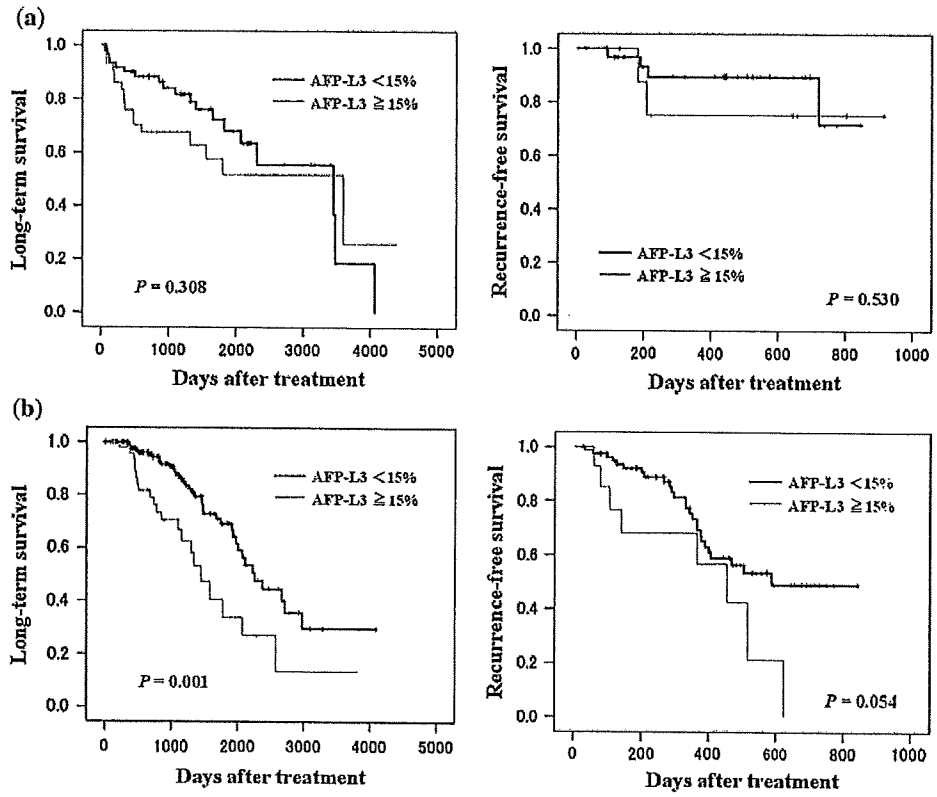

Fig. 1 Comparison of long-term survival rates and recurrence-free survival rates between patients with and without AFP-L3 elevation who underwent hepatectomy (a) and who underwent percutaneous ablation (b)

In our study, the pathological diagnosis was made by individual pathologists at each hospital. At Niigata University Hospital, 58 HCC patients underwent hepatectomy, of whom 23 had an elevated serum AFP-L3 level (≥15%) and the remaining 35 were negative for AFP-L3 (<15%). Among the 23 patients with AFP-L3 elevation, only two (8.7%) were diagnosed as having well-differentiated HCC on the basis of the resected specimens, 14 (60.9%) had moderately differentiated HCC, and seven (30.4%) had poorly differentiated HCC. In contrast, among the 35 patients who were negative for AFP-L3, 7 (20.0%) were diagnosed as having well-differentiated HCC, 24 (68.6%) had moderately differentiated HCC, and only four (11.4%) had poorly differentiated HCC. Although no statistically significant differences were observed by Fisher's exact test, the group showing AFP-L3 elevation tended to have a poorer histopathological grading ($P = 0.141$). Only eight out of 331 patients who underwent percutaneous ablative therapy were diagnosed as having HCC on the basis of histological findings in four hospitals. Therefore, we were unable to investigate whether poorly differentiated tumors were more frequent in the groups who underwent percutaneous ablative therapy and hepatectomy. Portal vein invasion was investigated similarly in 58 patients, and was found to be present in six of 23 AFP-L3-positive patients and six of 35 AFP-L3-negative patients. No significant difference was observed between AFP-L3 and portal vein invasion in this limited investigation.

We demonstrated here in a multicenter retrospective study that AFP-L3 status was a significant prognostic factor affecting the long-term survival of patients who underwent percutaneous ablative therapy. In addition, to evaluate the prognostic influence of AFP-L3 in two subgroups comparable for tumor extension, we performed a multicenter prospective study to identify the prognostic factors for recurrence-free survival in patients with early stage HCC. Although this evaluation was conducted over a short period of time, we confirmed that AFP-L3 status was a significant prognostic predictor of recurrence-free survival in patients who underwent percutaneous ablative therapy, but it did not affect the prognosis of patients who underwent hepatectomy.

A number of studies have shown that AFP-L3 status is an independent prognostic factor in patients with HCC [12, 13, 15]. We previously reported that AFP-L3-positive (>15%) patients had a lower survival rate than negative (<15%) patients in subgroups with a low serum AFP concentration. Moreover, the statistically significant differences were more distinct in the subgroups with lower AFP concentrations [20]. However, the patients in these studies had received various treatments such as hepatectomy, RFA, and transcatheter arterial embolization, and

there have been few reports of the relationship between AFP-L3 status and prognosis in subgroups of HCC patients receiving different therapeutic modalities. Tateishi et al. [15] demonstrated that pre-treatment AFP-L3 positivity (>15%) was a significant predictor of HCC recurrence in patients who underwent curative ablation, and that AFP-L3 positivity after ablation was the strongest predictor of HCC recurrence by multivariate analysis. Although their study was performed in only one center and did not evaluate long-term survival, their results are compatible with ours.

Treatment of HCC patients with cirrhosis faces a dilemma in that minimization of damage to noncancerous liver tissue improves long-term survival, but incomplete treatment of subsequent HCC recurrences results in a poor prognosis. Accordingly, if a useful indicator of choice of therapeutic modality were to be available before the initial therapy, there would be several advantages in not only the treatment, but also the follow-up, of patients with HCC.

In conclusion, present results revealed that AFP-L3 had different impacts on prognosis in patients with HCC who underwent percutaneous ablative therapy and hepatectomy. Although this study was not a randomized control trial, AFP-L3 might be a promising scale to improve the prognostic estimate and appraisal of therapeutic outcome in patients with HCC.

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ORIGINAL ARTICLE—LIVER, PANCREAS, AND BILIARY TRACT

Efficacy and safety of addition of minor bloodletting (petit phlebotomy) in hepatitis C virus-infected patients receiving regular glycyrrhizin injections

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Abstract

Background Hepatoprotective therapies that include regular glycyrrhizin injections (GIs) are beneficial for chronic hepatitis C patients, but are sometimes insufficient for normalizing serum alanine aminotransferase (ALT) levels. Here, we evaluated whether the addition of minor bloodletting, named petit phlebotomy (PP), prior to each GI could further reduce serum ALT concentrations in such patients.

Methods Seventy-six hepatitis C virus (HCV)-infected patients receiving regular GI, with persistently abnormal serum ALT levels, were randomly divided into GI + PP and GI groups and monitored for 12 months. PP was performed before every GI to a total 60 ml of blood a week. The primary PP endpoint was a serum ferritin level of less than 20 ng/ml. PP was suspended upon reaching the endpoint, but was resumed as needed. The efficacy of the addition of PP was evaluated by measuring changes in serum ALT levels.

Results Two patients in each group dropped out because of the appearance of hepatocellular carcinoma. The remainder completed the 12-month treatment with no serious adverse events. Serum ALT and ferritin levels were significantly decreased in the GI + PP group (from 67 ± 34 to 44 ± 14 U/l and from 163 ± 127 to 25 ± 21 ng/ml, respectively, both $P < 0.001$), but these changes were not seen in the GI group. Although 20 patients in the GI + PP group had compensated cirrhosis, no significant reductions in serum albumin concentrations were observed.

Conclusions The addition of PP is effective and safe for improving serum aminotransferase levels in HCV-infected patients receiving regular GI, even in those with compensated cirrhosis.

Keywords Petit phlebotomy · Hepatitis C · Liver cirrhosis · Glycyrrhizin injection · Ferritin · Albumin

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Introduction

Chronic hepatitis due to persistent hepatitis C virus (HCV) infection may lead to liver cirrhosis (LC) and eventually hepatocellular carcinoma (HCC) [1]. To prevent these complications, maintaining serum alanine aminotransferase (ALT) levels as low as possible is considered very important [2].

Interferon therapy has been demonstrated to reduce the risk of development of LC and HCC and improve the survival of HCV-infected patients. However, some patients are unwilling to continue interferon therapy because of its unpleasant side effects and cost. Furthermore, interferon therapy has the potential to cause serious adverse effects, especially in elderly patients and in patients having arteriosclerosis, thrombocytopenia, or neuropsychological disorders. For such patients, hepatoprotective therapies, including regular intake of ursodeoxycholic acid (UDCA) and/or glycyrrhizin injection (GI), are considered, but these are sometimes insufficient for maintaining serum ALT levels within the normal range.

Recently, the usefulness of iron reduction therapy for HCV-infected patients has been established [3–7]. We hypothesized that the addition of minor bloodletting, named petit phlebotomy (PP), prior to each GI could adequately reduce serum ALT concentrations, and we planned a 12-month randomized prospective study to evaluate this.

Patients, material, and methods

Patients

From October 2004 to September 2007, 76 HCV-infected patients treated with regular GI at Showa Inan General Hospital and Iida Municipal Hospital were enrolled in this study after informed consent was obtained. All patients were positive for serum HCV-RNA and had demonstrated persistent elevation of serum ALT levels (>45 U/l) for more than 6 months. The exclusion criteria at entry were: (1) previous interferon therapy within 6 months; (2) serum ferritin levels of less than 20 ng/ml; (3) hemoglobin values of less than 11 g/dl; (4) serum albumin concentrations of less than 3.6 g/dl[7]; (5) decompensated LC; (6) malignancy complications or cardiac, pulmonary, renal, or hematological disease; and (7) pregnancy. All patients were treated with UDCA in addition to regular GI for more than 6 months before entry. These agents were not changed after entry. None of the patients had received regular administration of branched-chain amino acids, diuretics, or albumin infusion. The enrolled patients were assigned randomly to GI + PP or GI groups using sealed opaque envelopes.

All patients were advised to reduce their intake of iron-rich foods and were counseled by a registered dietitian. To aid with compliance, each patient was given a comprehensive list of iron-rich foods, as well as instructions on how to complete dietary records, which required a listing of all food and drink consumed over a 3-day period once every 4 months throughout treatment. Iron intake was assessed based on dietary records using the nutrition-analysis software BASIC-4 for Windows version 2.0 (Kagawa Nutrition University Publishing Division, Tokyo, Japan).

Body mass index (BMI) was calculated at entry. Patients were considered to have hypertension if their systolic/diastolic pressure was greater than 140/90 mmHg, or if they were taking anti-hypertensive drugs. Patients were considered to have diabetes if they had a fasting glucose level equal to or higher than 126 mg/dl, or if they were taking insulin or oral hypoglycemic drugs [8–10].

Diagnosis of LC

The initial diagnosis of LC was made from the histological findings of a percutaneous liver biopsy. In patients who refused liver biopsy, the diagnosis was made using a formula for estimating LC proposed by Ikeda et al. [11]. Diagnoses were then further corroborated by imaging findings, such as the presence of liver surface irregularities, swelling of the left or caudal lobes, the presence of splenomegaly, and the development of esophageal and/or gastric varices.

Procedure of PP

PP was performed before each regular GI for 12 months, using a 24- or 26-gauge needle. The volume of blood drawn was set at a total 1-week volume of 60 ml, divided equally by the number of GIs given; for example, in patients receiving GI twice a week, 30 ml of blood was removed prior to each GI. The primary PP endpoint was a serum ferritin level of less than 20 ng/ml and the secondary PP endpoint was a hemoglobin value of less than 11 g/dl. Upon reaching these endpoints, PP was suspended and conventional hepatoprotective therapies only were continued. If serum ferritin levels began to increase, PP was resumed to again lower them to 20 ng/ml or less. Other discontinuance criteria were the appearance of peripheral edema, ascites, HCC or other serious adverse events, or the patient's refusal to continue PP.

Laboratory examination

Complete blood counts, including hemoglobin values and platelet counts, and biochemical parameters were measured monthly using standard automated analyzers. The amount of serum HCV-RNA was measured by the Amplicor monitoring method (Roche Diagnostic Systems, Basel, Switzerland).

Assessment of adverse effects

The presence of any adverse effects from PP was verified prior to every GI by a member of the nursing staff and

monitored once a month by a medical interview and physical and blood examinations by a doctor.

Ethics

This study was carried out in accordance with the World Medical Association Helsinki Declaration, and was approved by the ethics committees of the applicable hospitals.

Statistics

Statistical analyses were performed using SPSS software 11.0J for Windows (SPSS, Chicago, IL, USA). Qualitative variables were expressed as percentages and were compared using the χ^2 test. Quantitative data were expressed as mean ± SD and were compared using a paired or unpaired two-tailed Student's t test. A P value of less than 0.05 was considered to be statistically significant.

Results

Effects of addition of PP

Of the 76 patients enrolled in this study, 2 patients in each group withdrew because of the appearance of HCC. The remaining 72 patients (36 in each group) completed the 12-month treatment. There were no differences in the baseline characteristics between the groups (Table 1). Twenty patients (56%) in the GI + PP group reached serum ferritin levels of less than 20 ng/ml at 7.2 months on average after starting PP. At the end of the study period, significant differences in serum aspartate aminotransferase (AST) and ALT levels, hemoglobin values, and iron profiles, were found between the groups (Table 2). Serum ALT levels were significantly decreased in the GI + PP group after the treatment period (from 67 ± 34 to 44 ± 14 U/l, $P < 0.001$; Fig. 1), but remained unchanged in the GI group (from 75 ± 33 to 71 ± 28 U/l). Due to the addition of the PP regime, hemoglobin values and serum ferritin levels were also decreased (from 14.0 ± 1.6 to 12.3 ± 1.7 g/dl and from 163 ± 127 to 25 ± 21 ng/ml, respectively, both $P < 0.001$). Serum albumin, cholinesterase (ChE), cholesterol, and α-fetoprotein levels did not differ between the two groups at the end of the treatment course (Table 2).

In chronic hepatitis C patients receiving regular GI, the addition of PP significantly lowered serum ALT and ferritin levels (from 79 ± 46 to 43 ± 14 U/l and from 186 ± 172 to 26 ± 21 ng/ml, respectively, both $P = 0.002$; Table 3).

Table 1 Baseline characteristics of the patients

	GI + PP ($n = 36$)	GI ($n = 36$)	P
Age (years)	70 ± 8	68 ± 8	0.310
Male (n)	19 (53%)	13 (36%)	0.236
BMI (kg/m^2)	23.4 ± 3.1	22.0 ± 2.5	0.061
Hypertension (n)	18 (50%)	17 (47%)	1.000
Diabetes (n)	5 (14%)	2 (6%)	0.429
Liver cirrhosis (n)	20 (56%)	26 (72%)	0.220
UDCA intake (mg/day)	558 ± 105	531 ± 128	0.337
Glycyrrhizin injection (ml/week)	111 ± 68	127 ± 54	0.272
Dietary iron intake (mg/day)	7.3 ± 2.1	7.3 ± 1.8	0.840
Hemoglobin (g/dl)	14.0 ± 1.6	13.8 ± 1.6	0.568
Platelet count (×10^3/μl)	118 ± 44	105 ± 46	0.239
Albumin (g/dl)	4.0 ± 0.3	3.9 ± 0.3	0.676
Bilirubin (mg/dl)	0.7 ± 0.3	0.8 ± 0.4	0.569
AST (U/l)	68 ± 27	70 ± 24	0.707
ALT (U/l)	67 ± 34	75 ± 33	0.201
ChE (U/l)	194 ± 97	170 ± 109	0.520
Cholesterol (mg/dl)	153 ± 26	163 ± 30	0.446
Iron (μg/dl)	131 ± 52	143 ± 56	0.456
Transferrin saturation (%)	39 ± 17	42 ± 21	0.712
Ferritin (ng/ml)	163 ± 127	120 ± 91	0.086
AFP (ng/ml)	26 ± 53	22 ± 27	0.709

Qualitative data are expressed as percentages and quantitative data are expressed as mean ± SD. P values were calculated using either the χ^2 test or the unpaired two-tailed Student's t test

GI glycyrrhizin injection, *PP* petit phlebotomy, *BMI* body mass index, *UDCA* ursodeoxycholic acid, *AST* aspartate aminotransferase, *ALT* alanine aminotransferase, *ChE* cholinesterase, *AFP* alpha-fetoprotein

Effects of the addition of PP in patients with compensated LC

The additive effects of PP were also investigated in patients with HCV-related compensated LC. Serum ALT levels, as well as serum AST and ferritin levels and hemoglobin values, were markedly decreased in the GI + PP group after the treatment period (Table 4). These decreases were not seen in the 26 patients with LC in the GI group (Table 4). Significant reductions in serum albumin, ChE, or cholesterol concentrations were not observed by the addition of PP (Table 4).

Adverse effects of PP

PP was well tolerated. One patient complained of transient faintness just after PP, but immediately recovered without

Table 2 Characteristics of the patients at the end of the study

	GI + PP ($n = 36$)	GI ($n = 36$)	P
Total blood phlebotomized (ml)	2206 ± 878		
BMI (kg/m^2)	23.3 ± 3.0	22.2 ± 2.6	0.079
Total glycyrrhizin administered (ml)	5234 ± 3257	5458 ± 2110	0.732
Dietary iron intake (mg/day)	6.0 ± 0.7	6.0 ± 0.6	0.886
Hemoglobin (g/dl)	12.3 ± 1.7	13.7 ± 1.6	**0.003**
Platelet count (×10^3/μl)	133 ± 53	109 ± 46	0.086
Albumin (g/dl)	3.9 ± 0.4	3.9 ± 0.4	0.609
AST (U/l)	52 ± 18	69 ± 24	**0.002**
ALT (U/l)	44 ± 14	71 ± 28	**<0.001**
ChE (U/l)	217 ± 104	229 ± 91	0.781
Cholesterol (mg/dl)	148 ± 28	142 ± 14	0.405
Iron (μg/dl)	81 ± 59	146 ± 68	**0.034**
Transferrin saturation (%)	20 ± 18	43 ± 21	**0.046**
Ferritin (ng/ml)	25 ± 21	131 ± 102	**<0.001**
AFP (ng/ml)	12 ± 12	28 ± 53	0.189

P values less than 0.05 are in bold

Clinical and biochemical parameters at the end of the 12-month treatment were compared between the GI + PP and GI groups. Quantitative data are expressed as mean ± SD. *P* values were calculated using the unpaired two-tailed Student's *t* test

GI glycyrrhizin injection, *PP* petit phlebotomy, *BMI* body mass index, *AST* aspartate aminotransferase, *ALT* alanine aminotransferase, *ChE* cholinesterase, *AFP* alpha-fetoprotein

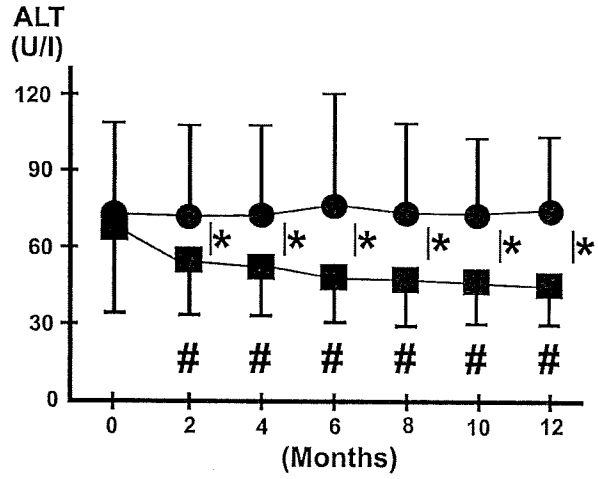

Fig. 1 Changes in serum alanine aminotransferase (*ALT*) levels. Data are expressed as mean ± SD. Statistical analysis was conducted using the two-tailed Student's *t* test. *Filled squares*, Glycyrrhizin injection (GI) + petit phlebotomy (PP) group ($n = 36$); *filled circles*, GI group ($n = 36$); *$P < 0.05$ compared with the GI group at the same time point (unpaired *t* test); #$P < 0.05$ compared with the baseline in the GI + PP group (paired *t* test)

any treatment. No patients in the GI + PP group showed new signs of peripheral edema or ascites due to decreased serum albumin concentrations throughout the treatment.

Discussion

This study clearly demonstrates the usefulness of the addition of PP in HCV-infected patients with persistently abnormal ALT levels, regardless of repeated GI. Long-term GI is believed to be a very effective hepatoprotective therapy, but may sometimes be insufficient to normalize serum ALT levels. Our results show that the addition of PP to regular GI can further lower serum AST and ALT levels and reinforce hepatoprotection, with few adverse effects and at relatively little cost. Thus, PP can be performed in most HCV-infected patients, including elderly patients and those with arteriosclerosis, thrombocytopenia, or neuropsychological disorders.

From the standpoint of iron reduction therapy, this procedure is already considered to be quite useful. In a previous report [6], regular biweekly phlebotomies of 200 or 400 ml were successfully performed in chronic hepatitis C patients until serum ferritin levels reached 10 ng/ml. However, unpleasant symptoms associated with sudden blood loss, such as general fatigue, dizziness, and orthostatic hypotension, may sometimes appear, especially in elderly or female patients. Moreover, the thick needles (16–18 gauge) used in regular phlebotomies often cause stress from puncture pain. In contrast, a thin needle (24–26 gauge) was used for PP in our study, and the volume of blood removed was too small to noticeably affect systemic circulation (20–60 ml). Therefore, we presume that this method will be easily accepted and adhered to by many patients, even elderly ones and those with delicate superficial veins. We are now planning a future trial to compare the efficacy and adherence rates between PP and conventional phlebotomy.

It is noteworthy that PP was continued safely for 1 year in patients with compensated LC. In cirrhotic patients, conventional repeated phlebotomies are sometimes discontinued because of decreases in serum albumin concentrations; some adjustments, such as a reduction of the removed blood volume or extension of intervals between extractions, are required [7]. On the other hand, the extent of decreases in serum albumin, ChE, and cholesterol levels was minimal in PP. Thus, PP is presumed to be a safe and effective iron reduction therapy for patients with compensated LC as well.

Lastly, it has been reported that long-term intermittent GI successfully reduces the incidence of HCC in patients with HCV-related chronic liver disease [12]. Similar results have been found for long-term iron reduction therapy [13, 14]. Based on these findings, it is plausible that the

Table 3 Changes in the laboratory data of patients with chronic hepatitis C

	GI + PP ($n = 16$)			GI ($n = 10$)		
	Before	After	*P*	Before	After	*P*
Hemoglobin (g/dl)	14.2 ± 1.6	12.6 ± 1.7	**0.002**	14.5 ± 1.4	14.6 ± 1.4	0.442
Platelet count ($\times 10^3/\mu l$)	147 ± 45	173 ± 38	**<0.001**	151 ± 33	147 ± 34	0.223
Albumin (g/dl)	4.0 ± 0.3	3.9 ± 0.3	0.058	4.2 ± 0.2	4.2 ± 0.3	0.862
AST (U/l)	76 ± 34	53 ± 19	**0.008**	70 ± 23	66 ± 25	0.836
ALT (U/l)	79 ± 46	43 ± 14	**0.002**	95 ± 32	81 ± 29	0.276
ChE (U/l)	197 ± 91	237 ± 101	0.381	213 ± 135	280 ± 41	0.549
Cholesterol (mg/dl)	158 ± 31	141 ± 37	0.302	176 ± 22	148 ± 14	0.404
Iron (μg/dl)	142 ± 60	76 ± 45	**<0.001**	136 ± 50	151 ± 52	0.694
Transferrin saturation (%)	43 ± 20	19 ± 14	**<0.001**	34 ± 18	42 ± 24	0.701
Ferritin (ng/ml)	186 ± 172	26 ± 21	**0.002**	154 ± 57	155 ± 82	0.788
AFP (ng/ml)	14 ± 13	9 ± 5	0.110	14 ± 16	28 ± 61	0.183

P values less than 0.05 are in bold

Data are expressed as mean ± SD. *P* values were calculated using the paired two-tailed Student's *t* test

GI glycyrrhizin injection, *PP* petit phlebotomy, *AST* aspartate aminotransferase, *ALT* alanine aminotransferase, *ChE* cholinesterase, *AFP* alpha-fetoprotein

Table 4 Changes in the laboratory data of patients with HCV-related cirrhosis

	GI + PP ($n = 20$)			GI ($n = 26$)		
	Before	After	*P*	Before	After	*P*
Hemoglobin (g/dl)	13.8 ± 1.5	12.0 ± 1.5	**<0.001**	13.8 ± 1.6	13.7 ± 1.6	0.262
Platelet count ($\times 10^3/\mu l$)	95 ± 27	98 ± 36	0.246	105 ± 46	109 ± 46	0.241
Albumin (g/dl)	3.9 ± 0.3	3.8 ± 0.4	0.121	3.9 ± 0.3	3.9 ± 0.4	0.512
AST (U/l)	61 ± 18	51 ± 14	**0.038**	70 ± 24	69 ± 24	0.833
ALT (U/l)	57 ± 15	43 ± 14	**<0.001**	67 ± 22	71 ± 28	0.157
ChE (U/l)	192 ± 105	204 ± 104	0.476	170 ± 109	229 ± 91	0.685
Cholesterol (mg/dl)	149 ± 20	153 ± 16	0.334	163 ± 30	140 ± 14	0.691
Iron (μg/dl)	123 ± 43	85 ± 69	**0.022**	142 ± 56	146 ± 68	0.675
Transferrin saturation (%)	37 ± 15	21 ± 20	**0.003**	42 ± 21	43 ± 31	0.507
Ferritin (ng/ml)	144 ± 73	25 ± 21	**<0.001**	107 ± 91	122 ± 102	0.229
AFP (ng/ml)	40 ± 69	15 ± 16	0.118	22 ± 27	28 ± 52	0.460

P values less than 0.05 are in bold

Data are expressed as mean ± SD. *P* values were calculated using the paired two-tailed Student's *t* test

GI glycyrrhizin injection, *PP* petit phlebotomy, *AST* aspartate aminotransferase, *ALT* alanine aminotransferase, *ChE* cholinesterase, *AFP* alpha-fetoprotein

combination of GI and PP could reduce the risk of HCC development more than GI alone. A long-term follow-up of the patients enrolled in the present study is needed to address this issue.

In conclusion, this study confirmed that addition of PP to regular GI successfully reduced serum aminotransferase and ferritin levels in patients with HCV-related chronic liver disease. Serious adverse events did not appear from PP, even in patients with compensated LC. For HCV-infected patients with persistently abnormal serum aminotransferase levels in spite of regular GI, the addition of PP just prior to every GI could become a promising therapeutic option.

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nature genetics

Genome-wide association of *IL28B* with response to pegylated interferon-α and ribavirin therapy for chronic hepatitis C

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The recommended treatment for patients with chronic hepatitis C, pegylated interferon-α (PEG-IFN-α) plus ribavirin (RBV), does not provide sustained virologic response (SVR) in all patients. We report a genome-wide association study (GWAS) to null virological response (NVR) in the treatment of patients with hepatitis C virus (HCV) genotype 1 within a Japanese population. We found two SNPs near the gene *IL28B* on chromosome 19 to be strongly associated with NVR (rs12980275, $P = 1.93 \times 10^{-13}$, and rs8099917, 3.11×10^{-15}). We replicated these associations in an independent cohort (combined P values, 2.84×10^{-27} (OR = 17.7; 95% CI = 10.0–31.3) and 2.68×10^{-32} (OR = 27.1; 95% CI = 14.6–50.3), respectively). Compared to NVR, these SNPs were also associated with SVR (rs12980275, $P = 3.99 \times 10^{-24}$, and rs8099917, $P = 1.11 \times 10^{-27}$). In further fine mapping of the region, seven SNPs (rs8105790, rs11881222, rs8103142, rs28416813, rs4803219, rs8099917 and rs7248668) located in the *IL28B* region showed the most significant associations ($P = 5.52 \times 10^{-28}$–2.68×10^{-32}; OR = 22.3–27.1). Real-time quantitative PCR assays in peripheral blood mononuclear cells showed lower *IL28B* expression levels in individuals carrying the minor alleles ($P = 0.015$).

Hepatitis C is a global health problem that affects a significant proportion of the world's population. The World Health Organization estimated that in 1999, there were 170 million HCV carriers worldwide, with 3–4 million new cases appearing each year. HCV infection affects more than 4 million people in the United States, where it represents the leading cause of cirrhosis and hepatocellular carcinoma as well as the leading cause of liver transplantation[1]. The American Gastroenterological Association estimated that drugs are the largest direct costs of hepatitis C[1].

The most effective current standard of care in patients with chronic hepatitis C, a combination of PEG-IFN-α with ribavirin, does not produce SVR in all patients treated. Large-scale studies on 48-week-long PEG-IFN-α/RBV treatment in the United States and Europe showed that 42–52% of patients with HCV genotype 1 achieved SVR[2–4], and similar results were found in Japan. However, older patients (greater than 50 years of age) had a significantly lower rate of SVR due to poor adherence resulting from adverse events and laboratory-detectable abnormalities such as neutropenia and thrombocytopenia[5,6]. Specifically, various well-described side effects (such as a flu-like syndrome, hematologic abnormalities and adverse neuropsychiatric events) often necessitate dose reduction, and 10–14% of patients require premature withdrawal from interferon-based therapy[7]. To avoid these side effects in patients who will not be helped by the treatment, as well as to reduce the substantial cost of PEG-IFN-α/RBV treatment, it would be useful to be able to predict an individual's response before or early in treatment. Several viral factors, such as genotype 1, high baseline viral load, viral

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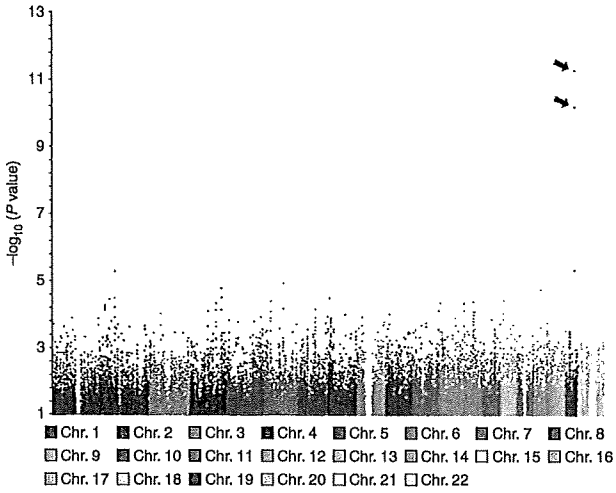

Figure 1 Genome-wide association results with PEG-IFN-α/RBV treatment in 142 Japanese patients with HCV (78 NVR and 64 VR samples). *P* values were calculated by using a χ^2 test for allele frequencies. The dots with arrows for chromosome 19 denote SNPs that showed significant genome-wide associations ($P < 8.05 \times 10^{-8}$) with response to PEG-IFN-α/RBV treatment.

kinetics during treatment, and amino acid pattern in the interferon sensitivity–determining region, have been reported to be significantly associated with the treatment outcome in a number of independent studies[8–10]. Studies have also provided strong evidence that ~20% of patients with HCV genotype 1 and 5% of patients with genotype 2 or 3 have a null response to PEG-IFN-α/RBV. No definite predictor of this resistance is currently available that make it possible to bypass the initial 12–24 weeks' treatment before deciding whether treatment should be continued. If a reliable predictor of non-response were identified for use in patients before treatment initiation, then an estimated 20%, including those who have little or no chance to achieve SVR, could be spared the side effects and cost of treatment.

Host factors, including age, sex, race, liver fibrosis and obesity, have also been reported to be associated with PEG-IFN-α/RBV therapy outcome[11,12]. However, little is known about the host genetic factors that might be associated with the response to therapy: thus far only a few candidate genes, including those encoding type I interferon receptor-1 (*IFNAR1*) and mitogen-activated protein kinase–activated protein kinase 3 (*MAPKAPK3*), have been reported to be associated with treatment response[13,14]. We describe here a GWAS for response to PEG-IFN-α/RBV treatment.

We conducted this GWAS to identify host genes associated with response to PEG-IFN-α/RBV treatment in 154 Japanese patients with HCV genotype 1 (82 with NVR and 72 with virologic response (VR), based on the selection criteria as described in Online Methods). We used the Affymetrix SNP 6.0 genome-wide SNP typing array for 900,000 SNPs. A total of 621,220 SNPs met the following criteria: (i) SNP call rate ≥95%, (ii) minor allele frequency (MAF) ≥1% and (iii) deviation from Hardy-Weinberg equilibrium (HWE) $P \geq 0.001$ in VR samples. After excluding 4 NVR and 8 VR samples that showed quality control (QC) call rates of <95%, 78 NVR and 64 VR samples were included in the association analysis. **Figure 1** shows a genome-wide view of the single-point association data based on allele frequencies. Two SNPs located close to *IL28B* on chromosome 19 showed strong associations, with a minor allele dominant model (rs12980275, $P = 1.93 \times 10^{-13}$, and rs8099917, $P = 3.11 \times 10^{-15}$, respectively), with NVR to PEG-IFN-α/RBV treatment (**Table 1**). The rs8099917 lies between *IL28B* and *IL28A*, ~8 kb downstream from *IL28B* and ~16 kb upstream from *IL28A*. These associations reached genome-wide levels of significance for both SNPs in this initial GWAS cohort (Bonferroni criterion $P < 8.05 \times 10^{-8}$ (0.05/621,220)). The frequencies of minor allele–positive patients were much higher in the NVR group than in the VR group for both SNPs (74.3% in NVR, 12.5% in VR for rs12980275; 75.6% in NVR, 9.4% in VR for rs8099917). Notably, individuals homozygous for the minor allele were observed only in the NVR group. The VR group, as compared to the NVR group, showed genotype frequencies closer to those in the healthy Japanese population[15], yet the minor allele frequencies were slightly higher in the transient virologic response (TVR) group (23.1%, 15.4%) than in the SVR group (9.8%, 7.8%) (**Table 1**). We applied the Cochrane-Armitage test on all the SNPs and found a genetic inflation factor, λ, of 1.029 for the GWAS stage (**Supplementary Fig. 1**). We also carried out principal component analysis in 142 samples for the GWAS stage together with the HapMap samples (CEU, YRI, CHB and JPT) (**Supplementary Fig. 2**); this suggested that the effect of population stratification was negligible.

Table 1 Significant association of two SNPs (rs12980275 and rs8099917) with response to PEG-IFN-α/RBV treatment

					Null responder (NVR[a], *n* = 128)			Responder (VR[a], *n* = 186)			Responder (SVR[a], *n* = 140)			NVR vs. VR		NVR vs. SVR	
dbSNP rsID	Nearest gene	MAF[b] (allele)	Allele (1/2)	Stage	11	12	22	11	12	22	11	12	22	OR (95% CI)[c]	*P* value[d]	OR (95% CI)[c]	*P* value[d]
rs12980275	*IL28B*	0.15 (G)	A/G	GWAS	20 (25.6)	54 (69.2)	4 (5.1)	56 (87.5)	8 (12.5)	0 (0.0)	46 (90.2)	5 (9.8)	0 (0.0)	20.3 (8.3–49.9)	1.93×10^{-13}	26.7 (9.3–76.5)	7.41×10^{-13}
				Replication	10 (20.0)	37 (74.0)	3 (6.0)	101 (82.8)	21 (17.2)	0 (0.0)	73 (82.0)	16 (18.0)	0 (0.0)	19.2 (8.3–44.4)	5.46×10^{-15}	18.3 (7.6–44.0)	8.37×10^{-13}
				Combined	30 (23.4)	91 (71.1)	7 (5.5)	157 (84.4)	29 (15.6)	0 (0.0)	119 (85.0)	21 (15.0)	0 (0.0)	17.7 (10.0–31.3)	2.84×10^{-27}	18.5 (10.0–34.4)	3.99×10^{-24}
rs8099917	*IL28B*	0.12 (G)	T/G	GWAS	19 (24.4)	56 (71.8)	3 (3.8)	58 (90.6)	6 (9.4)	0 (0.0)	47 (92.2)	4 (7.8)	0 (0.0)	30.0 (11.2–80.5)	3.11×10^{-15}	36.5 (11.6–114.6)	5.00×10^{-14}
				Replication	11 (22.0)	37 (74.0)	2 (4.0)	108 (88.5)	14 (11.5)	0 (0.0)	78 (87.6)	11 (12.4)	0 (0.0)	27.4 (11.5–65.3)	9.47×10^{-18}	25.1 (10.0–63.1)	1.00×10^{-14}
				Combined	30 (23.4)	93 (72.7)	5 (3.9)	166 (89.2)	20 (10.8)	0 (0.0)	125 (89.3)	15 (10.7)	0 (0.0)	27.1 (14.6–50.3)	2.68×10^{-32}	27.2 (13.9–53.4)	1.11×10^{-27}

[a]NVR, null virologic response; VR, virologic response; SVR, sustained virologic response. The 186 VRs consisted of 46 transient virologic response (TVRs) and 140 SVRs. [b]Minor allele frequency and minor allele in 184 healthy Japanese individuals[15]. The MAF of the SNPs in SVR is similar to that of TVR group, whereas that of NVR is much higher (76.6%). [c]Odds ratio for the minor allele in a dominant model. [d]*P* value by χ^2 test for the minor allele dominant model.

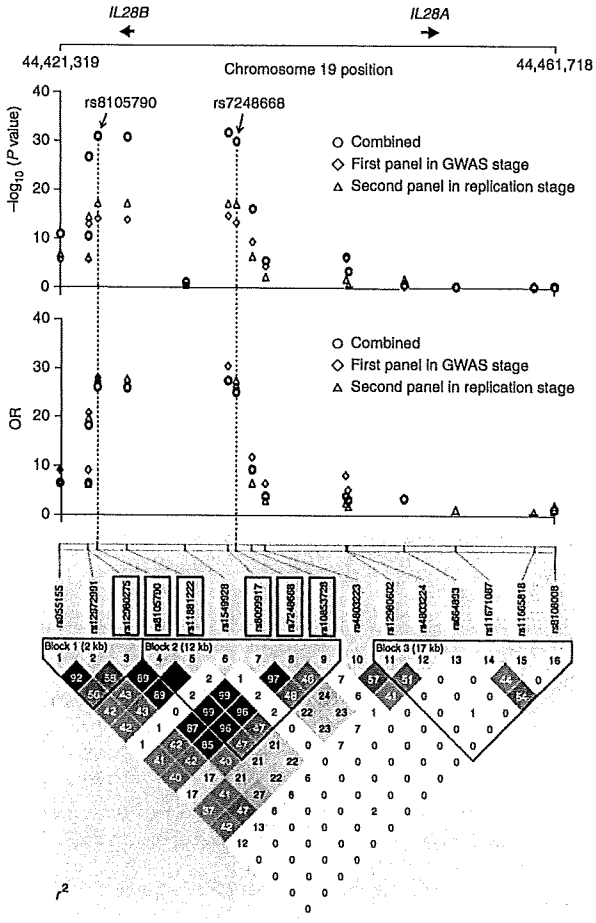

Figure 2 Genomic structure, *P* value and OR plots in association analysis and LD map around *IL28B* and *IL28A* (chr.19, nucleotide positions 44421319–44461718; build 35). *P* values by the χ^2 test for minor allele dominant effect model are shown for the first panel of 142 samples in the GWAS stage, the second panel of 172 samples in the replication stage, and the combined analysis. Below are estimates of pairwise r^2 for 16 SNPs selected in the replication study using a total of 314 Japanese patients with HCV treated with PEG-IFN-α/RBV. Boxes indicate the significantly associated SNPs with response to PEG-IFN-α/RBV treatment both in the GWAS stage and in the replication stage. Dotted lines indicate the region with the strongest associations from the positions of rs8105790 to rs7248668.

We analyzed the region of ~40 kb (chr. 19, nucleotide positions 44421319–44461718; build 35) containing the significantly associated SNPs (rs12980275 and rs8099917) using Haploview software for linkage disequilibrium (LD) and haplotype structure based on the HapMap data for individuals of Japanese ancestry. The LD blocks were analyzed using the four-gamete rule, and four blocks were observed (**Supplementary Fig. 3**). We selected 16 SNPs for both replication study and high-density association mapping, including tagging SNPs estimated on the basis of the haplotype blocks, one SNP located within *IL28B* (rs11881222) and the significantly associated SNPs from the GWAS stage (rs12980275 and rs8099917) (**Supplementary Table 1**).

To validate the results of the GWAS stage, 16 SNPs selected for the replication stage, including the original SNPs, were genotyped using the DigiTag2 assay in an independent set of 172 Japanese patients with HCV treated with PEG-IFN-α/RBV treatment (50 NVR and 122 VR samples), together with the first panel of 142 samples analyzed in the GWAS stage (**Supplementary Table 1**). The associations of the original SNPs were replicated in the replication cohort of 172 patients ($P = 5.46 \times 10^{-15}$, OR = 19.2 for rs12980275; $P = 9.47 \times 10^{-18}$, OR = 27.4 for rs8099917; **Table 1**). The combined *P* values for both stages reached 2.84×10^{-27} (OR = 17.7; 95% CI = 10.0–31.3) and 2.68×10^{-32} (OR = 27.1; 95% CI = 14.6–50.3), respectively (**Table 1**). Notably, when we compared the SVR ($n = 140$) with the NVR group ($n = 128$), the original two SNPs (rs12980275 and rs8099917) again showed strong associations: both *P* values and ORs were similar to those observed in the comparison between VR and NVR, and the combined *P* values for both stages reached 3.99×10^{-24} (OR = 18.5; 95% CI = 10.0–34.4) and 1.11×10^{-27} (OR = 27.2; 95% CI = 13.9–53.4), respectively (**Table 1**). Comparing SVR ($n = 140$) versus NVR plus TVR ($n = 174$), we again found that these SNPs were significantly associated ($P = 1.71 \times 10^{-16}$, OR = 8.8; 95% CI 5.1–15.4 for rs12980275; $P = 1.18 \times 10^{-18}$, OR = 12.1; 95% CI 6.5–22.4 for rs8099917, **Supplementary Table 2**), suggesting that these SNPs would predict NVR as well as SVR before PEG-IFN-α/RBV therapy.

Among the newly analyzed SNPs in the replication study, six (rs12980275, rs8105790, rs11881222, rs8099917, rs7248668 and rs10853728) showed significant associations both in the GWAS stage ($P < 8.05 \times 10^{-8}$) and in the replication stage ($P < 0.0031$ (0.05/16)) after Bonferroni correction. These SNPs are located within a 15.7-kb region that includes *IL28B* (**Fig. 2** and **Supplementary Table 1**). In particular, the strongest associations with NVR were observed for four SNPs, rs8105790, rs11881222, rs8099917 and rs7248668, that are located in the downstream flanking region, the third intron and the upstream flanking region of *IL28B*. The combined *P* values for these polymorphisms were 1.98×10^{-31} (OR = 25.7; 95% CI = 13.9–47.6), 2.84×10^{-31} (OR = 25.6; 95% CI = 13.8–47.3), 2.68×10^{-32} (OR = 27.1; 95% CI = 14.6–50.3) and 1.84×10^{-30} (OR = 24.7; 95% CI = 13.3–45.8), respectively (**Supplementary Table 1**). We then sequenced this region to identify further variants and found three SNPs (rs8103142, rs28416813 and rs4803219) located in the third exon, the first intron and the upstream flanking region of *IL28B*, and a few infrequent variations. These SNPs also showed strong associations in the combined dataset of 128 NVR and 186 VR samples ($P = 1.40 \times 10^{-29}$, OR = 26.6 for rs8103142; $P = 5.52 \times 10^{-28}$, OR = 22.3 for rs28416813; $P = 2.45 \times 10^{-29}$, OR = 23.3 for rs4803219; **Supplementary Table 3**). We also performed LD and haplotype analyses with seven SNPs. These SNPs were in strong LD, and the risk haplotype showed a level of association similar to those of individual SNPs ($P = 1.35 \times 10^{-25}$, OR = 11.1; 95% CI = 6.6–18.6) (**Table 2**). These results suggest that the association with NVR was primarily driven by one of these SNPs.

Table 2 Association analysis of response to treatment by *IL28B* haplotype

SNP							Frequencies			
rs8105790	rs11881222	rs8103142	rs28416813	rs4803219	rs8099917	rs7248668	NVR group	VR group	*P* value	OR (95% CI)
T	A	T	C	C	T	G	0.543	0.942	1.81×10^{-32}	0.1 (0.04–0.12)
C	G	C	G	T	G	A	0.387	0.054	1.35×10^{-25}	11.1 (6.6–18.6)

Association analysis of haplotypes consisting of seven SNPs with response to PEG-IFN-α/RBV treatment in 314 Japanese patients with HCV. Boldface letters: rs11881222 (third intron); rs8103142 (third exon).

Table 3 Factors associated with NVR by logistic regression model

Factors	Odds ratio	95% CI	*P* value
rs8099917 (G allele)	37.68	16.71–83.85	<0.0001
Age	1.02	0.98–1.07	0.292
Gender (Female)	3.32	1.49–7.39	0.003
Re-treatment[a]	1.12	0.55–2.33	0.750
Platelet count	0.93	0.87–1.01	0.080
Aminotransferase level	1.00	0.99–1.00	0.735
Fibrosis stage[20]	1.10	0.73–1.66	0.658
HCV-RNA level	1.01	0.99–1.02	0.139

[a]Re-treatment, non-response to previous treatment with interferon-α (plus RBV).

To examine the relative contribution of factors associated with NVR, we used a logistic regression model. One tagging SNP located within *IL28B* (minor allele of rs8099917) was the most significant factor for predicting NVR, followed by gender (**Table 3**). Clinically, viral factors such as HCV genotype and HCV RNA level are important for the outcome of PEG-IFN-α/RBV therapy. Indeed, mean HCV-RNA level was significantly lower in SVR (SVR versus TVR, $P = 0.002$; SVR versus NVR, $P = 0.016$; **Supplementary Table 4**). Mean platelet count and the proportion of mild fibrosis (F1–F2) were significantly higher in SVR than in NVR.

Real-time quantitative PCR assays in peripheral blood mononuclear cells revealed a significantly lower level of *IL28* mRNA expression in individuals with the minor alleles (**Fig. 3**), suggesting that variant(s) regulating *IL28* expression is associated with a response to PEG-IFN-α/RBV treatment. *IL28B* encodes a cytokine distantly related to type I (α and β) interferons and the interleukin (IL)-10 family. This gene and *IL28A* and *IL29* (encoding IL-28A and IL-29, respectively) are three closely related cytokine genes that encode proteins known as type III IFNs (IFN-λs) and that form a cytokine gene cluster at chromosomal region 19q13 (ref. 16). The three cytokines are induced by viral infection and have antiviral activity[16,17]. All three interact with a heterodimeric class II cytokine receptor that consists of IL-10 receptor beta (IL10Rβ) and IL-28 receptor alpha (IL28Rα, encoded by *IL28RA*)[16,17], and they may serve as an alternative to type I IFNs in providing immunity to viral infection.

Notably, a recent report showed that the strong antiviral activity evoked by treating mice with TLR3 or TLR9 agonists was significantly reduced in both $IL28RA^{-/-}$ and $IFNAR^{-/-}$ mice, indicating that IFN-λ is important in mediating antiviral protection by ligands for TLR3 and TRL9 (ref. 18). IFN-λ induced a steady increase in the expression of a subset of IFN-stimulated genes, whereas IFN-α induced the same genes with more rapid and transient kinetics[19]. Therefore, it is possible that IFN-λ induces a slower but more sustained response that is important for TLR-mediated antiviral protection. This might be one of the ways that a genetic variant regulating *IL28* expression influences the response to PEG-IFN-α/RBV treatment. Further research will be required to fully understand the specific mechanism by which a genotype might affect the response to treatment.

In conclusion, the strongest associations with NVR were observed for seven SNPs, rs8105790, rs11881222, rs8103142, rs28416813, rs4803219, rs8099917 and rs7248668, that are located in the downstream flanking region, the third intron, the third exon, the first intron and the upstream flanking region of *IL28B*. Further studies following our report of this robust genetic association to NVR may make it possible to develop a pre-treatment predictor of which individuals are likely to respond to PEG-IFN-α/RBV treatment. This would remove the need for the initial 12–24 weeks of treatment that is currently used as a basis for a clinical decision about whether treatment should be continued. That would allow better targeting of PEG-IFN-α/RBV treatment, avoiding the unpleasant side effects that commonly accompany the treatment where it is unlikely to be beneficial, and reduce overall treatment costs. Because of the small number of samples in this study, we plan to conduct a further prospective multicenter study to establish these SNPs as a clinically useful marker.

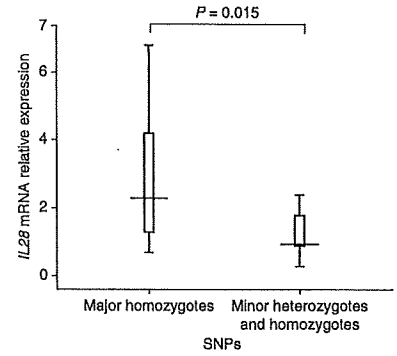

Figure 3 Quantification of *IL28* mRNA expression. The expression level of *IL28* genes was determined by real-time quantitative RT-PCR using RNA purified from peripheral blood mononuclear cells. Distribution of relative gene expression levels was compared between the individuals homozygous for major alleles ($n = 10$) and the heterozygous or homozygous individuals carrying minor alleles ($n = 10$) of rs8099917 by using the Mann-Whitney *U*-test. The bars indicate the median. All samples were obtained from HCV-infected patients before PEG-IFN-α/RBV therapy.

METHODS

Methods and any associated references are available in the online version of the paper at http://www.nature.com/naturegenetics/.

Note: Supplementary information is available on the Nature Genetics website.

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AUTHOR CONTRIBUTIONS

Study design and discussion: Y.T., N.N., N.M., K.T., M.M.; sample collection: Y.T., M.K., K.M., N.S., M.N., M.K., K.H., S.H., Y.I., E.M., E.T., S.M., Y.M., M.H., A.S., Y.H., S.N., I.S., M.I., K.I., K.Y., F.S., N.I.; genotyping: N.N.; statistical analysis: N.N., A.K., K.I.; quantitative RT-PCR: M.S.; manuscript writing: Y.T., N.N., K.T., M.M.

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ONLINE METHODS

Study cohorts. From April 2007 to April 2009, samples were obtained from 314 patients with chronic HCV (genotype 1) infection who were treated at 15 multicenter hospitals (liver units with hepatologists) throughout Japan. Each patient was treated with PEG-IFN-α2b (1.5 μg per kg body weight (μg/kg) subcutaneously once a week) or PEG-IFN-α2a (180 μg/kg once a week) plus RBV (600–1,000 mg daily depending on body weight). As a reduction in the dose of PEG-IFN-α and RBV can contribute to a less sustained virological response[21], only patients with an adherence of >80% dose for both drugs during the first 12 weeks were included in this study. HBsAg-positive and/or anti-HIV–positive individuals were excluded from this study.

NVR (seen in ~20% of total treated patients) was defined as less than a 2-log-unit decline in the serum level of HCV RNA from the pre-treatment baseline value within the first 12 weeks and detectable viremia 24 weeks after treatment. VR was defined as the achievement of SVR or transient TVR in this study; SVR was defined as undetectable HCV RNA in serum 6 months after the end of treatment, whereas TVR was defined as a reappearance of HCV RNA in serum after treatment was discontinued in a patient who had undetectable HCV RNA during the therapy or on completion of the therapy. Of 878 patients with HCV genotype 1 treated by PEG-IFN-α/RBV at 14 hospitals, only 114 (13.0%) met the criteria for NVR in this study. For the GWAS stage of the study, a case-control study was conducted comparing individuals with NVR (82 individuals) and VR (72 individuals). For the replication stage, an independent cohort of samples from 172 Japanese patients with HCV genotype 1, including 50 with NVR and 122 with VR, was obtained from an independent cohort study at Tokyo Medical and Dental University Hospital (Ochanomizu Liver Conference Study Group) and Musashino Red Cross Hospital. Clinical data from the combined cohorts, with a total of 140 SVR, 46 TVR and 128 NVR patients, are shown in **Supplementary Table 4**.

Informed consent was obtained from each patient who participated in the study. The study protocol conforms to the relevant ethical guidelines as reflected in *a priori* approval by the ethics committees of all the participating universities and hospitals.

SNP genotyping and data cleaning. In the GWAS stage, we genotyped 154 Japanese patients with HCV receiving PEG-IFN-α/RBV treatment using the Affymetrix Genome-Wide Human SNP Array 6.0 according to the manufacturer's instructions. After exclusion of 4 NVR samples and 8 SVR samples with QC call rates <95%, the remaining 142 samples were recalled using the Birdseed version 3 software (Affymetrix). The average overall call rate of 78 NVR and 64 VR samples reached 99.46% and 99.46%, respectively. We then applied the following thresholds for QC in data cleaning: SNP call rate ≥95% for all samples, MAF ≥1% for all samples and HWE P value ≥0.001 for VR group[22,23]. A total of 621,220 SNPs on autosomal chromosomes passed the QC filters and were used for association analysis. All cluster plots for the SNPs showing $P < 0.001$ in association analyses by comparing allele frequencies in NVR and VR groups were checked by visual inspection. SNPs with ambiguous genotype calls were excluded. **Supplementary Table 5** shows SNPs that might be weakly associated with NVR ($P < 10^{-4}$).

Although the 12 samples noted above were excluded from the GWAS stage by data cleaning, their quality was good enough for the SNP typing in the replication study, and thus they were included in the replication stage. In the subsequent replication stage with high-density association mapping, SNP genotyping in the independent set of 172 patients was completed using the DigiTag2 assay[24] and direct sequencing using the Applied Biosystems 3730 DNA Analyzer (Applied Biosystems). In addition, strongly associated SNPs identified in the GWAS stage were also genotyped for the GWAS samples using the DigiTag2 assay, and the results were 100% concordant to those from the GWAS platform.

Screening for new polymorphisms. To determine possible genomic variants in the region of *IL28B* and its promoter, we sequenced the 3.3-kb region in a total of 48 Japanese patients with HCV (28 NVR and 20 VR). We selected 7 samples from NVR patients who were minor allele homozygotes for 2 SNPs (rs12980275 and rs8099917), 11 samples from NVR and 10 samples from VR heterozygotes, and 10 samples from NVR and 10 samples from VR major allele homozygotes. The sequencing primers were designed using the Visual OMP Nucleic Acid software (**Supplementary Table 6**). PCR was carried using TaKaRa LA *Taq* polymerase (Takara Biochemicals) under the following thermal cycler conditions: stage 1, 94 °C for 1 min; stage 2, 98 °C for 10 s, 68 °C for 15 min, for a total of 30 cycles; stage 3, 72 °C for 10 min. A 50-μl PCR analysis was performed using 2.5 U TaKaRa LA *Taq* with 1× LA PCR buffer II, 0.4 mM dNTP, 10 pmol of each primer and 10 ng of genomic DNA. For sequencing, 7.0 μl of the PCR products were incubated with 3 μl of Exonuclease I/Shrimp Alkali Phosphatase (Takara Biochemicals) first for 90 min at 37 °C and then for another 10 min at 80 °C. Sequencing reactions were performed with the use of a BigDye Terminator Cycle Sequencing FS Ready Reaction Kit (Applied Biosystems). After purification with MultiScreen-HV (Millipore) and Sephadex G-50 Fine (GE Healthcare UK Ltd.), the reaction products were applied to the Applied Biosystems 3730 DNA Analyzer.

In the variation screening, three SNPs (rs8103142, rs28416813 and rs4803219) and a few infrequent variations were detected. We then typed these SNPs in all of the 314 patients.

Statistical analysis. The observed association between a SNP and response to PEG-IFN-α/RBV treatment was assessed by χ^2 test with a two-by-two contingency table in three genetic models: allele frequency model, dominant-effect model and recessive-effect model. SNPs on the X chromosome were removed because gender was not matched between the NVR group and the VR group. A total of 621,220 SNPs passed the QC filters in the GWAS stage; therefore, significance levels after the Bonferroni correction for multiple testing were $P = 8.05 \times 10^{-8}$ (0.05/621,220) in the GWAS stage and $P = 0.0031$(0.05/16) in the replication stage. None of the 16 markers genotyped in the replication stage showed deviations from Hardy-Weinberg equilibrium in the VR group ($P > 0.05$).

The inflation factor λ was estimated based on the median χ^2 and revealed to be 1.029 (median) and 1.011 (mean), suggesting that the population substructure should not have any substantial effect on the statistical analysis (**Supplementary Fig. 1**). In addition, the principal component analysis on the 142 patients (78 NVR samples and 64 VR samples) analyzed in the GWAS stage together with the HapMap samples also revealed that the effect of population stratification was negligible (**Supplementary Fig. 2**).

For the replication study and the high-density association mapping, 16 SNPs were selected from the region of ~40 kb (chr. 9, nucleotide positions 44421319–44461718; build 35) containing the significantly associated SNPs (rs12980275 and rs8099917) in the GWAS stage by analyzing, using Haploview software, LD and haplotype structure based on the HapMap data for individuals of Japanese descent. These SNPs included tagging SNPs estimated on the basis of haplotype blocks, SNPs located within the *IL28B* and *IL28A* genes (rs11881222 and rs576832, respectively) and the significantly associated SNPs identified in the GWAS stage (**Supplementary Table 1**). On the basis of the genotype data from the total of 314 patients in the GWAS stage and replication stages, haplotype blocks were estimated using the four-gamete rule, and three blocks were observed (**Fig. 2**). Association of haplotype with response to PEG-IFN-α/RBV treatment was analyzed using Haploview software.

The logistic regression model was used to assess the factors associated with NVR. STATA 10 (Statacorp LP) was used for all analysis. Age, platelet count, and aminotransferase (ALT) and HCV-RNA levels were applied as continuous variables.

Real-time quantitative RT-PCR for *IL28* gene. A layer of mononuclear cells was collected via Ficoll from peripheral blood. Total RNA was isolated using the RNeasy Mini Kit and the RNase-Free DNase Set (Qiagen) according to the manufacturer's protocol. First-strand cDNA was synthesized using SuperScript II reverse transcriptase with Oligo $(dT)_{12-18}$ primer (Invitrogen). The relative quantification of the target gene was determined using Custom TaqMan Gene Expression Assays, and the expression of glyceraldehyde-3-phosphate dehydrogenase was used to normalize the gene expression level (Applied Biosystems) according to the manufacturer's protocol. The data were analyzed by the $2[-\Delta\Delta C_t]$ method using Sequence Detector version 1.7 software (Applied Biosystems). A standard curve was prepared by serial tenfold dilutions of

human cDNA. The curve was linear over 7 logs with a correlation coefficient of 0.998. The specific detection of *IL28B* in real-time PCR is hard to establish, because the nucleotide differences between *IL28A* and *IL28B* consist of only 9 nucleotides scattered throughout the gene. Primers and probes are designed for the *IL28* gene (**Supplementary Table 6**).

URLs. The results of the present GWAS have been registered at a public database: https://gwas.lifesciencedb.jp/cgi-bin/gwasdb/gwas_top.cgi.

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Journal of Gastroenterology

Epidemiology of hepatocellular carcinoma in Japan

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Primary liver cancer, 95% of which is hepatocellular carcinoma (HCC), is ranked third in men and fifth in women as a cause of death from malignant neoplasms in Japan. The number of deaths and death rate of HCC began to increase sharply in 1975. These numbers peaked at 34510 and 27.4/100000, respectively, in 2004, but decreased to 33662 annual deaths and a 26.7/100000 death rate in 2006. Although hepatitis B virus (HBV) and hepatitis C virus (HCV) infection are both major causes of HCC, HCV-related HCC represents 70% of all cases. The incidence of HCC without hepatitis B surface antigen (HBsAg) or antibodies to HCV (anti-HCV) accounts for 8%–15% of HCC patients nationwide. Geographically, HCC is more frequent in western than eastern Japan, and death rates of HCC in each prefecture correlate with anti-HCV, but not HBsAg, prevalence. Interferon therapy for chronic hepatitis C reduces the risk of development of HCC, especially among patients with sustained virological response. Further research should focus on the mechanisms of carcinogenesis by HCV and HBV, development of more effective treatments, and establishment of early detection and preventative approaches. Better understanding of HCC unrelated to HCV and HBV, possibly caused by steatohepatitis and diabetes, should also be a major concern in future studies.

Key words: HCC, HCV, HBV, nonalcoholic steatohepatitis (NASH), interferon

Introduction

The three leading causes of death in Japan since 1981 are malignant neoplasms, cardiovascular diseases, and cerebrovascular diseases. For the past 30 years, liver cancer has been the third leading cause of death from malignant neoplasms in men, following lung and stomach cancer. In women, liver cancer has ranked fifth as a cause of death during the past decade, following colon, stomach, lung, and breast cancer. Primary liver cancer can be classified into three types according to the cell from which the cancer originated, namely, hepatocellular carcinoma (HCC), cholangiocellular carcinoma, and other. As HCC accounts for up 95% of all primary cancer cases, the term "liver cancer" usually means HCC.[1]

Hepatitis B virus (HBV) and hepatitis C virus (HCV) are the two major causes of HCC in Japan.[2,3] The increase in incidence of HCC in Japan, however, is largely attributable to the increase of HCV infection in the general population during the past 50 to 60 years.[2]

Changes in deaths and death rates of primary liver cancer

Changes in annual deaths from primary liver cancer among different age groups between 1958 and 2006 are shown in Fig. 1. The total number of deaths from HCC was stable at fewer than 10000 persons/year until 1975 before showing a sharp increase. The spike in 1995 resulted from a change in the International Classification of Disease (ICD) code from ICD 9 to ICD 10, which included intrahepatic bile duct cancer, accounting for approximately 5% of HCC deaths.

The majority of HCC mortalities were in patients below the age of 69 until 1999, when this age reached 70 years. In 2006, 66% of patients with HCC were over 70. The number of deaths from HCC reached 34510 in 2004, but decreased to 33662 in 2006.

The death rates of liver cancer by sex (Fig. 2) are consistently higher in men than in women. A sharp rise in the death rate of primary liver cancer in men began

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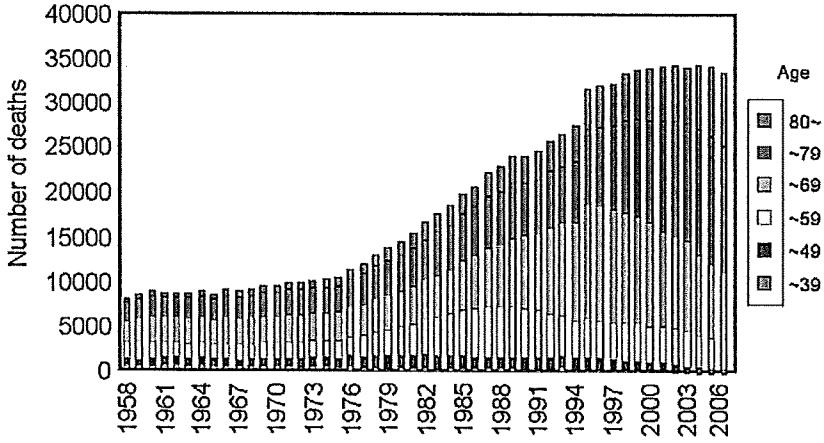

Fig. 1. Changes in annual deaths of patients (by age, in years) with primary liver cancer between 1958 and 2006. (Taken from the Vital Statistics of Japan, released every year by the Ministry of Health, Labour, and Welfare)

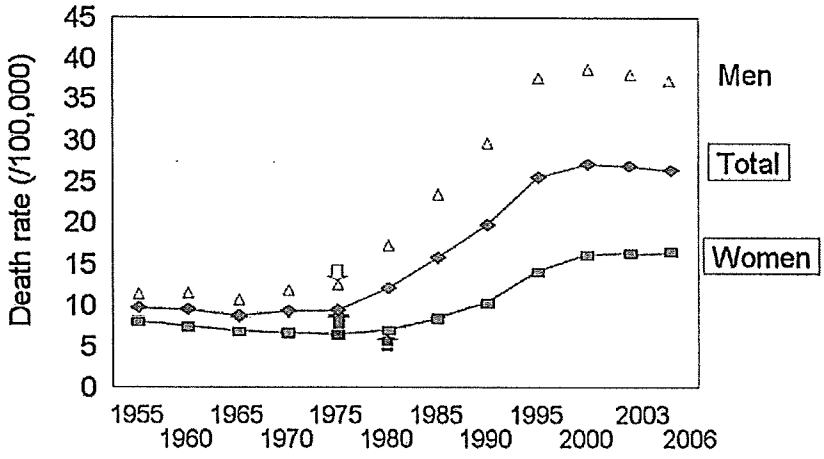

Fig. 2. Changes in the death rate of primary liver cancer in men (*triangles, yellow*), women (*rectangles, pink*), and in total (*diamonds, blue*)

in 1975, and a more gradual rise in women commenced in 1980. The total age-adjusted death rate peaked in 2002 (27.5/100000 persons in 2002), and decreased to 27.0 in 2003. In 2006, the total age-adjusted death rate stood at 26.7/100000, which is caused by a decrease in death rate (36.7) in men, but offset by an increase in women to 17.2.

Age and sex in HCC

Changes in the mean age of HCC patients and male/female ratio every 2 years between 1984 and 2003 are shown in Fig. 3. In that period, the mean age of female HCC patients was higher than that of males, and the mean ages of both sexes progressively increased. As reported previously, however, HBV-related HCC was stable from 1982 to 2003, implying that this change originated from HCV-related HCC patients. The male/female ratio was 4.5 in 1984–1985 and 2.5 in 2002–2003 (see Fig. 3), showing that the proportion of female patients with HCC had increased. This increase in female patients is also considered as a consequence of increased HCV-related HCC.

Changes in etiology of HCC in Japan

A nationwide survey on primary liver cancer has been conducted every 2 years since 1968 by the Liver Cancer Study Group of Japan.[1,4–9] Five serological surveys performed between 1990 and 2001 have documented that most patients with HCC are positive for either HBsAg or antibodies to HCV (anti-HCV). Tests for HBsAg became available in 1975 and those for anti-HCV in 1990. HBsAg-positive cases of HCC constituted 42% of patients in 1977–1978, but only 15.5% in 2002–2003 (Fig. 4). In contrast, anti-HCV-positive cases of HCC accounted for more than 70% of cases diagnosed until 2000–2001. However, this number dipped to 69.6% in 2002–2003, and has since remained at less than 70%. In contrast, HCV of unknown origin and other cases of HCC have been increasing gradually, and constituted 14.9% of all cases in 2002–2003.

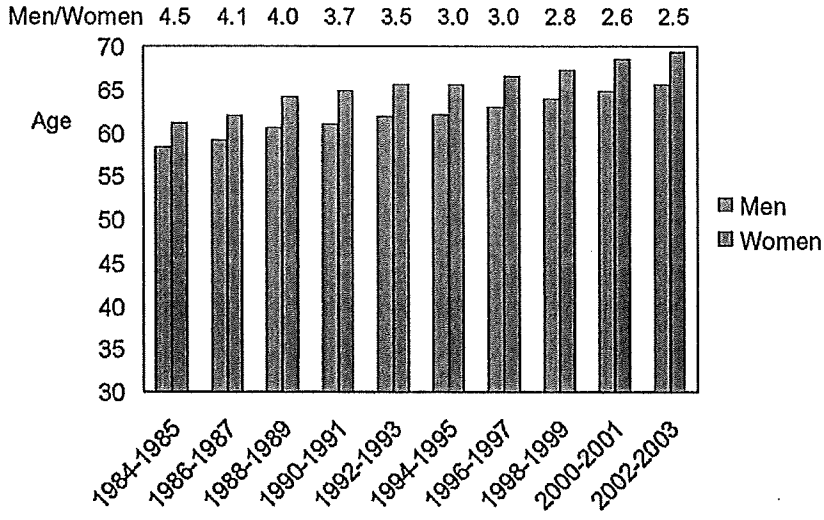

Fig. 3. Changes in the mean age (in years) of men (*blue bars*) and women (*pink bars*) patients with hepatocellular carcinoma (HCC) between 1984 and 2003

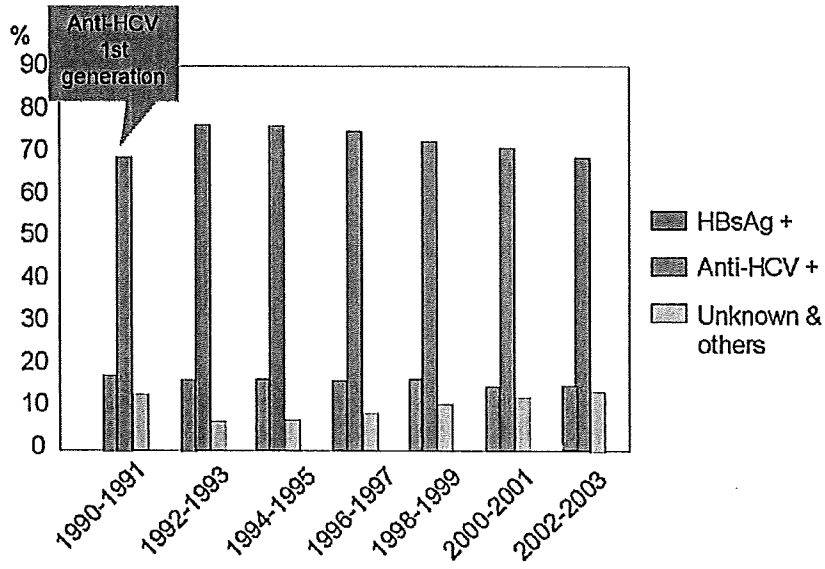

Fig. 4. Changes in the etiology of HCC between 1990 and 2003: hepatitis B surface antigen (*HBsAg+, pink*), antihepatitis C virus (*anti-HCV+, blue*), and *unknown* and *others* (*green*)

In cross-sectional studies conducted at Shinshu University Hospital, HCV-related HCC was found in the majority of cases (72%) (Fig. 5). Non-B non-C HCC (NBNC-HCC) accounted for 10% of cases in 2002–2007. In these 28 patients, nonalcoholic steatohepatitis (NASH) accounted for 14%.

Geographic variation of liver cancer and HBV/HCV infection

Although Japan is a relatively small country with a homogeneous population, the incidence of HCC varies greatly among different regions. The Vital Statistics of Japan for 2005 published in 2007 by the Japanese Ministry of Health, Labour, and Welfare on the incidence of deaths as a result of HCC in its 48 prefectures shows a steady increase in death rates of HCC from east to west in Japan. The average age-adjusted death rate of HCC among 48 prefectures was 27.2 per 100000 persons in 2005 (Fig. 6). Furthermore, nationwide health screening for HBsAg and anti-HCV in citizens over 40 years of age has been performed since 2002, and the prevalence rates of these markers have been analyzed for each prefecture in Japan. In 2006, the average HBsAg and anti-HCV prevalences were 1.0% and 0.7%, respectively, in this group (see Fig. 6). There was a highly significant association between the death rate of HCC and prevalence of anti-HCV in each prefecture (Fig. 7; correlation coefficient = 0.66; $P < 0.001$,

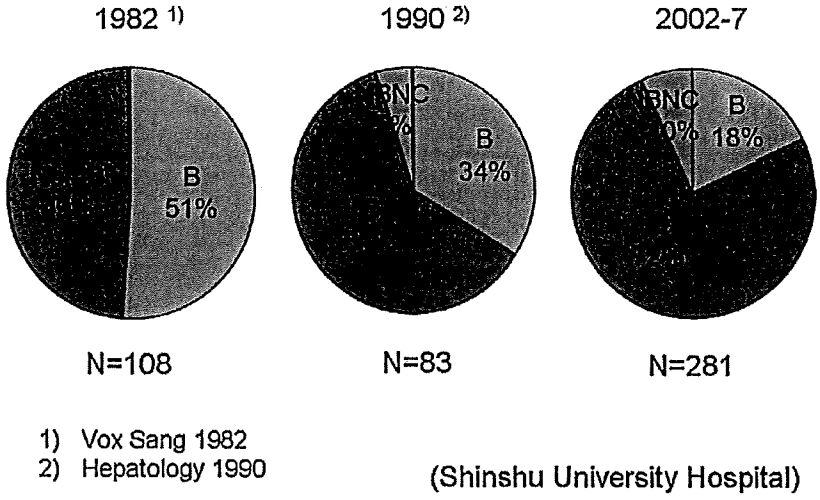

Fig. 5. Clinical features of hepatitis B (*B*) virus (HBV)- and hepatitis C (*C*) virus (HCV)-related HCC in 1982, 1990, and 2002–2005 at Shinshu University Hospital. *NBNC*, non-B non-C

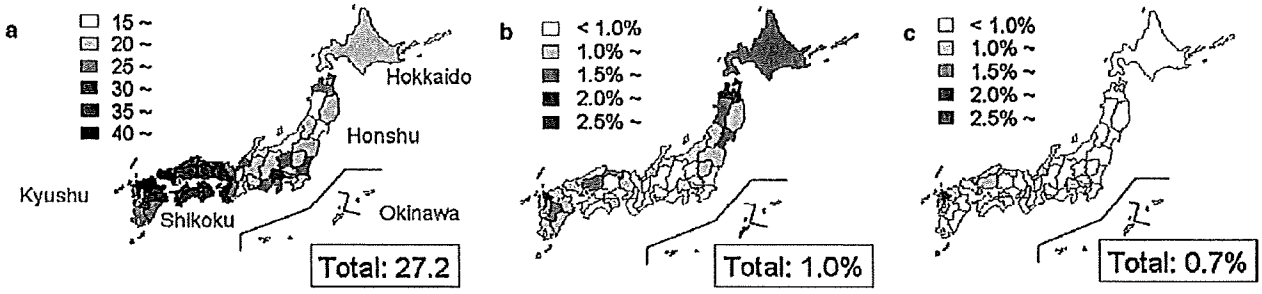

Fig. 6. **a** Death rate of primary liver cancer was 27.2 per 100000 in 2005 among people over 40 years of age in 48 prefectures in Japan. In the same group in 2006, HBsAg prevalence was 1.0% (**b**) and anti-HCV prevalence was 0.7% (**c**)

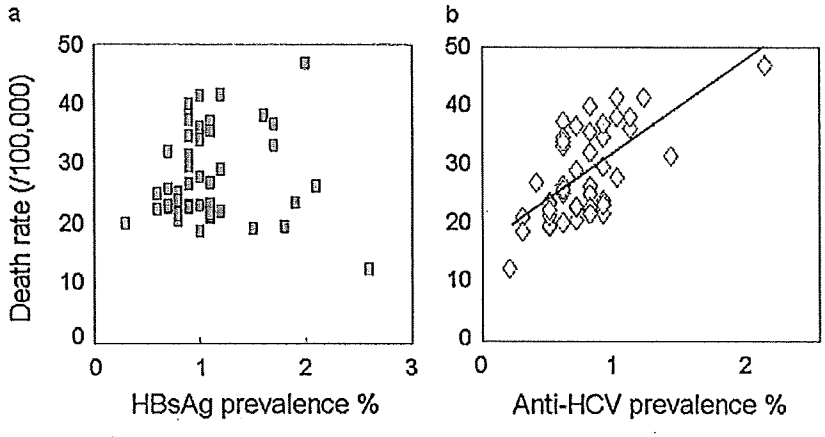

Fig. 7. Relationship between the death rate of primary liver cancer and prevalence of (**a**) HBsAg ($r = 0.02$, P = NS) and (**b**) anti-HCV ($r = 0.66$, $P < 0.001$, $y = 16.3x + 16.1$) among the general population over 40 years of age in 2006

$y = 16.3x + 16.1$), but no correlation with the prevalence of HBsAg was seen (Fig. 6). For instance, although Okinawa Prefecture had the highest prevalence of HBsAg (2.6%), its HCC death rate was the lowest (12.5/100000 persons). A possible explanation for this discrepancy is that the HBV genotype Bj, which shows good clinical prognosis,[10,11] is the dominant HBV genotype in Okinawa. In contrast, areas with high rates of anti-HCV, especially in western Japan, had high death rates from HCC. HCV appears to be the major contributor to primary liver cancer in these regions; Saga Prefecture shows both the highest HCC death rate (46.9/100000) and highest prevalence rate of anti-HCV (2.1%) in Japan.

Table 1. Summary of findings in representative studies on the incidence of hepatocellular carcinoma (HCC) among patients with chronic hepatitis C virus (HCV) infection treated with interferon alone in Japan

	Treated							
	Untreated		Non-SVR		SVR		Total	
Author	No. HCC/no. cases	%	No. HCC/no. cases	%	No. HCC/no. cases	%	No. HCC/no. cases	%
Kasahara[12]			41/709	5.8	5/313	1.6	46/1022	4.5
Imai[13]	19/140	13					18/419	4.3
Ikeda[14]	67/452	15	23/730	3.2	5/461	1.1	28/1191	2.4
Yoshida[15]	67/395	17	214/1556	13.8	27/836	3.2	241/2392	10.1
Okanoue[16]			119/849	14.0	8/397	2.0	127/1246	10.2
Ikeda[17]	59/352	17	34/171	19.9	1/53	1.9	94/576	16.3
Total	212/1339	16	432/4015	10.8	46/2060	2.2	554/6846	8.1

SVR: sustained virological response

Antiviral therapy suppresses the incidence of HCC

As described in prior sections, HCV infection is the major cause of HCC in Japan, suggesting that eradication of HCV may decrease the incidence of HCC. A summary of different studies on the incidence of HCC among patients with chronic hepatitis C who were treated with interferon in Japan can be found in Table 1.[12–17] These studies show a moderate decrease in the risk of HCC in patients with chronic hepatitis C treated with interferon, especially in patients with sustained virological response as compared with nonresponders and nontreated patients.

Recently, Ikeda et al. prospectively studied patients with chronic HCV infection and evidence of occult HBV infection [negative results for HBsAg and HBV DNA but positive results for antibodies to hepatitis B core antigen (anti-HBc) in serological testing].[17] Patients with HCV-related cirrhosis and positive results for anti-HBc were at high risk for HCC, even in patients with a sustained virological response to interferon (IFN) therapy. Thus, anti-HBc positivity is a marker of high risk for HCC among patients with HCV-related cirrhosis.

Between 1992 and 2001, approximately 300000 patients with chronic hepatitis C received IFN monotherapy in Japan. As shown in Fig. 1, it is remarkable that the number of deaths and the death rate of HCC began to decrease in 2005. These phenomena suggest that antiviral treatment indeed reduces the risk of HCC in patients with HCV infection.

Conclusion

The number of deaths and death rate of HCC showed a sharp increase from 1975 onward but had begun to decrease in 2006. Although both HBV and HCV infection play a major role in HCC in Japan, HCV-related HCC represents 70% of all cases. The incidence of HCC without HBsAg or anti-HCV accounts for 7%–15% in Japan, and half of NBNC-HCC cases are of unknown origin. Geographically, HCC is more frequent in western than eastern Japan, and the death rates of HCC in each prefecture correlate with anti-HCV, but not HBsAg, prevalence. IFN therapy for chronic hepatitis C reduces the risk of development of HCC, especially in patients with sustained viral response.

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