PNPLA3 genetic variants determine hepatic steatosis in non-obese chronic hepatitis C patients

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The influence of patatin-like phospholipase domain-containing 3 (PNPLA3) genetic variants in the development of liver steatosis in Asian chronic hepatitis C patients remains elusive. A total of 1018 biopsy-proven chronic hepatitis C patients were enrolled for evaluation. The proportions of PNPLA3 rs738409 GG genotype carriage were 7.8% (44/563), 15.8% (58/367) and 19.3% (17/88) in patients with no (liver fat content <5%), mild (5–33%) and moderate/severe (>66%) hepatic steatosis, respectively (trend P < 0.001). Stepwise logistic regression analysis revealed that the strongest factor independently associated with steatosis was the carriage of the PNPLA3 rs738409 GG genotype (odds ratio [OR]/95% confidence intervals [CI]:2.34/1.557–3.515, P < 0.001). Among the patients with BMI < 24 kg/m², carriage of the rs738409 GG genotype was the only factor associated with hepatic steatosis (OR/CI:3.44/1.824–6.500, P < 0.001). PNPLA3 genetic variants had minimal effects on hepatic steatosis among overweight or obese patients. Compared to patients with BMI <24 kg/m²/non-GG genotype, those with BMI >24 kg/m²/GG genotype were more likely to have hepatic steatosis (OR/CI:3.87/2.392–6.524, P < 0.001). In conclusions, both PNPLA3 genetic variants and BMI played important roles in hepatic steatosis among Asian chronic hepatitis C patients. However, the genetic effect was mainly restricted to non-obese patients.

Hepatic steatosis is more frequently observed in patients with chronic hepatitis C virus (CHC) infection than in the general population. The frequency of significant steatosis in CHC patients who carry
variable attributable factors ranges between 40% and 80%, which is approximately twofold higher than in
the natural course of CHC. In addition, it may also determine the treatment efficacy of interferon based
anti-viral therapy. Two major mechanisms account for the high prevalence of hepatic steatosis in CHC,
which include the direct cytopathic effect of the hepatitis C virus genotype 3 (HCV-3) viral protein and
the indirect effect of metabolic derangement in patients with HCV non-3 infection.

Beyond the issue of virological and environmental factors, host genomes also play a role in hepatic
steatosis. Genome-wide association study (GWAS) has demonstrated that a single nucleotide polymor-
phism (SNP) of patatin-like phospholipase domain-containing 3 (PNPLA3) gene was associated with
nonalcoholic fatty liver disease (NAFLD). In addition, PNPLA3 genetic variants have also been shown
to be associated with hepatic steatosis in CHC patients. However, the result was not consistent in
different cohorts. This finding raised the question of whether the association between PNPLA3 SNP
and hepatic steatosis in CHC patients varies across ethnicities. Meanwhile, it has been reported that the
determination of PNPLA3 SNPs in NAFLD was not universal in the same population with different
metabolic profiles. Taken collectively, the impact of PNPLA3 genetic variants on liver steatosis in Asian
CHC patients with different characteristics has never been studied. We herein recruited a large CHC
cohort with histologically proven steatosis and well-characterized demographics, and we aimed to
determine the association of PNPLA3 genetic variants with hepatic steatosis in an Asian CHC population.
Importantly, we also sought to determine whether the influence of this gene differs among subpopula-
tions with different characteristics.

**Methods**

A total of 1,018 CHC patients who received pre-antiviral evaluation were consecutively recruited in a
medical center and two core regional hospitals in Taiwan from 2001 to 2013. Anti–HCV antibodies
were detected using a third-generation, commercially available enzyme-linked immunosorbent assay kit
(AxSYM 3.0, Abbott Laboratories, Chicago, IL, USA). Serum HCV RNA was detected using qualitative
real-time polymerase chain reaction (PCR) (COBAS AMPLICOR Hepatitis C Virus Test, ver. 2.0; Roche,
Branchburg, NJ, USA, detection limit: 501U/ml) and quantification branched DNA assay (Versant HCV
RNA 3.0, Bayer, Tarrytown, New Jersey, USA; quantification limit: 615 IU/ml) before 2011. The HCV
genotypes were determined using the Okamoto method before 2011. Both the HCV RNA and geno-
type were detected using real-time PCR assay (RealTime HCV; Abbott Molecular, Des Plaines IL, USA;
detection limit: 12 IU/ml) since 2011. All of the patients received liver biopsies before initiating anti-
viral therapy. Patients with current or past history of alcohol abuse (>20g daily) were excluded in the
current cohort. The liver histology was graded and staged according to the scoring system described by
Knodell and Scheuer. Hepatic steatosis was evaluated with an H&E stain with the definition of no (liver
fat content <5%), mild (5–33%), moderate (33–66%) and severe (>66%) hepatic steatosis. The diagnosis
of diabetes was based on 1) laboratory tests with twice the fasting plasma glucose levels >126mg/dL
or hemoglobin A1C >6.5%, or 2) medical history of previously established diagnosis of diabetes. All
patients gave written informed consent before enrollment. The study was approved by the ethics com-
mittee of Kaohsiung Medical University Hospital and was performed according to the guidelines of the
International Conference on Harmonization for Good Clinical Practice.

**PNPLA3 genotyping.** The PNPLA3 rs738409 was selected as the candidate SNP, and the genotype
was determined using the methods described previously.

**Statistical analyses.** The frequency was compared between groups using the χ² test with the Yates
correction or Fisher’s exact test. Group means, presented as the mean values and standard deviations,
were compared using the analysis of variance and Student’s t test or the Mann-Whitney U test. The serum
HCV RNA levels were expressed after logarithmic transformation of the original values. The influence
of PNPLA3 in liver steatosis was calculated using dominant (genotype CC vs. CG+GG) and recessive
(genotype GG vs. CG+CC) genetic models of inheritance. A stepwise logistic regression analysis was
performed to evaluate the independent factors associated with steatosis by analyzing the co-variants with
P values <0.05 in the univariate analysis. The statistical analyses were performed using the SPSS 12.0
statistical package (SPSS, Chicago, IL, USA). All statistical analyses were based on two-tailed hypothesis
tests with a significance level of p < 0.05.

**Results**

**Patients.** The mean age of the patients included in the study population was 51.8 years and 56.6%
of the patients were male (Table 1). The majority of the patients weighed between 18.5kg/m² and
30kg/m². Hepatic steatosis was observed in 455 (44.7%) of the CHC patients. The proportion of no,
mild, moderate and severe hepatic steatosis was 55.3% (n = 563), 36.1% (n = 367), 7.8% (n = 79) and
0.9% (n = 9), respectively. PNPLA3 rs738409 CC, CG, and GG genotypes accounted for 41.5%, 46.9%
and 11.7% of the population, respectively. The majority of patients were infected with HCV-1 and HCV-
2, whereas only one patient in the current cohort was infected with HCV-3.
### Table 1. Univariate analysis of factors associated with hepatic steatosis

Note: *data available in 694 patients. BMI: body mass index; r-GT: r-glutamyltransferase; AST: alanine aminotransferase; ALT: aspartate aminotransferase; HBsAg: hepatitis B surface antigen; PNPLA3: patatin-like phospholipase domain-containing 3.

|                      | All patients (N = 1018) | Steatosis (−) (n = 563) | Steatosis (+) (n = 455) | P value |
|----------------------|-------------------------|-------------------------|-------------------------|---------|
| Age (years, mean ± SD) | 51.8 ± 11.3             | 50.9 ± 11.8             | 53.0 ± 10.6             | 0.004   |
| Male gender, n (%)    | 576 (56.6)              | 318 (56.5)             | 258 (56.7)             | 0.94    |
| BMI (kg/m², mean ± SD)| 24.9 ± 3.5              | 24.3 ± 3.5              | 25.7 ± 3.4              | <0.001  |
| BMI, <18.5, 18.5–24, 24–27, 27–30, >30 (kg/m², %) | 1.9%, 39.8%, 33.5%, 16.8%, 8.1% | 3.2%, 46.5%, 29.8%, 14.6%, 5.9% | 0.2%, 31.4%, 38.0%, 19.6%, 10.8% | <0.001  |
| Diabetes, n (%)       | 158 (15.5)              | 72 (12.8)              | 86 (18.9)              | 0.007   |
| Cholesterol (mg/dL, mean ± SD)* | 167 ± 33                   | 167 ± 33                   | 166 ± 34                   | 0.53    |
| Triglyceride (mg/dL, mean ± SD)* | 103 ± 57                       | 109 ± 64                        | 96 ± 49                        | 0.004   |
| Platelet count (x10³/μL, mean ± SD) | 164 ± 58                       | 165 ± 56                       | 162 ± 59                       | 0.44    |
| AST (IU/L, mean ± SD) | 104 ± 62                  | 104 ± 67                  | 103 ± 54                  | 0.96    |
| ALT (IU/L, mean ± SD) | 157 ± 103                 | 159 ± 114                 | 155 ± 86                  | 0.58    |
| Ferritin (ng/ml, mean ± SD) | 406 ± 472                  | 400 ± 554                 | 415 ± 344                 | 0.61    |
| r-GT (IU/L, mean ± SD) | 67.2 ± 63.3               | 62.3 ± 62.5               | 73.3 ± 63.7               | 0.005   |
| HCV genotype 1, n/N (%) | 604/1012 (59.7)           | 344/562 (61.2)           | 260/450 (59.5)           | 0.27    |
| HCV RNA (log IU/mL, mean ± SD) | 5.38 ± 0.98                | 5.37 ± 1.03               | 5.39 ± 0.92               | 0.81    |
| HBsAg(+), n (%)       | 90 (8.8)                  | 53 (9.4)                  | 37 (8.1)                  | 0.47    |
| Fibrosis 3–4, n (%)   | 308 (30.3)                | 162 (28.8)               | 146 (32.1)               | 0.25    |

**PNPLA3 rs738409 genotype**

|                         | CC/CG/GG, n (%) | Recessive model GG, n (%) | Dominant model GG+GC, n (%) |
|-------------------------|-----------------|---------------------------|-----------------------------|
| All patients (N = 1018) | 422 (41.5)/477 (46.9)/119 (11.7) | 119 (11.7) | 596 (58.5) |
| 119 (11.7)              | 247 (43.9)/272 (48.3)/44 (7.8) | 44 (7.8) | 316 (56.1) |
| 175 (38.5)/205 (45.3)/75 (16.5) |                  | 75 (16.5) | 280 (61.5) |

|                         | P value |
|-------------------------|---------|
| Recessive model GG, n (%) | <0.001  |
| Dominant model GG+GC, n (%) | 0.08    |

### Factors associated with hepatic steatosis in CHC patients

In the univariate analysis, the factors associated with fatty liver included older age, high body mass index (BMI), the presence of diabetes, a high r-glutamyltransferase (r-GT) level and carriage of the PNPLA3 rs738409 GG genotype, using the recessive model. CHC patients with hepatic steatosis had numerically higher proportions of CG/GG genotype carriage when compared to those without, as determined by using the dominant model (P = 0.08). Six-hundred and ninety-four patients had available cholesterol and triglyceride (TG) data. Patients with steatosis had significantly higher TG level (109 ± 64 mg/dL vs. 96 ± 49 mg/dL, P = 0.004). Stepwise logistic regression analysis was performed to evaluate factors independently associated with hepatic steatosis. If the variable of TG was not taken into consideration, the strongest factor independently associated with steatosis was carriage of the PNPLA3 rs738409 GG genotype (odds ratio [OR]/95% confidence intervals [CI]: 2.34/1.557–3.515, P < 0.001), followed by body mass index (BMI, OR/CI: 1.12/1.082–1.167, P < 0.001) and age (OR/CI: 1.02/1.004–1.028, P = 0.007) by using the recessive model. By using the dominant model, the factors associated with steatosis included carriage of the PNPLA3 rs738409 CG/GG genotype (OR/CI: 1.31/1.013–1.703, P = 0.04), BMI (OR/CI: 1.13/1.084–1.169, P < 0.001) and age (OR/CI: 1.02/1.004–1.028, P = 0.006). If the variable of TG was taken into account, the strongest factor associated with liver steatosis remains the carriage of PNPLA3 rs738409 GG genotype (OR/CI: 2.37/1.408–3.983, P = 0.001) (Table 2). The PNPLA3 genotype distribution did not differ between patients with or without available TG level either by recessive model (GG genotype: 11.1% vs. 13.0%, P = 0.39) or dominant model (CG/GG genotype: 58.8% vs. 58.0%, P = 0.82).

### Role of PNPLA3 genetic variants in determining hepatic steatosis among patients with different BMIs

Because BMI and PNPLA3 genetic variants are both important determinants of hepatic...
steatosis, we further explored the influence of the PNPLA3 SNP in steatosis among patients with different BMIs. Patients were categorized into normal or underweight (<24 kg/m²), overweight (24–27 kg/m²) or obese (>27 kg/m²) according to the definition of the Health Promotion Administration of the Ministry of Health and Welfare in Taiwan. Among the patients with normal body weights, hepatic steatosis was associated with a higher r-GT level, a lower proportion of HBsAg seropositivity and a higher proportion of PNPLA3 rs738409 G genotype carriage in univariate analysis (Table 3). In multivariate analysis, carriage of the rs738409 GG genotype was the only factor associated with hepatic steatosis (OR/CI: 3.44/1.82–6.500, P < 0.001) by using the recessive model, whereas factors associated with steatosis were the rs738409 GG/GC genotype (OR/CI: 1.69/1.10–2.614, P = 0.02) and HBV dual infection (OR/CI: 0.42/0.18–0.940, P = 0.04) using the dominant model (Table 4). Among the overweight patients (BMI between 24–27 kg/m²), the univariate analysis revealed that the patients with steatosis were older and more likely to have diabetes, lower platelet counts and a higher r-GT level; while the steatotic patients had a substantially higher proportion of rs738409 GG genotype carriage, the difference was not significant (P=0.06) (Table 3). Multivariate analysis revealed that the factors associated with

| Variables       | OR  | 95% C.I.     | P value |
|-----------------|-----|--------------|---------|
| Age             |     |              |         |
| Per 1 year increase | 1.02 | 1.004–1.028 | 0.007   |
| BMI             |     |              |         |
| Per 1 kg/m² increase | 1.12 | 1.082–1.167 | <0.001  |
| PNPLA3 rs738409 genotype |
| CC/CG           |     |              |         |
| GG              | 2.34 | 1.557–3.515 | <0.001  |

Table 2. Logistic regression analysis of factors associated with hepatic steatosis. Note: OR: odds ratio; C.I.: confidence intervals; BMI: body mass index. PNPLA3: patatin-like phospholipase domain-containing 3.
steatosis in overweight CHC patients included age (OR/CI:1.023/1.001–1.045, \( P = 0.04 \)) and diabetes (OR/CI:2.201/1.055–3.875, \( P = 0.03 \)), but not \( PNPLA3 \) rs738409 genotype variants (Table 4). For obese patients, hepatic steatosis was only associated with a higher \( r\)-GT level in the univariate analysis (Table 3), although no factors were associated with hepatic steatosis in obese CHC patients in the multivariate analysis. The impact of the \( PNPLA3 \) rs738409 genotype on fatty liver varied among patients with different BMIs (Supplementary Fig 1). We further explored the role of the SNP in patients with different degrees of hepatic steatosis. The proportion of \( PNPLA3 \) rs738409 GG genotype carriage was 7.8% (44/563), 15.8% (83/56) and 19.3% (17/88) in patients with no, mild, and moderate/severe hepatic steatosis, respectively (trend \( P < 0.001 \)) (fig. 1).

### Interaction of \( PNPLA3 \) rs738409 genotype and BMI in hepatic steatosis.

Both the \( PNPLA3 \) genetic variants and BMI determined hepatic steatosis. We further analyzed their interactions in contributing to fatty liver. There was a significantly increased proportion of mild and moderate/severe hepatic steatosis in patients with BMI\( > 24 \) kg/m\(^2\) and the \( PNPLA3 \) rs738409 GG genotype compared to those with BMI\( < 24 \) kg/m\(^2\) and/or non-GG genotype (\( P < 0.001 \)) (fig. 2). The proportion of hepatic steatosis was 30.5%, 51.8% and 63.5% in patients with BMI\( < 24 \) kg/m\(^2\)/non-GG genotype, BMI\( < 24 \) kg/m\(^2\)/GG genotype or BMI\( > 24 \) kg/m\(^2\)/non-GG genotype, and BMI\( > 24 \) kg/m\(^2\)/GG genotype, respectively. Compared to patients with BMI\( < 24 \) kg/m\(^2\)/non-GG genotype, those with BMI\( > 24 \) kg/m\(^2\)/GG genotype were more likely to have hepatic steatosis (OR/CI:3.87/2.292–6.524, \( P < 0.001 \)), followed by those patients with BMI\( < 24 \) kg/m\(^2\)/GG genotype or BMI\( > 24 \) kg/m\(^2\)/non-GG genotype (OR/CI:2.43/1.849–3.202, \( P < 0.001 \)) (Table 5).

### Discussion

In the current large-scale study, we demonstrated that the influence of the \( PNPLA3 \) genetic variants in hepatic steatosis remains consistent in Asian CHC populations, and the effect is independent of other metabolic disorders. The association was particularly enhanced through the use of the recessive model. CHC patients who carried the \( PNPLA3 \) rs738409 GG genotype had a 2.3-fold risk of developing hepatic...
steatosis when compared to their counterparts. Both PNPLA3 genetic variants and BMI played important roles in hepatic steatosis in CHC patients. Importantly, we identified that the host genetic effect was mainly restricted to non-obese patients and not obese patients. For patients with BMI < 24 kg/m², the carriage of the PNPLA3 rs738409 GG genotype increased the risk of hepatic steatosis 3.4-fold when compared to those individuals carrying the C allele.

PNPLA3 participates in the restoration of lipid homeostasis upon aberrant intracellular lipid accumulation. The determination of the role of the PNPLA3 SNP in NAFLD was established in 2008. Later, several reports have linked the genetic variants to other spectrums of liver disease such as HBV and HCV infection. Due to the direct steatogenic effect of the HCV-3 protein, the determination of the host genome in hepatic steatosis has been restricted to non-HCV-3 infection. Most of the studies have originated from the West, where the genetic effect has been fully explored in Caucasians. Nevertheless, Nakamura et al. recently reported that there was no association between the PNPLA3 rs738409 genotype and fatty changes in sonography in Japanese patients with HCV-1 and HCV-2 infections. It is therefore imperative to validate the effect of PNPLA3 in another cohort with different ethnicities and patient characteristics in which the phenotype is clearly defined by liver biopsy.

| BMI   | Variables                  | OR   | 95% C.I.       | P value |
|-------|----------------------------|------|----------------|---------|
| <24 kg/m² | Recessive Model          |      |                |         |
|       | PNPLA3 rs738409 genotype |      |                |         |
|       | CC/CG                      | 1    |                |         |
|       | GG                         | 3.44 | 1.824–6.500    | <0.001  |
|       | Dominant Model             |      |                |         |
|       | HBsAg                      | 1    |                |         |
|       | Positive                   | 0.42 | 0.188–0.940    | 0.04    |
|       | PNPLA3 rs738409 genotype  |      |                |         |
|       | CC                         | 1    |                |         |
|       | GG+GC                      | 1.69 | 1.101–2.614    | 0.02    |
| 24–27 kg/m² | Age                      |      |                |         |
|       | Per 1 year increase        | 1.023| 1.001–1.045    | 0.04    |
|       | Diabetes                   |      |                |         |
|       | No                         | 1    |                |         |
|       | Yes                        | 2.201| 1.055–3.875    | 0.03    |

Table 4. Logistic regression analysis of factors associated with hepatic steatosis in patients with different BMI. Note: OR: odds ratio; C.I.: confidence intervals; BMI: body mass index. PNPLA3: patatin-like phospholipase domain-containing 3; HBsAg: hepatitis B surface antigen.

Figure 1. Percentage of PNPLA3 rs38409 genotype in patients with hepatic steatosis <5%, 5–33% and >33%, respectively.
CHC patients had hepatic steatosis in the current cohort, which was similar to the prevalence in some Western reports. However, the mean BMI was only 24.9 kg/m² and less than one tenth of the patients had hepatic steatosis > 33% in the current population. We confirmed that the *PNPLA3* genetic variants consistently play a role in hepatic steatosis in Asian patients with HCV-1 and HCV-2 infections. The determined power was similar to the reports from the West where patients homozygous for the risk G allele had an approximately 2-fold higher risk for hepatic steatosis.

As HCV infection increases the risk for liver steatosis and metabolic derangement, the relationship between HBV infection and hepatic steatosis remains conflicting. We identified that lean CHC patients with HBV co-infection had a lower proportion of liver steatosis compared to those with HCV mono-infection. The finding was in agreement with some reports that HBV infection protects against fatty liver rather than promoting it. This finding may be attributed to a lower frequency of metabolic disorders in HBV carriers. In addition, hepatic steatosis may accelerate hepatitis B surface antigen clearance. Whether the mechanism supports the inverse relationship between HBV infection and steatosis awaits further confirmation. Hepatic steatosis may promote liver fibrosis progression, although we did not observe this association in the current study. Because fibrotic tissue may replace liver fat content as the disease progresses, the linkage might be masked from cross-sectional observation.

Interestingly, we observed that the role of the *PNPLA3* genetic variants in hepatic steatosis was particularly enhanced in non-obese patients. The odds ratios further increased compared with those in the whole population, and the *PNPLA3* genotype was the strongest predictor for hepatic steatosis in non-obese subjects. The reason why some lean CHC patients developed fatty liver was not fully understood. The current study in part provided a clue from the perspective of the host genetic profile. In contrast, the *PNPLA3* SNP did not independently determine hepatic steatosis in overweight or obese patients. The higher the BMI, the less the genetic composition impacted the degree of hepatic steatosis.

### Figure 2. Percentage of patients with hepatic steatosis <5%, 5–33% and >33%, respectively, stratified by body mass index and *PNPLA3* rs38409 genotype. Supplementary Figure 1 Proportion of hepatic steatosis in patients with different *PNPLA3* rs38409 genotypes, stratified by BMI.

### Table 5. Interaction of *PNPLA3* rs738409 genotype and body mass index in hepatic steatosis. Note: adjust age and sex; BMI: body mass index.

| BMI (kg/m²) & *PNPLA3* rs738409 genotype | Steatosis (%) | OR  | 95% CI     | Adjusted P value* | Trend P value |
|----------------------------------------|--------------|-----|------------|-------------------|--------------|
| BMI < 24/non-GG                         | 30.5%        | 1   | Ref        | <0.001            |              |
| BMI < 24/GG or BMI > 24/non-GG          | 51.8%        | 2.43| 1.84-3.202 | <0.001            |              |
| BMI > 24/GG                            | 63.5%        | 3.87| 2.29-6.524 | <0.001            |              |
The current study focusing on CHC patients may echo the report that the PNPLA3 rs738409 GG genotype increases the risk of NAFLD in the general population without metabolic disorder.²

The current study was limited by the absence of metabolic profiles, which may interfere with the final results. Nevertheless, we demonstrated the associations between the PNPLA3 SNP and other simple demographic characteristics with hepatic steatosis. These findings should facilitate more direct clinical relevance in the interpretation of the association. In conclusion, we demonstrated that the PNPLA3 genetic variants determined the risk of development of hepatic steatosis in Asian CHC patients. However, the effect was not universal and was mainly restricted to non-obese patients. Whether the latter finding is generalizable to other ethnicities awaits further validation.

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