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Genome-Wide Association Study Identifies *TLL1* Variant Associated With Development of Hepatocellular Carcinoma After Eradication of Hepatitis C Virus Infection

Kentaro Matsuura,[1,2] Hiromi Sawai,[3] Kazuho Ikeo,[4] Shintaro Ogawa,[1] Etsuko Iio,[1,2] Masanori Isogawa,[1] Noritomo Shimada,[5] Atsumasa Komori,[6] Hidenori Toyoda,[7] Takashi Kumada,[7] Tadashi Namisaki,[8] Hitoshi Yoshiji,[8] Naoya Sakamoto,[9,10] Mina Nakagawa,[10,11] Yasuhiro Asahina,[10,12] Masayuki Kurosaki,[13] Namiki Izumi,[13] Nobuyuki Enomoto,[14] Atsunori Kusakabe,[15] Eiji Kajiwara,[16] Yoshito Itoh,[17] Tatsuya Ide,[18] Akihiro Tamori,[19] Misako Matsubara,[19] Norifumi Kawada,[19] Ken Shirabe,[20] Eiichi Tomita,[21] Masao Honda,[22] Shuichi Kaneko,[22] Sohji Nishina,[23] Atsushi Suetsugu,[24] Yoichi Hiasa,[25] Hisayoshi Watanabe,[26] Takuya Genda,[27] Isao Sakaida,[28] Shuhei Nishiguchi,[29] Koichi Takaguchi,[30] Eiji Tanaka,[31] Junichi Sugihara,[32] Mitsuo Shimada,[33] Yasuteru Kondo,[34] Yosuke Kawai,[35] Kaname Kojima,[35] Masao Nagasaki,[35] Katsushi Tokunaga,[3] and Yasuhito Tanaka,[1] on behalf of the Japanese Genome-Wide Association Study Group for Viral Hepatitis

[1]Department of Virology and Liver Unit, and [2]Department of Gastroenterology and Metabolism, Nagoya City University Graduate School of Medical Sciences, Nagoya, Japan; [3]Department of Human Genetics, Graduate School of Medicine, The University of Tokyo, Tokyo, Japan; [4]Center for Information Biology, National Institute of Genetics, Mishima, Japan; [5]Division of Gastroenterology and Hepatology, Otakanomori Hospital, Kashiwa, Japan; [6]Clinical Research Center, National Nagasaki Medical Center, Omura, Japan; [7]Department of Gastroenterology and Hepatology, Ogaki Municipal Hospital, Ogaki, Japan; [8]Third Department of Internal Medicine, Nara Medical University, Kashihara, Japan; [9]Department of Internal Medicine, Hokkaido University Graduate School of Medicine, Sapporo, Japan; [10]Department of Gastroenterology and Hepatology, [11]Center for Interprofessional Education, and [12]Department of Liver Disease Control, Tokyo Medical and Dental University, Tokyo, Japan; [13]Division of Gastroenterology and Hepatology, Musashino Red Cross Hospital, Musashino, Japan; [14]First Department of Internal Medicine, University of Yamanashi, Chuo, Japan; [15]Division of Gastroenterology, Japanese Red Cross Nagoya Daini Hospital, Nagoya, Japan; [16]Department of Hepatology, Steel Memorial Yahata Hospital, Kitakyushu, Japan; [17]Molecular Gastroenterology and Hepatology, Kyoto Prefectural University of Medicine, Kyoto, Japan; [18]Division of Gastroenterology, Department of Medicine, Kurume University, Kurume, Japan; [19]Department of Hepatology, Graduate School of Medicine, Osaka City University, Osaka, Japan; [20]Department of Surgery and Science, Graduate School of Medical Sciences, Kyushu University, Fukuoka, Japan; [21]Department of Gastroenterology, Gifu Municipal Hospital, Gifu, Japan; [22]Department of Gastroenterology, Kanazawa University Graduate School of Medicine, Kanazawa, Japan; [23]Department of Hepatology and Pancreatology, Kawasaki Medical School, Kurashiki, Japan; [24]Department of Gastroenterology, Gifu University Graduate School of Medicine, Gifu, Japan; [25]Department of Gastroenterology and Metabology, Ehime University Graduate School of Medicine, Toon, Japan; [26]Department of Gastroenterology, Yamagata University Faculty of Medicine, Yamagata, Japan; [27]Department of Gastroenterology and Hepatology, Juntendo University Shizuoka Hospital, Shizuoka, Japan; [28]Department of Gastroenterology and Hepatology, Yamaguchi University Graduate School of Medicine, Ube, Japan; [29]Department of Internal Medicine, Hyogo College of Medicine, Nishinomiya, Japan; [30]Department of Hepatology, Kagawa Prefectural Central Hospital, Takamatsu, Japan; [31]Department of Medicine, Shinshu University School of Medicine, Matsumoto, Japan; [32]Department of Gastroenterology, Gifu Prefectural General Medical Center, Gifu, Japan; [33]Department of Surgery, The University of Tokushima, Tokushima, Japan; [34]Division of Gastroenterology, Tohoku University Graduate School of Medicine, Sendai, Japan; and [35]Department of Integrative Genomics, Tohoku Medical Megabank Organization, Tohoku University, Sendai, Japan

See Covering the Cover synopsis on page 1251; see editorial on page 1282.

BACKGROUND & AIMS: There is still a risk for hepatocellular carcinoma (HCC) development after eradication of hepatitis C virus (HCV) infection with antiviral agents. We investigated genetic factors associated with the development of HCC in patients with a sustained virologic response (SVR) to treatment for chronic HCV infection. **METHODS:** We obtained genomic DNA from 457 patients in Japan with a SVR to interferon-based treatment for chronic HCV infection from 2007 through 2015. We conducted a genome-wide association study (GWAS), followed by a replication analysis of 79 candidate single nucleotide polymorphisms (SNPs) in an independent set of 486 patients in Japan. The study end point was HCC diagnosis or confirmation of lack of HCC (at follow-up examinations until December 2014 in the GWAS cohort, and until January 2016 in the replication cohort). We collected clinical and laboratory data from all patients. We analyzed expression levels of candidate gene variants in human hepatic stellate cells, rats with steatohepatitis caused by a choline-deficient L-amino acid-defined diet, and a mouse model of liver injury caused by administration of carbon tetrachloride. We also analyzed

EDITOR'S NOTES

BACKGROUND AND CONTEXT

There is still a risk for the development of hepatocellular carcinoma (HCC) after eradication of hepatitis C virus (HCV) by antiviral therapy.

NEW FINDINGS

The researchers identified a host genetic variant that was associated with the development of HCC after eradication of HCV by antiviral therapy.

LIMITATIONS

This study was designed as a retrospective case-control study with selected population.

IMPACT

Genetic testing of the variant located within *TLL1* would be useful for implementing personalized surveillance of HCC after eradication of HCV.

CLINICAL LIVER

expression levels in liver tissues of patients with chronic HCV infection with different stages of fibrosis or tumors vs patients without HCV infection (controls). **RESULTS:** We found a strong association between the SNP rs17047200, located within the intron of the tolloid like 1 gene (*TLL1*) on chromosome 4, and development of HCC; there was a genome-wide level of significance when the results of the GWAS and replication study were combined (odds ratio, 2.37; $P = 2.66 \times 10^{-8}$). Multivariate analysis showed rs17047200 AT/TT to be an independent risk factor for HCC (hazard ratio, 1.78; $P = .008$), along with male sex, older age, lower level of albumin, advanced stage of hepatic fibrosis, presence of diabetes, and higher post-treatment level of α-fetoprotein. Combining the rs17047200 genotype with other factors, we developed prediction models for HCC development in patients with mild or advanced hepatic fibrosis. Levels of *TLL1* messenger RNA (mRNA) in human hepatic stellate cells increased with activation. Levels of *Tll1* mRNA increased in liver tissues of rodents with hepatic fibrogenesis compared with controls. Levels of *TLL1* mRNA increased in liver tissues of patients with progression of fibrosis. Gene expression levels of *TLL1* short variants, including isoform 2, were higher in patients with rs17047200 AT/TT. **CONCLUSIONS:** In a GWAS, we identified the association between the SNP rs17047200, within the intron of *TLL1*, and development of HCC in patients who achieved an SVR to treatment for chronic HCV infection. We found levels of *Tll1/TLL1* mRNA to be increased in rodent models of liver injury and liver tissues of patients with fibrosis, compared with controls. We propose that this SNP might affect splicing of *TLL1* mRNA, yielding short variants with high catalytic activity that accelerates hepatic fibrogenesis and carcinogenesis. Further studies are needed to determine how rs17047200 affects *TLL1* mRNA levels, splicing, and translation, as well as the prevalence of this variant among other patients with HCC. Tests for the *TLL1* SNP might be used to identify patients at risk for HCC after an SVR to treatment of HCV infection.

Keywords: Genetics; Liver Cancer; Mutation; Metalloprotease.

Chronic hepatitis C virus (HCV) infection is at significant risk for progressive hepatic fibrosis, subsequent liver cirrhosis (LC) and development of hepatocellular carcinoma (HCC). It is estimated that 28% of cases of LC and 26% of cases of HCC are attributable to HCV, resulting in 499,000 deaths worldwide in 2010.[1] In the United States, the numbers of LC and HCC are predicted to increase to peak levels around 2020; furthermore, HCV-related mortality is estimated to continue to increase until 2022.[2] Thus, HCV infection will continue to be a serious social concern for several decades.

Recently developed interferon (IFN)-free oral regimens combining direct-acting antiviral agents have achieved remarkably high rates of HCV eradication even in patients with LC.[3] However, the risk of developing HCC does not completely disappear even after sustained virologic response (SVR): the 3- and 5-year HCC incidence rates after achieving SVR by IFN-based therapy were 0.5%–2.0% and 2.3%–8.8%, respectively.[4] Therefore, developing HCC after achieving SVR is still a critical issue, even in the era of direct-acting antiviral agents. Previous studies have identified several pre- or post-treatment risk factors for developing HCC after achieving SVR: older age, male sex, advanced hepatic fibrosis, alcohol intake, diabetes, lower platelet count and albumin level, higher aspartate aminotransferase-to-platelet ratio index (APRI), α-fetoprotein (AFP), and γ-glutamyl transpeptidase levels.[5–15] Intriguingly, viral factors, such as HCV genotype 3 or genotype 1 accompanied by amino acid substitutions at position 70 in the HCV core region were associated with developing HCC even if HCV was eradicated.[9,16] Thus, various viral, host, and environmental factors have been identified as influencing the development of HCC after the eradication of HCV. Novel predictive biomarkers of HCC development are therefore desired for implementing personalized treatment and surveillance of HCC.

Recent genome-wide association studies (GWAS) identified *IL28B* variants strongly associated with the response to pegylated IFN plus ribavirin (RBV) therapy for chronic HCV genotype 1 infection,[17–19] as well as genetic variations associated with the progression of hepatic fibrosis[20,21] or

Abbreviations used in this paper: **AFP, α-fetoprotein; anti-HBc, antibody to hepatitis B core antigen; BMP1, bone morphogenetic protein 1; CCl_4, carbon tetrachloride; CDAA, choline-deficient L-amino acid-defined; CHC, chronic hepatitis C; CI, confidence interval; CSAA, choline-sufficient L-amino acid-defined; EOT, end of treatment; GWAS, genome-wide association study; HBV, hepatitis B virus; HCC, hepatocellular carcinoma; HCV, hepatitis C virus; HR, hazard ratio; HSC, hepatic stellate cell; IFN, interferon; LC, liver cirrhosis; LD, linkage disequilibrium; mRNA, messenger RNA; NASH, non-alcoholic steatohepatitis; OR, odds ratio; RBV, ribavirin; SNP, single nucleotide polymorphism; SVR, sustained virologic response; TGF-β, transforming growth factor–β; TLD, tolloid; TLL, tolloid-like.**

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hepatocarcinogenesis[22,23] in chronic hepatitis C (CHC), and hepatocarcinogenesis in chronic hepatitis B.[24–27] However, to the best of our knowledge, there are no GWAS data on hepatocarcinogenesis specifically in CHC patients after the eradication of HCV.

Therefore, we conducted a GWAS in the Japanese population to identify genetic variants associated with HCC occurrence after the eradication of HCV by IFN-based therapy.

Methods

Patients

From 2007 through 2015, genomic DNA samples were obtained for GWAS from 457 Japanese patients in whom eradication of HCV had been achieved by IFN-based therapy and for the replication study from an independent cohort of 486 patients, from 44 hospitals (liver units with hepatologists) throughout Japan. Patients had been treated with pegylated IFN/RBV, IFN/RBV, IFN monotherapy, or pegylated IFN/RBV/protease inhibitor, according to standard regimens. SVR was defined as no detectable HCV RNA 24 weeks after the end of treatment (EOT). Surveillance methodology and diagnostic criteria for HCC are provided in the Supplementary Methods. The end point was the date when HCC was diagnosed in patients who developed HCC, whereas it was the date when lack of HCC was confirmed at the last follow-up to December 2014 in the GWAS cohort, and to January 2016 in the replication cohort in patients who did not develop HCC. The period of observation was defined as from the EOT to the end point. Patients with a history of HCC; decompensated cirrhosis; other causes of liver disease, such as autoimmune hepatitis and primary biliary cirrhosis; or co-infection with hepatitis B virus (HBV) or human immunodeficiency virus were excluded from this study. Written informed consent was obtained from all individual participants and the study protocol conformed to the ethics guidelines of the Declaration of Helsinki and was approved by the Institutional Ethics Review Committees for our human genome projects (no. 67).

Selection Criteria of Case-Control Groups in the Discovery Genome-Wide Association Study and Replication Cohort

We designed an original case-control study with the following inclusion criteria: In the discovery GWAS, patients who developed HCC at ≥1 year since EOT (designated the case group, G-Case), or who did not develop HCC at ≥5 years since EOT (designated the control group, G-CTRL [≥5 years]). In the replication cohort, the criterion for the case group (R-Case) was the same as for the G-Case group, whereas the replication control group was patients who did not develop HCC at ≥3 years since EOT (R-CTRL [≥3 years]), and then was further stratified by the same criterion as for the GWAS stage (R-CTRL [≥5 years]) (Table 1 and Supplementary Figure 1).

Additional information about methods is provided in Supplementary Methods.

Results

Genetic Variants Associated with Development of Hepatocellular Carcinoma After Eradication of Hepatitis C Virus

We genotyped 456 Japanese patients who either developed HCC (G-Case, n = 123) or did not develop HCC (G-CTRL [≥5 years], n = 333) after they had achieved SVR, and carried out GWAS in these 2 groups (Supplementary Figure 1). The characteristics of these patients and those in the replication cohort are summarized in Table 1. Associations were found between 70 SNPs and development of HCC at $P < 10^{-4}$ in the allele frequency model after excluding SNPs with ambiguous genotype calls by visual inspection of the cluster (Supplementary Table 1). No significant deviation from expectation was observed on a quantile−quantile plot (genomic inflation factor $\lambda = 1.020$) (Supplementary Figure 2). We also carried out principal component analysis in the 456 samples for the discovery GWAS, together with the HapMap phase III samples (http://hapmap.ncbi.nlm.nih.gov/index.html.en) (Supplementary Figure 3). These results indicated a low probability of false-positive associations resulting from population stratification. A genome-wide view of the single-point association data based on allele frequencies in the GWAS is shown in Supplementary Figure 4. Two SNPs located downstream of *C6orf118* on chromosome 6 showed strong associations with a genome-wide level of significance (rs4709076, odds ratio [OR], 2.66; 95% confidence interval [CI], 1.89−3.74; $P = 1.17 \times 10^{-8}$ and rs4709927, OR, 2.63; 95% CI, 1.86−3.70; $P = 1.95 \times 10^{-8}$, in the allele frequency model). Furthermore, the 3 SNPs: rs922231, rs922232, and rs11073757 located within *NTRK3* on chromosome 15 also showed strong associations. We then selected the 70 SNPs with $P < 10^{-4}$ and included an additional 9 SNPs, which had been reported to be associated with the progression of hepatic fibrosis[20,21,28,29] or hepatocarcinogenesis[22,23] in CHC, for the replication study using an independent set of 486 patients consisting of R-Cases (n = 130), R-CTRLs (≥5 years) (n = 210), and R-CTRLs (≥3 years) (n = 356). We were able to genotype 74 SNPs using DigiTag2 assays in the replication cohort with sufficient amounts of DNA available (Supplementary Table 2). In this way, we identified three promising SNPs, rs17047200, rs1932439, and rs597533, with $P < .05$ by comparing R-Cases vs. R-CTRLs (≥5 years). We then further performed genotyping of these SNPs in the remaining samples of the replication cohort by TaqMan SNP Genotyping Assays. The distribution of the 3 SNPs in the entire replication cohort showed that rs17047200, located within the intron of the Tolloid-like 1 (*TLL1*) gene on chromosome 4 (Supplementary Figure 5), had the strongest association when comparing R-Cases with R-CTRLs (≥5 years) (OR, 2.35; 95% CI, 1.48−3.75; $P = 2.33 \times 10^{-4}$, in the allele frequency model) (Supplementary Table 3). This remained significant in the replication cohort after Bonferroni correction for multiple testing. Furthermore, we analyzed the cumulative incidence of HCC up to 10 years after EOT in patients of the discovery GWAS and the entire

Table 1. Clinical Characteristics of Patients in the Discovery Genome-Wide Association Study and the Replication Cohort

Characteristics	Discovery GWAS		Replication cohort		
	G-Case (n = 123)	G-CTRL (≥5 y) (n = 333)	R-Case (n = 130)	R-CTRL (≥5 y) (n = 210)	R-CTRL (≥3 y) (n = 356)
Pretreatment					
Sex, male / female	93 / 30	176 / 157	100 / 30	106 / 104	175 / 181
Age, *y*	60 (57–66)	58 (51–64)	61 (56–66)	58 (50–65)	59 (50–65)
Hemoglobin, *g/dL*	13.8 (12.9–14.7)	14.1 (13.3–15.0)	13.9 (13.1–14.8)	13.8 (12.8–15.0)	13.8 (12.8–14.8)
Platelet count, $\times 10^9$/L	127 (104–155)	165 (132–198)	125 (106–145)	170 (131–204)	170 (134–208)
ALT, *IU/L*	76 (49–113)	58 (35–100)	73 (48–124)	56 (34–103)	47 (31–92)
γ-GTP, *IU/L*	41 (31–91)	31 (19–55)	51 (29–89)	38 (23–58)	33 (19–55)
Albumin, *g/dL*	3.9 (3.7–4.1)	4.2 (3.9–4.4)	4.0 (3.8–4.2)	4.1 (3.9–4.4)	4.1 (3.9–4.4)
AFP, *ng/mL*	7.7 (4.5–18.0)	4.8 (3.1–7.4)	10.3 (6.0–19.0)	4.1 (2.3–7.2)	4.1 (2.7–7.4)
APRI	1.65 (0.91–2.49)	0.90 (0.52–1.44)	1.35 (0.98–2.26)	0.81 (0.50–1.43)	0.72 (0.43–1.41)
FIB-4	3.73 (2.59–4.92)	2.30 (1.48–3.27)	3.87 (2.51–5.00)	2.16 (1.49–3.23)	2.15 (1.31–3.27)
HCV RNA, *log IU/mL*	5.4 (4.7–6.3)	6.2 (5.7–6.6)	5.5 (4.6–6.3)	6.1 (5.3–6.7)	6.1 (5.3–6.8)
HCV genotype, 1 / 2 / 3 / NA	64 / 37 / 0 / 22	246 / 86 / 0 / 1	68 / 45 / 0 / 17	116 / 88 / 1 / 5	205 / 143 / 1 / 7
Liver fibrosis, F0–2 / F3-4 / NA	39 / 48 / 36	239 / 64 / 30	40 / 52 / 38	137 / 39 / 34	214 / 62 / 80
Body mass index	22.7 (21.1–24.6)	23.2 (21.5–25.3)	23.5 (21.1–25.6)	23.0 (20.9–25.2)	22.8 (20.7–24.9)
Diabetes, + / − / NA	25 / 65 / 33	30 / 242 / 61	24 / 81 / 25	19 / 184 / 7	29 / 305 / 22
Anti-HBc, + / − / NA	49 / 47 / 27	75 / 157 / 101	51 / 55 / 24	53 / 123 / 34	96 / 213 / 47
6 months after the end of treatment					
ALT, IU/L	21 (15–28)	16 (13–20)	23 (15–36)	18 (14–26)	17 (12–24)
AFP, ng/mL	4.4 (2.8–6.5)	3.1 (2.1–4.4)	4.2 (3.0–6.6)	3.0 (2.0–4.0)	3.0 (2.1–4.2)
APRI	0.57 (0.38–0.90)	0.37 (0.28–0.52)	0.60 (0.44–0.85)	0.38 (0.27–0.54)	0.34 (0.26–0.51)
FIB-4	2.76 (2.00–3.83)	1.87 (1.32–2.44)	2.68 (1.98–3.81)	1.79 (1.22–2.54)	1.79 (1.21–2.47)
Regimen					
PEG-IFN+RBV / IFN+RBV / IFN mono / PI+PEG-IFN+RBV / NA	52 / 13 / 54 / 4 / 0	298 / 17 / 18 / 0 / 0	53 / 6 / 64 / 4 / 3	167 / 5 / 37 / 1 / 0	298 / 5 / 42 / 11 / 0
Period of observation					
Period from end of treatment to diagnosis of HCC, months	61 (31–96)	—	62 (38–116)	—	—
Period from end of treatment to last follow-up, months	—	89 (78–119)	—	71 (64–119)	61 (42–89)

NOTE. Data are expressed as numbers for categorical data or the median (first–third quartiles) for noncategorical data.
ALT, alanine aminotransaminase; APRI, aspartate aminotransferase-to-platelet ratio index; CTRL, control; FIB-4, fibrosis-4; γ-GTP, γ-glutamyl transpeptidase; IFN mono, interferon monotherapy; NA, not available; PEG-IFN, pegylated interferon; PI, protease inhibitor; RBV, ribavirin.

replication cohort, showing significant differences according to rs17047200 genotype in both groups ($P = .009$ for the GWAS, $P < .001$ for the replication cohort, $P < .001$ for the combined set) (Figure 1). However, there were no differences according to rs1932439 and rs597533 genotypes in the latter (Supplementary Figure 6). Consequently, we conclude that the *TLL1* variant of rs17047200 is significantly associated with the development of HCC after eradication of HCV by IFN-based therapy at a genome-wide level of significance (OR, 2.37; 95% CI, 1.74–3.23; $P = 2.66 \times 10^{-8}$, in the allele frequency model) when the results of the discovery GWAS and the replication cohort were combined (Table 2).

Imputation-Based Fine Mapping of the Tolloid-Like 1 Region

To further characterize the *TLL1* locus, we scrutinized pairwise linkage disequilibrium (LD) diagrams in the HapMap Data phase II (JPT), but found no SNP in strong LD ($r^2 \geq 0.8$) with rs17047200 (Supplementary Figure 5). Next, we performed SNP imputation in a 2-Mb genomic region around rs17047200 using a phased reference panel of 1070 healthy Japanese individuals (1KJPN panel). We then conducted an association test for imputed data. However, no SNPs more promising than rs17047200 including any in the exon or promoter regions of *TLL1* were observed, although several intronic SNPs were in strong LD with rs17047200 (data not shown).

Risk Factors for Developing Hepatocellular Carcinoma After Eradication of Hepatitis C Virus

The clinical characteristics of patients developing or not developing HCC suggest that the rs17047200 genotype did not strongly and directly influence other parameters related to hepatic fibrosis or inflammation (Supplementary Tables 4 and 5). Next, we performed multivariate analysis with stepwise Cox proportional hazard modeling to identify the risk factors for developing HCC in the entire set of patients. This showed that rs17047200 AT/TT was an independent risk factor (hazard ratio [HR], 1.78; 95% CI, 1.17–2.70; $P = .008$). Other risk factors included male sex, older age,

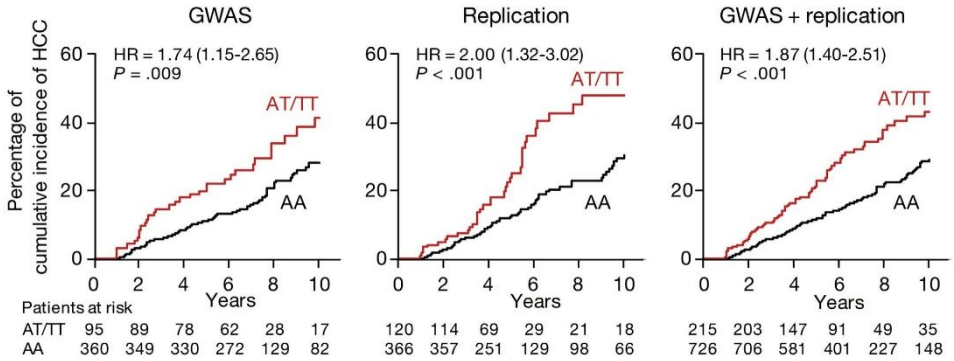

Figure 1. Cumulative incidence of HCC occurring after the eradication of HCV by IFN-based therapy according to rs17047200 genotype in patients of the discovery GWAS, the replication cohort and both combined. HR (95% CI) up to 10 years after the end of treatment were calculated by the Cox proportional hazard method. *P* values were calculated by log-rank testing. Data of subjects whose genotypes were not determined were excluded.

lower albumin level, advanced hepatic fibrosis stage, presence of diabetes, and higher post-treatment AFP level (Table 3).

In many previous studies, the advanced state of hepatic fibrosis was found to be the major risk factor for developing HCC, therefore, we considered it valuable to evaluate risk factors in patients stratified by hepatic fibrosis stage. We sorted the patients into 2 groups according to their METAVIR fibrosis stage: a mild fibrosis group with F0−2 (including only patients with a platelet count $\geq 130 \times 10^9$/L and albumin level ≥ 4.0 g/dL) and an advanced fibrosis group with F3−4. Multivariate analysis with stepwise Cox proportional hazard modeling in the mild fibrosis group indicated that older age and rs17047200 AT/TT were independent risk factors for developing HCC (HR, 2.90; 95% CI, 1.52−5.54; $P = .001$ for older age [by every 10 years]; HR, 4.26; 95% CI, 1.56−11.70; $P = .005$ for rs17047200 AT/TT) (Supplementary Table 6). The cutoff for age was determined by the Kaplan−Meier method, which indicated that setting it at 57 years yielded the best model with the lowest *P* value by log-rank testing (data not shown). The cumulative incidence of HCC up to 10 years after EOT in the mild fibrosis group stratified by these 2 risk factors showed that patients ≥57 years old with rs17047200 AT/TT were at the highest risk of developing HCC, whereas those with rs17047200 AA were at intermediate risk, and the remainder were at low risk ($P < .001$) (Figure 2*A*). Additionally, multivariate analysis with stepwise Cox proportional hazard modeling in the advanced fibrosis group identified higher post-treatment AFP level, lower albumin level, and rs17047200 AT/TT as independent risk factors for the development of HCC (HR, 1.90; 95% CI, 1.37−2.65; $P < .001$ for higher post-treatment AFP level [by every 10 ng/mL]; HR, 0.43; 95% CI, 0.25−0.76; $P = .004$ for lower albumin level [by every 1 g/dL]; and HR, 1.86; 95% CI, 1.12−3.09; $P = .017$ for rs17047200 AT/TT) (Supplementary Table 7). The cutoff values for post-treatment AFP and albumin levels were determined by the Kaplan−Meier method, which indicated that setting post-treatment AFP at 6 ng/mL and albumin at 3.8 g/dL yielded the best models with the lowest *P* values by log-rank testing (data not shown). The cumulative incidence of HCC up to 10 years after the EOT in the advanced group stratified by these 3 risk factors showed that patients with ≥2 risk factors were at significantly greater risk relative to those with ≤1 risk factor ($P < .001$) (Figure 2*B*).

Table 2. SNP Associated With the Development of Hepatocellular Carcinoma in Patients After Eradication of Hepatitis C Virus

dbSNP rsID	Chr	Nearest gene	Risk allele	Allele (1/2)	Stage	Case (n = 253) 11	12	22	CTRL (≥5 y) (n = 543) 11	12	22	OR (95% CI)	*P* value
rs17047200	4	*TLL1*	T	T/A	GWAS	7	31	85	0	57	275	2.38 (1.56−3.64)	3.74×10^{-5}
					Replication	5	37	88	1	34	175	2.35 (1.48−3.75)	2.33×10^{-4}
					Combined[a]	12	68	173	1	91	450	2.37 (1.74−3.23)	2.66×10^{-8}

NOTE. The results were obtained by genotyping using DigiTag2 assays and/or the Affymetrix Axiom Genome-Wide ASI 1 Array Plate for the discovery GWAS, and DigiTag2 assays or TaqMan SNP Genotyping Assays for the replication cohort. Allele distribution data represent numbers. Data of subjects whose genotypes were not determined were excluded. OR and *P* value by the χ^2 test for the allele frequency model.
Chr, chromosome; CTRL, control.
[a]Allele distribution in GWAS and Replication were combined.

Table 3. Factors Associated With Development of Hepatocellular Carcinoma in Patients After Eradication of Hepatitis C Virus

Risk factor	HR (95% CI)	*P* value
Univariate analysis		
Sex, male / female	1.99 (1.49–2.67)	<.001
Age (by every 10 y)	1.65 (1.42–1.93)	<.001
Platelet count (by every 10^{10} /L)	0.85 (0.83–0.88)	<.001
γ-GTP (by every 40 IU/L)	1.12 (1.06–1.19)	<.001
Albumin (by every 1 g/dL)	0.25 (0.17–0.37)	<.001
Pre-ALT (by every 40 IU/L)	1.02 (0.95–1.09)	.643
Pre-AFP (by every 10 ng/mL)	1.02 (1.00–1.03)	.020
Pre-APRI (by every 1 unit)	1.29 (1.19–1.41)	<.001
Pre-FIB-4 (by every 1 unit)	1.33 (1.27–1.40)	<.001
Fibrosis, F3–4 / F0-2	3.23 (2.39–4.32)	<.001
HCV genotype, 1 / 2	0.85 (0.64–1.12)	.234
rs17047200, AT+TT / AA	1.81 (1.39–2.37)	<.001
Body mass index (by every 1 unit)	0.99 (0.95–1.04)	.828
Diabetes, + / −	2.62 (1.89–3.62)	<.001
Anti-HBc, + / −	1.57 (1.19–2.08)	.001
Post-ALT (by every 40 IU/L)	1.39 (1.11–1.74)	.004
Post-AFP (by every 10 ng/mL)	2.52 (2.02–3.13)	<.001
Post-APRI (by every 1 unit)	1.88 (1.59–2.23)	<.001
Post-FIB-4 (by every 1 unit)	1.18 (1.13–1.22)	<.001
Multivariate analysis		
Sex, male / female	2.19 (1.39–3.44)	<.001
Age (by every 10 y)	1.44 (1.13–1.83)	.003
Albumin (by every 1 g/dL)	0.31 (0.19–0.53)	<.001
Fibrosis, F3–4 / F0-2	1.63 (1.07–2.48)	.024
rs17047200, AT+TT / AA	1.78 (1.17–2.70)	.008
Diabetes, + / −	1.91 (1.21–3.03)	.006
Post-AFP (by every 10 ng/mL)	2.37 (1.71–3.30)	<.001

NOTE. HRs were calculated by the Cox proportional hazard method. Sex, age, γ-GTP levels, albumin levels, pre-AFP levels, stage of fibrosis, rs17047200 genotypes, presence of diabetes, presence of anti-HBc, post-ALT levels and post-AFP levels were included as covariates in the multivariate stepwise Cox model, whereas platelet count, pre- and post-treatment APRI and FIB-4 were not included as covariates in this analysis, because these factors were strong confounders for stage of hepatic fibrosis.
ALT, alanine aminotransaminase; APRI, aspartate aminotransferase-to-platelet ratio index; γ-GTP, γ-glutamyl transpeptidase; Pre, pre-treatment; FIB-4, fibrosis-4; Post, post-treatment (24 wk after the end of treatment).

Another well-known risk factor for developing HCC is male sex. We evaluated the impact of rs17047200 genotype on developing HCC in patients stratified by sex in the entire cohorts. It was found that the cumulative incidence of HCC up to 10 years after the EOT was significantly higher in patients with rs17047200 AT/TT in females as well as males (Supplementary Figure 7).

Tolloid-Like 1 Messenger RNA Expression Analyses Using Human Hepatic Stellate Cells, Animal Models of Hepatic Fibrosis, and Human Samples

Mammalian TLL1 is 1 of the 4 members of the bone morphogenetic protein 1/tolloid (BMP1/TLD)-like proteinase family. Bioinformatic analysis on protein–protein interaction networks indicated that the TLL1 and BMP1 exert several biological roles in regulating extracellular matrix assembly[30–34] and in transforming growth factor (TGF)−β signaling[35] (Supplementary Figure 8). Chronic liver pathologies, including HCV infection, result in up-regulation of TGF-β signaling and subsequently activate human hepatic stellate cells (HSCs), causing excessive accumulation of the various extracellular matrix proteins in the liver.[36,37] These findings led us to hypothesize that TLL1 may be involved in hepatic fibrogenesis and thereby carcinogenesis. Therefore we investigated the expression of *TLL1* and *BMP1* using in vitro and in vivo models, as well as human samples.

First, we evaluated the phenotype of human HSCs (HHSteC; ScienCell Research Laboratories, San Diego, CA) after treatment with recombinant TGF-β1 (Supplementary Figure 9*A*). The expression of α-smooth muscle actin, which is a surrogate marker for activated HSCs, and the level of *ACTA2* messenger RNA (mRNA) were high in TGF-β1–treated cells, lower in mock-treated cells, and lowest of all in cells cultured in media with supplement (Supplementary Figure 9*B* and *C*). Then, we investigated levels of *TLL1/BMP1* mRNA in these differently activated cells. *TLL1* mRNA was significantly up-regulated on activation of HSCs by TGF-β1, whereas *BMP1* mRNA levels were only modestly increased on activation (Figure 3*A*).

Second, we examined *Tll1/Bmp1* mRNA expression in livers of animals with hepatic fibrosis: a rat model of nonalcoholic steatohepatitis (NASH) which was fed choline-deficient L-amino acid-defined (CDAA) diets and its control, which was fed choline-sufficient L-amino acid-defined (CSAA) diets, and a mouse model of liver injury caused by administration of carbon tetrachloride (CCl_4). The pathologic fibrosis stage in each group was as follows: F0/2/3–4 in CSAA 10W/CDAA 10W/CDAA 30W, respectively (Figure 3*B*); F0/2-3/3-4 in CCl_4 0W/6W/12W (data not shown). In CDAA-fed rats, levels of mRNA for *Tll1* increased in parallel with the progression of hepatic fibrosis, whereas *Bmp1* mRNA levels were similar in all 3 groups (Figure 3*C*). *Tll1* mRNA levels were increased in CCl_4-treated mice relative to the controls (CCl_4 0W), but did not differ according to progression of fibrosis (CCl_4 6W vs 12W), whereas *Bmp1* levels were again similar in the 3 groups (Supplementary Figure 10).

Thirdly, we investigated *TLL1/BMP1* mRNA levels in human liver tissues. The characteristics of the patients in this analysis are shown in Supplementary Table 8. We found that levels of *TLL1* mRNA increased in parallel with the progression of hepatic fibrosis in CHC patients (Figure 4*A*), and although *BMP1* mRNA levels were higher in the liver tissues of CHC patients than in normal livers, they did not change with fibrosis progression (F1–2 vs F3–4) (Supplementary Figure 11*A*). In the analysis of paired tumorous and the surrounding nontumorous tissues of patients developing HCC after achieving SVR, *TLL1* mRNA levels were significantly lower in the tumor than the nontumor tissues (Figure 4*B*). However, *BMP1* mRNA levels were again not different (Supplementary Figure 11*B*). Meanwhile, the stage of fibrosis did not correlate with