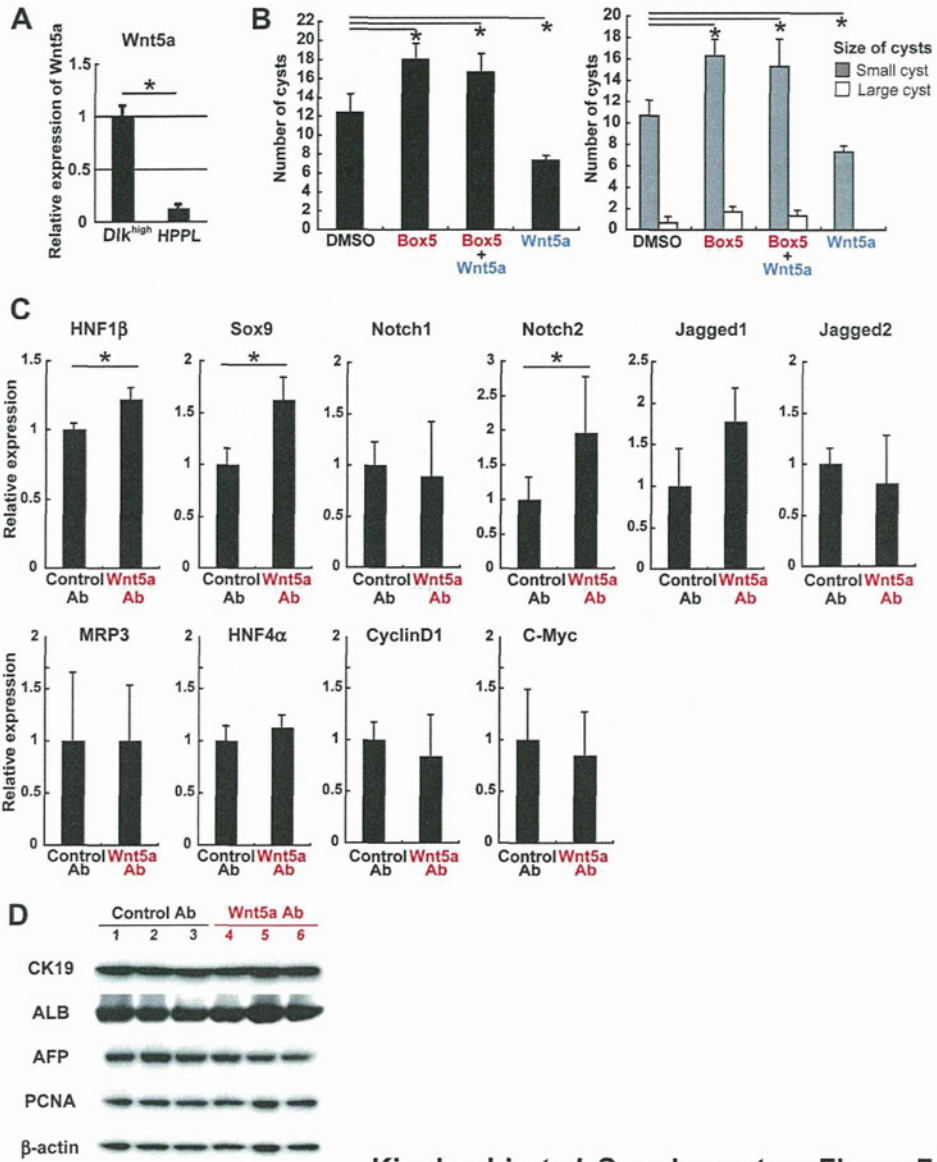


A : Colony without clear lumina
 B : Small cyst
 (50-100 μm diameter with clear lumina)
 C : Large cyst
 (>100 μm diameter with clear lumina)

Kiyohashi *et al.* Supplementary Figure 6

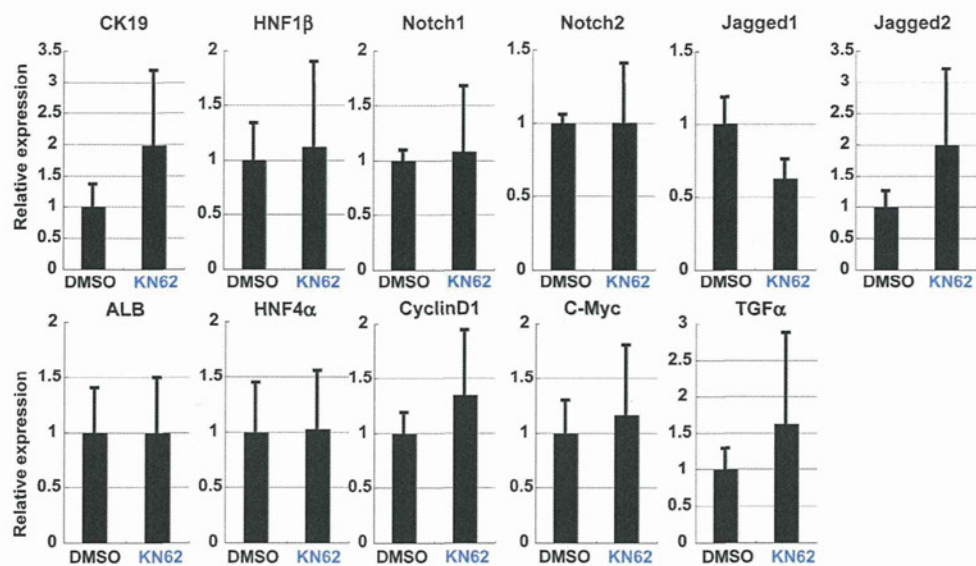
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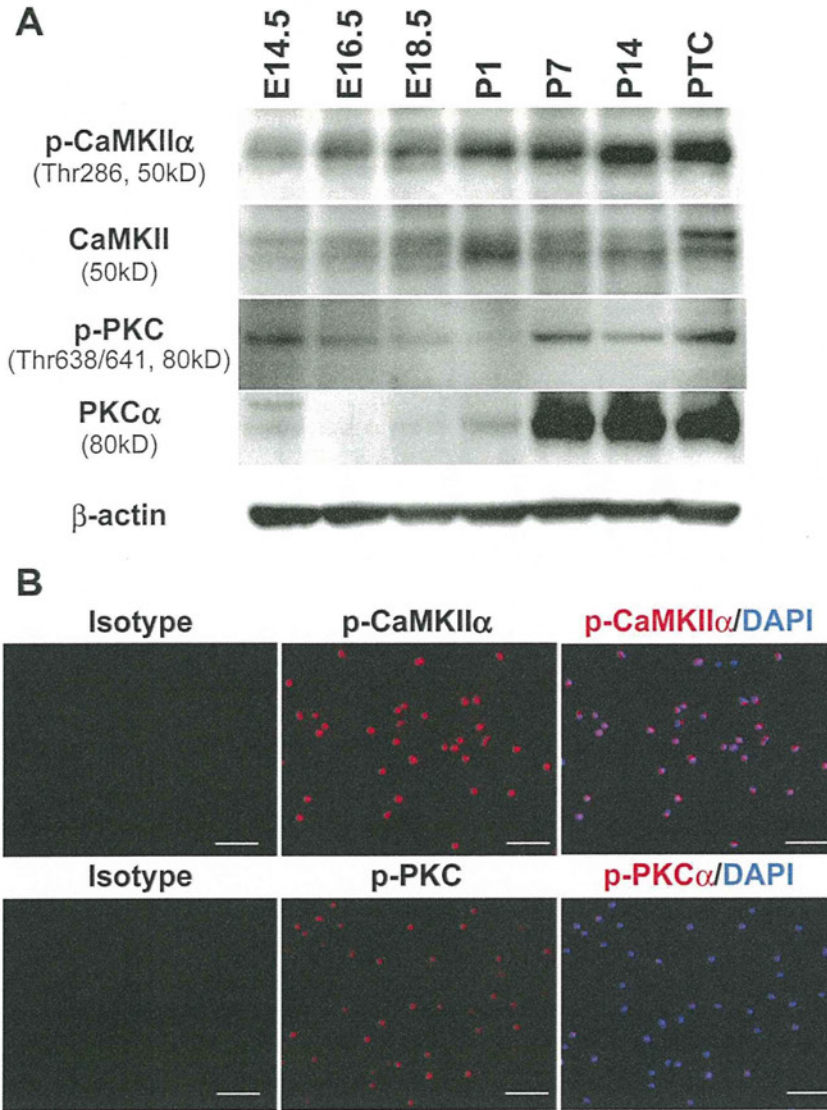
Kiyohashi *et al.* Supplementary Figure 7

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Kiyohashi *et al.* Supplementary Figure 8

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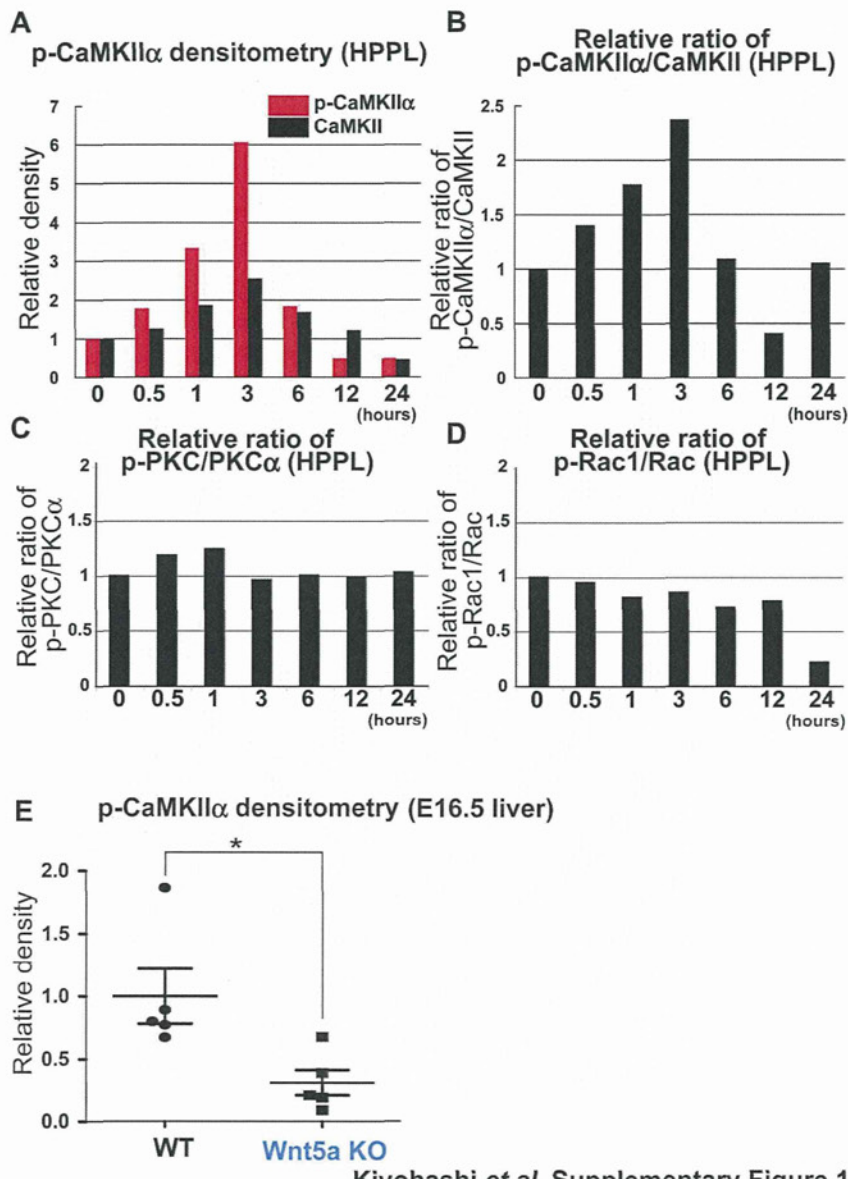
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Kiyohashi *et al.* Supplementary Figure 9

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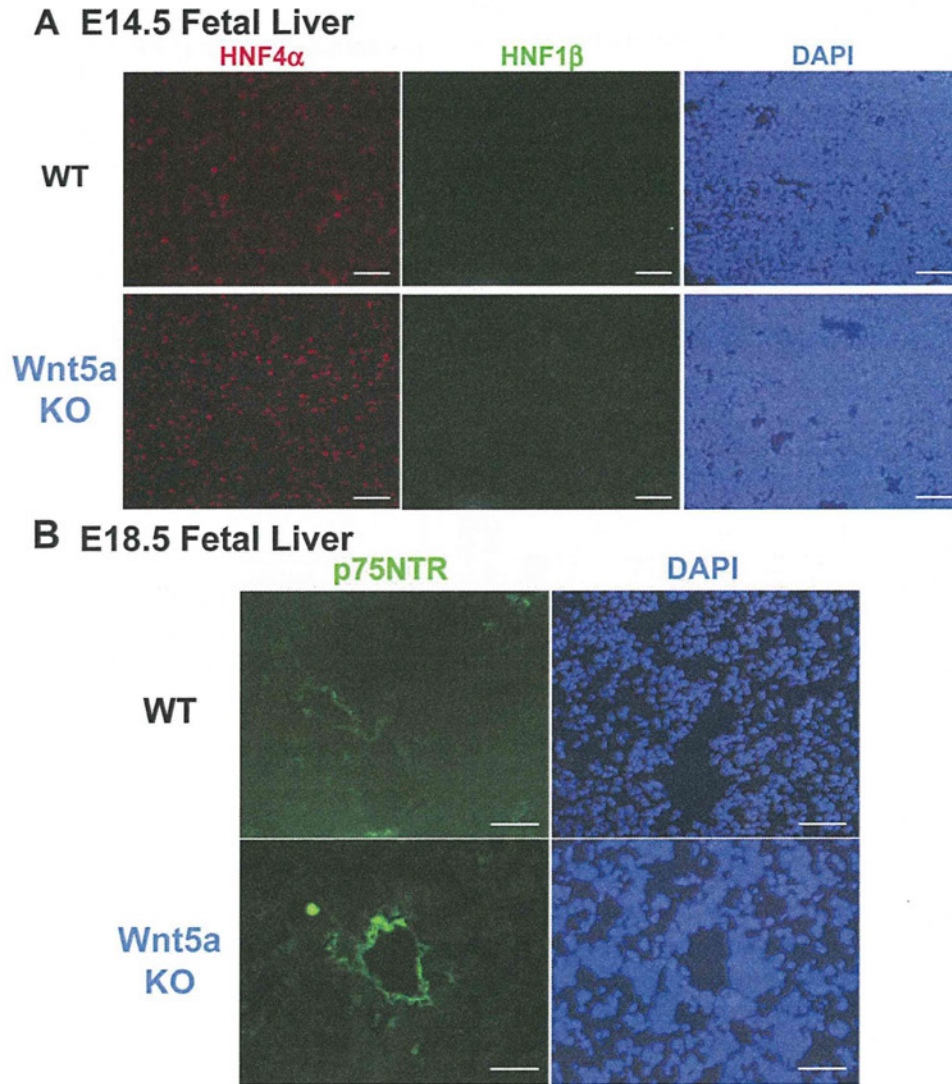
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Kiyohashi et al. Supplementary Figure 10

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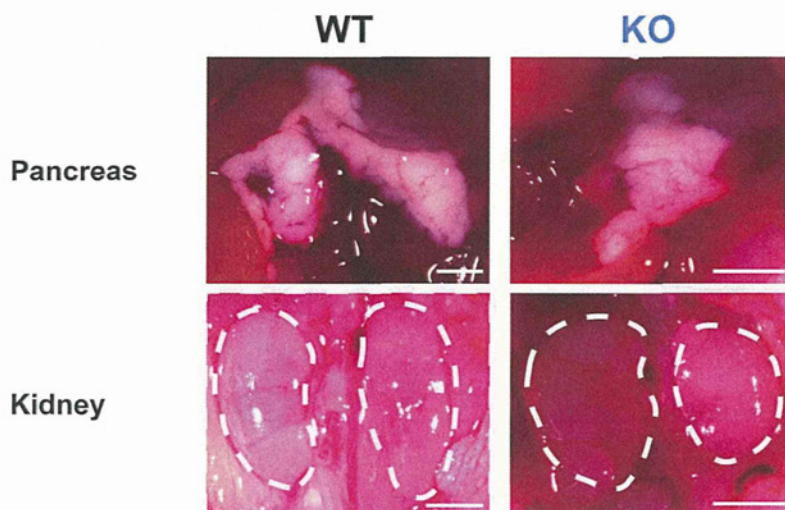


Kiyohashi *et al.* Supplementary Figure 11

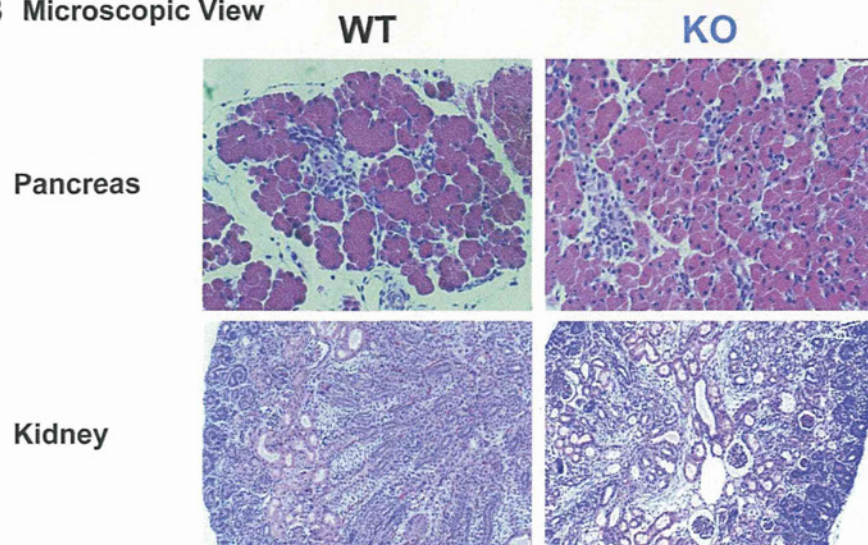
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A Stereoscopic View



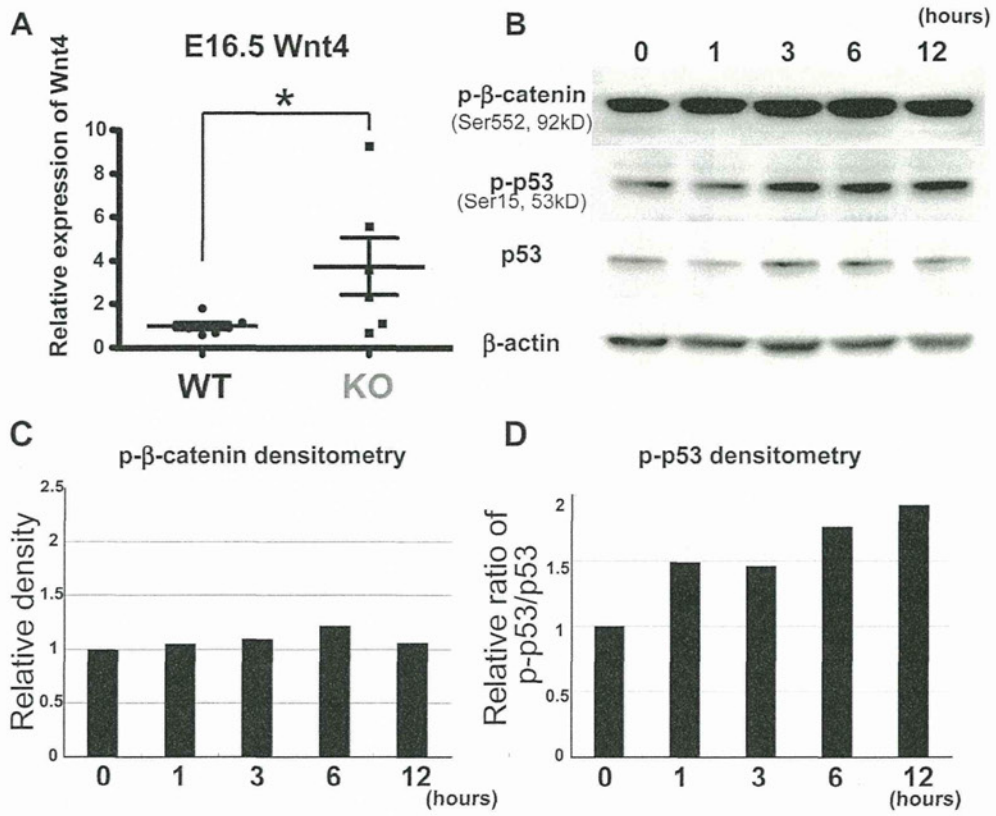
B Microscopic View



Kiyohashi *et al.* Supplementary Figure 12

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Kiyohashi *et al.* Supplementary Figure 13

203x180mm (300 x 300 DPI)

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Inhibition of hepatocellular carcinoma by PegIFN α -2a in patients with chronic hepatitis C: a nationwide multicenter cooperative study

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Received: 23 April 2012 / Accepted: 25 June 2012
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Abstract

Background We investigated whether the administration of maintenance doses of interferon prevented hepatocellular carcinoma (HCC) in patients with chronic hepatitis C. **Methods** Study 1: A multicenter, retrospective, cooperative study was carried out to determine whether long-term administration of low-dose peginterferon alpha-2a

(PegIFN α -2a) prevented HCC development in patients with chronic hepatitis C. In total, 594 chronic hepatitis C patients without a history of HCC were enrolled and treated with 90 μ g PegIFN α -2a administered weekly or bi-weekly for at least 1 year. Study 2: HCC developed in 16 of 99 additional patients without PegIFN α -2a treatment during 3.8 years of observation. A propensity-matched control study was then carried out to compare the incidence of

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HCC between the 59 patients who received low-dose PegIFN α -2a (PegIFN α -2a group) and 59 patients who did not receive PegIFN α -2a treatment (control group), matched for sex, age, platelet count, and total bilirubin levels.

Results Study 1: HCC developed in 49 patients. The risk of HCC was lower in patients with undetectable hepatitis C virus RNA, ≤ 40 IU/L alanine aminotransferase (ALT), or ≤ 10 ng/L alpha-fetoprotein (AFP) 24 weeks after the start of therapy. Study 2: The incidence of HCC was significantly lower in the PegIFN α -2a group than in the control group.

Conclusions Low-dose and long-term maintenance administration of PegIFN α -2a decreased the incidence of HCC in patients with normalized ALT and AFP levels at 24 weeks compared with patients without normal ALT and AFP levels.

Keywords Chronic hepatitis C · Hepatocellular carcinoma · Peginterferon

Introduction

Hepatocellular carcinoma (HCC), the sixth most common cancer worldwide, often develops because of long-term hepatitis B or C virus infection [1, 2]. In particular, chronic hepatitis C and hepatic cirrhosis increase the risk of HCC; the annual incidence of tumor development in such patients may be as high as 2–4 % [3–5]. The incidence of HCC decreases in patients who achieve a sustained virological response (SVR) to interferon (IFN) treatment, although the incidence remains high in non-SVR patients [6–9]. A detailed analysis of HCC development revealed that chronic hepatitis C patients aged 65 years or more, especially those with advanced fibrosis of the liver, were at an increased risk of developing HCC [10]. For patients

65 years or older with advanced liver fibrosis, the dose of ribavirin is often reduced or the agent is discontinued, resulting in lower SVR rates in those with discontinuation of ribavirin. Establishing an effective treatment strategy for preventing the development of HCC is important for these high-risk patients.

Factors related to the development of HCC have been analyzed in patients who did not achieve an SVR even after IFN treatment; advanced fibrosis of the liver and high levels of serum alanine aminotransferase (ALT), and alpha-fetoprotein (AFP) are risk factors for HCC development [11, 12]. A randomized controlled trial was conducted in Western countries to determine whether combined peginterferon and ribavirin treatment with weekly administration of 90 μ g peginterferon alpha-2a (PegIFN α -2a) could prevent HCC in non-responders. A 3.5-year follow up showed that administration of a maintenance dose of PegIFN α -2a did not reduce tumor incidence in these patients [13]. However, after 8.5 years of observation, the incidence of HCC was decreased among those in the PegIFN α -2a group with cirrhosis [14]. Meanwhile, Bruix et al. [15] reported that maintenance therapy with PegIFN α -2b did not prevent HCC in chronic hepatitis C patients with cirrhosis. In Japan, long-term low-dose administration of natural IFN has been reported to decrease the incidence of HCC [16]. In light of these conflicting results, investigations should be carried out in a large number of patients with chronic hepatitis C to resolve the question of whether IFN treatment prevents the development of HCC.

We carried out a multicenter retrospective cooperative study of patients with chronic hepatitis C to determine whether those treated with 90 μ g PegIFN α -2a without ribavirin had a reduced incidence of HCC compared with those not treated with IFN.

Patients and methods

Study 1: analysis of risk factors for HCC in patients treated with long-term low-dose-PegIFN α -2a

In total, at 21 hepatitis centers throughout Japan, 743 patients with hepatitis C who had received 90 μ g of PegIFN α -2a therapy weekly or bi-weekly for 1 year or more without having received the full dose (180 μ g) since December 2003 were examined retrospectively for the development of HCC. The end of enrollment in this study was the end of December 2008 and the end of follow up was the end of December 2010. Patients with a history of HCC before the start of therapy and those with a therapy period of less than 48 weeks were excluded, leaving 594 patients who had undergone long-term administration of PegIFN α -2a for analysis. At the 21 centers involved in this

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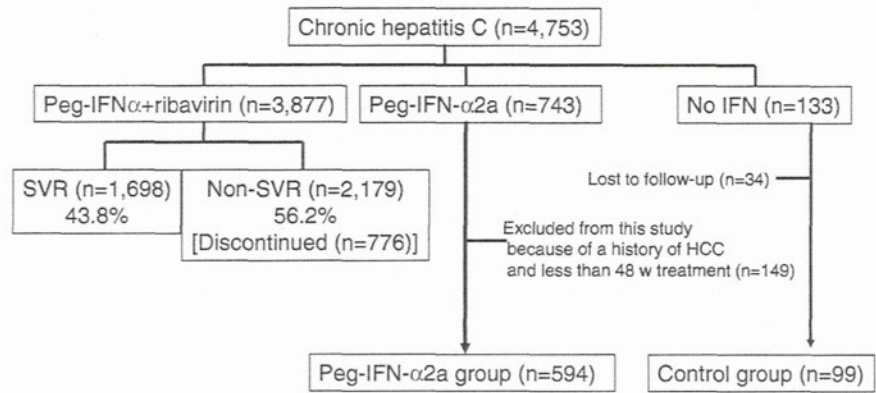
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Fig. 1 Flow diagram of the patients' enrollment in the study. Peg-IFN α pegylated interferon α , SVR sustained viral response, HCC hepatocellular carcinoma, w week



study, 4,753 patients with chronic hepatitis C had been treated; Peg-IFN and ribavirin combination treatment had been administered to 3,877 patients, 743 patients had received Peg-IFN alone, and 133 patients had not agreed to receive IFN (a flow diagram of the enrollment of patients in this study is shown in Fig. 1). In the patients with Peg-IFN and ribavirin combination treatment, the SVR rate was 43.8 %; SVR was not achieved in 2,179 patients, and in 776 of these patients, the combination therapy was discontinued owing to adverse events or the patient's choice. Patients who failed to achieve an SVR were not included in this study, because the incidence of HCC is known to be reduced even in non-responders to IFN [17].

The backgrounds of the 594 patients studied are shown in Table 1. Findings from the liver biopsies of the patients were classified according to international standards [18]. Long-term PegIFN α -2a treatment is approved by the Japanese Medical Insurance system. Written informed consent was obtained from all patients prior to participation in this study. The study design was approved by the regional ethics committees of the 21 centers involved in this study, including the Musashino Red Cross Hospital, in accordance with the Helsinki Declaration. The 743 patients treated with PegIFN α -2a alone were not indicated for Peg-IFN α and ribavirin combination therapy because of anemia or heart disease. The 133 patients who did not agree to receive IFN served as the control group (see Fig. 1). A large proportion of the 594 study patients had advanced fibrosis of the liver and active inflammation. A dose of 90 μ g PegIFN α -2a was administered to 512 and 82 patients weekly and biweekly, respectively, according to the patients' wishes. There were no significant differences between the weekly and biweekly groups in the patients' background data (data not shown).

The median duration of follow up in the PegIFN α -2a group was 1,273 days (range 228–2,768 days) and HCC was observed in 49 of the 594 patients (Table 1). Pre-treatment and on-treatment factors associated with the development of HCC were analyzed by Student's *t*-test, the

Table 1 Background data of patients treated with PegIFN α -2a (n = 594)

	n = 594
Age (years)	61.7 \pm 11.7
Sex (male/female)	258/336
BMI	23.2 \pm 3.3
Genotype (1/2)	443/151
Diagnosis (ASC/CH/LC)	4/460/130
History of excess alcohol consumption (\geq 60 g/day; yes/no)	118/376
Fibrosis (F0, 1, 2/F3, 4)	443/151
Inflammatory activity (A0, 1/A2, 3)	469/125
Diabetes mellitus (no/yes)	499/95
LDL cholesterol (mg/dL)	94.2 \pm 31.1
Fasting blood sugar (mg/dL)	106.3 \pm 28.5
White blood cell count (/mm ³)	4,360 \pm 1,470
Red blood cell count ($\times 10^6/\mu$ L)	423.8 \pm 56.4
Hemoglobin (g/dL)	13.3 \pm 1.8
Platelet count ($\times 10^3/\mu$ L)	137 \pm 56
Albumin (g/dL)	4.0 \pm 0.5
Total bilirubin (mg/dL)	0.8 \pm 0.6
AST (IU/L)	65.8 \pm 47.8
ALT (IU/L)	72.1 \pm 68.0
Gamma-GTP (IU/L)	55.2 \pm 51.3
Esophageal varices (no/yes)	344/31
Alpha fetoprotein (ng/L)	6.9 (4.2–13.8)
Once weekly or biweekly PegIFN α -2a	512:82
Baseline HCV RNA (KIU/mL)	1,024 (73–2,130)
Development of HCC (no/yes)	545/49

PegIFN pegylated interferon, BMI body mass index, ASC asymptomatic carrier, CH chronic hepatitis, LC liver cirrhosis, LDL low-density lipoprotein, AST aspartate aminotransferase, ALT alanine aminotransferase, GTP guanosine triphosphate, HCV hepatitis C virus, HCC hepatocellular carcinoma

Values are means \pm SD, with ranges in parentheses

Mann–Whitney *U*-test, and the χ^2 test (Table 2). Independent factors for the development of HCC were assessed by multivariate analysis using logistic regression. The

incidence of HCC was analyzed according to the ALT, AFP, and hepatitis C virus (HCV) RNA levels 24 weeks after the start of PegIFN α -2a administration by using the Kaplan–Meier method. The risk of HCC was analyzed, using the Kaplan–Meier method, only in the non-responders with detectable HCV RNA during PegIFN α -2a administration by dividing them according to the ALT and AFP levels 24 weeks after the start of therapy. The incidence of HCC was compared between the patients with ALT levels of <41 IU/L and those with levels of \geq 41 IU/L, and between patients with serum AFP levels of <10 ng/L and those with levels of \geq 10 ng/mL at 24 weeks after starting treatment, because at most of the centers participating in the this study, the upper normal range of serum ALT is set at 40 IU/L, and the most significant difference in the incidence of HCC was observed between the PegIFN α -2a and control group with the cut-off serum ALT set at 41 IU/L and cutoff serum AFP set at 10 ng/mL, 24 weeks after starting treatment. The HCV RNA level was measured using the Amplicor Monitor method with a lower detection limit of 50 IU/L (Roche Diagnostics, Tokyo, Japan). A history of excess alcohol consumption was determined as >60 g alcohol per day in order to exclude alcoholic liver disease.

An asymptomatic carrier was defined as a patient with a serum ALT level within the normal range and minimal inflammation or fibrosis in the biopsied tissues of the liver. Chronic hepatitis was defined as mild-to-severe fibrosis of the liver according to liver biopsy [18]. The diagnosis of liver cirrhosis was based on the results of histological examination of the biopsied liver tissues.

Study 2: incidence of HCC in the PegIFN α -2a therapy and non-administration (control) groups in comparison with propensity-matched controls

Ninety-nine of the 133 chronic hepatitis C patients who had not received IFN were examined as controls; patients in this group received liver-protective agents such as glycyrrhizin or were untreated, and the group was observed for more than 1 year. None of the individuals in the control groups had received IFN alone or PegIFN α and ribavirin combination treatment. They were treated for a median of 1,395 days (range 75–6,556 days). Fifty-nine of these patients underwent liver biopsy before the treatment and were considered the control group for the propensity-matched study. For the propensity-matched study, 59 patients were selected from the PegIFN α -2a group according to their age, sex, platelet count, and total bilirubin levels, which had been identified as independent pretreatment risk factors for the development of HCC in Study 1. The rates of HCC were analyzed using the Kaplan–Meier method, and the risk of HCC was analyzed particularly in patients with advanced fibrosis of the liver (F3 and F4).

Table 2 Comparison of HCC and non-HCC patients with long-term PegIFN α -2a administration ($n = 594$)

	Patients with or without development of HCC		<i>p</i> value
	With HCC ($n = 49$)	Without HCC ($n = 545$)	
Pretreatment parameters			
Age (years)	63.8 \pm 1.7	61.3 \pm 0.5	<0.05
Sex (male/female)	32/17	226/319	<0.01
BMI	24.0 \pm 0.5	23.1 \pm 0.2	n.s.
Genotype (1/2)	47/6	397/148	n.s.
History of excess alcohol consumption (\geq 60 g/day; yes/no)	11/38	107/338	n.s.
Fibrosis (F0, 1, 2/F3, 4)	25/24	418/127	<0.001
Inflammatory activity (A0, 1/A2, 3)	7/42	462/83	<0.001
Diabetes mellitus (no/yes)	38/11	461/84	n.s.
LDL cholesterol (mg/dL)	88.2 \pm 9.0	94.7 \pm 2.6	n.s.
White blood cell count (/mm ³)	4,355 \pm 210	4,360 \pm 64	n.s.
Red blood cell count ($\times 10^6/\mu$ L)	420.8 \pm 8.1	424.1 \pm 2.6	n.s.
Hemoglobin (g/dL)	13.6 \pm 0.3	13.3 \pm 0.1	n.s.
Platelet count ($\times 10^3/\mu$ L)	106 \pm 8	140 \pm 2	<0.001
Albumin (g/dL)	3.8 \pm 0.1	4.0 \pm 0.1	<0.001
Total bilirubin (mg/dL)	1.2 \pm 0.1	0.8 \pm 0.1	<0.001
AST (IU/L)	78.1 \pm 6.8	64.6 \pm 2.1	n.s.
ALT (IU/L)	72.8 \pm 9.7	72.0 \pm 2.9	n.s.
Gamma-GTP (IU/L)	68.7 \pm 7.5	53.9 \pm 2.3	n.s.
Alpha fetoprotein (ng/L)	17.1 (4.4–36.8)	16.7 (4.1–23.1)	n.s.
Esophageal varices	29.0 % (9/31)	6.4 % (22/344)	<0.01
On-treatment parameters			
ALT (IU/L)	59.4 \pm 5.7	44.6 \pm 1.8	<0.05
Alpha fetoprotein (ng/L)	9.8 (4.6–17.4)	5.5 (3.7–11.1)	<0.01
HCV RNA level (KIU/mL)	236 (<0.5–2,210)	21 (<0.5–1,780)	<0.05

n.s. not significant

Statistical analysis

Categorical data were compared using the χ^2 test or Fisher’s exact test. The distributions of continuous variables were analyzed using Student’s *t*-test and the Mann–Whitney *U*-test for two groups. Multivariate analysis was

conducted using logistic regression. The cumulative incidence curve was determined using the Kaplan–Meier method and differences between groups were assessed by the log-rank test. For all methods, the level of significance was set at $p < 0.05$. Multivariate analysis of the risk of HCC was carried out using the Cox proportional hazard model. Statistical analyses were performed using the Statistical Package for the Social Sciences software version 11.0 (SPSS, Chicago, IL, USA). In Study 1, age, sex, platelet count, and total bilirubin levels were identified as independent factors for the development of HCC; therefore, these factors were selected for the propensity-matched control study (Study 2) in which 59 patients from the PegIFN α -2a group were included.

Results

Study 1

We analyzed the factors involved in the development of HCC in patients who received 90 μ g PegIFN α -2a weekly or biweekly for more than a year. The incidence of HCC did not differ significantly between the groups treated with PegIFN α -2a weekly and biweekly (34 of 512 vs. 15 of 82, respectively). As shown in Table 2, univariate analysis revealed statistically significant differences in the pretreatment parameters including age, sex, fibrosis of the liver, platelet count, albumin level, and total bilirubin, between patients who developed HCC and those who did not. Endoscopy was carried out in 375 patients, and esophageal varices were noted in 31 of them. The incidence of HCC was higher in patients with esophageal varices than in those without varices [29.0 % (9 of 31) vs. 6.4 % (22 of 344)]. Assessment of on-treatment factors by univariate analysis revealed statistically significant differences in serum ALT, AFP, and HCV RNA levels 24 weeks after the start of PegIFN α -2a maintenance treatment (Table 2).

Multivariate analysis including pretreatment parameters revealed that age, sex, fibrosis of the liver, platelet count, and total bilirubin were independent risk factors for HCC development (Table 3). Multivariate analysis including on-treatment parameters identified ALT levels of ≥ 41 IU/L and AFP levels of ≥ 10 ng/L 24 weeks after the start of the PegIFN α -2a therapy as independent risk factors for HCC development (Table 3).

The incidence of HCC was significantly lower in patients with ALT levels of ≤ 40 IU/L than in those with ALT levels of ≥ 41 IU/L 24 weeks after the start of observation (Fig. 2). The incidence of HCC was also significantly lower in patients with AFP concentrations of < 10 ng/mL at 24 weeks after the start of observation than in those with AFP concentrations of

≥ 10 ng/mL (Fig. 3). The dose of PegIFN α -2a was reduced to 45 μ g in 16 patients because of neutropenia and thrombocytopenia. In addition, PegIFN α -2a was discontinued in 18 patients because of adverse events, including depression (7 patients), interstitial pneumonitis (3 patients), thrombocytopenia (3 patients), neutropenia (1 patient), itching (1 patient), and ascites (3 patients). No statistically significant differences were found between the patients with reduced dosage or treatment interruption and those without treatment modifications with respect to overall survival, HCC incidence, ascites formation, variceal bleeding, hepatic encephalopathy, and 2-point increases in the Child-Pugh score. No patients underwent liver transplantation.

Table 3 Independent risk factors for HCC development in patients treated with 90 μ g PegIFN α -2a weekly or bi-weekly, evaluated by multivariate analysis (logistic regression analysis)

	Multivariate analysis		
	Odds ratio	95 % Confidence interval (CI)	<i>p</i>
Age (years) (every 5 years)	2.24	1.76–9.33	<0.005
Sex (male/female)	3.16	1.56–10.7	<0.005
Fibrosis (F3, 4/F0, 1, 2)	1.69	1.18–5.2	<0.01
Platelet count ($< 120 \times 10^3/\mu$ L vs. $\geq 120 \times 10^3/\mu$ L)	3.24	1.44–27.6	<0.01
Total bilirubin (mg/dL)	1.59	1.09–2.58	<0.05
ALT (at 24 weeks) (≥ 41 vs. < 40 IU/L)	2.49	1.51–8.28	<0.05
AFP (at 24 weeks) (≥ 10 vs. < 10 ng/L)	3.78	1.92–11.8	<0.01

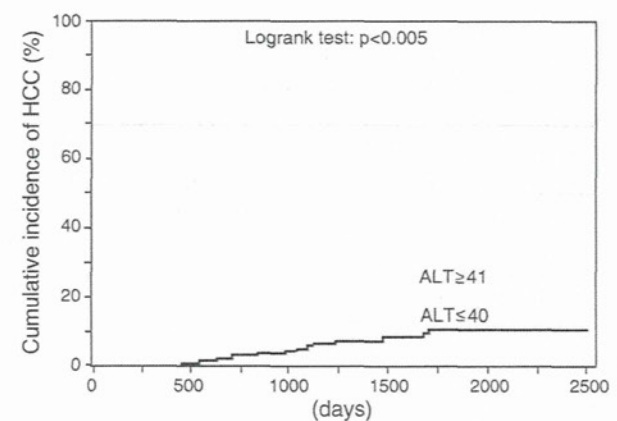


Fig. 2 Comparison of HCC rates in patients administered with PegIFN α -2a ($n = 594$) with respect to alanine aminotransferase (ALT) levels 24 weeks after the start of therapy. *Black line* patients with ALT ≥ 41 IU/L in the first 24 weeks, *gray line* patients with ALT ≤ 40 IU/L in the first 24 weeks

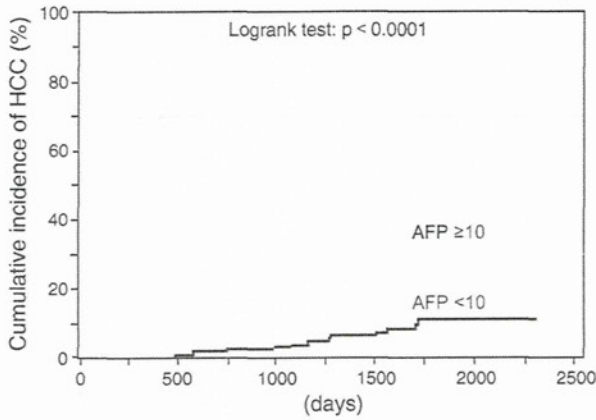


Fig. 3 Comparison of HCC rates in patients administered PegIFN α -2a ($n = 594$) with respect to alpha-fetoprotein (AFP) levels in the first 24 weeks after the start of therapy. *Black line* patients with AFP ≥ 10 ng/mL at 24 weeks, *gray line* patients with AFP < 10 ng/mL at 24 weeks

Study 2

We compared the incidence of HCC between 59 patients in the control group and the same number of patients in the PegIFN α -2a group using the matched-pair test. The backgrounds of the patients are shown in Table 4. The PegIFN α -2a group had higher rates of advanced fibrosis (F3 and F4) and active inflammation (A2 and A3). No other differences were found between the two groups, except for the white blood cell count (Table 4).

Development of HCC was observed in 2 patients in the PegIFN α -2a group and 8 in the control group. The incidence of HCC was compared between the two groups, using the Kaplan–Meier method. The incidence of HCC in the PegIFN α -2a group was significantly lower than that in the control group (log-rank test, $p = 0.0187$; Fig. 4). Among the patients with advanced fibrosis of the liver (F3 and F4), those in the PegIFN α -2a group had a lower incidence of HCC than those in the control group. The independent risk factors for the development of HCC were analyzed using the stepwise Cox proportional hazard model. Only PegIFN α -2a administration and age were identified as independent risk factors for the development of HCC (Table 5).

Discussion

The number of HCC cases resulting from HCV infection continues to increase worldwide [19]. To date, IFN therapy is the most effective preventive measure against HCC in patients with chronic hepatitis C; furthermore, the

Table 4 Backgrounds of the patients in the propensity-matched control study (PegIFN α -2a group, $n = 59$; control group, $n = 59$)

	PegIFN α -2a group ($n = 59$)	Control group ($n = 59$)	<i>p</i> value
Age (years)	60.5 \pm 13.0	63.3 \pm 10.5	n.s.
Gender (male/female)	24/35	25/34	n.s.
BMI	22.9 \pm 3.6	22.9 \pm 3.4	n.s.
Genotype (1/2)	49/10	46/13	n.s.
History of excess alcohol consumption (60 g/day; yes/no)	10/49	4/55	n.s.
Fibrosis (F0, 1, 2/F3, 4)	37/22	43/16	<0.05
Development of HCC (F0–2/F3, 4)	1/1	1/7	n.s.
Inflammatory activity (A0,1/A2, 3)	19/40	30/29	<0.05
Diabetes mellitus (no/yes)	57/2	56/3	n.s.
LDL cholesterol (mg/dL)	95.3 \pm 23.8	117.0 \pm 4.2	n.s.
White blood cell count (/mm ³)	4,260 \pm 1,239	5,193 \pm 2,078	<0.05
Red blood cell count ($\times 10^{-4}$ / μ L)	430 \pm 57.8	441 \pm 44.9	n.s.
Hemoglobin (g/dL)	13.6 \pm 1.5	13.6 \pm 1.9	n.s.
Platelet count ($\times 10^{-3}$ / μ L)	14.5 \pm 5.7	15.8 \pm 5.7	n.s.
Albumin (g/dL)	4.1 \pm 0.5	4.1 \pm 0.4	n.s.
Total bilirubin (mg/dL)	0.7 \pm 0.5	0.9 \pm 0.7	n.s.
AST (IU/L)	58.3 \pm 47.7	49.7 \pm 26.6	n.s.
ALT (IU/L)	63.6 \pm 68.7	58.0 \pm 39.2	n.s.
Gamma-GTP (IU/L)	78.3 \pm 81.3	55.3 \pm 75.1	n.s.
Baseline alpha-fetoprotein (AFP) (ng/L)	7.2 (4.3–14.2)	7.7 (3.9–13.8)	n.s.
Baseline HCV RNA level (KIU/mL)	1,230 (24–3,870)	1,024 (38–3,110)	n.s.

incidence of HCC is reduced in patients who achieve an SVR to IFN [6–9] Therefore, achieving an SVR is the most effective approach for reducing the risk of developing HCC. In Japan, the incidence of HCC is elevated in older patients with hepatitis C. Corroborating this finding, the results of a Japanese study show a higher risk of HCC in patients aged 65 years and more [10]. Therefore, prevention of HCC in aged patients is an important challenge.

In the present multicenter, cooperative, retrospective study conducted in Japan, the incidence of HCC was reduced in patients who received 90 μ g PegIFN α -2a weekly or biweekly and had AFP values of < 10 ng/mL and ALT values of ≤ 40 IU/L 24 weeks after the start of the treatment. The results of the matched case–control study of the PegIFN α -2a group and the non-IFN control group show that the incidence of HCC was significantly lower in the PegIFN α -2a group than in the control group, especially in patients with advanced fibrosis of the liver (F3 and F4). However, there could have been a selection bias between

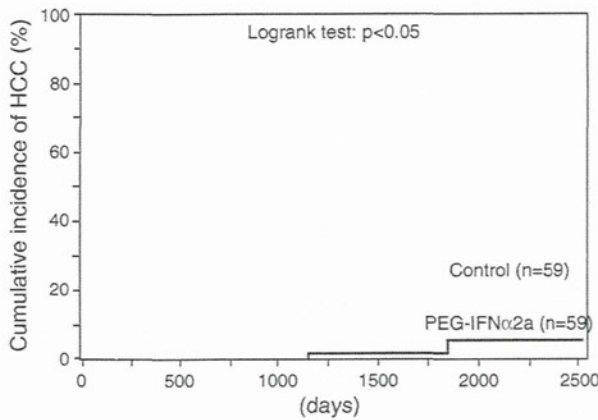


Fig. 4 Comparison of HCC rates between the long-term PegIFN α -2a administration group ($n = 59$) and non-administration group ($n = 59$) in the propensity-matched control study (Kaplan–Meier log-rank test, $p = 0.019$)

Table 5 Risk factors for HCC in the propensity-matched control study (Cox proportional hazard model)

Variables	Risk ratio	95 % CI	p value
PegIFN versus control	0.17	0.03–0.75	<0.05
Age (every 1 year)	1.12	1.02–1.25	<0.05
Fibrosis (F3, 4 vs. F0, 1, 2)	1.70	0.75–4.16	n.s.
Platelet count (every $10 \times 10^3/\mu\text{L}$)	0.89	0.73–1.09	n.s.
Albumin (every 1.0 g/dL)	0.80	0.10–6.68	n.s.
On-treatment AFP (<10 vs. ≥ 10 ng/L)	4.07	0.59–40.12	n.s.

the PegIFN α -2a group and the control group (patients who did not agree to receive IFN treatment), because this was a retrospective and non-randomized study. However, concordant with the findings of the HALT-C study [14], the present results show that PegIFN α -2a inhibits the development of HCC in patients with advanced fibrosis of the liver.

Recent studies show that polymorphisms in the host *IL28B* gene are important factors in the response to Peg-IFN α and ribavirin combination therapy [20, 21]. However, the mechanism of *IL28B* involvement in the response to PegIFN α and ribavirin has not been elucidated completely. A recent report has shown that *IL28B* is a significant factor in the development of HCC as well as in the response to IFN therapy [22]. Further studies are warranted to analyze the relationship between *IL28B* and inhibition of the development of HCC by PegIFN α in chronic hepatitis C.

Risk factors for the development of HCC have been discussed previously. Increased intrahepatic fat is involved in the development of HCC in chronic hepatitis C patients [23, 24]. In addition, diabetes-associated fat disorder [25,

26], hepatic iron overload [27], advanced fibrosis, older age, and fatty deposits in the liver are risk factors for HCC development [4]. Therefore, it is important to establish strategies to mitigate these risk factors to prevent the development of HCC and thus improve the outcomes of hepatitis C patients.

IFN therapy after HCC treatment is reported to inhibit the recurrence of tumors [28, 29], and a meta-analysis has revealed a trend toward inhibition of the recurrence of HCC [30, 31]. The prevention of HCC is an important issue that needs to be addressed to improve the survival of chronic hepatitis C patients. The findings of the present study and the HALT-C trial [14] indicate the effectiveness of long-term administration of maintenance IFN for preventing the development of HCC in chronic hepatitis C patients without an SVR. Improvement in ALT levels is also known to be an important predictor for the prevention of HCC [32]. A low AFP value during IFN administration is also recognized as a significant indicator of a lower risk of HCC [33, 34]. Recently, Osaki et al. [35] reported that a decrease of serum AFP during treatment with IFN was associated with a reduced incidence of HCC. Taking these findings and our own together, we conclude that maintenance administration of low-dose PegIFN α -2a weekly or biweekly to non-SVR patients with chronic hepatitis C decreases the incidence of HCC, especially in patients whose serum ALT and AFP levels are within the normal range 24 weeks after the start of treatment. The preventive effects of IFN against the development of HCC without elimination of the virus may be associated with its anti-carcinogenic effects [16, 35]; however, the precise mechanism should be investigated.

The limitations of the present study are that it is retrospective and multicentric; therefore, potentially there may have been a selection bias. However, the reduction of the rate of development of HCC by maintenance administration of PegIFN α -2a in the patients in whom serum ALT and AFP levels were within the normal ranges 24 weeks after the start of treatment may be attributable to the anticarcinogenic effects of IFN without elimination of the virus.

Conclusion

The incidence of HCC was lower in non-SVR patients with chronic hepatitis C who were administered with maintenance low-dose PegIFN α -2a; especially in those whose serum ALT and AFP levels were within the normal ranges 24 weeks after the start of treatment.

Acknowledgments This study was supported by a Grant-in-Aid from the Japanese Ministry of Health, Welfare, and Labor.

Conflict of interest Namiki Izumi received lecture fees from Chugai Co. and MSD Co. in 2011.

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References

- Parkin DM, Bray F, Ferlay J, Pisani P. Global cancer statistics, 2002. *CA Cancer J Clin*. 2005;55:74–108. doi:10.3322/canjclin.55.2.74.
- Llovet JM, Burroughs A, Bruix J. Hepatocellular carcinoma. *Lancet*. 2003;362:1907–17. doi:10.1016/S0140-6736(03)14964-1.
- Kiyosawa K, Sodeyama T, Tanaka E, Gibo Y, Yoshizawa K, Nakano K, et al. Interrelationship of blood transfusion, non-A, non-B hepatitis and hepatocellular carcinoma: analysis by detection of antibody to hepatitis C virus. *Hepatology*. 1990;12:671–5. doi:10.1002/hep.1840120409.
- Namiki I, Nishiguchi S, Hino K, Suzuki F, Kumada H, Itoh T, et al. Management of hepatitis C; Report of the consensus meeting at the 45th annual meeting of the Japan Society of Hepatology (2009). *Hepatol Res*. 2010;40:347–68. doi:10.1111/j.1872-034X.2010.00642.x.
- Tanaka Y, Hanada K, Mizokami M, Yeo AE, Shin JW, Gojbori T, et al. A comparison of the molecular clock of hepatitis C virus in the United States and Japan predicts that hepatocellular carcinoma incidence in the United States will increase over the next two decades. *Proc Natl Acad Sci USA*. 2002;99:11584–9. doi:10.1073/pnas.242608099.
- Ikedo K, Saitoh S, Arase Y, Chayama K, Suzuki Y, Kobayashi M, et al. Effect of interferon therapy on hepatocellular carcinoma in patients with chronic hepatitis type C: a long-term observation study of 1,643 patients using statistical bias correction with proportional hazard analysis. *Hepatology*. 1999;29:1124–30.
- Imai Y, Kawata S, Tamura S, Yabuuchi I, Noda S, Inada M, et al. Relation of interferon therapy and hepatocellular carcinoma in patients with chronic hepatitis C. *Ann Intern Med*. 1998;129:94–9.
- Bruno S, Stroffolini T, Colombo M, Bollani S, Benveguo L, Mazzella G, et al. Sustained virological response to interferon-alpha is associated with improved outcome in HCV-related cirrhosis: a retrospective study. *Hepatology*. 2007;45:579–87. doi:10.1002/hep.21492.
- Veldt BJ, Heathcote EJ, Wedemeyer H, Reichen J, Hofmann WP, Zeuzem S, et al. Sustained virological response and clinical outcomes in patients with chronic hepatitis C and advanced fibrosis. *Ann Intern Med*. 2007;147:677–84.
- Asahina Y, Tsuchiya K, Tamaki N, Hirayama I, Tanaka T, Sato M, et al. Effect of aging on risk for hepatocellular carcinoma in chronic hepatitis C virus infection. *Hepatology*. 2010;52:518–27. doi:10.1002/hep.23691.
- Amarapurkar D, Han KH, Chan HL, Ueno Y, Asia-Pacific working party on prevention of hepatocellular carcinoma. Application of surveillance programs for hepatocellular carcinoma in the Asia-Pacific Region. *J Gastroenterol Hepatol*. 2009;24:955–61. doi:10.1111/j.1440-1746.2009.05805.x.
- Tamura Y, Yamagiwa S, Aoki Y, Kurita S, Suda T, Ohkoshi S, et al. Serum alpha-fetoprotein levels during and after interferon therapy and the development of hepatocellular carcinoma in patients with chronic hepatitis C. *Dig Dis Sci*. 2009;54:2530–7.
- Di Bisceglie AM, Shiffman ML, Everson GT, Lindsay KL, Everhart JE, Wright EC, et al. Prolonged therapy of advanced chronic hepatitis C with low-dose peginterferon. *N Engl J Med*. 2008;359:2429–41. doi:10.1056/NEJMoa0707615.
- Lok AS, Everhart JE, Wright EC, Di Bisceglie AM, Kim HY, Sterling RK, et al. Maintenance peginterferon therapy and other factors associated with hepatocellular carcinoma in patients with advanced hepatitis C. *Gastroenterology*. 2011;140:840–9. doi:10.1053/j.gastro.2010.11.050.
- Bruix J, Poynard T, Colombo M, Schiff E, Burak K, Heathcote EJ, et al. Maintenance therapy with peginterferon alfa-2b does not prevent hepatocellular carcinoma in cirrhotic patients with chronic hepatitis C. *Gastroenterology*. 2011;140:1990–9. doi:10.1053/j.gastro.2010.11.050.
- Arase Y, Ikeda K, Suzuki F, Suzuki Y, Kobayashi M, Akuta N, et al. Prolonged-interferon therapy reduces hepatocarcinogenesis in aged-patients with chronic hepatitis C. *J Med Virol*. 2007;79:1095–102. doi:10.1002/jmv.20866.
- Poynard T, Moussali J, Ratziu V, Regimberu C, Opolan P. Effects of interferon therapy in “non-responder” patients with chronic hepatitis C. *J Hepatol*. 1999;31S:178–83. doi:10.1016/S0168-8278(99)80397-3.
- Desmet VJ, Gerber M, Hoofnagle JH, Manns M, Scheuer P. Classification of chronic hepatitis: diagnosis, grading and staging. *Hepatology*. 1994;19:1513–20. doi:10.1016/0270-9139(94)90250-X, doi:10.1002/hep.1840190629.
- Kanwal F, Hoang T, Kramer JR, Asch SM, Goetz MB, Zeringue A, et al. Increasing prevalence of HCC and cirrhosis in patients with chronic hepatitis C virus infection. *Gastroenterology*. 2011;140:1182–8. doi:10.1053/j.gastro.2010.12.032.
- Ge D, Fellay J, Thompson AJ, Simon JS, Shianna KV, Urban TJ, et al. Genetic variation in IL28B predicts hepatitis C treatment-induced viral clearance. *Nature*. 2009;461:399–401. doi:10.1038/nature08309.
- Tanaka Y, Nishida N, Sugiyama M, Kurosaki M, Matsuura K, Sakamoto N, et al. Genome-wide association of IL28B with response to pegylated interferon-alpha and ribavirin therapy for chronic hepatitis C. *Nature*. 2009;461:1105–9.
- Fabris C, Falletti E, Cussigh A, Bitetto D, Fontanini E, Bignulin S, et al. IL-28B rs 12979860 C/T allele distribution in patients with liver cirrhosis: role in the course of chronic viral hepatitis and the development of HCC. *J Hepatol*. 2011;54:716–22. doi:10.1016/j.jhep.2010.07.019.
- Kurosaki M, Hosokawa T, Matsunaga K, Hirayama I, Tanaka T, Sato M, et al. Hepatic steatosis in chronic hepatitis C is a significant risk factor for developing hepatocellular carcinoma independent of age, sex, obesity, fibrosis stage and response to interferon therapy. *Hepatol Res*. 2010;40:870–7. doi:10.1111/j.1872-034X.2010.00692.x.
- Koike K. Steatosis, liver injury, and hepatocarcinogenesis in hepatitis C viral infection. *J Gastroenterol*. 2009;44(Suppl 19):82–8. doi:10.1007/s00535-008-2276-4.
- Veldt BJ, Chen W, Heathcote EJ, Wedemeyer H, Reichen J, Hofman WP, et al. Increased risk of hepatocellular carcinoma among patients with hepatitis C cirrhosis and diabetes mellitus. *Hepatology*. 2008;47:1856–62. doi:10.1002/hep.22251.
- Lai MS, Hsieh MS, Chiu YH, Chen TH. Type 2 diabetes and hepatocellular carcinoma: a cohort study in high prevalence area of hepatitis virus infection. *Hepatology*. 2006;43:1295–302. doi:10.1002/hep.21208.
- Furutani T, Hino K, Okuda M, Gondo T, Nishina S, Kitase A, et al. Hepatic iron overload induces hepatocellular carcinoma in transgenic mice expressing the hepatitis C virus polyprotein. *Gastroenterology*. 2006;130:2087–98. doi:10.1053/j.gastro.2006.02.060.
- Kubo S, Nishiguchi S, Hirohashi K, Tanaka H, Shuto T, Kinoshita H. Randomized clinical trial of long-term outcome after resection of hepatitis C virus-related hepatocellular carcinoma by

- postoperative interferon therapy. *Br J Surg.* 2002;89:418–22. doi:10.1046/j.0007-1323.2001.02054.x.
29. Kudo M, Sakaguchi Y, Chung H, Hatanaka K, Hagiwara S, Ishikawa E, et al. Long-term interferon maintenance therapy improves survival in patients with HCV-related hepatocellular carcinoma after curative radiofrequency ablation. A matched case–control study. *Oncology.* 2007;72(Suppl 1):132–8. doi:10.1159/000111719.
30. Singal AK, Freeman DH Jr, Anand BS. Meta-analysis: interferon improves outcomes following ablation or resection of hepatocellular carcinoma. *Aliment Pharmacol Ther.* 2010;32:851–8. doi:10.1111/j.1365-2036.2010.04414.x.
31. Miyake Y, Takaki A, Iwasaki Y, Yamamoto K. Meta-analysis: interferon-alpha prevents the recurrence after curative treatment of hepatitis C virus-related hepatocellular carcinoma. *J Viral Hepat.* 2010;17:287–92. doi:10.1111/j.1365-2893.2009.01181.x.
32. Arase Y, Ikeda K, Suzuki F, Suzuki Y, Kobayashi M, Akuta N, et al. Interferon-induced prolonged biochemical response reduces hepatocarcinogenesis in hepatitis C virus infection. *J Med Virol.* 2007;79:1485–90. doi:10.1002/jmv.20925.
33. Nomura H, Kashiwagi Y, Hirano R, Tanimoto H, Tsutsumi N, Higashi M, et al. Efficacy of low dose long-term interferon monotherapy in aged patients with chronic hepatitis C genotype 1 and its relation to alpha-fetoprotein: a pilot study. *Hepatol Res.* 2007;37:490–7. doi:10.1111/j.1872-034X.2007.00073.x.
34. Chen TM, Huang PT, Tsai MH, Lin LF, Liu CC, Ho KS, et al. Predictors of alpha-fetoprotein elevation in patients with chronic hepatitis C, but not hepatocellular carcinoma, and its normalization after pegylated interferon alfa 2a-ribavirin combination therapy. *J Gastroenterol Hepatol.* 2007;22:669–75. doi:10.1111/j.1440-1746.2007.04898.x.
35. Osaki Y, Ueda Y, Marusawa H, Nakajima J, Kimura T, Kita R, et al. Decrease in alpha-fetoprotein levels predicts reduced incidence of hepatocellular carcinoma in patients with hepatitis C virus infection receiving interferon therapy: a single center study. *J Gastroenterol.* 2012;47:444–51.

Genome-Wide Association Study Confirming Association of HLA-DP with Protection against Chronic Hepatitis B and Viral Clearance in Japanese and Korean

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Abstract

Hepatitis B virus (HBV) infection can lead to serious liver diseases, including liver cirrhosis (LC) and hepatocellular carcinoma (HCC); however, about 85–90% of infected individuals become inactive carriers with sustained biochemical remission and very low risk of LC or HCC. To identify host genetic factors contributing to HBV clearance, we conducted genome-wide association studies (GWAS) and replication analysis using samples from HBV carriers and spontaneously HBV-resolved Japanese and Korean individuals. Association analysis in the Japanese and Korean data identified the *HLA-DPA1* and *HLA-DPB1* genes with $P_{meta} = 1.89 \times 10^{-12}$ for rs3077 and $P_{meta} = 9.69 \times 10^{-10}$ for rs9277542. We also found that the *HLA-DPA1* and *HLA-DPB1* genes were significantly associated with protective effects against chronic hepatitis B (CHB) in Japanese, Korean and other Asian populations, including Chinese and Thai individuals ($P_{meta} = 4.40 \times 10^{-19}$ for rs3077 and $P_{meta} = 1.28 \times 10^{-15}$ for rs9277542). These results suggest that the associations between the *HLA-DP* locus and the protective effects against persistent HBV infection and with clearance of HBV were replicated widely in East Asian populations; however, there are no reports of GWAS in Caucasian or African populations. Based on the GWAS in this study, there were no significant SNPs associated with HCC development. To clarify the pathogenesis of CHB and the mechanisms of HBV clearance, further studies are necessary, including functional analyses of the HLA-DP molecule.

Citation: Nishida N, Sawai H, Matsuura K, Sugiyama M, Ahn SH, et al. (2012) Genome-Wide Association Study Confirming Association of HLA-DP with Protection against Chronic Hepatitis B and Viral Clearance in Japanese and Korean. PLoS ONE 7(6): e39175. doi:10.1371/journal.pone.0039175

Editor: Anand S. Mehta, Drexel University College of Medicine, United States of America

Received: February 1, 2012; **Accepted:** May 16, 2012; **Published:** June 21, 2012

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Funding: This work was supported by Grants-in-Aid from the Ministry of Health, Labour, and Welfare of Japan (H22-kanen-005, H23-kanen-005), the Japan Science and Technology Agency (09038024), and the Miyakawa Memorial Research Foundation. Partial support by Grant-in-Aid for Young Scientists (B) (22710191) from the Ministry of Education, Culture, Sports, Science, and Technology is also acknowledged. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: AK is an employee of the Central Research Laboratory, Hitachi Ltd. There are no patents, products in development or marketed products to declare. This does not alter the authors' adherence to all the PLoS ONE policies on sharing data and materials, as detailed online in the guide for authors.

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Introduction

Overall, one-third of the world's population (2.2 billion) is infected with hepatitis B virus (HBV), and about 15% of these are chronic carriers. About 75% of the chronic carriers live in the east-south Asia and east pacific area, and there are 1.3–1.5 million chronic carriers living in Japan [1]. Of chronic carriers, 10–15% develop liver cirrhosis (LC), liver failure and hepatocellular carcinoma (HCC), and the remaining individuals eventually achieve a state of nonreplicative infection, resulting in hepatitis B surface antigen (HBsAg) negative and hepatitis B core antibody (anti-HBc) positive, i.e. HBV-resolved individuals [2–3]. In Japan, although the major route of HBV transmission was perinatal transmission and horizontal transmission in early childhood, infant HBV carriers have successfully been reduced since 1986 through a selective vaccination policy by the Japanese government [4–7]. However, the prevalence of HBV genotype A in acute HBV (AHB) infection has increased markedly since 2000, reaching approximately 52% in 2008 due to the lack of a universal HB vaccination, and around 10% of AHB cases could be persistent infection [8–9]. Viral factors, as well as host factors, are thought to be associated with persistent HB infection.

In 2009, significant associations between chronic hepatitis B (CHB) and a region including *HLA-DPA1* and *HLA-DPBI* were identified using 786 Japanese individuals having CHB and 2,201 control individuals through a two-stage genome-wide association study (GWAS) [10]. The same group was also subjected to a second GWAS using a total of 2,667 Japanese persistent HBV infection cases and 6,496 controls, which confirmed significant associations between the *HLA-DP* locus and CHB, in addition to associations with another two SNPs located in the genetic region including the *HLA-DQ* gene [11]. The associations between *HLA-DP* variants with HBV infection were replicated in other Asian populations, including Thai and Han Chinese individuals [10,12–13]. With regard to HBV clearance, the association between the human leukocyte antigen (HLA) class II allele and clearance of HBV was confirmed by the candidate gene approach in African, Caucasian and Asian populations [14–18]. However, in a previous GWAS using samples of Japanese CHB and control individuals, the clinical data on HBV exposure in the control individuals were unknown, and this may have led to bias. Moreover, there have been no reports of GWAS using samples from HBV carriers and HBV-resolved individuals to identify host genetic factors associated with HBV clearance other than HLA class II molecules.

Here, we performed a GWAS using samples from Japanese HBV carriers, healthy controls and spontaneously HBV-resolved individuals in order to confirm or identify the host genetic factors related to CHB and viral clearance. In the subsequent replication analysis, we validated the associated SNPs in the GWAS using two independent sets of Japanese and Korean individuals. In our study, healthy controls were randomly selected with clinically no evidence of HBV exposure, therefore, HBV-resolved individuals were prepared to clearly identify the host genetic factors related with CHB or HBV clearance.

Results

Protective Effects Against Chronic Hepatitis B in Japanese and Korean Individuals

In this study, we conducted a GWAS using samples from 181 Japanese HBV carriers (including asymptomatic carriers (ASC), CHB cases, LC cases and HCC cases, based on the criteria described in Materials and Methods) and 184 healthy controls in

order to identify the host genetic factors related to progression of CHB. All samples were genotyped using a genome-wide SNP typing array (Affymetrix Genome-Wide Human SNP Array 6.0 for 900 K SNPs). Figure 1a shows a genome-wide view of the single point association data based on allele frequencies using the SNPs that met the following filtering criteria: (i) SNP call rate $\geq 95\%$; (ii) minor allele frequency (MAF) $\geq 1\%$ for HBV carriers and healthy controls; and (iii) no deviation from Hardy-Weinberg equilibrium (HWE) $P \geq 0.001$ in healthy controls. We identified significant associations of protective effects against CHB with two SNPs (rs3077 and rs9277542) using the allele frequency model, both of which are located in the 3' UTR of *HLA-DPA1* and in the sixth exon of *HLA-DPBI*, respectively (rs3077, $P = 1.14 \times 10^{-7}$, and rs9277542, $P = 5.32 \times 10^{-8}$, respectively). The association for rs9277542 reached a genome-wide level of significance in the GWAS panel (Bonferroni criterion $P < 8.36 \times 10^{-8}$ (0.05/597,789)).

In order to validate the results of GWAS, a total of 32 SNPs, including the associated two SNPs (rs3077 and rs9277542), were selected for replication in two independent sets of HBV carriers and healthy controls (replication-1:256 Japanese HBV carriers and 236 Japanese healthy controls; and replication-2:344 Korean HBV carriers and 151 Korean healthy controls; Table 1). The associations for the original significant SNP (rs9277542) and marginal SNP (rs3077) on GWAS were replicated in both replication sets [replication-1 (Japanese); rs3077, $P = 2.70 \times 10^{-8}$, OR = 0.48 and rs9277542, $P = 3.33 \times 10^{-6}$, OR = 0.54; replication-2 (Korean); rs3077, $P = 2.08 \times 10^{-6}$, OR = 0.47 and rs9277542, $P = 8.29 \times 10^{-5}$, OR = 0.54, Table 2]. We conducted meta-analysis to combine these studies using the DerSimonian Laird method (random effects model) to incorporate variation among studies. As shown in Table 2, the odds ratios were quite similar across the three studies (GWAS and two replication studies) and no heterogeneity was observed ($P_{het} = 0.80$ for rs3077 and 0.40 for rs9277542). P_{meta} values were 4.40×10^{-19} for rs3077 (OR = 0.46, 95% confidence interval (CI) = 0.39–0.54), and 1.28×10^{-15} for rs9277542 (OR = 0.50, 95% CI = 0.43–0.60). Among the remaining 30 SNPs in the replication study, 27 SNPs were successfully genotyped by the DigiTag2 assay with SNP call rate $\geq 95\%$ and HWE p -value ≥ 0.01 . Two SNPs (rs9276431 and rs7768538), located in the genetic region including the *HLA-DQ* gene, were marginally replicated in the two sets of HBV carriers and healthy controls with Mantel-Haenszel P values of 2.80×10^{-7} (OR = 0.56, 95% CI = 0.45–0.70) and 1.09×10^{-7} (OR = 0.53, 95% CI = 0.42–0.67), respectively, when using additive, two-tailed Cochran Mantel-Haenszel (CMH) fixed-effects model with no evidence of heterogeneity ($P_{het} = 0.67$ for rs9276431 and 0.70 for rs7768538) (Table S1).

Meta-analysis using the random effects model across 6 independent studies, including 5 additional published data, showed $P_{meta} = 3.94 \times 10^{-45}$, OR = 0.55 for rs3077, $P_{meta} = 1.74 \times 10^{-21}$, OR = 0.61 for rs9277535 and $P_{meta} = 1.69 \times 10^{-15}$, OR = 0.51 for rs9277542, with the SNP rs9277535 being located about 4-kb upstream from rs9277542 and showing strong linkage disequilibrium of $r^2 = 0.955$ on the HapMap JPT (Table S2). As shown in Table S2, the odds ratio was very similar among the 6 studies, and heterogeneity was negligible with $P_{het} > 0.01$.

Moreover, based on GWAS using samples from 94 chronic HBV carriers with LC or HCC and 87 chronic HBV carriers without LC and HCC, we found no significant SNPs associated with CHB progression (Figure S1).

