elevated AFP level may be related to the histological grading. Parfitt et al. (24) reported that the histological grade of tumor differentiation and macroscopic vascular invasion were independent predictors of long-term survival after liver transplantation. However, the most significant risk factor in our cohort was early recurrence after initial RFA, suggesting that careful surveillance for recurrence is necessary even after complete local ablation, and if early recurrence occurs within 1 year, liver transplantation should be considered as soon as possible to avoid loss of the indication, even in patients in whom initial tumor size and number are small. Importantly, liver function tests, such as albumin level and prothrombin activity, were not identified as risk factors for recurrence exceeding the Milan criteria in our cohort, suggesting that preserved liver function itself does not necessarily indicate that there has been an adequate waiting time.

We here calculated the risk score from two simple factors: the initial tumor marker and early recurrence after initial complete RFA. The 3- and 5-year survival rates of patients with both risk factors were 33.5% and 22.6%, respectively, in spite of early stage at initial ablation. Conversely, the 3-and 5-year survival rates of patients with neither risk factor were 93.1% and 78.0%, respectively. The number of patients with both risk factors was small (12.1%); however, new therapeutic strategies (early transplantation or

repeated adjuvant therapy) were necessary to achieve long-term survival.

Takada et al. (25) reported that repeated nontransplant treatment for recurrent HCC, such as RFA and transluminal arterial embolization, prior to living donor liver transplantation (LDLT) might increase the risk of recurrence and impair the survival advantage conferred by LDLT. As our study focused mainly on recurrence exceeding the Milan criteria, we did not assess whether RFA performed prior to liver transplantation affected the final outcome of patients who actually received liver transplantation. Therefore, further controlled studies are warranted to confirm whether bridging therapy with RFA actually leads to better survival after transplantation. Nevertheless, liver transplantation should be considered before the patient exceeds the Milan criteria in order to achieve excellent survival after liver transplantation.

In conclusion, RFA presents a promising bridging therapy for liver transplantation in patients who are at low risk of tumor progression. However, patients with a higher AFP level at initial RFA and earlier recurrence even after successful RFA should be considered for timely liver transplantation or new adjuvant therapy. In these patients, the 3- and 5-year survival rates were below 50% although they were classified as early stage at initial therapy.

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Figure Legends

Figure 1. The cumulative recurrence rate exceeding the Milan criteria stratified by the number of risk factors. The cumulative recurrence rate exceeding the Milan criteria in patients with more risk factors was significantly higher than that in patients with fewer risk factors ($p < 0.0001$)

Figure 2. The cumulative survival rate stratified by the number of risk factors. The cumulative survival rate in patients with more risk factors was significantly lower than that in patients with fewer risk factors ($p < 0.0001$)

Table 1. Patient characteristics

Characteristics	Value
Patients, n	323
Age, years	66 ± 9
Duration of follow-up, years	4.0 (0.6-12.2)
Gender, male/female (%)	191 (59)/132 (41)
Clinical and laboratory data	
AFP, median (range), ng/mL	25.6 (1.2–76600)
PIVKA-II, median (range), mAU/mL	25 (7–10600)
Child–Pugh score, A/B (%)	256 (79)/67 (21)
Pathology	
Maximum diameter of HCC, mm	
≤20/21–30/31–50 (%)	117 (36)/158 (49)/48 (15)
Number of HCC nodules, n	
Single/multiple (%)	226 (70)/97 (30)
CLIP score, 0/1/2/3 (%)	173 (52)/114 (37)/32 (10)/3 (1)
Lymph node involvement	0
Metastasis	0

Major associated liver diseases	
HCV/HBV/HCV+HBV/others (%)	248 (76.8)/31 (9.6)/3 (0.9)/41 (12.7)

Table 2. Cox proportional hazards analysis for recurrence exceeding the Milan criteria (n = 323).

Factors	Univariate analysis	Multivariate analysis	
	p value	p value	(HR, 95% CI)
Age, >65 years	0.644		
Child–Pugh score (B compared with A)	0.098		
AFP, >100 ng/mL	0.0006	0.0059	(1.59, 1.14–2.23)
PIVKA-II, >100 mAU/mL	0.0004	0.211	(1.26, 0.87–1.84)
Tumor size, > 20mm	0.0033	0.012	(1.54, 1.09–2.16)
Tumor number, >2	0.291		
Early recurrence (within 1 year after RFA)	<0.0001	<0.0001	(2.76, 2.05–3.71)

HR = hazard ratio; CI = confidence interval

Table 3. Cox proportional hazards analysis for overall survival (n = 323).

Factors	Univariate analysis	Multivariate analysis
	p value	p value (HR, 95%CI)
Age, >65 years	0.644	
Child–Pugh score (B compared with A)	<0.0001	<0.0001 (2.42, 1.61–3.64)
AFP, >100 ng/mL	<0.0001	0.0003 (2.03, 1.37–3.00)
PIVKA-II, ≥100 mAU/mL	0.136	
Tumor size, > 20mm	0.943	
Tumor number, >2	0.0037	0.056 (1.45, 0.99–2.13)
Early recurrence (within 1 year after RFA)	<0.0001	0.0001 (2.09, 1.43–3.03)

HR = hazard ratio; CI = confidence interval

Figure 1

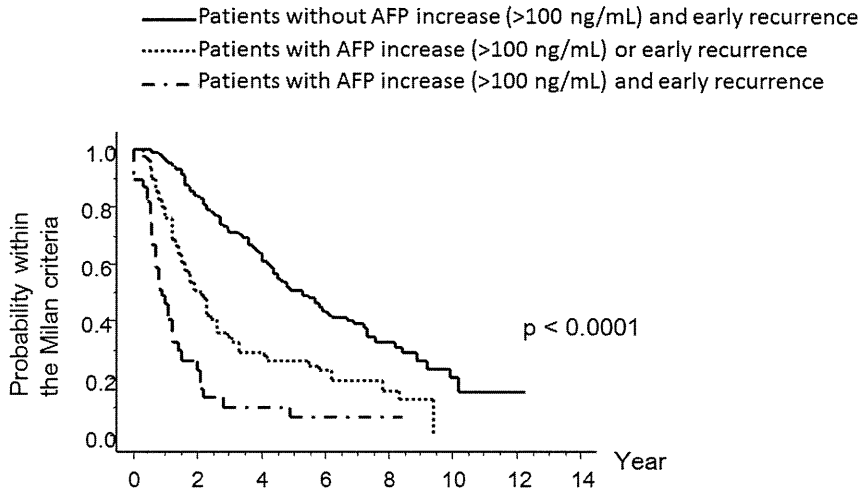

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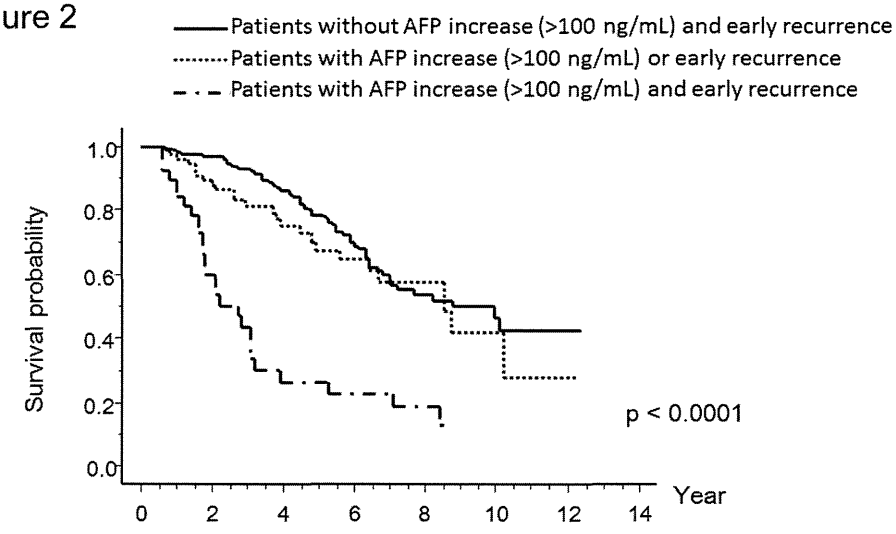
Figure 2



254x190mm (300 x 300 DPI)

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ORIGINAL ARTICLE—Liver, Pancreas, and Biliary Tract

Genetic variation near interleukin 28B and the risk of hepatocellular carcinoma in patients with chronic hepatitis C

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Abstract
Background We aimed to clarify the association between single nucleotide polymorphism (SNP) located near *interleukin 28B* and hepatocellular carcinoma (HCC).
Methods A cohort comprising 792 patients treated with interferon for chronic hepatitis C was investigated. SNPs at rs8099917 and rs12979860 were determined. Cumulative incidence and HCC risk were analyzed by Kaplan–Meier and Cox proportional hazard analyses for a mean follow-up period of 4.9 years. Fibrosis progression rate (FPR) was determined in these patients with a known time of infection ($n = 294$).
Results Cumulative HCC incidence was significantly higher in rs8099917 nonTT (minor homozygote or heterozygote) patients than in rs8099917 TT (major homozygote) patients (20.8 vs. 10.5 % over 10 years, logrank test, $p = 0.002$). This difference was notable in patients infected with genotype 1 and those treated with pegylated interferon and ribavirin. Among nonSVRs, interferon had a limited effect in suppressing alanine aminotransferase (ALT) and/or α-fetoprotein (AFP) levels in nonTT patients. The suppression of these values after interferon therapy was associated with a lower incidence of HCC. FPR were similar in TT and nonTT patients.
Conclusions rs8099917 nonTT is related to higher HCC development in patients with HCV genotype 1 and those treated with pegylated interferon and ribavirin. Higher HCC incidence observed in nonTT patients partly results from the limited suppression of ALT and/or AFP by interferon in these patients.

Keywords Hepatocarcinogenesis · Fibrosis · Interferon · Alanine aminotransferase · α-Fetoprotein

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Introduction

Hepatitis C virus (HCV) infection is one of the major causes of chronic hepatitis, which progresses to hepatocellular carcinoma (HCC) in many patients [1]. In the last two decades, interferon (IFN) therapy has been used to treat chronic hepatitis C (CH-C) with the goal of altering its natural progression. Although HCV eradication with IFN therapy in CH-C patients reportedly prevents HCC development [2–4], factors responsible for HCC development in IFN-treated patients are difficult to determine because of the prolonged clinical course of CH-C.

Recent studies demonstrated that single nucleotide polymorphisms (SNPs) near *interleukin (IL) 28B* were strongly associated with the virological response to pegylated IFNα (PEG-IFNα) and ribavirin (RBV) combination therapy [5–7]. However, it remains unclear if the SNPs near *IL28B* are associated with further consequences of CH-C, such as HCC and liver fibrosis, in IFN-treated patients because of the paucity of adequate cohort studies. To address the important question of whether SNPs near *IL28B* are associated with the development of HCC, we analyzed the influence of this polymorphism on HCC risk in a large-scale, long-term cohort of IFN-treated patients.

Methods

Patients

Patients chronically infected with HCV who had histologically proven chronic hepatitis or cirrhosis and had undergone IFN treatment between 1992 and 2010 were enrolled in the original cohort [8]. In this cohort comprising 1,818 patients, a subgroup of 792 patients who were available for genotyping of the SNPs near *IL28B* (rs8099917 and rs12979860) was assessed in the present study. Patients were excluded from the original cohort if they had a history of HCC at the time of liver biopsy, autoimmune hepatitis, primary biliary cirrhosis, excessive alcohol consumption (≥50 g/day), hepatitis B surface antigen, or anti-human immunodeficiency virus antibody. HCC was definitively ruled out by ultrasonography, dynamic computed tomography (CT), and/or magnetic resonance imaging (MRI) on enrollment. Written informed consent was obtained from all patients, and the Ethical Committee of Musashino Red Cross Hospital approved this study, which was conducted in accordance with the Declaration of Helsinki.

Genotyping for SNPs near *IL28B* (rs8099917 and rs12979860)

Genetic polymorphisms in tagged SNPs located near *IL28B* (rs8099917 and rs12979860) were determined by direct sequencing of PCR-amplified DNA, as reported previously [9].

Histological evaluation

Laparoscopic or ultrasound-guided liver biopsy was undertaken using 13-gauge or 15-gauge needles, respectively. The median length of specimens was 18 mm (range 11–40 mm), and the median number of portal tracts was 18 (range 9–34). Fibrosis stage and grade of inflammatory activity were scored by two pathologists according to Desmet et al.'s classification [10]. In case of interobserver disagreement in histological staging or grading, the diagnosis was confirmed by consensus.

IFN therapy and definitions of response to IFN therapy

All patients had chronic HCV infection at liver biopsy, which was confirmed by the presence of HCV-RNA in serum. All IFN therapies were initiated within 48 weeks after liver biopsy. Of 792 patients, 71 patients received IFNα or IFNβ monotherapy for 24 weeks, 54 received IFNα/RBV combination therapy for 24 weeks, 118 received PEG-IFNα monotherapy for 48 weeks, and 549 received PEG-IFNα/RBV combination therapy for 48–72 weeks.

Patients negative for serum HCV-RNA 24 weeks after IFN therapy completion were defined as sustained virological responders (SVRs). Patients who remained positive for HCV-RNA 24 weeks after therapy completion were defined as nonSVRs. HCV-RNA was determined by the qualitative Amplicor or TaqMan HCV assay (Roche Molecular Diagnostics, Tokyo, Japan).

Data collection and patient follow up

At primary liver biopsy, patient characteristics and biochemical, hematological, virological, and histological data were evaluated. Age at primary liver biopsy was determined. Patients were examined for HCC by abdominal ultrasonography, dynamic computed tomography, and/or magnetic resonance imaging every 3–6 months. Serum alanine aminotransferase (ALT) and α-fetoprotein (AFP) levels were measured every 1–6 months. Surveillance protocols were in accordance with the standard of care in Japan. If HCC was suspected on the basis of the screening examination, additional procedures (e.g., dynamic CT, dynamic MRI, CT during hepatic arteriography, CT during arterial portography, contrast-enhanced ultrasonography, and tumor biopsy) were used to confirm the diagnosis. HCC diagnosis was confirmed by needle biopsy, histology of surgically resected specimens, or characteristic radiological findings. To evaluate the effect of changes in serum ALT and AFP levels during IFN therapy on

hepatocarcinogenesis, mean integration values of ALT and AFP in each patient were calculated before and after IFN therapy. In patients who developed HCC, data obtained more than 1 year prior to HCC development were used to exclude AFP elevation caused by HCC itself.

Follow-up was between the date of primary liver biopsy and HCC development or the last medical attendance until June 2011. The mean follow-up period was 4.9 years (range 1.0–18.6 years).

Determination of changes in fibrosis stage over time

Changes in fibrosis stage over time were determined in patients who showed evidence of a single blood transfusion as a known time of HCV infection. Two hundred ninety-four patients had a single blood transfusion before 1992, indicating the known time of HCV infection (rs8099917 TT, $n = 217$; rs8099917 nonTT, $n = 77$). In this subgroup, 221 (75.2 %) patients were infected with HCV genotype 1. Annual fibrosis progression rate (FPR) was calculated as the fibrosis stage at liver biopsy divided by HCV infection duration, which was determined by the period between blood transfusion and liver biopsy (mean duration, 35.1 years; range 12.0–60.0 years).

Statistical analyses

Categorical data were compared by Chi-square or Fisher's exact tests. Continuous variable distributions were analyzed with Student's *t*- or Mann–Whitney *U* test. All tests of significance were two-tailed. $p < 0.05$ was considered significant. The cumulative incidence curve was determined by the Kaplan–Meier method, and differences between groups were assessed using the logrank test. Factors associated with HCC risk were determined by the Cox proportional hazard model. As covariates in the multivariate stepwise Cox model, age, sex, stage of liver fibrosis, pre- and post-IFN treatment ALT and AFP levels, virological response, and *IL28B* genotype were included. HCC development was the dependent variable. Time zero was defined as the time of primary liver biopsy. The proportional assumption was supported by log[−log(survival)] vs. log(time) plots, which showed parallel lines. Statistical analyses were performed using the Statistical Package for the Social Sciences software (version 18.0) (SPSS Inc., Chicago, IL, USA).

Results

Patient characteristics and the SNPs near *IL28B*

Patient characteristics are demonstrated in Table 1. Frequency of the rs8099917 genotype was as follows: major homozygote (TT), 74.2 % (588/792); heterozygote (TG), 24.2 % (192/792); and minor homozygote (GG), 1.5 % (12/792). Genotypic distribution of this SNP was consistent with that in a recent report on Japanese patients [5]. The frequency of the rs12979860 genotype was as follows: major homozygote (TT), 73.4 % (581/792); heterozygote (TG), 25.1 % (199/792); and minor homozygote (GG), 1.5 % (12/792). The genotypic discrepancy between rs8099917 and rs12979860 was found only in seven patients. Therefore, the genotypes of the two SNPs (rs8099917 and rs12979860) were 99.1 % identical. All seven patients had a major homozygote (TT) in rs8099917 but a heterozygote (CT) in rs12979860, and HCC developed in one of seven patients at 2.2 years after initiation of the follow-up.

Response to IFN therapy

The final responses to IFN therapy (SVR or nonSVR) were determined in all patients. SVR rate was significantly higher in TT patients than in nonTT patients (58.3 vs. 27.9 %, $p < 0.001$) (Table 1). SVR rates for each therapeutic regimen in TT and nonTT patients, respectively, were as follows: IFN monotherapy, 35.7 % (20/56) vs. 26.7 % (4/15), $p = 0.759$; IFNα/RBV combination therapy, 46.3 % (19/41) vs. 15.4 % (2/13), $p = 0.057$; PEG-IFNα monotherapy, 63.2 % (55/87) vs. 35.5 % (11/31), $p = 0.008$; PEG-IFNα/RBV combination therapy, 61.6 % (249/404) vs. 27.6 % (40/145), $p < 0.001$.

Factors associated with the SNPs near *IL28B*

NonTT patients were significantly associated with higher γ-glutamyl transpeptidase levels, lower low-density lipoprotein cholesterol levels, higher hepatic steatosis frequency, glutamine or histidine mutations at amino acid position 70 (70QH) in the HCV core region, and one or no mutation in the IFN sensitivity-determining region in the HCV nonstructural 5A gene (Table 1).

Cumulative incidence of HCC according to the SNPs near *IL28B*

During follow-up, 53 patients developed HCC (Table 1). At 3, 5, and 10 years, the overall cumulative incidence of HCC was 3.4, 7.4, and 13.1 %, respectively. The cumulative incidence of HCC at 5 and 10 years was significantly higher in nonTT patients than in TT patients (13.0 and 20.8 % vs. 5.4 and 10.5 %, respectively; logrank test, $p = 0.002$) (Fig. 1a). Among SVRs, no significant difference was found in the cumulative HCC incidence between TT and nonTT patients (Fig. 1b). However, the cumulative incidence of HCC among nonSVRs was significantly

Table 1 Characteristics of patients and comparison between the SNPs near *IL28B*

Characteristics	Total	rs8099917 TT	rs8099917 nonTT	*p* value*
Patients, *n*	792	588	204	
Sex, *n* (%)				0.329†
Male	310 (39.1)	236 (40.1)	74 (36.3)	
Female	482 (60.9)	352 (59.9)	130 (63.7)	
Age (SD), year	58.6 (10.7)	58.5 (10.6)	58.8 (11.0)	0.684‡
BMI (SD), kg/m^2	22.8 (3.2)	22.9 (3.2)	22.7 (3.3)	0.382‡
Fibrosis stage, *n* (%)				0.751†
F1/2	612 (77.3)	456 (77.6)	156 (76.5)	
F3/4	180 (22.7)	132 (22.4)	48 (23.5)	
%Severe steatosis (≥10%)	25.3	21.4	35.4	<0.001†
ALT level (SD), IU/L	63.4 (52.5)	64.9 (50.1)	59.0 (42.9)	0.170‡
γ-GTP level (SD), IU/L	45.9 (45.3)	41.5 (43.5)	58.3 (47.9)	<0.001‡
LDL-C level (SD), mg/dL	99.8 (26.8)	102.0 (26.6)	93.6 (26.8)	0.034‡
AFP level (SD), ng/mL	10.3 (26.7)	8.24 (12.2)	16.4 (47.9)	<0.001‡
Platelet counts (SD), ×10^3/μL	164 (52)	163 (51)	167 (56)	0.422‡
HCV load (SD), KIU/mL	1550 (1465)	1612 (1465)	1392 (1457)	0.107‡
HCV genotype, *n* (%)[a]				0.065†
1a	8 (1.0)	5 (0.9)	3 (1.5)	
1b	588 (74.8)	422 (72.4)	166 (81.7)	
2a	118 (15.0)	96 (16.5)	22 (10.8)	
2b	63 (8.0)	52 (8.9)	11 (5.4)	
Others	9 (1.1)	8 (1.4)	1 (0.5)	
%Core 70 a.a. mutation[b]	34.5	26.2	57.1	<0.001†
%ISDR wild or 1 mutation[c]	67.4	64.0	76.1	0.005†
Duration (SD), year	4.9 (3.0)	5.0 (3.1)	4.8 (2.8)	0.480‡
IFN regimen, *n* (%)				0.798†
IFN mono	71 (9.0)	56 (9.5)	15 (7.4)	
IFN + RBV	54 (6.8)	41 (7.0)	13 (6.4)	
PEG-IFN mono	118 (14.9)	87 (14.8)	31 (15.2)	
PEG-IFN + RBV	549 (69.3)	404 (68.7)	145 (71.1)	
SVR, *n* (%)	400 (50.5)	343 (58.3)	57 (27.9)	<0.001†
HCC, *n* (%)	53 (6.7)	30 (5.1)	23 (11.3)	0.002†

* Comparison between *IL28B* major and minor genotypes

† Chi-square test

‡ Student's *t*-test

[a] HCV genotype was determined in 786 patients (*n*: *IL28B* major = 583, minor = 203)

[b] HCV core mutation was determined in 313 patients with genotype 1b

[c] ISDR was determined in 585 patients with genotype 1b

higher in nonTT patients than in TT patients (15.5, and 24.8 vs. 7.2 %, and 15.4 % at 5, and 10 years, respectively; logrank test, $p = 0.016$) (Fig. 1c). Similar results were obtained when the rs12979860 genotype was used as a reference. That is, the cumulative incidences of HCC at 5 and 10 years in overall patients were 13.1 and 20.5 % in nonCC patients and 5.2 and 10.4 % in CC patients (logrank test, $p = 0.001$); those in SVRs were 3.8 and 4.9 % in CC patients and 4.9 and 4.9 % in nonCC patients; and those in nonSVRs were 15.9 and 25.1 % in nonCC patients and 6.8 and 15.0 % in CC patients (logrank test, $p = 0.008$).

Ten subjects [rs8099917 TT, $n = 9$; nonTT, $n = 1$: SVR, $n = 8$; nonSVR, $n = 2$: mean follow-up period = 4.3 years (range 1.1–8.3 years)] were lost to follow-up during the last 2 years. These patients were censored from the cumulative incidence analyses at the time of the last visit.

In this study cohort, only three (one TT, $n = 1$; nonTT, $n = 2$) patients died during follow-up, and no patient underwent liver transplantation. These deaths were HCC-related. Therefore, it is unlikely that competing risks would have affected our results regarding differences in HCC incidence between TT and nonTT patients.

Because the SNPs near *IL28B* affects treatment responses particularly in patients infected with HCV genotype 1 and/or those treated with PEG-IFNα/RBV combination therapy, the cumulative incidences of HCC were analyzed in a subgroup of the patients. In patients infected with HCV genotype 1 ($n = 596$), the cumulative incidence of HCC was significantly higher in nonTT patients than in TT patients (15.2 and 24.9 % vs. 6.4 and 10.5 % at 5, and 10 years, respectively; logrank test, $p = 0.001$) (Fig. 1d). In patients treated with PEG-IFNα/RBV combination therapy ($n = 549$), the cumulative incidence of HCC was also