Authors declare that there are no conflicts of interest in association with this study.

## Authors' Contributions

HE designed the study, analyzed the data, and wrote the manuscript. NA, NI, TT, YY, MO, RY, SK, KY, SO, RN, HS, and TN collected and interpreted the data; HI collected the data and supervised the study. All of the Authors reviewed and edited the manuscript and approved the final version of the manuscript.

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