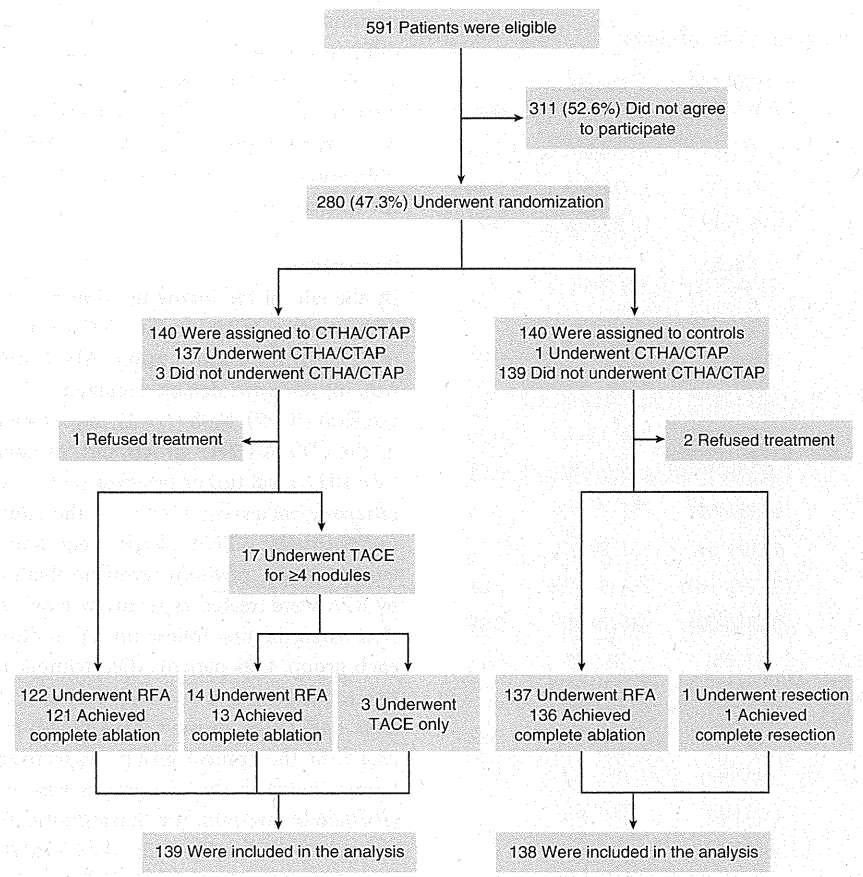

Figure 1. Patient enrollment and outcomes. CTAP, computed tomography during arterial portography; CTHA, computed tomography during hepatic arteriography; RFA, radiofrequency ablation; TACE, transarterial chemoembolization.

in univariate analysis. Data at entry were used for the analyses. A *post hoc* analysis comparing the recurrence-free survival of those with and without newly diagnosed HCC in the CTHA/CTAP group was performed.

All analyses were performed on an intention-to-treat basis. Differences with a two-sided P value of <0.05 were considered statistically significant. Data processing and analysis were performed with S-PLUS ver. 7 (TIBCO Software, Palo Alto, CA). Finally, all authors had access to the study data and had reviewed and approved the final manuscript.

RESULTS

Patient enrollment

According to the study protocol, the registration started from September 2004 for 5 years and the follow-up was censored in February 2011 when 2 years had passed after the enrollment of patient 280. During the study period, 280 of 591 (47.4%) eligible patients agreed to participate in the trial, and 140 of these were randomly assigned to undergo CTHA/CTAP before RFA. Three patients declined to undergo CTHA/CTAP after assignment. A total of 140 patients were randomly assigned to the control group. One patient assigned to the control group received CTHA/CTAP because of strong preference (**Figure 1**).

Treatment

In 45 (32.4%) patients, 75 nodules with a median diameter of 8 mm (range, 2–20) were additionally diagnosed by experienced radiologists as definite HCC on CTHA/CTAP. The detailed characteristics of newly diagnosed nodules have been reported previously (33). In 17 patients, the number of HCC nodules exceeded 3 after CTHA/CTAP, and TACE was performed subsequently. We intended to ablate all nodules by RFA including additionally detected nodules. In 122 patients, there were ≤3 HCC nodules, and complete ablation was obtained in 121 patients (99.2%). Among 17 patients treated with TACE, 14 (82.4%) subsequently underwent RFA and complete ablation was obtained in 13 (92.9%) patients. The remaining 3 patients (17.6%) did not undergo RFA because of tumor nodule multiplicity in 2 patients and simultaneously diagnosed malignant B-cell lymphoma in the third patient. Among 140 patients who were assigned to the control group, 137 (97.9%) were treated with RFA, and complete ablation was obtained in 136 (99.3%) patients. One patient withdrew consent and underwent hepatic resection. Two patients refused to receive

Table 1. Baseline characteristics of the patients[a]

Characteristics	CTHA/CTAP (*N*=139)	Control (*N*=138)	*P* value
Age, years	70 (63–74)	70 (64–75)	0.43
Male, *n* (%)	93 (67)	86 (62)	0.42
Alcohol >80 g/day, *n* (%)	23 (17)	20 (15)	0.82
BMI (kg/m^2)	23.1 (21.4–25.1)	23.4 (21.2–25.3)	0.48
Viral markers			
HCVAb positive, *n* (%)	104 (75)	99 (72)	0.59
HBsAg positive, *n* (%)	21 (15)	20 (14)	1
Serum albumin (g/dl)	3.8 (3.6–4.1)	3.9 (3.6–4.1)	0.20
Total bilirubin (mg/dl)	0.8 (0.6–1.0)	0.8 (0.6–1.0)	0.31
AST (IU/l)	56 (34–69)	57 (33–70)	0.84
ALT (IU/l)	54 (29–63)	57 (27–73)	0.61
Platelet count (×10^3/μl)	128 (89–163)	130 (91–159)	0.88
Prothrombin activity (%)	80 (72–90)	81 (74–87)	0.39
Treatment-naive case, *n* (%)	77 (55)	74 (54)	0.81
Previously treated case, n *(%)*	62 (45)	64 (46)	
Resection, *n* (%)[b]	15 (24)	16 (25)	0.27
RFA, *n* (%)[b]	46 (74)	45 (70)	
Ethanol injection, *n* (%)[b]	10 (16)	3 (4.6)	
TACE, *n* (%)[b]	11 (18)	7 (11)	
Tumor size (cm)	1.6 (1.2–2.0)	1.7 (1.2–2.0)	0.91
Single nodule, *n* (%)	101 (73)	98 (71)	0.76
AFP >100 ng/ml, *n* (%)	23 (17)	24 (17)	0.85
DCP >100 mAU/ml, *n* (%)	16 (12)	22 (16)	0.28
AFP-L3 >15%, *n* (%)	16 (12)	15 (11)	0.86

AFP, α-fetoprotein; AFP-L3, lens culinaris agglutinin-reactive fraction of AFP; ALT, alanine aminotransferase; AST, aspartate aminotransferase; BMI, body mass index; CTHA/CTAP, computed tomography during hepatic arteriography and arterial portography; DCP, des-γ-carboxy prothrombin; HBsAg, hepatitis B surface antigen; HCVAb, hepatitis C virus antibody; RFA, radiofrequency ablation, TACE, transarterial chemoembolization.

[a]Data are expressed as median (25th–75th percentiles) or number (percent).

[b]Including overlap.

any treatment and were lost to follow-up. Finally, 139 (99.3%) patients in the CTHA/CTAP group and 138 (98.6%) patients in the control group were included in the analysis.

Patient characteristics

There was no statistically significant difference in patient characteristics between the groups (**Table 1**). Median age at enrollment was 70 years, and approximately two-thirds of patients were male. Approximately 55% of patients were treatment-naive cases and the remaining patients had a history of previous treatment. Among those previously treated patients, the median interval between the initial treatment and the study enrollment was 42 (interquartile range, 22–65) months in the CTHA/CTAP group and 30 (20–61) months in the control group. There was no statistically significant difference between the two groups ($P=0.72$). The total number of HCC nodules detected in original contrast-enhanced dynamic CT was 197 (101 patients were uninodular and the rest were multinodular) in the CTHA/CTAP group and 196 (98 patients were uninodular and the rest were multinodular) in the control group.

Recurrence

By the end of the follow-up, tumor recurrence was identified in 109 patients (78.4%) in the CTHA/CTAP group and 112 patients (81.2%) in the control group. The distribution of recurrent site was intrahepatic distant recurrence ($N=98$), local tumor progression ($N=7$), both ($N=1$), and extrahepatic metastasis ($N=3$) in the CTHA/CTAP group and intrahepatic distant recurrence ($N=103$), local tumor progression ($N=4$), both ($N=2$), and extrahepatic metastasis ($N=3$) in the control group. Five patients (3.6%) in the CTHA/CTAP group and 1 patient (0.7%) in the control group in whom complete ablation could not be obtained by RFA were treated as recurrence on 120 days after randomization when the first follow-up CT would have been scheduled. In each group, four patients died without recurrence. The cumulative recurrence-free survival rates at 1, 2, and 3 years were 60.1, 29.0, and 18.9% in the CTHA/CTAP group and 52.2, 29.7, and 23.1% in the control group, respectively (**Figure 2a**). The difference between the two groups was not statistically significant ($P=0.66$ by log-rank test; hazard ratio, 0.94 for CTHA/CTAP vs. control; 95% CI, 0.73–1.22). The CTHA/CTAP group showed better recurrence-free survival with marginal statistical significance in the subgroups with higher AFP or AFP-L3 values (**Figure 3**).

Univariate Cox regression analysis identified older age ($P=0.01$), hepatitis C virus antibody positivity ($P=0.001$), lower albumin level ($P=0.04$), recurrent cases ($P<0.001$), multinodular HCC ($P<0.001$), and higher AFP level ($P=0.02$) as significant predictors for recurrence-free survival (**Table 2**). Adjusted hazard ratio of the CTHA/CTAP group vs. the control group by multivariate Cox regression analysis was 0.86 (95% CI, 0.67–1.12; $P=0.27$, **Table 3**).

Overall survival

By the end of the follow-up, 51 patients (36.7%) in the CTHA/CTAP group and 45 patients (32.6%) in the control group died. The cumulative overall survival rates at 3 and 5 years were 79.7 and 56.4% in the CTHA/CTAP group and 86.8 and 60.1% in the control group, respectively (**Figure 2b**). There was no statistically significant difference between the groups ($P=0.50$ by log-rank test; hazard ratio, 1.15, 95% CI, 0.77–1.71).

Safety

No procedural complications attributable to CTHA/CTAP or TACE were observed. Major complications related to RFA were observed in 2 patients (1.4%) in the CTHA/CTAP group (2 with neoplastic seeding) and in 3 patients (2.2%) in the control group (1 each with hepatic infarction, hemothorax, and neoplastic seeding). There was no procedure-related death.

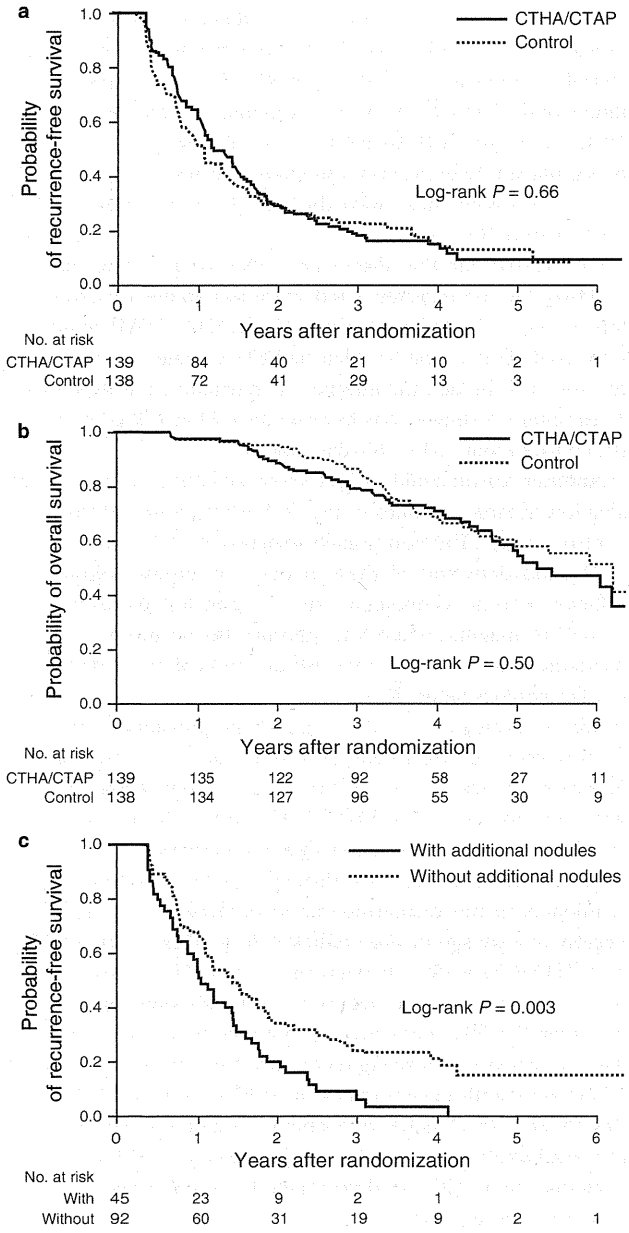

Figure 2. Kaplan–Meier estimate of the recurrence-free survival and overall survival. (**a**) The cumulative recurrence-free survival rates at 1, 2, and 3 years were 60.1, 29.0, and 18.9% in the CTHA/CTAP group and 52.2, 29.7, and 23.1% in the control group, respectively. (**b**) The cumulative overall survival rates at 3 and 5 years were 79.7 and 56.4% in CTHA/CTAP group and 86.8 and 60.1% in the control group, respectively. (**c**) Patients with an additional nodule detected by CTHA/CTAP showed significantly poorer recurrence-free survival than those without an additional nodule. CTAP, computed tomography during arterial portography; CTHA, computed tomography during hepatic arteriography.

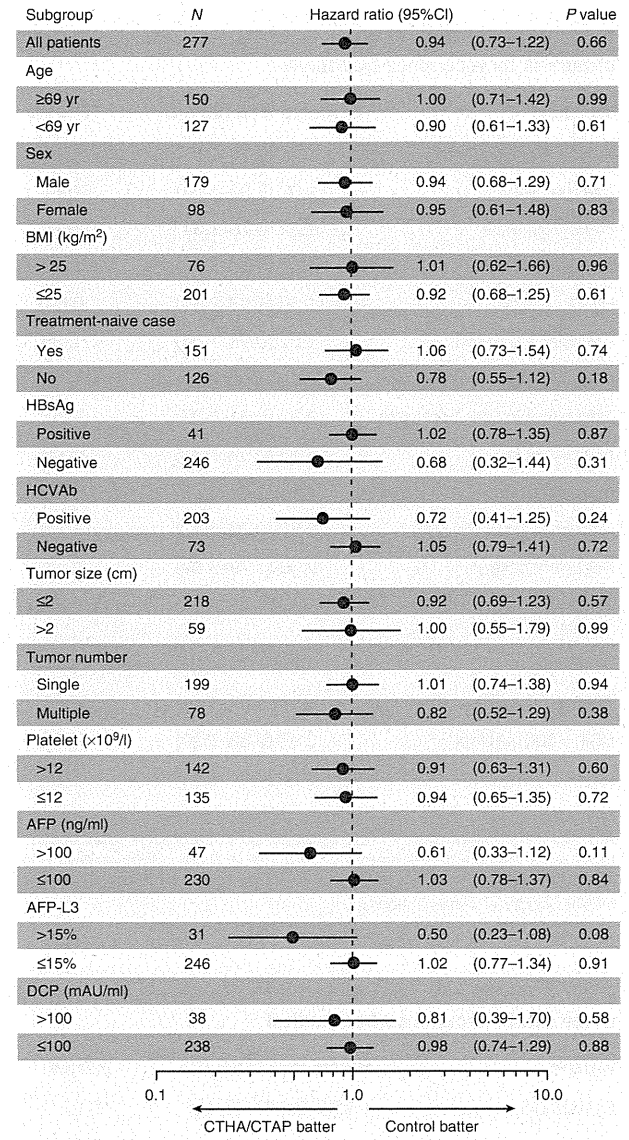

Figure 3. Recurrence-free survival of subgroups by Cox proportional hazard regression according to clinical characteristics at study entry. AFP, α-fetoprotein; BMI, body mass index; CI, confidence interval; CT, computed tomography; CTAP, computed tomography during arterial portography; CTHA, computed tomography during hepatic arteriography; DCP, des-γ-carboxy prothrombin; HBsAg, hepatitis B surface antigen; HCVAb, hepatitis C virus antibody; yr, year.

Recurrence-free survival between those with and without additional nodules in CTHA/CTAP group

As a *post hoc* analysis, we compared the recurrence-free survival between those with ($N=45$) and without ($N=92$) additional HCC nodules diagnosed by CTHA/CTAP. As compared with those in whom additional HCC nodules were not detected by CTHA/CTAP, those with additional nodules included more HBsAg-negative patients (97.7 vs. 78.3%, $P=0.002$), previously treated patients (62.2 vs. 23.9%, $P=0.006$), and patients with multiple HCC nodules on dynamic CT (44.4 vs. 17.4%, $P=0.002$). Patients with additional nodule by CTHA/CTAP showed significantly poorer

Table 2. Univariate Cox's proportional hazard regression analysis of the risk for recurrence-free survival

Variable	Hazard ratio (95% CI)	*P* value
CTHA/CTAP vs. control	0.94 (0.73–1.22)	0.66
Age (per year)	1.02 (1.00–1.04)	0.01
Female vs. male	1.02 (0.78–1.34)	0.88
Alcohol >80 g/day	1.02 (0.88–1.17)	0.81
HCVAb positive	1.69 (1.23–2.31)	0.001
BMI (per 1.0 kg/m^2)	1.02 (0.98–1.06)	0.35
Albumin (per 1.0 g/dl)	0.72 (0.52–0.98)	0.04
Total bilirubin (per 1.0 mg/dl)	1.02 (0.97–1.07)	0.51
AST >40 IU/l	1.14 (0.99–1.31)	0.07
ALT >40 IU/l	1.05 (0.92–1.20)	0.45
Platelet count >10×10^3/μl	0.89 (0.78–1.01)	0.08
Recurrent case	2.33 (1.79–3.02)	<0.001
Tumor size of maximal nodule >2.0 cm	0.97 (0.85–1.10)	0.62
Multinodular	1.38 (1.20–1.59)	<0.001
AFP >100 ng/ml	1.21 (1.03–1.43)	0.02
DCP >100 mAU/ml	0.99 (0.82–1.20)	0.93
AFP-L3 >15%	1.20 (0.99–1.46)	0.07

AFP, α-fetoprotein; AFP-L3, lens culinaris agglutinin-reactive fraction of AFP; ALT, alanine aminotransferase; AST, aspartate aminotransferase; BMI, body mass index; CI, confidence interval; CTHA/CTAP, computed tomography during hepatic arteriography and arterial portography; DCP, des-γ-carboxy prothrombin; HCVAb, hepatitis C virus antibody.

Table 3. Multivariate Cox's proportional hazard regression analysis of the risk for recurrence-free survival

Variable	Hazard ratio (95% CI)	*P* value
CTHA/CTAP vs. control	0.86 (0.0.67–1.12)	0.27
Age (per year).	1.01 (0.99–1.02)	0.36
HCVAb positive	1.36 (0.98–1.89)	0.07
Albumin (per 1.0 g/dl)	0.75 (0.53–1.07)	0.11
Recurrent case	2.21 (1.69–2.89)	<0.001
Multinodular	1.69 (1.27–2.25)	<0.001
AFP >100 ng/ml	1.41 (0.996–1.98)	0.052

AFP, α-fetoprotein; CI, confidence interval; CTHA/CTAP, computed tomography during hepatic arteriography and arterial portography; HCVAb, hepatitis C virus antibody.

recurrence-free survival than those without additional nodules ($P=0.003$, **Figure 2c**).

DISCUSSION

An advance in diagnostic technology generally indicates improved sensitivity or specificity, which corresponds to the detection of smaller lesions with a clearer view in imaging modalities. In our previous study, we showed that 75 nodules with a mean diameter of 8.7 mm (range, 2–20 mm) in 45 (33%) of 139 patients who underwent CTHA/CTAP were additionally diagnosed as definite HCC, compared with dynamic CT examination (33). However, no significant difference was observed in terms of recurrence-free survival between those who did and did not undergo CTHA/CTAP before RFA.

One reason for this discrepancy may be that the impact of CTHA/CTAP on recurrence reduction was diluted by a long-term follow-up of >2 years. It is unlikely that CTHA/CTAP could detect small nodules that would be detected ≥2 years later by conventional dynamic CT. In fact, the number of recurrences identified within 1 year after enrollment was lower in the CTHA/CTAP group than the control group (54 vs. 65, data not shown).

Another reason could be that fewer patients achieved complete ablation of target nodules in the CTHA/CTAP group than in the control group. The additionally diagnosed HCC nodules were small, and detection of these nodules by ultrasonography was difficult. Recent technologies such as contrast ultrasonography or fusion imaging, which can improve the accuracy of ablation techniques (34–36), may increase the probability of detection of smaller nodules before RFA.

Precise evaluation of the stage of progression is important for deciding on treatment procedures in HCC management. Seventeen patients in the CTHA/CTAP group were diagnosed with ≥4 nodules by CTHA/CTAP, which is not considered suitable for RFA according to widely used criteria.

In our previous study, we showed that recurrence as opposed to initial occurrence, multinodularity on dynamic CT, and HBsAg negativity were significant predictors for finding additional HCC by CTHA/CTAP (33). In fact, the CTHA/CTAP group showed better outcomes in the subgroups with HBsAg-negative cases, previously treated patients, and multinodular HCC. However, *post hoc* analysis comparing recurrence-free survival of those with and without additional nodules detected by CTHA/CTAP showed that those with a higher probability of additional nodules were also at a higher risk of recurrence. The advantage of CTHA/CTAP in finding more HCC nodules might be counter balanced by the higher risk of recurrence.

This study has several limitations. First, the additional nodules detected by CTHA/CTAP were not confirmed histologically. Therefore, we cannot exclude the possibility of overdiagnosis. Second, 45% of the patients had a history of previous treatment including resection, RFA, and TACE. Those previous treatments might substantially alter the hemodynamic status in the liver and affect the accuracy of CTHA/CTAP. On the other hand, in the previously treated cases, the radiologists could refer to the past series of dynamic CT during performing CTHA/CTAP, which might improve the accuracy of CTHA/CTAP as compared with treatment-naive cases. Third, 17 patients in the CTHA/CTAP group underwent TACE as a salvage treatment because total number of HCC nodules exceeded 3 after CTHA/CTAP. This might affect the recurrence-free and overall survival in the CTHA/CTAP group.

Our results may be extrapolated to other imaging modalities including gadoxetic acid–enhanced magnetic resonance imaging and second-generation contrast ultrasonography (37,38). These newly developed modalities also make possible the detection of small nodules that are invisible by dynamic CT. However, better diagnosis does not necessarily lead to better primary outcome.

In conclusion, CTHA/CTAP before RFA resulted in improved HCC diagnosis and detection of additional nodules in one-third of the study participants. However, it did not improve recurrence-free survival. The indications for CTHA/CTAP should be evaluated carefully.

Study protocol URL: https://upload.umin.ac.jp/cgi-open-bin/ctr/ctr.cgi?function=brows&action=brows&recptno=R000000117&type=summary&language=E.

CONFLICT OF INTEREST

Guarantor of the article: Ryosuke Tateishi, MD, PhD.

Specific author contributions: Conception and design: R.T., M.A., N.Y., T.G., S.S., H.Y., Y.M., and M.O.; analysis: R.T. and Y.M.; treatment and data collection: T.O., R.T., M.A., S.M., M.S., K.U., T.A., K.E., Y.K., T.G., and S.S.; drafting article: T.O.; critical revision: R.T., M.A., H.Y., K.O., and K.K.

Financial support: This work was supported by Health Sciences Research grants of The Ministry of Health, Labour and Welfare of Japan (Research on Hepatitis). No additional external funding was received for this study. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Potential competing interests: None.

Study Highlights

WHAT IS CURRENT KNOWLEDGE

- ✓ Computed tomography with hepatic arteriography and arterial portography (CTHA/CTAP) give higher hepatocellular carcinoma (HCC) detection sensitivity than conventional dynamic enhanced CT.
- ✓ CTHA/CTAP is an invasive procedure requiring the insertion of an intraarterial catheter through a femoral puncture.
- ✓ The indication for CTHA/CTAP can be justified only when the expected benefits exceed the risks and cost of the procedure.

WHAT IS NEW HERE

- ✓ Our study is the first randomized controlled trial (RCT) to evaluate the utility of CTHA/CTAP before radiofrequency ablation (RFA) in patients with HCC in the whole world.
- ✓ The best candidates for CTHA/CTAP were patients with multinodular HCC, and recurrent cases after previous treatment.
- ✓ However, CTHA/CTAP before RFA did not improve cumulative recurrence-free survival or overall survival.
- ✓ These observations are clinically important as the technique had limited utility and highlights the observation that patient outcomes are probably not related to the presence of small liver nodules.
- ✓ These findings reinforce the notion of genetic determinants of HCC recurrence.

REFERENCES

1. El-Serag HB, Mason AC. Rising incidence of hepatocellular carcinoma in the United States. N Engl J Med 1999;340:745–50.
2. Kiyosawa K, Umemura T, Ichijo T *et al.* Hepatocellular carcinoma: recent trends in Japan. Gastroenterology 2004;127:S17–26.
3. Itai Y, Matsui O. Blood flow and liver imaging. Radiology 1997;202:306–14.
4. Matsui O. Detection and characterization of hepatocellular carcinoma by imaging. Clin Gastroenterol Hepatol 2005;3:S136–40.
5. Teratani T, Yoshida H, Shiina S *et al.* A novel display of reconstruction computed tomography for the detection of small hepatocellular carcinoma. Liver Int 2004;24:619–24.
6. Fujishima T, Yoshida H, Obi S *et al.* Analysis of factors influencing hepatocellular carcinoma detection: efficient use of computed tomography during arterial portography and during hepatic arteriography. J Gastroenterol 2005;40:266–73.
7. Sangiovanni A, Manini MA, Iavarone M *et al.* The diagnostic and economic impact of contrast imaging techniques in the diagnosis of small hepatocellular carcinoma in cirrhosis. Gut 2010;59:638–44.
8. Murakami T, Oi H, Hori M *et al.* Helical CT during arterial portography and hepatic arteriography for detecting hypervascular hepatocellular carcinoma. AJR Am J Roentgenol 1997;169:131–5.
9. Kanematsu M, Hoshi H, Imaeda T *et al.* Detection and characterization of hepatic tumors: value of combined helical CT hepatic arteriography and CT during arterial portography. AJR Am J Roentgenol 1997;168:1193–8.
10. Irie T, Takeshita K, Wada Y *et al.* CT evaluation of hepatic tumors: comparison of CT with arterial portography, CT with infusion hepatic arteriography, and simultaneous use of both techniques. AJR Am J Roentgenol 1995;164:1407–12.
11. Small WC, Mehard WB, Langmo LS *et al.* Preoperative determination of the resectability of hepatic tumors: efficacy of CT during arterial portography. AJR Am J Roentgenol 1993;161:319–22.
12. Soyer P, Levesque M, Elias D *et al.* Detection of liver metastases from colorectal cancer: comparison of intraoperative US and CT during arterial portography. Radiology 1992;183:541–4.
13. Soyer P, Levesque M, Elias D *et al.* Preoperative assessment of resectability of hepatic metastases from colonic carcinoma: CT portography vs sonography and dynamic CT. AJR Am J Roentgenol 1992;159:741–4.
14. Matsui O, Kadoya M, Suzuki M *et al.* Work in progress: dynamic sequential computed tomography during arterial portography in the detection of hepatic neoplasms. Radiology 1983;146:721–7.
15. Sherman M, Klein A. AASLD single-topic research conference on hepatocellular carcinoma: conference proceedings. Hepatology 2004;40:1465–73.
16. Tateishi R, Shiina S, Ohki T *et al.* Treatment strategy for hepatocellular carcinoma: expanding the indications for radiofrequency ablation. J Gastroenterol 2009;44 (Suppl 19): 142–6.
17. Ebara M, Ohto M, Sugiura N *et al.* Percutaneous ethanol injection for the treatment of small hepatocellular carcinoma. Study of 95 patients. J Gastroenterol Hepatol 1990;5:616–26.
18. Livraghi T, Giorgio A, Marin G *et al.* Hepatocellular carcinoma and cirrhosis in 746 patients: long-term results of percutaneous ethanol injection. Radiology 1995;197:101–8.
19. Shiina S, Tagawa K, Niwa Y *et al.* Percutaneous ethanol injection therapy for hepatocellular carcinoma: results in 146 patients. AJR Am J Roentgenol 1993;160:1023–8.
20. Seki T, Wakabayashi M, Nakagawa T *et al.* Percutaneous microwave coagulation therapy for patients with small hepatocellular carcinoma: comparison with percutaneous ethanol injection therapy. Cancer 1999;85:1694–702.
21. Rossi S, Di Stasi M, Buscarini E *et al.* Percutaneous RF interstitial thermal ablation in the treatment of hepatic cancer. AJR Am J Roentgenol 1996;167:759–68.
22. Allgaier HP, Deibert P, Zuber I *et al.* Percutaneous radiofrequency interstitial thermal ablation of small hepatocellular carcinoma. Lancet 1999;353:1676–7.
23. Livraghi T, Goldberg SN, Lazzaroni S *et al.* Small hepatocellular carcinoma: treatment with radio-frequency ablation versus ethanol injection. Radiology 1999;210:655–61.
24. Curley SA, Izzo F, Ellis LM *et al.* Radiofrequency ablation of hepatocellular cancer in 110 patients with cirrhosis. Ann Surg 2000;232:381–91.
25. Shiina S, Tateishi R, Arano T *et al.* Radiofrequency ablation for hepatocellular carcinoma: 10-year outcome and prognostic factors. Am J Gastroenterol 2012;107:569–77; quiz 578.
26. Jang HJ, Lim JH, Lee SJ *et al.* Hepatocellular carcinoma: are combined CT during arterial portography and CT hepatic arteriography in addition to

triple-phase helical CT all necessary for preoperative evaluation? Radiology 2000;215:373–80.
27. Rennie D. CONSORT revised--improving the reporting of randomized trials. JAMA 2001;285:2006–7.
28. Efron B. Forcing a sequential experiment to be balanced. Biometrika 1971;58:403.
29. Kitao A, Zen Y, Matsui O *et al.* Hepatocarcinogenesis: multistep changes of drainage vessels at CT during arterial portography and hepatic arteriography--radiologic-pathologic correlation. Radiology 2009;252:605–14.
30. Tateishi R, Shiina S, Teratani T *et al.* Percutaneous radiofrequency ablation for hepatocellular carcinoma. An analysis of 1000 cases. Cancer 2005;103:1201–9.
31. Goldberg SN, Grassi CJ, Cardella JF *et al.* Image-guided tumor ablation: standardization of terminology and reporting criteria. J Vasc Interv Radiol 2009;20:S377–90.
32. Sacks D, McClenny TE, Cardella JF *et al.* Society of Interventional Radiology clinical practice guidelines. J Vasc Interv Radiol 2003;14:S199–202.
33. Ohki T, Tateishi R, Akahane M *et al.* Characteristics of hepatocellular carcinoma nodules newly detected by computed tomography during arteriography and arterial portography: preliminary report of a randomized controlled trial. Hepatol Int 2011 (e-pub ahead of print).
34. Minami Y, Kudo M. Review of dynamic contrast-enhanced ultrasound guidance in ablation therapy for hepatocellular carcinoma. World J Gastroenterol 2011;17:4952–9.
35. Numata K, Fukuda H, Morimoto M *et al.* Use of fusion imaging combining contrast-enhanced ultrasonography with a perflubutane-based contrast agent and contrast-enhanced computed tomography for the evaluation of percutaneous radiofrequency ablation of hypervascular hepatocellular carcinoma. Eur J Radiol 2012;81:2746–53.
36. Masuzaki R, Shiina S, Tateishi R *et al.* Utility of contrast-enhanced ultrasonography with Sonazoid in radiofrequency ablation for hepatocellular carcinoma. J Gastroenterol Hepatol 2011;26:759–64.
37. Ichikawa T, Saito K, Yoshioka N *et al.* Detection and characterization of focal liver lesions: a Japanese phase III, multicenter comparison between gadoxetic acid disodium-enhanced magnetic resonance imaging and contrast-enhanced computed tomography predominantly in patients with hepatocellular carcinoma and chronic liver disease. Invest Radiol 2010;45:133–41.
38. Hatanaka K, Kudo M, Minami Y *et al.* Differential diagnosis of hepatic tumors: value of contrast-enhanced harmonic sonography using the newly developed contrast agent, Sonazoid. Intervirology 2008; 51 (Suppl 1): 61–9.

OPEN ACCESS Freely available online

PLOS | ONE

Impact of IL28B Genetic Variation on HCV-Induced Liver Fibrosis, Inflammation, and Steatosis: A Meta-Analysis

Masaya Sato[1], Mayuko Kondo[1], Ryosuke Tateishi[1]*, Naoto Fujiwara[1], Naoya Kato[2], Haruhiko Yoshida[1], Masataka Taguri[3], Kazuhiko Koike[1]

1 Department of Gastroenterology, Graduate School of Medicine, The University of Tokyo, Bunkyo-ku, Tokyo, Japan, **2** Unit of Disease Control Genome Medicine, Institute of Medical Science, The University of Tokyo, Minato-ku, Tokyo, Japan, **3** Department of Biostatistics and Epidemiology, Yokohama City University Medical Center, Yokohama, Kanagawa, Japan

Abstract

Background & Aims: IL28B polymorphisms were shown to be strongly associated with the response to interferon therapy in chronic hepatitis C (CHC) and spontaneous viral clearance. However, little is known about how these polymorphisms affect the natural course of the disease. Thus, we conducted the present meta-analysis to assess the impact of IL28B polymorphisms on disease progression.

Methods: A literature search was conducted using MEDLINE, EMBASE, and the Cochrane Library. Integrated odds ratios (OR) were calculated with a fixed-effects or random-effects model based on heterogeneity analyses.

Results: We identified 28 studies that included 10,024 patients. The pooled results indicated that the rs12979860 genotype CC was significantly associated (vs. genotype CT/TT; OR, 1.122; 95%CI, 1.003–1.254; P = 0.044), and that the rs8099917 genotype TT tended to be (vs. genotype TG/GG; OR, 1.126; 95%CI, 0.988–1.284; P = 0.076) associated, with an increased possibility of severe fibrosis. Both rs12979860 CC (vs. CT/TT; OR, 1.288; 95%CI, 1.050–1.581; P = 0.015) and rs8099917 TT (vs. TG/GG; OR, 1.324; 95%CI, 1.110–1.579; P = 0.002) were significantly associated with a higher possibility of severe inflammation activity. Rs8099917 TT was also significantly associated with a lower possibility of severe steatosis (vs. TG/GG; OR, 0.580; 95%CI, 0.351–0.959; P = 0.034), whereas rs12979860 CC was not associated with hepatic steatosis (vs. CT/TT; OR, 1.062; 95%CI, 0.415–2.717; P = 0.901).

Conclusions: IL28B polymorphisms appeared to modify the natural course of disease in patients with CHC. Disease progression seems to be promoted in patients with the rs12979860 CC and rs8099917 TT genotypes.

Citation: Sato M, Kondo M, Tateishi R, Fujiwara N, Kato N, et al. (2014) Impact of IL28B Genetic Variation on HCV-Induced Liver Fibrosis, Inflammation, and Steatosis: A Meta-Analysis. PLoS ONE 9(3): e91822. doi:10.1371/journal.pone.0091822

Editor: Ming-Lung Yu, Kaohsiung Medical University Hospital, Kaohsiung Medical University, Taiwan

Received October 3, 2013; **Accepted** February 15, 2014; **Published** March 17, 2014

Funding: This study was supported by the Global COE Program, "Center of Education and Research for Advanced Genome-Based Medicine: For personalized medicine and the control of worldwide infectious diseases"; the Ministry of Education, Culture, Sports, Science and Technology, Japan; by grants from the Leading Project of the Ministry of Education, Culture, Sports, Science and Technology, Japan; and by Health and Labor Sciences Research Grants for Research on Hepatitis from the Ministry of Health, Labor and Welfare, Japan. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

* E-mail: tateishi-tky@umin.ac.jp

Introduction

Hepatitis C virus (HCV) infection is a major cause of chronic hepatitis, liver cirrhosis, and hepatocellular carcinoma (HCC) [1]. In epidemiological studies of chronic HCV infection, age, duration of infection, alcohol consumption, coinfection with human immune deficiency virus, low CD4 count, male gender, and HCV genotype 3 have been shown to be associated with histological activity [2–7]. Although these factors explain part of the extreme variability seen in the progression of fibrosis among HCV-infected patients, they do not completely account for the differences. Genetic host factors have long been suspected to play a role in chronic hepatitis C (CHC) [8–10]. Two genome-wide association studies recently reported the susceptible loci for the progression of liver cirrhosis [11,12].

Currently, patients with CHC are treated with a combination of peg-interferon (peg-IFN) and ribavirin [13,14]. Telaprevir and boceprevir, two protease inhibitors, were recently approved for patients with genotype 1 in combination with peg-IFN and ribavirin. This combination has been shown to lead to substantial improvement in the sustained virologic response rate [15,16]. Genetic variations near the interleukin 28B (IL28B) gene, encoding type III IFN-λ3, were shown to be strongly associated with the response to peg-IFN and ribavirin treatment in patients with CHC [17–20] and with spontaneous clearance of HCV [21]. Host immune cells produce IFN and other cytokines in response to viral infection. In response to HCV, cellular sensors detect the double-stranded RNA via retinoic acid-inducible gene-I and toll-like receptor 3 and activate a pathway to produce antiviral cytokines, including alpha and beta IFNs that trigger an antiviral response to eradicate the virus [22,23].

Polymorphisms of genes involved in innate immunity are likely to influence the strength and nature of this defense system [24]. Moreover, IL28B polymorphisms were shown to be associated with lipid metabolism [25]. Thus, this genetic factor is thought to influence the natural course of HCV infection including liver fibrosis, inflammation activity, or steatosis. However, associations between IL28B polymorphisms and the state of background liver disease (fibrosis, inflammation activity, or steatosis) in patients with CHC remain controversial. Single studies may have limited statistical power to detect the modest effects of IL28B polymorphisms on disease progression.

Thus, we conducted the present meta-analysis to integrate the results of eligible studies and provide statistically reliable evidence of the role of IL28B polymorphisms in patients with CHC.

Materials and Methods

2.1 Search strategy

An electronic search was conducted in MEDLINE, EMBASE, and the Cochrane Library for articles published prior to 30 April, 2012. Search terms included *IL28B*, *IL28*, *IL-28B*, *interleukin-28B*, *interleukin 28B*, *rs12979860*, and *rs8099917*. The search was limited to the English language.

2.2 Inclusion criteria

A study was included in the current analysis if it satisfied the following criteria: (1) It evaluated the associations between IL28B polymorphisms (rs12979860 or rs8099917) and liver fibrosis, inflammation activity, or steatosis. We also included studies that evaluated fibrosis or inflammation activity using the aminotransferase platelet ratio index or ALT. (2) It provided sufficient published data for estimating odds ratios (OR) with 95% confidence intervals (CIs). In case of multiple studies based on the same population, we selected the study with the largest number of participants. A study was excluded if (1) it dealt only with co-infection of HCV and human immunodeficiency virus, (2) it dealt only with patients with a specific condition such as a comorbid disease (e.g., thalassemia) or status after liver transplantation, or (3) it only used a recessive hereditary model (rs12979860 CC + CT vs. TT, or rs8099917 TT +TG vs. GG).

2.3 Data extraction

Two authors (M.S. and M.K.) independently screened titles and abstracts for potential eligibility and full texts for final eligibility. Disagreements were resolved by consultation with a third author (R.T.). The following information was extracted or calculated from each study: first author, year of publication, country of origin, ethnicity, sex, HCV genotype, and background liver information (fibrosis, inflammation activity, or steatosis) for each genotype. The analysis was based on the dominant model (CC vs. CT and TT in rs12979860; TT vs. TG and GG in rs8099917).

2.4 Definition

In some studies, mild or severe fibrosis or inflammation activity was not defined. To compare results among studies on these outcomes, we defined Ishak level F4 to F6; METAVIR, Ludwig Batts, and Inuyama level F3 to F4; and Knodell histology activity index as severe fibrosis. We also defined METAVIR A2 to A3 as severe inflammation activity.

2.5 Statistical analysis

The association of liver fibrosis, inflammation activity, or steatosis with the IL28B genotype in patients with CHC was assessed by summary ORs and corresponding 95% CIs. Heterogeneity among studies was examined with I^2 statistics interpreted as the proportion of total variation contributed by between-study variation [26]. If there was no or low statistical heterogeneity among studies ($I^2<50\%$ and $P>0.05$), the ORs and 95% CIs were calculated by the fixed-effects model. Otherwise, the random-effects model was adopted. When significant heterogeneity was observed, we performed a meta-regression analysis to investigate relationships between the effect of IL28B polymorphisms on liver fibrosis, inflammation activity, or steatosis; and continuous variables (proportion of patients with genotype 1 or 4 virus infection, proportion of males; and proportion of Caucasian, African-American, and Asian patients) to explore the possible reason for heterogeneity between studies [27,28]. To check for publication bias, we used the linear regression approach described by Egger et al. [29]. All calculations were performed using Comprehensive Meta-Analysis software (Biostat, Englewood, NJ).

Results

3.1 Characteristics of articles

Figure 1 shows the literature search and study selection procedures. A total of 471 potentially relevant publications up to 30 April, 2012, were initially identified through MEDLINE, EMBASE, and the Cochrane Library, 443 of which were excluded because they did not meet our inclusion criteria. Therefore, 28 studies involving a total number of 10,024 patients were included in the meta-analysis. Study characteristics are shown in Table 1. There were 5616 males and 3974 females, and the sex was not reported in the remaining 434 patients (1 study). Nineteen studies (7542 patients) evaluated liver fibrosis according to rs12979860 polymorphism and 16 studies (5052 patients) according to rs8099917 polymorphism; four studies (2301 patients) evaluated inflammation activity according to rs12979860 polymorphism and eight studies (2904 patients) according to rs8099917 polymorphism; and four studies (962 patients) evaluated steatosis according to rs12979860 polymorphism and five studies (1308 patients) according to rs8099917 polymorphism.

3.2 Fibrosis

For rs12979860, the between-study heterogeneity was not significant ($I^2=25\%$, $P=0.147$); thus, the fixed-effects model was applied. The pooled results indicated that IL28B rs12979860 genotype CC was associated with an increased possibility of severe fibrosis (OR, 1.122; 95%CI, 1.003–1.254; $P=0.044$) (Fig. 2-a). For rs8099917, there was no or low heterogeneity ($I^2=31\%$, $P=0.111$), and IL28B rs8099917 genotype TT tended to be associated with a higher possibility of severe fibrosis; however, the difference did not reach statistical significance (OR, 1.126; 95%CI, 0.988–1.284; $P=0.076$) (Fig. 2-b). Egger's test showed no evidence for publication biases for either rs12979860 ($P=0.839$) or rs8099917 ($P=0.342$). When restricted to studies in which only treatment-naïve patients were included, 12 studies (5865 patients) according to rs12979860 polymorphism and eight studies (3333 patients) according to rs8099917 polymorphism were extracted. The between-study heterogeneities were not significant for rs12979860 ($I^2=0\%$, $P=0.615$) and rs8099917 ($I^2=16\%$, $P=0.304$). For rs12979860, fixed-effect model analyses showed a higher probability of severe fibrosis in genotype CC (OR, 1.184; 95%CI, 1.040–1.348; $P=0.010$) (Fig. 2-c), and for rs8099917, genotype TT tended to be associated with a higher possibility of severe fibrosis; however, the difference was not statistically significant (OR, 1.154; 95%CI, 0.985–1.351; $P=0.076$) (Fig. 2-d). Egger's test showed no evidence of publication bias ($P=0.394$ for rs12979860 and $P=0.295$ for rs8099917).

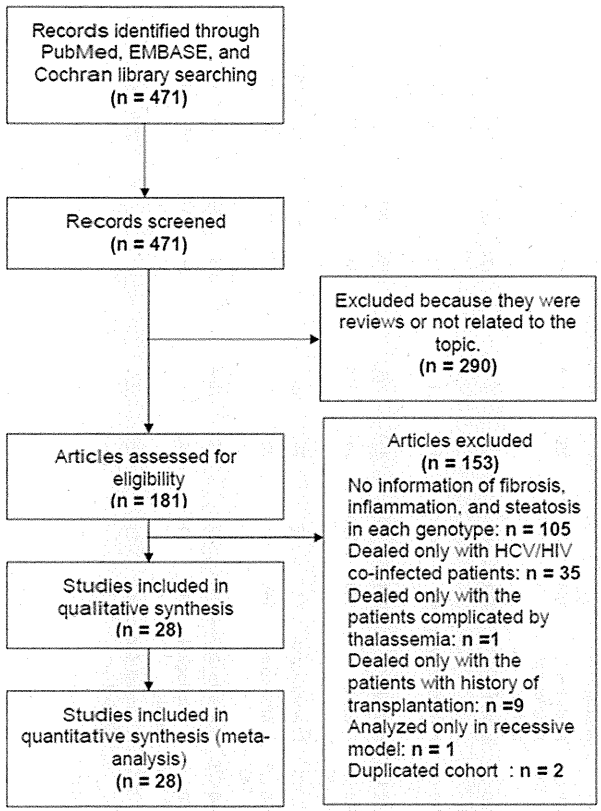

Figure 1. Literature search and study selection process. Twenty-eight individual studies that met all of the inclusion and exclusion criteria.
doi:10.1371/journal.pone.0091822.g001

3.3 Inflammation activity

The between-study heterogeneity was not significant ($I^2 = 35\%$, $P = 0.204$) for rs12979860. In the fixed-effects model, the pooled results indicated that IL28B rs12979860 genotype CC was associated with a higher possibility of severe inflammation activity (OR, 1.288; 95%CI, 1.050–1.581; $P = 0.015$) (Fig. 3-a). For rs8099917, there was no or low heterogeneity ($I^2 = 0\%$, $P = 0.598$), and IL28B rs8099917 genotype TT was also associated with a higher possibility of severe inflammation activity (OR, 1.324; 95%CI, 1.110–1.579; $P = 0.002$) (Fig. 3-b). Egger's test showed no evidence of publication biases for rs12979860 ($P = 0.448$) and rs8099917 ($P = 0.531$). When restricted to studies in which only treatment-naïve patients were included, three studies (2192 patients) according to rs12979860 polymorphism and two studies (1769 patients) according to rs8099917 polymorphism were extracted. Significant heterogeneities were found for rs12979860 ($I^2 = 53\%$, $P = 0.120$); thus, the random-effect model was applied. The pooled results indicated that IL28B rs12979860 genotype was not associated with inflammatory activity (OR, 1.340; 95%CI, 0.938–1.916; $P = 0.108$) (Fig. 3-c). For rs8099917, the between-study heterogeneity was not significant ($I^2 = 0\%$, $P = 0.585$). In the fixed-effects model, genotype TT tended to be associated with a higher possibility of severe inflammation activity (OR, 1.217; 95%CI, 0.978–1.515; $P = 0.079$) (Fig. 3-d). Egger's test showed no evidence of publication bias in rs12979860 ($P = 0.646$). For rs8099917, Egger's test was not applicable because only 2 studies were included. We also performed a meta-regression analysis for rs12979860 because significant heterogeneities were observed. Table 2 shows the results of these meta-regression analyses. Significant correlation was observed between rs12979860 polymorphisms and the proportion of patients with genotype 1 or 4 virus (slope, 2.992 ± 1.497; $P = 0.046$).

3.4 Steatosis

Significant heterogeneities were found for rs12979860 ($I^2 = 86\%$, $P<0.001$) and rs8099917 ($I^2 = 52\%$, $P = 0.082$); thus, we applied the random-effects model for this outcome. The pooled results indicated that IL28B rs12979860 genotype CC was not associated with hepatic steatosis (OR, 1.062; 95%CI, 0.415–2.717, $P = 0.901$) (Fig. 4-a), whereas rs8099917 TT was significantly associated with a lower possibility of severe steatosis (OR, 0.580; 95%CI, 0.351–0.959; $P = 0.034$) (Fig. 4-b). Egger's test showed no evidence of publication biases for rs12979860 ($P = 0.238$) or rs8099917 ($P = 0.182$). We also performed a meta-regression analysis because significant heterogeneities were observed. Table 3 shows the results of these meta-regression analyses. In terms of the effect of rs12979860 on steatosis, significant correlations were observed between the proportion of patients with genotype 1 or 4 virus (slope, -4.947 ± 1.086; $P<0.001$), the proportion of Caucasian patients (slope, 7.361 ± 1.569; $P<0.001$), and the proportion of African-American patients (slope, -8.996 ± 1.918; $P<0.001$). We also observed a significant correlation between the effect of rs8099917 polymorphism on steatosis and the proportion of male patients (slope, 6.225 ± 2.530; $P = 0.014$) (Fig. 5). Finally, we observed significant correlations between rs8099917 polymorphisms and the proportion of patients with genotype 1 or 4 virus (slope, -2.704 ± 1.277; $P = 0.034$), the proportion of Caucasian patients (slope, 1.168 ± 0.422; $P = 0.006$), and the proportion of Asian patients (slope, -1.049 ± 0.398; $P = 0.008$). When restricted to studies in which only treatment-naïve patients were included, two studies (495 patients) according to rs12979860 polymorphism and four studies (812 patients) according to rs8099917 polymorphism were extracted. The between-study heterogeneities were not significant for rs12979860 ($I^2 = 0\%$, $P = 0.823$) and rs8099917 ($I^2 = 41\%$, $P = 0.166$). For rs12979860, fixed-effect model analyses showed that rs12979860 genotype CC was significantly associated with a higher possibility of severe steatosis (OR, 1.708; 95%CI, 1.047–2.787; $P = 0.032$) (Fig. 4-c), whereas rs8099917 TT was significantly associated with a lower possibility of severe steatosis (OR, 0.675; 95%CI, 0.474–0.960; $P = 0.026$) (Fig. 4-d). Egger's test showed no evidence of publication bias in rs8099917 ($P = 0.554$). For rs12979860, Egger's test was not applicable because only 2 studies were included.

Discussion

In the present study, we evaluated the association between IL28B polymorphisms and the background liver disease (fibrosis, inflammation activity, or steatosis) in patients with CHC. The rs12979860 CC genotype was significantly associated with a higher probability of severe fibrosis (Fig. 2-c), and the rs8099917 TT genotype tended to be associated with a higher possibility of severe fibrosis (Fig. 2-d). The accumulation of liver inflammation promotes liver fibrosis, and these polymorphisms are associated with the effect of IFN-based treatment; therefore, past treatment might alter the results. Thus, we also analyzed studies involving only patients without a history of IFN-based treatment; however, the results were not changed.

The rs12979860 CC and rs8099917 TT genotypes were also associated with a higher possibility of severe inflammation activity. Genetic variations near the IL28B gene were originally reported as

Table 1. Main characteristics of all studies included in the meta-analysis.

First author (year)	Ref.	Population ethnicity, region	IL-28B SNP rsID, Allele	Outcome measure F(Fibrosis) A(Activity) S(Steatosis)	Patients*			HCV genotype	Genotype for patients rs12979860		Genotype for patients rs8099917	
					Male	Female	Total		CC	CT/TT	TT	TG/GG
Abe (2010)	[48]	Asian, Japan	rs8099917 T/G	F, A: Inuyama	212	152	364	1/2			265	99
Honda (2010)	[49]	Asian, Japan	rs8099917 T/G	F, A: Inuyama	58	33	91	1			60	31
Lotrich (2010)	[50]	Mixed (African-American/Caucasian), USA	rs12979860 C/T	F: Ishak	101	32	133	1/2	57	76		
Monte (2010)	[51]	Caucasian, Spain	rs12979860 C/T	F: Scheuer	166	117	283	1–4	129	154		
Thompson (2010)	[52]	Mixed (African-American/Caucasian/Asian/Hispanic), USA	rs12979860 C/T	F: METAVIR	986	642	1628	1	538	1090		
Bochud (2011)	[53]	Caucasian, Switzerland	rs12979860 C/T rs8099917 T/G	F: Ishak, A: ALT S: Histological finding	163	79	242	1–3	90	150	150	92
Dill MT (2011)	[54]	Caucasian, Switzerland	rs12979860 C/T rs8099917 T/G	F, A: METAVIR	30	79	109	1–4	33	96	52	57
Fabris (2011)	[44]	Caucasian, Italy	rs12979860 C/T	F: Ishak	N.A	N.A	434	1–4	133	301		
Falleti (2011)	[55]	Caucasian, Italy	rs12979860 C/T	F: Ishak	357	272	629	1–4	205	424		
Kurosaki (2011)	[56]	Asian, Japan	rs8099917 T/G	F: METAVIR S: Histological finding	250	246	496	1			269	106
Lagging (2011)	[57]	Caucasian, Sweden	rs12979860 C/T rs8099917 T/G	F: Ishak S: Histological finding	169	83	252	1–4	93	159	153	99
Lin (2011)	[58]	Asian, Taiwan	rs12979860 C/T rs8099917 T/G	F: METAVIR	123	68	191	1	171	20	170	21
Lindh (2011)-1	[59]	Mixed (Caucasian/Asian), Sweden	rs12979860 C/T rs8099917 T/G	F: Batts Ludwig	67	43	110	1	38	72	66	44
Lindh (2011)-2	[60]	Caucasian, Sweden	rs12979860 C/T	F: Ishak	204	137	341	2/3	150	191		
Marabita (2011)	[61]	Caucasian, Italy	rs12979860 C/T rs8099917 T/G	F: Ishak	129	118	247	1–4	88	159	131	116
Miyamura (2011)	[62]	Asian, Japan	rs8099917 T/G	F, A: Inuyama	37	42	79	1			53	26
Moghaddam(2011)	[63]	Caucasian, Norway	rs12979860 C/T rs8099917 T/G	F: APRI score	166	115	281	3	129	152	201	80
Rueda (2011)	[64]	Caucasian, Spain	rs12979860 C/T	F, A: Scheuer	246	177	423	1–4	83	184		
Tillman (2011)	[35]	Mixed (African-American/Caucasian/Asian), USA	rs12979860 C/T rs8099917 T/G	S: Histological finding	215	110	325	1	88	237	97	67
Yu (2011)	[65]	Asian, Taiwan	rs8099917 T/G	F: Knodell and Scheuer	264	218	482	2			315	34
Asahina (2011)	[66]	Asian, Japan	rs12979860 C/T rs8099917 T/G	F: Inuyama	28	60	88	1	54	34	54	34

Table 1. Cont.

First author (year)	Ref.	Population ethnicity, region	IL-28B SNP rsID, Allele	Outcome measure F(Fibrosis) A(Activity) S(Steatosis)	Patients*			HCV genotype	Genotype for patients rs12979860		Genotype for patients rs8099917	
					Male	Female	Total		CC	CT/TT	TT	TG/GG
Bochud (2012)	[47]	Caucasian, Switzerland	rs12979860 C/T rs8099917 T/G	F, A: METAVIR	870	657	1527	1–4	534	993	855	672
Mach (2012)	[67]	Slav: Poland	rs12979860 C/T	F: Batts Ludwig	82	60	142	1	38	104		
Miyashita (2012)	[68]	Asian, Japan	rs8099917 T/G	F, A: Desmet	88	132	220	1/2			155	63
Ohnishi (2012)	[69]	Asian, Japan	rs8099917 T/G	S: Histological finding	83	70	153	1			116	37
Rembeck (2012)	[70]	Caucasian, Sweden	rs12979860 C/T	F: Ishak	199	140	339	2/3	144	179		
Tolmane (2012)	[71]	Caucasian, Latvia	rs12979860 C/T	F: Knodell histology activity index S: Histological finding	84	58	142	1–3	41	80		
Toyoda (2012)	[72]	Asian, Japan	rs8099917 T/G	F, A: METAVIR	139	133	272	1			187	59

*Patients included in the original study.
Thus, patients without information regarding IL28B polymorphism were also included.
APRI, aminotransferase platelet ratio index.
doi:10.1371/journal.pone.0091822.t001

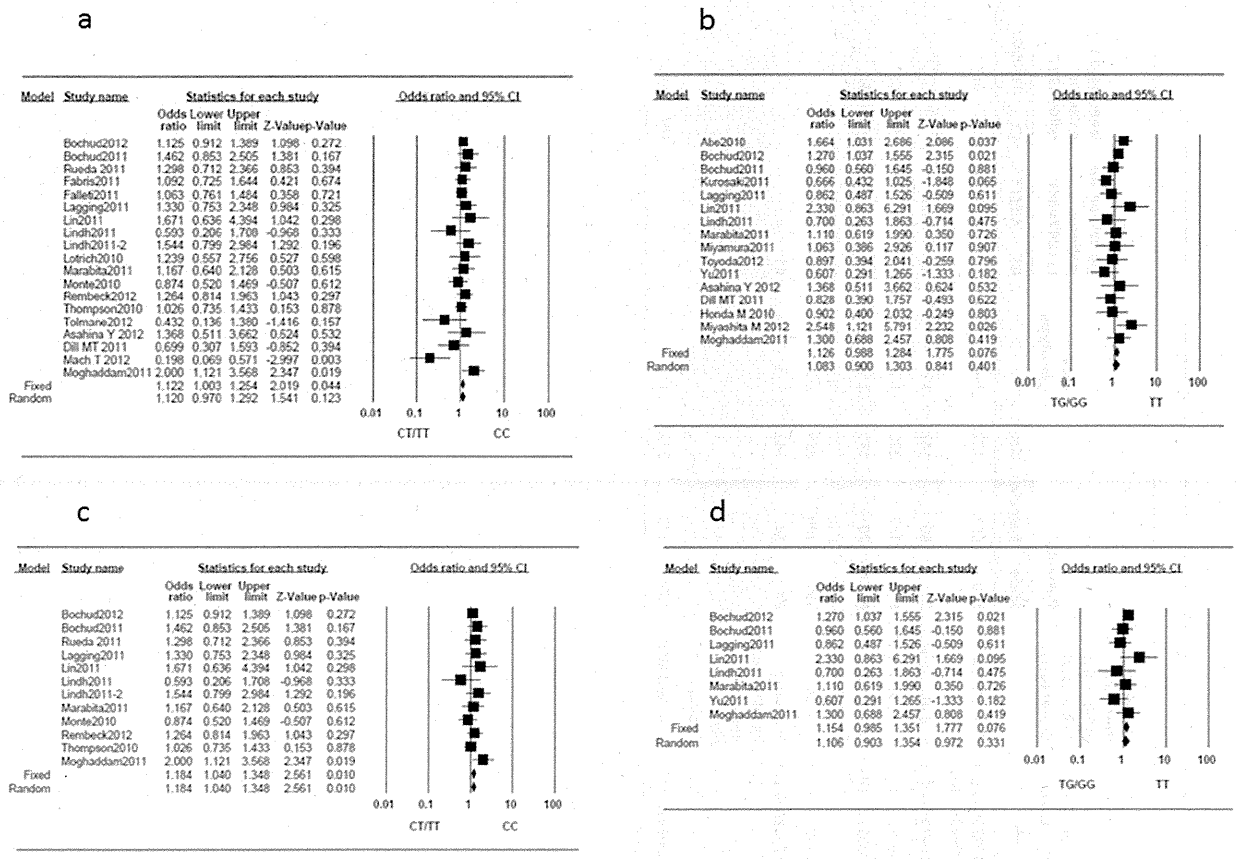

Figure 2. Forest plot of the IL28B genotypes and the risk of severe fibrosis. (a) rs12979860 in all patients, (b) rs8099917 in all patients, (c) rs12979860 in treatment-naïve patients, and (d) rs8099917 in treatment-naïve patients.
doi:10.1371/journal.pone.0091822.g002

strong predictors of a sustained viral response [17–20] or spontaneous clearance of HCV [21]. The level of IL28B gene transcripts is reportedly higher in patients homozygous for the IFN responsive allele [18,19]. Therefore, in patients with the rs12979860 CC and rs8099917 TT genotype, IL28B production, which induces expression of interferon-stimulated genes, including some inflammatory cytokines, was thought to be increased. This may be the underlying cause of the higher inflammation activity and progressed fibrosis in patients with the IFN responsive allele. In analysis with the studies involving only patients without a history of IFN-based treatment, rs12979860 CC and rs8099917 TT genotypes were associated with higher possibility of having severe inflammation activity; however, the differences did not reach to the significant level. Only three studies according to rs12979860 polymorphism and two studies according to rs8099917 polymorphism were included when restricted to studies with only treatment-naïve patients, and may be underpowered to detect the effects of IL28B polymorphisms on inflammation activity. The further analyses with larger sample are needed to confirm this association. Additionally, meta-regression analysis showed that the effect of the rs12979860 polymorphism was influenced by viral genotype distribution. This result may imply a different influence of rs12979860 polymorphism on immune response according to viral genotype in treatment-naïve patients.

IL28B polymorphisms were also shown to be associated with lipid metabolism [25]. In the present study, the rs8099917 TT genotype was significantly associated with a lower possibility of severe steatosis. This association still remained statistically significant after we restricted to studies in which only treatment-naïve patients were included. The lower hepatic steatosis in patients with the IFN responsive allele could be explained by a more efficient export of lipids from hepatocytes. Higher interferon expression was shown to lead to suppression of lipoprotein lipase, which would result in decreased conversion of VLDL to LDL and subsequent higher steatosis [30–33]. The difference in IL28B expression might cause an aberration of lipid metabolism in patients with CHC. We found no significant association of rs12979860 with steatosis. And when we restricted to treatment-naïve patients, rs12979860 CC genotype was significantly associated with a higher possibility of severe steatosis. Previous studies have shown that racial differences or viral genotypes make a difference in the effects of rs12979860 and rs8099917 polymorphisms [34,35]. This may explain the discrepancy between the effect of rs12979860 and rs8099917 on hepatic steatosis. However, only four studies (962 patients) were included in the analysis of rs12979860; or when it comes to the studies with only treatment-naïve patients, only two studies (495 patients) were extracted. Thus, we should not make any definite conclusion on this matter right now. Further studies with larger sample sizes are needed to identify their exact correlation.

According to the meta-regression analysis, the effect of rs8099917 polymorphisms on steatosis became smaller with the

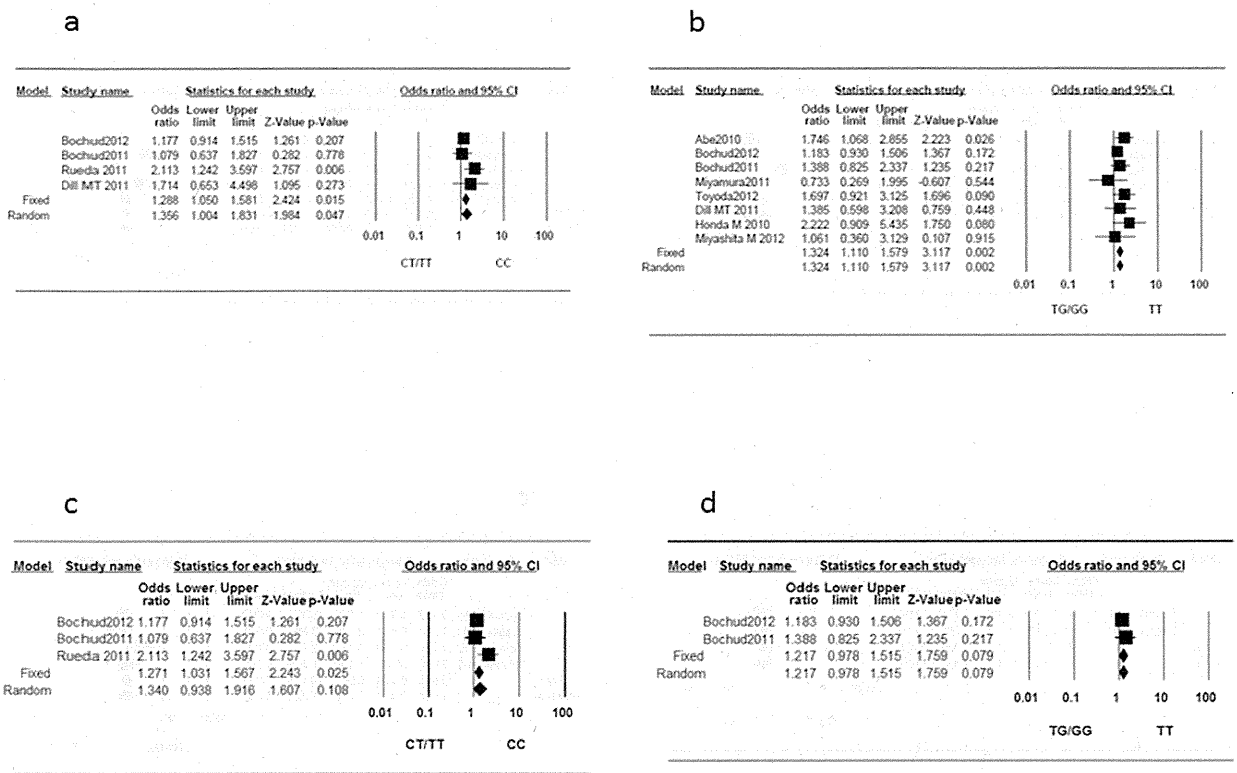

Figure 3. Forest plot of the IL28B genotypes and the risk of severe inflammation activity. (a) rs12979860 and (b) rs8099917. (c) rs12979860 in treatment-naïve patients, and (d) rs8099917 in treatment-naïve patients.
doi:10.1371/journal.pone.0091822.g003

increase in the male proportion (Fig. 5), suggesting that a sexual dimorphism might be involved in the effect of rs8099917 polymorphisms on the liver fat content. Although the present study cannot explain the interaction between the polymorphism and sex, immune systems responding to IFN are reportedly controlled by estrogenic sex hormones [36,37]. Differences in IL28B expression mediated by sex hormones could be a possible mechanism for the sexual dimorphism in the effect of rs8099917 polymorphisms on liver steatosis.

The rs738409 genotype within the patatin-like phospholipase domain containing 3 locus was also reported to be associated with hepatic steatosis in patients with CHC [38–40]. Notably, previous meta-analysis evaluating the effect of patatin-like phospholipase domain containing 3 polymorphisms on steatosis also reported a

Table 2. Meta-regression analysis between each continuous variable among the studies (only treatment- naïve patients were included) and the effect (log odds ratio) of IL28B polymorphisms on inflammation activity.

Variables	Slope*	Standard error	P-value
Proportion of patients with genotype 1 or 4 virus, per 1% increase			
rs12979860	2.992	1.497	0.046
Proportion of male patients, per 1% increase			
rs12979860	−2.963	5.802	0.610
Proportion of Caucasian patients, per 1% increase			
rs12979860†	–	–	–
Proportion of African-American patients, per 1% increase			
rs12979860†	–	–	–
Proportion of Asian patients, per 1% increase			
rs12979860†	–	–	–

*Positive (negative) slope values indicate that the proportions of patients with the rs12979860 CC genotype with severe inflammation activity are increasing (decreasing) as the values of each contentious variable (proportions of genotype 1 or 4 virus, male, or each race) is increasing.
†We could not perform meta-regression analyses for these outcomes because only caucasian patients were included in all 3 studies included in this analysis.
doi:10.1371/journal.pone.0091822.t002

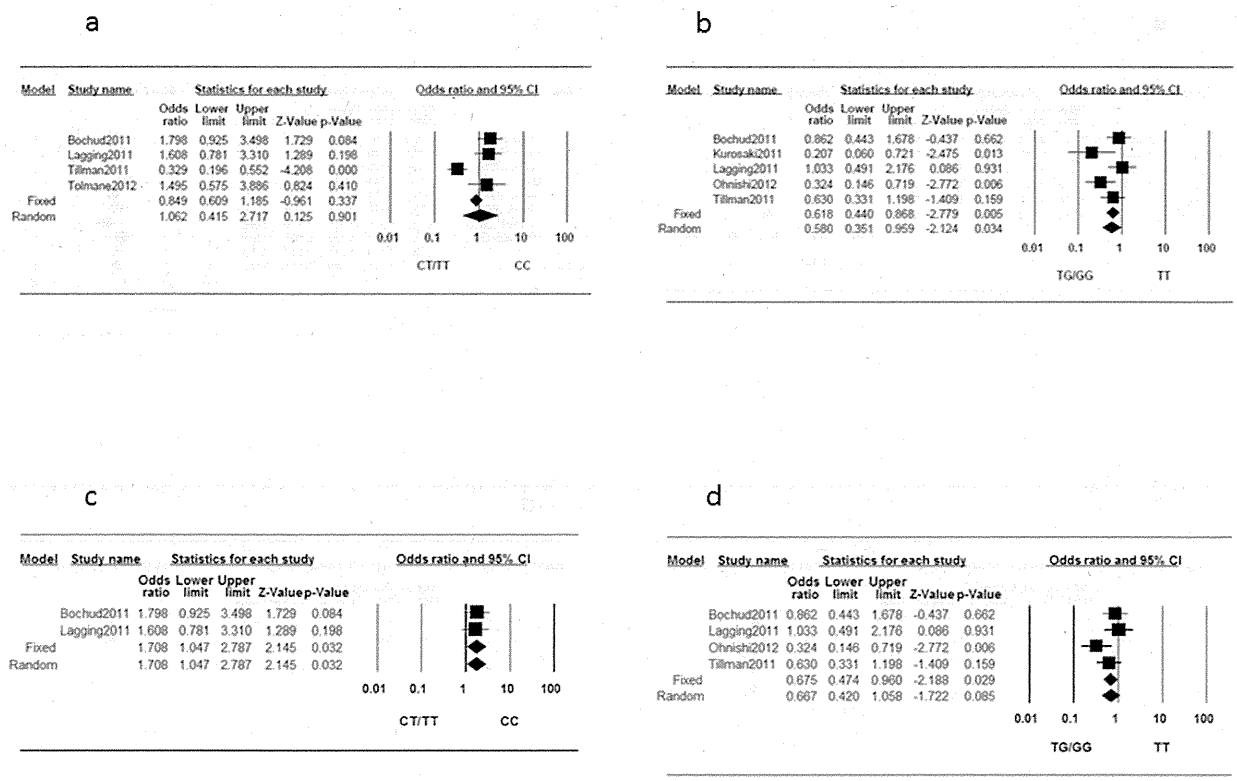

Figure 4. Forest plot of the IL28B genotypes and the risk of hepatic steatosis. (a) rs12979860 and (b) rs8099917. (c) rs12979860 in treatment-naïve patients, and (d) rs8099917 in treatment-naïve patients.
doi:10.1371/journal.pone.0091822.g004

Table 3. Meta-regression analysis between each continuous variable among the studies and the effect (log odds ratio) of IL28B polymorphisms on steatosis.

Variables	Slope*	Standard error	P-value
Proportion of patients with genotype 1 or 4 virus, per 1% increase			
rs12979860	−4.947	1.086	<0.001
rs8099917	−2.704	1.277	0.034
Proportion of male patients, per 1% increase			
rs12979860	−2.899	16.577	0.861
rs8099917	6.225	2.530	0.014
Proportion of Caucasian patients, per 1% increase			
rs12979860	7.361	1.569	<0.001
rs8099917	1.168	0.422	0.006
Proportion of African-American patients, per 1% increase			
rs12979860	−8.996	1.918	<0.001
rs8099917	0.142	2.147	0.947
Proportion of Asian patients, per 1% increase			
rs12979860†	–	–	–
rs8099917	−1.049	0.398	0.008

*Positive (negative) slope values indicate that the proportions of patients with the rs12979860 CC or rs8099917 TT genotypes with severe steatosis are increasing (decreasing) as the values of each contentious variable (proportions of genotype 1 or 4 virus, male, or each race) is increasing.
†We could not perform a meta-regression analysis for this outcome because only one patient was included in the corresponding studies.
doi:10.1371/journal.pone.0091822.t003

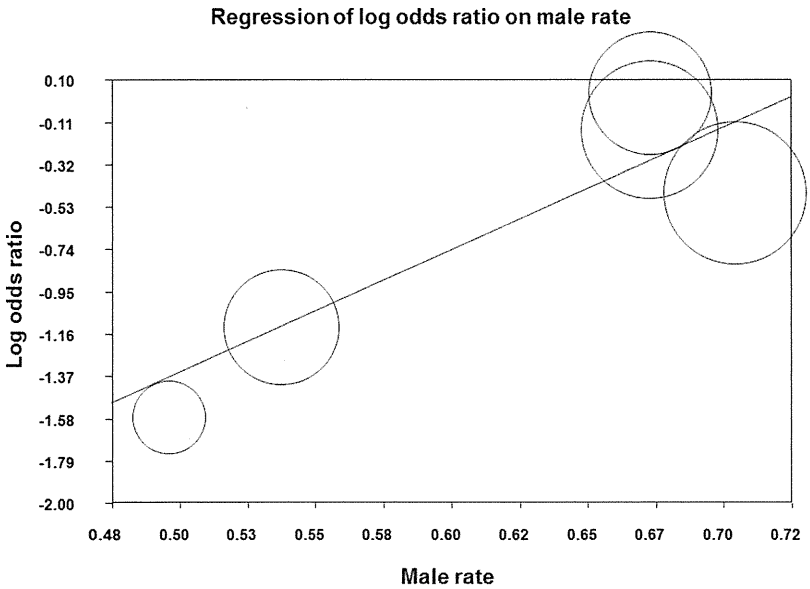

Figure 5. Meta-regression plot for log odds ratios in rates of patients with severe hepatic steatosis by proportion of males (%) in rs8099917.
doi:10.1371/journal.pone.0091822.g005

negative correlation between the male proportion and the effect of rs738409 on the liver fat content in nonalcoholic fatty liver disease [41]. Interestingly, the meta-regression analysis in the present study showed that the effect of the IL28B (rs12979860 and rs8099917) polymorphisms on steatosis was also influenced by racial and viral genotype distributions.

In the present study, we included studies that did not report the associations between IL28B genotypes and background liver diseases as study outcomes, but provided raw data that allowed us to calculate the OR for each outcome, which may have minimized potential publication bias. In fact, no publication bias was observed in the present study. The Human Genome Epidemiology Network highlighted the necessity of meta-analysis before evidence for a particular association can be regarded as strong [42]. The impact of IL28B genotypes on the disease progression found in the present meta-analysis may provide clinically important information in the follow-up of patients with CHC. The effect of IL28B polymorphisms on hepatocarcinogenesis, which is also crucial information in the HCC screening of patients with CHC, remains controversial [43–47]. Further analysis with larger sample sizes may be needed to elucidate the exact effect of IL28B polymorphisms on hepatocarcinogenesis.

A potential limitation of this study is inter-study variability in the outcome measure and the definition of "severe" among studies, where some discrepancies among studies exist. The studies without a pathological diagnosis, using laboratory data as surrogates, were also included. These studies may have diminished the accuracy of our research results concerning liver disease severity.

In conclusion, the present study highlighted the impact of IL28B polymorphisms on liver fibrosis, inflammation activity, and steatosis in patients with CHC. Disease progression appeared to be promoted in patients with rs12979860 CC or rs8099917 TT genotypes. The current findings may provide clinically important information in the follow-up of patients with CHC.

Supporting Information

Checklist S1 PRISMA 2009 Checklist.
(DOC)

Acknowledgments

The English in this document has been checked by at least two professional editors, both native speakers of English. For a certificate, please see: http://www.textcheck.com/certificate/IWcYpT.

Author Contributions

Conceived and designed the experiments: MS RT NK. Performed the experiments: MS MK RT. Analyzed the data: MS RT. Contributed reagents/materials/analysis tools: MS. Wrote the paper: MS RT HY. Critical revision of manuscript: NF MT KK.

References

1. Barrera JM, Bruguera M, Ercilla MG, Gil C, Celis R, et al. (1995) Persistent hepatitis C viremia after acute self-limiting posttransfusion hepatitis C. Hepatology 21: 639–644.
2. Poynard T, Bedossa P, Opolon P (1997) Natural history of liver fibrosis progression in patients with chronic hepatitis C. The OBSVIRC, METAVIR, CLINIVIR, and DOSVIRC groups. Lancet 349: 825–832.
3. Hourigan LF, Macdonald GA, Purdie D, Whitehall VH, Shorthouse C, et al. (1999) Fibrosis in chronic hepatitis C correlates significantly with body mass index and steatosis. Hepatology 29: 1215–1219.
4. Powell EE, Edwards-Smith CJ, Hay JL, Clouston AD, Crawford DH, et al. (2000)Host genetic factors influence disease progression in chronic hepatitis C. Hepatology 31: 828–833.
5. Massard J, Ratziu V, Thabut D, Moussalli J, Lebray P, et al. (2006) Natural history and predictors of disease severity in chronic hepatitis C. J Hepatol 44: S19–24.
6. Bochud PY, Cai T, Overbeck K, Bochud M, Dufour JF, et al. (2009) Genotype 3 is associated with accelerated fibrosis progression in chronic hepatitis C. J Hepatol 51: 655–666.

7. De Nicola S, Aghemo A, Rumi MG, Colombo M (2009) HCV genotype 3: an independent predictor of fibrosis progression in chronic hepatitis C. J Hepatol 51: 964–966.
8. Thursz M, Yallop R, Goldin R, Trepo C, Thomas HC (1999) Influence of MHC class II genotype on outcome of infection with hepatitis C virus. The HENCORE group. Hepatitis C European Network for Cooperative Research. Lancet 354: 2119–2124.
9. Pradat P, Tillmann HL, Sauleda S, Braconier JH, Saracco G, et al. (2007) Long-term follow-up of the hepatitis C HENCORE cohort: response to therapy and occurrence of liver-related complications. J Viral Hepat 14: 556–563.
10. Kato N, Ji G, Wang Y, Baba M, Hoshida Y, et al. (2005) Large-scale search of single nucleotide polymorphisms for hepatocellular carcinoma susceptibility genes in patients with hepatitis C. Hepatology 42: 846–853.
11. Urabe Y, Ochi H, Kato N, Kumar V, Takahashi A, et al. (2013) A genome-wide association study of HCV-induced liver cirrhosis in the Japanese population identifies novel susceptibility loci at the MHC region. J Hepatol; 58 (5): 875–82
12. Patin E, Kutalik Z, Guergnon J, Bibert S, Nalpas B, et al. (2012) Genome-Wide Association Study Identifies Variants Associated with Progression of Liver Fibrosis from HCV Infection. Gastroenterology; 143 (5): 1244–52
13. Hadziyannis SJ, Sette H Jr, Morgan TR, Balan V, Diago M, et al. (2004) Peginterferon-alpha2a and ribavirin combination therapy in chronic hepatitis C: a randomized study of treatment duration and ribavirin dose. Ann Intern Med 140: 346–355.
14. Manns MP, McHutchison JG, Gordon SC, Rustgi VK, Shiffman M, et al. (2001) Peginterferon alfa-2b plus ribavirin compared with interferon alfa-2b plus ribavirin for initial treatment of chronic hepatitis C: a randomised trial. Lancet 358: 958–965.
15. McHutchison JG, Everson GT, Gordon SC, Jacobson IM, Sulkowski M, et al. (2009) Telaprevir with peginterferon and ribavirin for chronic HCV genotype 1 infection. N Engl J Med 360: 1827–1838.
16. Poordad F, McCone J Jr, Bacon BR, Bruno S, Manns MP, et al. (2011) Boceprevir for untreated chronic HCV genotype 1 infection. N Engl J Med 364: 1195–1206.
17. Ge D, Fellay J, Thompson AJ, Simon JS, Shianna KV, et al. (2009) Genetic variation in IL28B predicts hepatitis C treatment-induced viral clearance. Nature 461: 399–401.
18. Tanaka Y, Nishida N, Sugiyama M, Kurosaki M, Matsuura K, et al. (2009) Genome-wide association of IL28B with response to pegylated interferon-alpha and ribavirin therapy for chronic hepatitis C. Nat Genet 41: 1105–1109.
19. Suppiah V, Moldovan M, Ahlenstiel G, Berg T, Weltman M, et al. (2009) IL28B is associated with response to chronic hepatitis C interferon-alpha and ribavirin therapy. Nat Genet 41: 1100–1104.
20. Rauch A, Kutalik Z, Descombes P, Cai T, Di Iulio J, et al. (2010) Genetic variation in IL28B is associated with chronic hepatitis C and treatment failure: a genome-wide association study. Gastroenterology 138: 1338–1345, 1345 e1331–1337.
21. Thomas DL, Thio CL, Martin MP, Qi Y, Ge D, et al. (2009) Genetic variation in IL28B and spontaneous clearance of hepatitis C virus. Nature 461: 798–801.
22. Yoneyama M, Kikuchi M, Natsukawa T, Shinobu N, Imaizumi T, et al. (2004) The RNA helicase RIG-I has an essential function in double-stranded RNA-induced innate antiviral responses. Nat Immunol 5: 730–737.
23. Moriyama M, Kato N, Otsuka M, Shao RX, Taniguchi H, et al. (2007) Interferon-beta is activated by hepatitis C virus NS5B and inhibited by NS4A, NS4B, and NS5A. Hepatol Int 1: 302–310.
24. Li CZ, Kato N, Chang JH, Muroyama R, Shao RX, et al. (2009) Polymorphism of OAS-1 determines liver fibrosis progression in hepatitis C by reduced ability to inhibit viral replication. Liver Int 29: 1413–1421.
25. Li JH, Lao XQ, Tillmann HL, Rowell J, Patel K, et al. (2010) Interferon-lambda genotype and low serum low-density lipoprotein cholesterol levels in patients with chronic hepatitis C infection. Hepatology 51: 1904–1911.
26. Borenstein M, Hedges LV, Higgins JP, Rothstein HR (2009) Introduction to Meta-analysis. West Sussex: John Wiley & Sons Ltd.
27. Baker WL, White CM, Cappelleri JC, Kluger J, Coleman CI (2009) Understanding heterogeneity in meta-analysis: the role of meta-regression. Int J Clin Pract 63: 1426–1434.
28. Thompson SG, Sharp SJ (1999) Explaining heterogeneity in meta-analysis: a comparison of methods. Stat Med 18: 2693–2708.
29. Egger M, Davey Smith G, Schneider M, Minder C (1997) Bias in meta-analysis detected by a simple, graphical test. BMJ 315: 629–634.
30. Schectman G, Kaul S, Mueller RA, Borden EC, Kissebah AH (1992) The effect of interferon on the metabolism of LDLs. Arterioscler Thromb 12: 1053–1062.
31. Ehnholm C, Aho K, Huttunen JK, Kostiainen E, Mattila K, et al. (1982) Effect of interferon on plasma lipoproteins and on the activity of postheparin plasma lipases. Arteriosclerosis 2: 68–73.
32. Shinohara E, Yamashita S, Kihara S, Hirano K, Ishigami M, et al. (1997) Interferon alpha induces disorder of lipid metabolism by lowering postheparin lipases and cholesteryl ester transfer protein activities in patients with chronic hepatitis C. Hepatology 25: 1502–1506.
33. Andrade RJ, Garcia-Escano MD, Valdivielso P, Alcantara R, Sanchez-Chaparro MA, et al. (2000) Effects of interferon-beta on plasma lipid and lipoprotein composition and post-heparin lipase activities in patients with chronic hepatitis C. Aliment Pharmacol Ther 14: 929–935.
34. Sarrazin C, Susser S, Doehring A, Lange CM, Muller T, et al. (2011) Importance of IL28B gene polymorphisms in hepatitis C virus genotype 2 and 3 infected patients. J Hepatol 54: 415–421.
35. Tillmann HL, Patel K, Muir AJ, Guy CD, Li JH, et al. (2011) Beneficial IL28B genotype associated with lower frequency of hepatic steatosis in patients with chronic hepatitis C. J Hepatol; 55 (6): 1195–200
36. Nakaya M, Tachibana H, Yamada K (2006) Effect of estrogens on the interferon-gamma producing cell population of mouse splenocytes. Biosci Biotechnol Biochem 70: 47–53.
37. Siracusa MC, Overstreet MG, Housseau F, Scott AL, Klein SL (2008) 17beta-estradiol alters the activity of conventional and IFN-producing killer dendritic cells. J Immunol 180: 1423–1431.
38. Cai T, Dufour JF, Muellhaupt B, Gerlach T, Heim M, et al. (2011) Viral Genotype-Specific Role of PNPLA3, PPARG, MTTP and IL28B in Hepatitis C Virus-Associated Steatosis. J Hepatol.
39. Trepo E, Pradat P, Potthoff A, Momozawa Y, Quertinmont E, et al. (2011) Impact of patatin-like phospholipase-3 (rs738409 C>G) polymorphism on fibrosis progression and steatosis in chronic hepatitis C. Hepatology 54: 60–69.
40. Valenti L, Rumi M, Galmozzi E, Aghemo A, Del Menico B, et al. (2011) Patatin-like phospholipase domain-containing 3 I148M polymorphism, steatosis, and liver damage in chronic hepatitis C. Hepatology 53: 791–799.
41. Sookoian S, Pirola CJ (2011) Meta-analysis of the influence of I148M variant of patatin-like phospholipase domain containing 3 gene (PNPLA3) on the susceptibility and histological severity of nonalcoholic fatty liver disease. Hepatology 53: 1883–1894.
42. Ioannidis JP, Boffetta P, Little J, O'Brien TR, Uitterlinden AG, et al. (2008) Assessment of cumulative evidence on genetic associations: interim guidelines. Int J Epidemiol 37: 120–132.
43. Asahina Y, Tanaka K, Suzuki Y, Tamaki N, Hoshioka T, et al. (2011) Association between IL28B gene variation and development of hepatocellular carcinoma after interferon therapy in patients with chronic hepatitis c. Journal of Hepatology 54: S37.
44. Fabris C, Falleti E, Cussigh A, Bitetto D, Fontanini E, et al. (2011) IL-28B rs12979860 C/T allele distribution in patients with liver cirrhosis: role in the course of chronic viral hepatitis and the development of HCC. J Hepatol 54: 716–722.
45. Joshita S, Umemura T, Katsuyama Y, Ichikawa Y, Kimura T, et al. (2011) Association of IL28B gene polymorphism with development of hepatocellular carcinoma in Japanese patients with chronic hepatitis C virus infection. Hum Immunol; 73 (3): 298–300
46. Miura M, Maekawa S, Kadokura M, Sueki R, Komase K, et al. (2011) Analysis of viral amino acids sequences and the IL28B SNP influencing the development of hepatocellular carcinoma in chronic hepatitis C. Hepatol Int; Aug 17 [Epub ahead of print].
47. Bochud PY, Bibert S, Kutalik Z, Patin E, Guergnon J, et al. (2011) IL28B alleles associated with poor hepatitis C virus (HCV) clearance protect against inflammation and fibrosis in patients infected with non-1 HCV genotypes. Hepatology; 55 (2): 384–94
48. Abe H, Ochi H, Maekawa T, Hayes CN, Tsuge M, et al. (2010) Common variation of IL28 affects gamma-GTP levels and inflammation of the liver in chronically infected hepatitis C virus patients. J Hepatol 53: 439–443.
49. Honda M, Sakai A, Yamashita T, Nakamoto Y, Mizukoshi E, et al. (2010) Hepatic ISG expression is associated with genetic variation in interleukin 28B and the outcome of IFN therapy for chronic hepatitis C. Gastroenterology 139: 499–509.
50. Lotrich FE, Loftis JM, Ferrell RE, Rabinovitz M, Hauser P (2010) IL28B Polymorphism Is Associated with Both Side Effects and Clearance of Hepatitis C During Interferon-Alpha Therapy. J Interferon Cytokine Res; Dec 6 [Epub ahead of print].
51. Montes-Cano MA, Garcia-Lozano JR, Abad-Molina C, Romero-Gomez M, Barroso N, et al. (2010) Interleukin-28B genetic variants and hepatitis virus infection by different viral genotypes. Hepatology 52: 33–37.
52. Thompson AJ, Muir AJ, Sulkowski MS, Ge D, Fellay J, et al. (2010) Interleukin-28B polymorphism improves viral kinetics and is the strongest pretreatment predictor of sustained virologic response in genotype 1 hepatitis C virus. Gastroenterology 139: 120–129 e118.
53. Bochud PY, Bibert S, Negro F, Haagmans B, Soulier A, et al. (2011) IL28B polymorphisms predict reduction of HCV RNA from the first day of therapy in chronic hepatitis C. J Hepatol; 55 (5): 980–8.
54. Dill MT, Duong FH, Vogt JE, Bibert S, Bochud PY, et al. (2011) Interferon-induced gene expression is a stronger predictor of treatment response than IL28B genotype in patients with hepatitis C. Gastroenterology 140: 1021–1031.
55. Falleti E, Bitetto D, Fabris C, Cussigh A, Fornasiere E, et al. (2011) Role of Interleukin 28B rs12979860 C/T Polymorphism on the Histological Outcome of Chronic Hepatitis C: Relationship with Gender and Viral Genotype. J Clin Immunol; 31 (5): 891–9.
56. Kurosaki M, Tanaka Y, Nishida N, Sakamoto N, Enomoto N, et al. (2011) Pre-treatment prediction of response to pegylated-interferon plus ribavirin for chronic hepatitis C using genetic polymorphism in IL28B and viral factors. J Hepatol 54: 439–448.
57. Lagging M, Askarieh G, Negro F, Bibert S, Soderholm J, et al. (2011) Response prediction in chronic hepatitis C by assessment of IP-10 and IL28B-related single nucleotide polymorphisms. PLoS One 6: e17232.

58. Lin CY, Chen JY, Lin TN, Jeng WJ, Huang CH, et al. (2011) IL28B SNP rs12979860 is a critical predictor for on-treatment and sustained virologic response in patients with hepatitis C virus genotype-1 infection. PLoS One 6: e18322.
59. Lindh M, Lagging M, Arnholm B, Eilard A, Nilsson S, et al. (2011) IL28B polymorphisms determine early viral kinetics and treatment outcome in patients receiving peginterferon/ribavirin for chronic hepatitis C genotype 1. J Viral Hepat 18: e325–331.
60. Lindh M, Lagging M, Farkkila M, Langeland N, Morch K, et al. (2011) Interleukin 28B gene variation at rs12979860 determines early viral kinetics during treatment in patients carrying genotypes 2 or 3 of hepatitis C virus. J Infect Dis 203: 1748–1752.
61. Marabita F, Aghemo A, De Nicola S, Rumi MG, Cheroni C, et al. (2011) Genetic variation in the interleukin-28B gene is not associated with fibrosis progression in patients with chronic hepatitis C and known date of infection. Hepatology 54: 1127–1134.
62. Miyamura T, Kanda T, Nakamoto S, Wu S, Fujiwara K, et al. (2011) Hepatic STAT1-nuclear translocation and interleukin 28B polymorphisms predict treatment outcomes in hepatitis C virus genotype 1-infected patients. PLoS ONE; 6 (12): e28617.
63. Moghaddam A, Melum E, Reinton N, Ring-Larsen H, Verbaan H, et al. (2011) IL28B genetic variation and treatment response in patients with hepatitis C virus genotype 3 infection. Hepatology 53: 746–754.
64. de Rueda PM, Lopez-Nevot MA, Saenz-Lopez P, Casado J, Martin-Casares A, et al. (2011) Importance of Host Genetic Factors HLA and IL28B as Predictors of Response to Pegylated Interferon and Ribavirin. Am J Gastroenterol.
65. Yu ML, Huang CF, Huang JF, Chang NC, Yang JF, et al. (2011) Role of interleukin-28B polymorphisms in the treatment of hepatitis C virus genotype 2 infection in Asian patients. Hepatology 53: 7–13.
66. Asahina Y, Tsuchiya K, Muraoka M, Tanaka K, Suzuki Y, et al. (2011) Association of gene expression involving innate immunity and genetic variation in IL28B with antiviral response. Hepatology.
67. Mach T, Ciesla A, Sanak M, Golwacki M, Warunek W, et al. (2012) The importance of IL28B polymorphism in response to pegylated interferon (alpha) and ribavirin in chronic hepatitis caused by HCV genotype 1b. Przeglad Gastroenterologiczny 7: 38–42.
68. Miyashita M, Ito T, Sakaki M, Kajiwara A, Nozawa H, et al. (2012) Genetic polymorphism in cyclooxygenase-2 promoter affects hepatic inflammation and fibrosis in patients with chronic hepatitis C. J Viral Hepat 19: 608–614.
69. Ohnishi M, Tsuge M, Kohno T, Zhang Y, Abe H, et al. (2012) IL28B polymorphism is associated with fatty change in the liver of chronic hepatitis C patients. J Gastroenterol 47: 834–844.
70. Rembeck K, Alsio A, Christensen PB, Farkkila M, Langeland N, et al. (2012) Impact of IL28B-related single nucleotide polymorphisms on liver histopathology in chronic hepatitis C genotype 2 and 3. PLoS One 7: e29370.
71. Tolmane I, Rozentale B, Keiss J, Ivancenko L, Subnikova N, et al. (2012) Interleukin 28B Gene Polymorphism and Association with Chronic Hepatitis C Therapy Results in Latvia. Hepat Res Treat: 324090.
72. Toyoda H, Kumada T, Tada T, Hayashi K, Honda T, et al. (2012) Predictive value of early viral dynamics during peginterferon and ribavirin combination therapy based on genetic polymorphisms near the IL28B gene in patients infected with HCV genotype 1b. J Med Virol 84: 61–70.

Hepatology Research

Hepatology Research 2014 doi: 10.1111/hepr.12258

Original Article

Impact of *PNPLA3* polymorphisms on the development of hepatocellular carcinoma in patients with chronic hepatitis C virus infection

Masaya Sato,[1] Naoya Kato,[2] Ryosuke Tateishi,[1] Ryosuke Muroyama,[2] Norie Kowatari,[2] Wenwen Li,[2] Kaku Goto,[2] Motoyuki Otsuka,[1] Shuichiro Shiina,[1] Haruhiko Yoshida,[1] Masao Omata[3] and Kazuhiko Koike[1]

[1]*Department of Gastroenterology, Graduate School of Medicine,* [2]*Unit of Disease Control Genome Medicine, Institute of Medical Science, The University of Tokyo, Tokyo, and* [3]*Yamanashi Prefectural Hospital Organization, Kofu, Japan*

Aim: The *PNPLA3* rs738409 C>G polymorphism (encoding for I148M) has recently been identified as a susceptibility factor for steatosis-mediated liver damage. We evaluated the influence of this polymorphism on hepatocarcinogenesis in patients with chronic hepatitis C (CHC) virus infection.

Methods: We genotyped the rs738409 single nucleotide polymorphism in 358 hepatitis C-associated hepatocellular carcinoma (HCC) patients and correlated the age at onset of HCC and the interval between hepatitis C virus (HCV) infection and the development of HCC in patients with each genotype.

Results: The frequencies of CC, CG and GG genotypes were 27.9% (100/358), 49.2% (176/358) and 22.9% (82/358), respectively, and were in Hardy–Weinberg equilibrium. The median age at onset of HCC for the GG genotype was significantly younger compared to for non-GG genotypes (67.81 vs 69.87 years, $P < 0.001$), and the median interval between HCV infection and the development of HCC was significantly shorter in patients with the GG genotype (39.96 vs 40.85 years, $P = 0.008$). *PNPLA3* GG genotype was also associated with a higher aspartate aminotransferase level (69.5 vs 59.0 IU/L, $P = 0.02$), lower prothrombin time (73.0% vs 78.0%, $P = 0.008$) and a higher prevalence of histological steatosis (40.0% *vs.* 22.2%, $P = 0.01$) at the time of HCC onset.

Conclusion: The *PNPLA3* genotype GG may be associated with accelerated hepatocarcinogenesis in CHC patients through increased steatosis in the liver.

Key words: fibrosis, hepatocarcinogenesis, risk allele, rs738409, steatosis

INTRODUCTION

HEPATITIS C VIRUS (HCV) infection is a major health burden, with 130–170 million people infected, representing nearly 3% of the world's population.[1] HCV infection is one of the major causes of chronic hepatitis, liver cirrhosis and hepatocellular carcinoma (HCC).[2]

In epidemiological studies of chronic HCV infection (CHC), age, duration of infection, alcohol consumption, co-infection with HIV, low CD4 count, male sex and HCV genotype 3 have been shown to be associated with histological activity.[3–8] We also reported higher body mass index (BMI) as an independent risk factor for HCC development in CHC patients.[9] Although these factors explain part of the extreme variability seen in fibrosis progression among HCV-infected patients, they do not completely account for the differences. Genetic host factors have long been suspected to play a role in CHC.[10–12] Recently, two genome-wide association studies (GWAS) carried out in Japan reported genetic factors, MICA locus (rs2596542) and DEPDC5 locus (rs1012068), associated with HCV-related HCC.[13,14]

Correspondence: Dr Naoya Kato, Unit of Disease Control Genome Medicine, Institute of Medical Science, The University of Tokyo, 4-6-1 Shirokanedai, Minato-ku, Tokyo 108-8639, Japan. Email: kato-2im@ims.u-tokyo.ac.jp

The English in this document has been checked by at least two professional editors, both native speakers of English. For a certificate, please see: http://www.textcheck.com/certificate/TgwNHQ.

Conflict of interest: None of the authors have any conflicts of interest.

Received 30 August 2013; revision 2 October 2013; accepted 4 October 2013.

Because of the global epidemic of obesity, non-alcoholic fatty liver disease (NAFLD) is rapidly becoming the most common liver disorder worldwide.[15–18] Liver steatosis also has gained increasing attention as a modifier of CHC progression. In fact, hepatic steatosis is a common histological feature of CHC, seen in more than half of patients, and has been associated with fibrosis progression and increased risk of HCC via overproduction of reactive oxygen species.[19–21]

Adiponutrin encoded by *PNPLA3* has been reported to have both lipolytic and lipogenic properties.[22] Recently, independent GWAS identified a single nucleotide polymorphism (SNP; rs738409 C>G) in the *PNPLA3* gene on chromosome 22, encoding an isoleucine to methionine substitution (p.I148M) of patatin-like phospholipase A3 as a genetic determinant of liver fat content or disease severity.[23,24] A recent meta-analysis showed that this polymorphism has been related, in NAFLD, to inflammatory activity and progression of fibrosis.[25] The previous basic research showed that the *PNPLA3* I148M impairs hydrolytic activity against triacylglycerol *in vitro* and is thought to lead to accumulation of triacylglycerol.[26] Other studies using mice showed that the inactivation of *PNPLA3* has no effect on hepatic fat accumulation,[27] but the overexpression of *PNPLA3* I148M causes an increase in hepatic triacylglycerol content.[28] The rs738409 polymorphism was also found to be associated not only with elevated liver enzymes or prevalence of fatty liver histology in healthy subjects,[29,30] but also with disease severity and fibrosis in NAFLD,[25,31,32] alcoholic liver disease[33,34] and CHC.[35,36] However, the influence of *PNPLA3* (rs738409 C>G) polymorphism on HCV-related HCC still remains controversial.[34,36,37] In the present study, we focused on the association between the rs738409 SNP and the age at onset of HCC and the interval between HCV infection and the development of HCC to evaluate the influence of the *PNPLA3* polymorphism on hepatocarcinogenesis in CHC patients.

METHODS

Patients

THIS RESEARCH PROJECT was approved by the ethics committees of the University of Tokyo (no. 400). The patients analyzed in the present study were derived from a HCV study cohort of the University of Tokyo Hospital. All patients visited the liver clinic at our institution between August 1997 and August 2009 and agreed to provide blood samples for human genome studies along with written informed consent according with the Declaration of Helsinki. We enrolled patients who had developed HCC and received initial therapy for HCC at our institution by 31 January 2010, and with samples available for genotyping. Exclusion criteria were positivity for hepatitis B surface antigen and presence of biliary disease. We also excluded patients without information on BMI, daily alcohol intake, HCV genotype and HCV viral load. Finally, 358 patients were enrolled, and all subjects were Japanese. We analyzed the association of rs738409 C>G polymorphism with the age at onset of HCC and the interval between HCV infection and the development of HCC. Because we lacked knowledge of the exact date of hepatitis C seroconversion, the duration of HCV infection was estimated indirectly, based on the year of the first transfusion.

Diagnosis of HCC

Hepatocellular carcinoma was diagnosed by dynamic computed tomography, and hyperattenuation in the arterial phase with washout in the late phase was considered a definite sign of HCC. When the diagnosis of HCC was ambiguous, an ultrasound-guided tumor biopsy was performed, and a pathological diagnosis was made based on the Edmondson and Steiner criteria.[38]

Genotyping

Human genomic DNA was extracted from the whole blood of each patient. Genotyping for the *PNPLA3* rs738409 C/G polymorphism was performed by polymerase chain reaction (PCR) using the TaqMan predesigned SNP Genotyping Assay (Applied Biosystems, Foster City, CA), as recommended by the manufacturer. Allele-specific primers were labeled with fluorescent dye (6-carboxyfluorescein or hexachloro-6-carboxyfluorescein) and used in the PCR reaction. Aliquots of the PCR products were genotyped using an allele-specific probe of the SNP on a real-time PCR thermocycler (MX3000P; Stratagene, La Jolla, CA, USA). Samples were subjected to 45 cycles of denaturation for 15 s at 95 °C, annealing of primers for 30 s at 60 °C and elongation for 30 s at 60 °C.

Study end-point

We analyzed the relationship between host factors, including *PNPLA3* (rs738409 C>G) polymorphisms, sex, BMI, alcohol consumption and HCV genotype, and the age at onset of HCC or the interval between HCV infection and the development of HCC (the primary end-points of this study). We also examined the relationship between rs738409 polymorphisms and clinical

findings at the onset of HCC (the secondary end-point), such as biochemical markers and histological findings. The histological grade of disease activity and the histological stage of fibrosis were assessed using the reproducible METAVIR scoring system as follows: grades A1 to A3 for the degree of necroinflammatory activity (A1 = mild to A3 = marked), and stages F0 to F4 for the degree of fibrosis (F0 = no fibrosis to F4 = cirrhosis).[39,40] The presence of steatosis was studied as a qualitative (<5% vs ≥5%) variable.

Statistical analysis

Continuous variables are presented as medians with 1st and 3rd quartiles, whereas categorical variables are expressed as frequencies (%). Categorical data were analyzed using the χ^2-test, and stepwise logistic regression analyses were used to adjust the influence of the *PNPLA3* genotype by other covariates such as sex, BMI (<25 or not) and alcohol consumption (<50 g/day or not). For continuous data, the univariate associations were evaluated using Student's *t*-test or the non-parametric Wilcoxon rank sum test as appropriate. Because the age at onset of HCC and the length of time between HCV infection and the development of HCC (the primary end-points of this study) satisfied the assumption of normal distribution (Kolmogorov–Smirnov test, $P > 0.05$), we used stepwise regression analysis for multivariate analyses. We evaluated the association between the rs738409 mutant G allele and each outcome using a recessive model of inheritance, comparing G allele homozygotes (GG genotype) with patients carrying one copy or no copies of the G allele (CG or CC genotypes) because this was suggested to be the most appropriate one by studies of the impact of rs738409 on CHC liver damage.[36,41] The Jonckheere–Terpstra trend test for continuous variables and the Cochran–Armitage trend test for categorical variables were used to evaluate the increasing or decreasing tendency of the findings across rs738409 CC, CG and GG genotypes. All statistical analyses were two-sided, and the threshold of the reported *P*-values for significance was less than 0.05. All statistical analyses were performed using the R version 2.13.1 software (http://www.r-project.org).

RESULTS

Patient characteristics

PATIENT CHARACTERISTICS ARE shown in Table 1. Frequencies of the rs738409 CC, CG and GG genotypes were 27.9% (100/358), 49.2% (176/358)

Table 1 Clinical characteristics and genotype distributions of the subjects ($n = 358$)

Parameter	Values
Median age at onset of HCC, years	69.76 (63.88–75.35)
Male sex	200 (55.9%)
BMI >25	67 (18.7%)
Alcohol consumption (>50 g/day)	75 (20.9%)
PNPLA3 genotype	
CC	100 (27.9%)
CG	176 (49.2%)
GG	82 (22.9%)
G allele frequency	0.47
HCV genotype	
Genotype 1	271 (75.7%)
Genotype 2	87 (24.3%)

Continuous variables are presented as medians with 1st and 3rd quartiles, and categorical variables as numbers and frequency (%).
BMI, body mass index; HCC, hepatocellular carcinoma; HCV, hepatitis C virus.

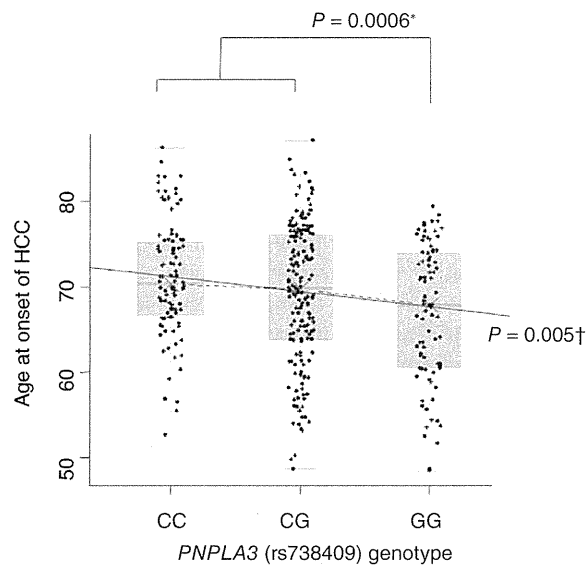

Figure 1 Box and whisker and dot plot: distributions of the age at onset of hepatocellular carcinoma (HCC) in each genotype. The dashed line connects the median value of each genotype, and the solid line shows the linear regression. The Jonckheere–Terpstra trend test showed a significant trend across the CC, CG and GG alleles ($P = 0.005$). **P*-values after adjustment for sex, body mass index and alcohol consumption. †*P*-value by the Jonckheere–Terpstra trend test.