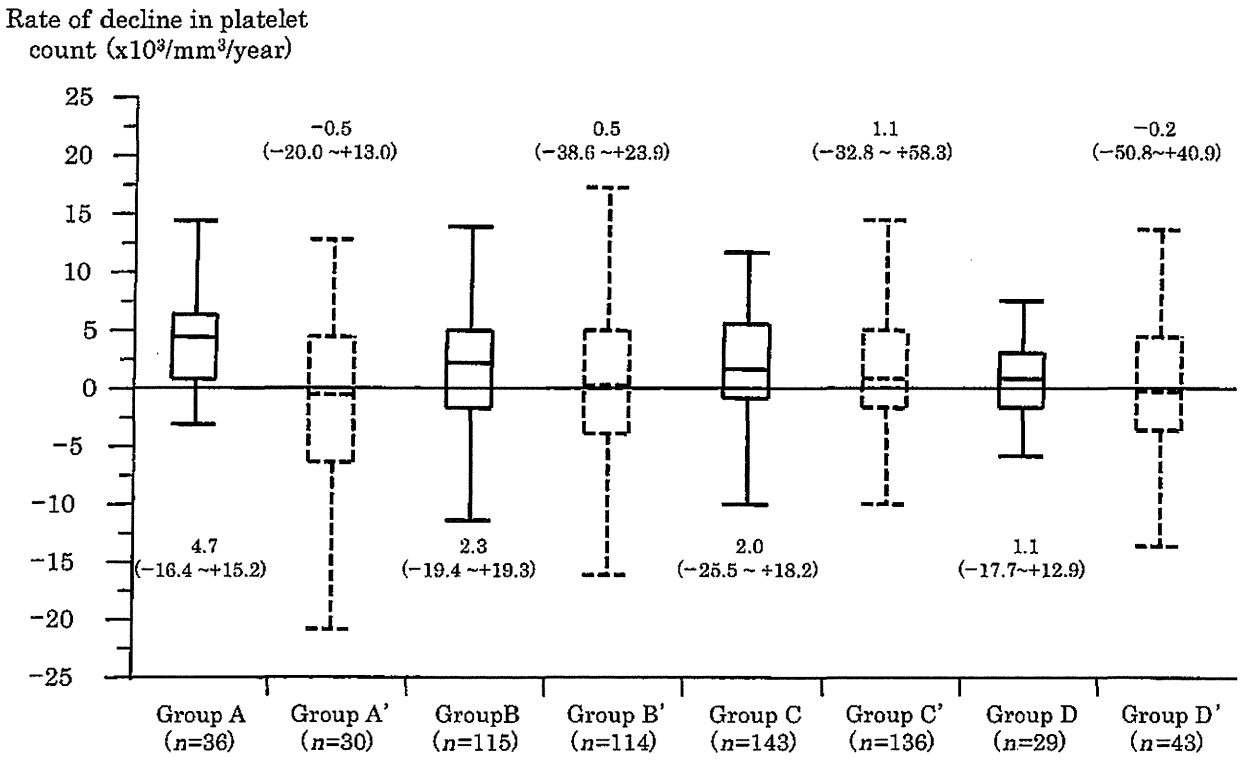

Figure 1 Rate of decline in platelet count prior to hepatocellular carcinoma (HCC) diagnosis in HCC patients and prior to the end of follow-up in control patients. The annual rate of platelet count decline in the period prior to HCC diagnosis was lower in the groups that were older at the time of HCC diagnosis. In control patients, there was no trend toward higher annual rates of platelet count decline in the period prior to the end of follow-up when the patients were classified by age ($P = 0.0247$ and 0.1571, respectively, Jonckheere-Terpstra Test). Group A, HCC diagnosed at age ≤ 60 years; group B, 61–70 years; group C, 71–80 years; group D, > 80 years. group A', control patients ≤ 60 years old at the end of follow-up; group B', 61–70 years; group C', 71–80 years; group D', > 80 years. The annual rate of platelet count decline was significantly lower in group A' than in group A ($P = 0.0039$); however, there were no significant differences when HCC patients in other age groups were compared to their respective matched controls.

lower in group A' than in group A ($P = 0.0039$), and there were no significant differences between group B and group B', group C and group C', and group D and group D'.

The average integration value of ALT in groups A, B, C, and D was 80.9 IU/L (25.3–179.3), 62.3 IU/L (14.5–167.9), 59.0 IU/L (9.9–134.1), and 44.9 IU/L (22.7–91.9), respectively. The average integration value of ALT was significantly lower in patients diagnosed with HCC at an older age (Fig. 2, $P < 0.0001$). There was a similar trend among control patients (Fig. 2, $P < 0.0001$). The average integration values of ALT in groups A', B', C', and D' were significantly lower than in groups A, B, C, and D, respectively ($P < 0.0001$).

Patient profiles at the time of HCC diagnosis are shown in Table 6. There were no significant differences in tumor characteristics and levels of tumor markers among the age groups. Fewer patients in Group D underwent hepatic resection ($P = 0.0293$).

Survival rates according to age at HCC diagnosis. Five and 10-year cumulative survival rates of groups A, B, C, and D were 44.2%, 58.2%, 44.3%, and 33.3% and 22.7%, 31.2%, 26.6%, and not available, respectively (Fig. 3). There were no significant differences in the cumulative survival rate among the four groups.

Discussion

In Japan, the average age of patients with chronic hepatitis, cirrhosis, or HCV-associated HCC is increasing. The number of deaths due to these diseases is also increasing. The age-specific prevalence of HCV seropositivity in the USA is about 30 years below that in Japan; thus, a majority of patients in the USA with chronic HCV infection will reach an advanced age in the near future.[3]

In our study, elderly HCC patients have high platelet counts and low ALT values. In addition, multivariate analysis using propensity-matched control patients revealed that the presence of cirrhosis and high ALT levels (> 20 IU/L) are significantly associated with the development of HCC. However, platelet count is not significantly associated with hepatocarcinogenesis in elderly HCV carriers (≳ 65 years). Physicians should be aware that patients aged 65 years or older could develop HCC regardless of their platelet count.

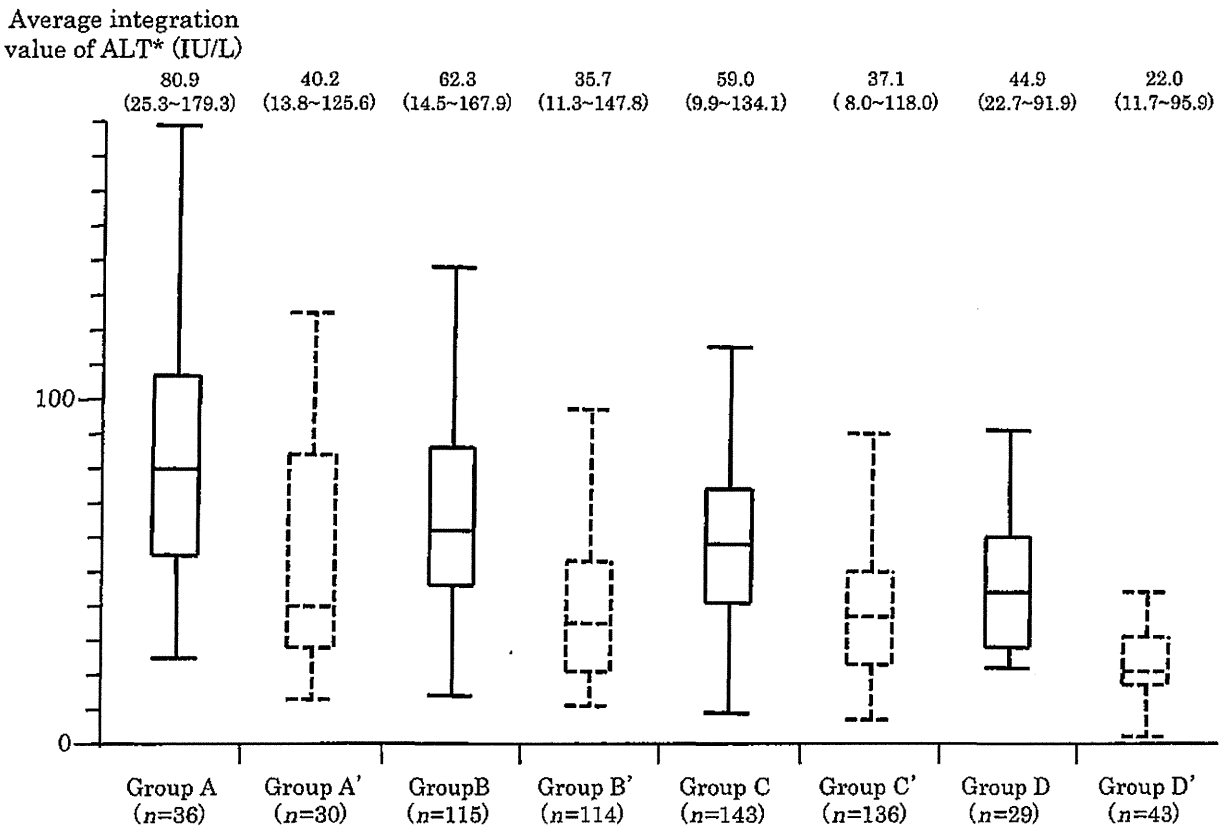

Figure 2 Average integration values of alanine aminotransferase (ALT) prior to HCC diagnosis in HCC patients and prior to the end of follow-up in control patients. Patients who were older at the time of HCC diagnosis had lower average integration values of ALT in the period prior to HCC diagnosis. In control patients, the average integration values of ALT in the period prior to the end of follow-up were lower in the groups that were older at the end of follow-up ($P < 0.0001$ and < 0.0001, respectively, Jonckheere-Terpstra Test). Average integration values of ALT in groups A', B', C', and D' were significantly lower than in groups A, B, C, and D, respectively ($P < 0.0001$).

Table 6 Profile of HCV-infected HCC patients at the time of HCC diagnosis

	Group A ($n = 36$)	Group B ($n = 115$)	Group C ($n = 143$)	Group D ($n = 29$)	*P*
AFP† (ng/mL)	23.9 (0.8–500)	19.8 (0.6–10500)	12.8 (0.8–12680)	17.8 (0.8–99720)	0.2347
AFP-L3† (%)	0 (0–89)	0 (0–87.2)	0 (0–81.0)	0 (0–40.7)	1.0000
DCP† (mAU/mL)	36 (10–36164)	35 (10–5941)	32 (10–50904)	24 (10–6229)	0.5650
Tumor size† (cm)	2.0 (0.8–10.0)	2.0 (0.3–8.8)	2.0 (0.6–11.4)	2.3 (1.0–9.0)	0.3754
Number of tumors†	1 (1–6)	1 (1–8)	1 (1–10)	1 (1–4)	1.0000
Portal thrombus (present/absent)	2/34	3/112	6/137	0/29	0.3293
Stage (I/II/III/IV)	14/15/5/2	41/53/21/0	50/61/29/3	10/12/7/0	0.4957
Initial treatment (HR/PT/TACE/none)	9/18/4/5	47/44/16/8	51/47/33/12	4/11/9/5	0.0293

†Expressed as median (range).

AFP, α-fetoprotein; AFP-L3, *lens culinaris* agglutinin–reactive fraction of AFP; DCP, des-γ-carboxy prothrombin; Group A, diagnosis of HCC at age ≤ 60 years; Group B, 61–70 years; Group C, 71–80 years; Group D, > 80 years; HCC, hepatocellular carcinoma; HCV, hepatitis C virus; HR, hepatic resection; PT; percutaneous treatment including ethanol injection therapy, microwave coagulation therapy, and radiofrequency ablation therapy; TACE, transcatheter arterial chemoembolization.

The male-to-female ratio of HCC patients in Japan has decreased from 4.5 in 1984–1985 to 2.5 in 2002–2003.[1] It is well known that the mean age of female HCC patients with HCV infection is higher than that of males.[18,19] The increased proportion of female patients is considered a result of more older patients with HCV-related HCC. In our study, the proportion of female patients was the highest in group D. Further investigation of the role of sex in hepatocarcinogenesis is needed.

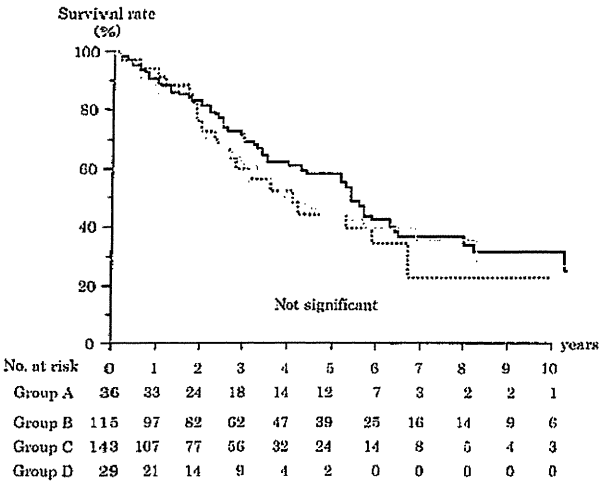

Figure 3 Cumulative survival rate of groups A, B, C, and D according to age at hepatocellular carcinoma (HCC) diagnosis. Kaplan-Meier curves showing the survival rate stratified by age at HCC diagnosis. There were no significant differences in the survival rate among the four groups. ——, A group (≤ 60 years, $n = 36$); ·····, B group (61–70 years, $n = 115$); , C group (71–80 years, $n = 143$); , D group (> 80 years, $n = 29$).

We previously reported that the average integration value of ALT was associated with the cumulative incidence of hepatocarcinogenesis and that minimizing ALT is necessary for the prevention of hepatocarcinogenesis.[20] In addition, we demonstrated a 6.242-fold higher (95% confidence interval: 1.499–25.987) cumulative incidence of hepatocarcinogenesis in patients with average ALT integration values between 20 and 40 IU/L (within the current normal range) than in patients with 20 IU/L or below.[21] In this study, the average integration value of ALT significantly decreased as the age at HCC diagnosis increased. Especially in group D, the average integration value of ALT was 44.9 IU/L (range, 22.7–91.9 IU/L), which is near the upper limit of the conventional reference range of ALT (40 IU/L). There was the same tendency in control patients; however, average integration values of ALT were lower in control patients than HCC patients in each corresponding age group. These data suggest close surveillance for HCC is important even if older patients (≥ 65 years) have low ALT values.

It is likely that low platelet counts account for a large proportion of patients with cirrhosis, consistent with the theory that HCC develops in patients with progressive or advanced liver disease. Cirrhosis is an established risk factor for HCC in patients with HCV.[22,23] It is generally accepted that platelet count is a surrogate marker of liver fibrosis.[24,25] Platelet counts were highest in group D, both at the start of follow-up and at the time of HCC diagnosis. In contrast, there were no differences in platelet counts among control patients without HCC. It is particularly worth noting that group D had the smallest annual decline in platelet count, at levels comparable to the control patients. A previous report showed that the rate of progression of fibrosis to cirrhosis was accelerated by aging.[24] The precise mechanism of this discrepancy is uncertain. Probably, differences in patient selection might account for this discrepancy. We hypothesize that in our study, the increased rate of annual decline in platelet count may be linked to accelerated carcinogenesis occurring in the younger patients. Group D also had the lowest values of AFP, which is considered a marker of hepatic regeneration as well as a HCC tumor marker in viral hepatitis.[26] Taken together, this suggests a weaker inflammatory response in older patients. Further investigation is necessary.

Why do elderly patients progress to HCC even though liver function appears stable? Aging is associated with a number of events at the molecular, cellular, and physiological level that influence carcinogenesis and subsequent cancer growth.[22] Age may be considered as a progressive loss of stress tolerance due to declines in the functional reserve of multiple organ systems.[27] It has been hypothesized that age-associated declines in DNA repair[28] contribute to the development of HCC. The precise relationship between aging and hepatocarcinogenesis remains uncertain. Further assessment of the role of aging in the progression of HCV is needed.

We found no difference in tumor stage among the four groups. The younger groups A and B tended to receive curative therapy more often than the older groups C and D. However, there were no significant differences in survival. We hypothesize that this is due to the aggressive multiple treatments received by elderly patients with good liver function.

One limitation of our study is that histological confirmation was available in only 234 patients (36.2%). However, it is not practical to perform biopsies on all patients because of potential complications. Lu *et al.* reported that the best cutoff platelet count for the diagnosis of cirrhosis is 150×10^3 /mm^3.[29] Therefore, we employed platelet count as a surrogate marker of liver fibrosis in this study.

In conclusion, we demonstrated that elderly HCV-positive patients (≥ 65 years old) with low ALT values developed HCC regardless of their platelet counts. This finding should be taken into account when designating the most suitable HCC surveillance protocol. The optimal screening interval for HCV-infected patients aged 65 years older should be three to four months like cirrhotic patients even in the absence of cirrhosis.

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Comparison of the Efficacy of Ribavirin Plus Peginterferon Alfa-2b for Chronic Hepatitis C Infection in Patients With and Without Coagulation Disorders

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Many patients with coagulation disorders are infected with hepatitis C virus (HCV) that advances to end stage liver disease, resulting in an increased number of deaths. The efficacy of ribavirin and peginterferon combination therapy for chronic HCV infection in patients with coagulation disorders has not been clarified fully. The aim of this study was to evaluate the efficacy and tolerability of combination therapy in this patient population compared with patients who are infected with HCV and do not have coagulation disorders. A total of 226 consecutive chronic hepatitis C patients were treated with combination therapy and divided into two groups: patients with (n = 23) and without coagulation disorders (n = 203). Clinical characteristics, sustained virological response rates obtained by an intention-to-treat analysis, and combination therapy discontinuation rates were compared between the two groups. The sustained virological response rates did not differ significantly between patients with and without coagulation disorders (65.2% vs. 47.8% by intention-to-treat analysis). According to a multivariate analysis, age, alanine aminotransferase, gamma-glutamyltransferase, and HCV genotype were associated significantly with a sustained virological response, whereas whether a patient had a coagulation disorder did not affect the sustained virological response. In conclusion, combination therapy for chronic hepatitis C was comparably effective between patients with and without coagulation disorders and did not result in adverse bleeding. ***J. Med. Virol. 85:228–234, 2013.*** © 2012 Wiley Periodicals, Inc.

KEY WORDS: chronic hepatitis C; interferon; ribavirin; coagulation disorders; hemophilia

INTRODUCTION

Hepatitis C virus (HCV) infection is a widespread viral infection that often leads to chronic hepatitis, cirrhosis, and hepatocellular carcinoma. Until the 1980s, most patients with coagulation disorders became infected with HCV because of the extensive use of untreated factor concentrate. Some of these patients were infected with both hepatitis C and human immunodeficiency virus (HIV) [Brettler et al., 1990; Troisi et al., 1993; Yee et al., 2000; Franchini et al., 2001]. These patients with liver diseases and persistent abnormal transaminase progress to end stage liver disease, resulting in an increased number of liver disease-related deaths. In cases of co-infection with the HIV, the progression of liver disease is more rapid [Sanchez-Quijano et al., 1995; Soto et al., 1997; Benhamou et al., 1999; Ragni and Belle, 2001; De Luca et al., 2002] with a higher mortality rate than

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during HCV monoinfection [Darby et al., 1997; Yee et al., 2000]. The need for treating infection with HCV in patients with coagulation disorders is increasing worldwide.

Sustained virological responders who are negative for serum HCV RNA 6 months after the end of treatment with interferon (IFN) are likely to remain in virological and biochemical remission with histologic improvement [Marcellin et al., 1997; Shiratori et al., 2000]. In addition, IFN therapy reduces the risk of hepatocellular carcinoma among virological or biochemical responders [Imai et al., 1998; Ikeda et al., 1999; Yoshida et al., 1999]. Ribavirin is now used generally in combination with IFN or pegIFN to treat chronic hepatitis C and combination therapy is more effective than IFN monotherapy [Lai et al., 1996; McHutchison et al., 1998; Poynard et al., 1998; Manns et al., 2001].

Previous studies have investigated the efficacy of IFN monotherapy in patients with coagulation disorders and chronic hepatitis C [Makris et al., 1991], and the efficacy of combination therapy with ribavirin and PegIFN in patients with coagulation disorders [Fried et al., 2002a; Mancuso et al., 2006; Posthouwer et al., 2007]. However, there are no reported comparisons of this combination therapy between patients infected with HCV with and without coagulation disorders. In this study, the efficacy and tolerability of ribavirin plus pegIFN were evaluated retrospectively in patients with coagulation disorders and chronic hepatitis C and the results were compared with the responses of patients infected with HCV but without coagulation disorders.

MATERIALS AND METHODS

Patients and Methods

A total of 226 consecutive patients with chronic hepatitis C and a high viral load (serum HCV RNA levels greater than 100 kilo-international units [KIU]) were treated with a combination of pegIFN and ribavirin between December 2004 and March 2007 at Nagoya University Hospital and Ogaki Municipal Hospital. These patients included 23 patients with coagulation disorders (17 with hemophilia A, 4 with hemophilia B, and 2 with von Willebrand disease). All patients were under 75 years old, were anti-HCV antibody-positive, and had serum HCV RNA levels greater than 100 KIU/ml by quantitative PCR assay (Amplicor GT-HCV Monitor Version 2.0; Roche Molecular Systems, Pleasanton, CA) within 12 weeks preceding the therapeutic period. Patients were excluded if they had pretreatment hemoglobin (Hb) levels <10 g/dl, tested positive for serum hepatitis B surface antigen, a history of drug addiction, alcohol abuse, autoimmune hepatitis, primary biliary cirrhosis, a serious psychiatric or medical illness, or were pregnant. To exclude patient bias, only complete cohorts from each hospital were enrolled. HCV genotypes were determined by PCR using genotype-specific primers [Okamoto et al., 1994; Simmonds et al., 1994].

All patients were treated with 1.5 μg/kg of pegIFN α-2b (Peg-Intron®, MSD, Tokyo, Japan) once weekly for 24 weeks in patients infected with HCV genotype 2 or 3 and for 48 weeks in patients infected with HCV genotype 1 or 4. For the 17 patients infected with HCV genotype 1, the treatment duration was extended to 72 weeks because of higher efficacy compared to that obtained after 48 weeks of treatment, but only in cases in which HCV RNA was positive at 12 weeks and negative at 24 weeks from the start of therapy. Treatment was discontinued when a patient's Hb concentration fell below 8.5 g/dl because of drug-induced hemolytic anemia or when a patient's white blood cell count fell below 1,000/mm^3, neutrophil count fell below 500/mm^3, or platelet count fell below 50,000/mm^3. Some patients discontinued treatment because the virus could not be eradicated after 24 weeks, as determined by the physician. The pegIFN alfa-2b dose was reduced to 50% of the assigned dose when the white blood cell count was below 1,500/mm^3, the neutrophil count below 750/mm^3 or the platelet count below 8,000/mm^3. Oral ribavirin (Rebetol®, MSD, Tokyo, Japan) was administered for the same duration as pegIFN at 600 mg/day for patients who weighed 60 kg or less, 800 mg/day for those who weighed more than 60 kg but less than 80 kg, and 1,000 mg/day for those who weighed more than 80 kg during the treatment period. The ribavirin dose was reduced by 200 mg/day when the patient's Hb concentration fell below 10 g/dl because of drug-associated hemolytic anemia. Ribavirin was discontinued when pegIFN therapy was discontinued. Informed consent was obtained from each patient and the study was performed in accordance with the 1975 Declaration of Helsinki.

Liver Histology

Pretreatment liver biopsy specimens were classified based on a fibrosis scale of F0 to F4 (F0, no fibrosis; F1, portal fibrosis without septa; F2, few septa; F3, numerous septa without cirrhosis; and F4, cirrhosis) and in terms of necroinflammatory activity on a scale of A0 to A3 (A0, no histological activity; A1, mild activity; A2, moderate activity; and A3, severe activity) [Bedossa and Poynard, 1996; Fried et al., 2002b]. In patients with coagulation disorders, a liver biopsy was performed using factor concentrate, provided the patients gave informed consent.

Assessment of Efficacy

The virological response was assessed by a qualitative HCV RNA assay with a lower sensitivity limit of 100 copies/ml (Amplicor HCV version 2.0; Roche Molecular Systems). According to the qualitative HCV RNA results, responses were defined as a sustained virological response if no HCV RNA was detected at the end of the 24-week follow-up period after the treatment was completed. A patient was considered to have an end of treatment virological response if no HCV RNA was detected at the end of treatment.

Comparison of Characteristics and Treatment Efficacy Between Patients With and Without Coagulation Disorders

Sex ratio, age, body weight, body mass index (BMI), baseline serum alanine aminotransferase (ALT) levels, gamma-glutamyltransferase (GGT), pretreatment Hb level, platelet counts, HCV genotype and viral load, histologic activity, and fibrosis were compared between patients with and without coagulation disorders. The sustained virological response rates obtained by an intention-to-treat analysis and per-protocol analysis, ribavirin and pegIFN dose reduction rates, and combination therapy discontinuation rates were compared between the two groups. The end of treatment virological response rate was obtained by intention-to-treat and per-protocol analyses and then compared between the two groups. Next, the variable accession method in a multivariate analysis was used to examine factors associated with a sustained virological response after combination therapy, including the following factors: sex, age, BMI, baseline serum ALT, GGT, platelet counts, genotype, HCV RNA concentration, and presence of a coagulation disorder.

Because efficacy differed by the HCV genotype and the patient age, and since all coagulation disorder patients were male, the analysis focused on male, age-matched patients infected with HCV genotype 1. The characteristics and efficacy of treatment were compared in males, and age-matched patients with and without coagulation disorders who were infected with HCV-genotype 1.

Statistical Analysis

Values are expressed as the means ± SDs. Between-group differences in mean quantitative values were analyzed by Student's *t*-test, and differences in nonparametric data were analyzed by the Mann–Whitney *U*-test. Differences in proportions were examined by the Chi-squared test. Multiple logistic regression analysis was used to identify factors related to a sustained virological response. All statistical analyses were performed using SAS software (SAS Institute, Cary, NC). All P values were two-tailed, and $P < 0.05$ was considered statistically significant.

RESULTS

Patient Characteristics

The patients included 127 men and 99 women aged 22–74 years (mean ± SD, 54.7 ± 11.6). The mean age of patients without coagulation disorders was 56.3 ± 10.9 years and most patients were in their 50s and 60s. In contrast, the mean age of patients with coagulation disorders was 41.5 ± 9.8 years with an age distribution ranging from 20 to 50 years. The clinical characteristics of the two study groups are shown in Table I. All patients with coagulation disorders in this study were male because of inherited, sex-linked hemophilia, and two patients in this study had male von Willebrand disease. Patients with coagulation disorders were significantly younger than patients without coagulation disorders ($P < 0.0001$). Although body weight was not different between the two groups, patients with coagulation disorders had a significantly lower BMI than patients without coagulation disorders. Patients without coagulation disorders were infected with HCV genotypes that are not unique to Japan, such as genotypes 1a, 3a, and 4a. Four patients with coagulation disorders were infected with human immunodeficiency virus and one of these patients had achieved a sustained virological response.

Response to Therapy

The ribavirin dose reduction rate tended to be higher in patients without coagulation disorders than in patients with coagulation disorders ($P = 0.0643$). The treatment discontinuation rate did not differ significantly between the two groups. As a result, the sustained virological response rate by an intention-to-treat analysis did not differ significantly between the

TABLE I. Clinical Characteristics of Patients Treated With Combination Therapy

	Total patients (n = 226)	Patients without coagulation disorders (n = 203)	Patients with coagulation disorders (n = 23)	*P* value
Sex ratio (male/female)	127/99	104/99	23/0	<0.0001
Age (years)	54.7 ± 11.6	56.3 ± 10.9	41.5 ± 9.8	<0.0001
Body weight (kg)	60.2 ± 11.1	60.5 ± 11.5	60.5 ± 8.1	0.9972
Body mass index	22.9 ± 3.1	23.1 ± 3.1	21.5 ± 2.5	0.0226
Baseline serum ALT (IU/L)	63.3 ± 56.8	60.9 ± 54.9	84.4 ± 69.1	0.0598
GGT (IU/L)	54.2 ± 63.9	51.4 ± 62.2	78.6 ± 74.4	0.0526
Hemoglobin (g/dl)	14.1 ± 1.3	14.1 ± 1.3	14.4 ± 1.3	0.2714
Platelets ($\times 10^4/\mu l$)	17.8 ± 5.2	17.7 ± 5.2	19.0 ± 5.6	0.2597
Genotype (1a/1b/2a/2b/3a/4a)	7/160/40/15/3/1	0/150/39/14/0/0	7/10/1/1/3/1	<0.0001
HCV RNA (KIU/ml)	1,898.0 ± 1,448.3	1,923.1 ± 1,464.5	1,676.6 ± 1,305.1	0.4404
Activity (A0/A1/A2/A3)	2/108/71/11	2/101/64/11	0/7/7/0	0.3442
Fibrosis (F0/F1/F2/F3)	17/104/49/22	16/97/45/20	1/7/4/2	0.5351

ALT, alanine aminotransferase; GGT, gamma-glutamyltransferase; HCV RNA, hepatitis C virus RNA; KIU, kilo-international units.

TABLE II. Efficacy of Combination Therapy

	Total patients (n = 226)	Patients without coagulation disorders (n = 203)	Patients with coagulation disorders (n = 23)	*P* value
SVR rate (intention-to-treat)	49.6 (112/226)	47.8 (97/203)	65.2 (15/23)	0.1130
SVR rate (per-protocol)	54.4 (111/204)	52.7 (97/184)	70.0 (14/20)	0.1405
ETR rate (intention-to-treat)	84.1 (190/226)	84.7 (172/203)	78.3(18/23)	0.4218
ETR rate (per-protocol)	89.1 (179/201)	89.6 (163/182)	84.2 (16/19)	0.4772
Ribavirin dose reduction rate	44.2 (100/226)	46.3 (94/203)	26.1 (6/23)	0.0643
PegIFN dose reduction rate	34.1 (77/226)	33.5 (68/203)	39.1 (9/23)	0.5891
Combination therapy discontinuation rate	9.8 (22/226)	9.4 (19/203)	13.0 (3/23)	0.5722

SVR, sustained virological response; ETR, end of treatment virological response; PegIFN, peginterferon.

two groups. The sustained virological response rate of patients with coagulation disorders by a per-protocol analysis was higher than that of patients without coagulation disorders, but there was no significant difference. In addition, based on both intention-to-treat and per-protocol analyses, the end of treatment virological response rate did not differ significantly between the two groups (Table II).

Factors associated with a sustained virological response in combination therapy were determined by a multivariate analysis. HCV genotype 1 and 4 versus 2 and 3 ($P = 0.001$, odds ratio 4.353 [95% CI, 1.810–10.469]), baseline serum GGT ($P = 0.003$, odds ratio 1.018 [1.006–1.030]), age ($P = 0.006$, odds ratio 1.053 [1.015–1.093]), and baseline serum ALT ($P = 0.014$, odds ratio 0.991 [0.983–0.998]) were associated significantly with a sustained virological response, but whether or not a patient had a coagulation disorder was not associated significantly with a sustained virological response.

Characteristics and Response of Male, Age-Matched Patients Infected With HCV Genotype 1

The clinical characteristics of the two study groups in the male, age-matched patients infected with HCV genotype 1 are shown in Table III. Body weight, BMI, and Hb levels were significantly lower in patients with coagulation disorders than patients without coagulation disorders ($P = 0.0003$, 0.0027, and 0.0103, respectively).

The treatment discontinuation rate of patients with coagulation disorders did not differ between the two groups. The sustained virological response rate by intention-to-treat and per-protocol analyses did not differ significantly between the two groups (Table IV). Factors associated with a sustained virological response in the male, age-matched, genotype 1 patients treated with combination therapy were determined by a multivariate analysis. BMI ($P = 0.036$, odds ratio 1.810 [1.041–3.145]) and baseline serum GGT ($P = 0.037$, odds ratio 0.981 [0.963–0.999]) were associated significantly with a sustained virological response, but whether or not a patient had a coagulation disorder was not associated significantly with a sustained virological response.

Adverse Events

The reasons for discontinuing combination therapy and the times at which the therapy was discontinued are shown in Table V. Once treatment was discontinued, therapy was not restarted even after the initial symptoms or illness disappeared. There were no bleeding episodes in the patients with coagulation disorders, including patients who received a liver biopsy.

TABLE III. Clinical Characteristics of Male, Age-Matched Patients With Genotype 1 Treated With Combination Therapy

	Total patients (n = 36)	Patients without coagulation disorders (n = 18)	Patients with coagulation disorders (n = 18)	*P* value
Age (years)	42.8 ± 8.0	44.9 ± 5.9	40.7 ± 9.3	0.1136
Body weight (kg)	66.1 ± 11.0	73.4 ± 9.3	60.4 ± 8.7	0.0003
Body mass index	22.7 ± 2.8	24.3 ± 2.3	21.4 ± 2.5	0.0027
Baseline serum ALT (IU/L)	69.8 ± 54.3	63.5 ± 31.7	76.2 ± 70.5	0.4919
GGT (IU/L)	72.7 ± 64.2	74.3 ± 71.1	71.2 ± 58.5	0.8869
Hemoglobin (g/dl)	14.9 ± 1.2	15.4 ± 1.0	14.4 ± 1.2	0.0103
Platelets ($\times 10^4/\mu l$)	19.3 ± 5.4	18.8 ± 4.5	19.8 ± 5.6	0.5773
HCV RNA (KIU/ml)	2,050.8 ± 1,273.4	2,322.8 ± 1,249.1	1,778.8 ± 1,273.5	0.2044
Activity (A0/A1/A2/A3)	0/12/11/0	0/6/5/0	0/6/6/0	0.6723
Fibrosis (F0/F1/F2/F3)	2/11/8/2	1/5/4/1	1/6/4/1	0.9392

ALT, alanine aminotransferase; GGT, gamma-glutamyltransferase; HCV RNA, hepatitis C virus RNA; KIU, kilo-international unit.

TABLE IV. Efficacy of Combination Therapy in Male, Age-Matched Patients With Genotype 1

	Total patients (n = 36)	Patients without coagulation disorders (n = 18)	Patients with coagulation disorders (n = 18)	*P* value
SVR rate (intention-to-treat)	58.3 (21/36)	61.1 (11/18)	55.6 (10/18)	0.7353
SVR rate (per-protocol)	69.0 (20/29)	64.7 (11/17)	75.0 (9/12)	0.5551
ETR rate (intention-to-treat)	77.8 (28/36)	83.3 (15/18)	72.2 (13/18)	0.4227
ETR rate (per-protocol)	93.1 (27/29)	88.2 (15/17)	100.0 (12/12)	0.2182
Ribavirin dose reduction rate	22.2 (28/36)	16.7 (3/18)	27.8 (5/18)	0.7175
PegIFN dose reduction rate	36.1 (13/36)	27.8 (5/18)	44.4 (8/18)	0.2979
Combination therapy discontinuation rate	5.6 (2/36)	0 (0/18)	16.7 (3/18)	0.0704

SVR, sustained virological response; ETR, end of treatment virological response; PegIFN, peginterferon.

DISCUSSION

A previous randomized trial in patients infected with HCV with inherited bleeding disorders showed that the sustained virological response rate improved significantly for patients who were treated with IFN and ribavirin compared to those treated with IFN alone [Fried et al., 2002a]. In addition, both chronic hepatitis C patients with and without coagulation disorders responded similarly to pegIFN and ribavirin combination therapy [Franchini et al., 2006; Posthouwer et al., 2006]. However, the efficacy and tolerability of this combination therapy differed based on the HCV genotype as well as the age, gender, and race of the patients; therefore it is difficult to compare patients with and without coagulation disorders under the same conditions. No report has examined that patients infected chronic hepatitis C with and without coagulation disorders at the same institution and during the same observation period. In addition, there are no reports on the efficacy of combination therapy in patients with chronic hepatitis C with and without coagulation disorders in age-matched patients infected with HCV genotype 1. Therefore, a retrospective study was conducted to evaluate the efficacy and tolerability of ribavirin plus pegIFN in chronic hepatitis C patients with and without coagulation disorders. In the per-protocol analysis, there were no significant differences, but the sustained virological response rate was higher in patients with coagulation disorders than in patients without coagulation disorders. Mancuso et al. [2006] reported that combination therapy with pegIFN alfa-2b plus ribavirin is highly efficacious in hemophiliacs with chronic hepatitis C. In an overall analysis, patients with coagulation disorders had a lower mean age than patients without coagulation disorders. In addition, the BMI of the patients with coagulation disorders was lower than that of patients without coagulation disorders. A multivariate analysis showed that the HCV genotype, baseline serum GGT, age, and baseline ALT were factors associated significantly with a sustained virological response and whether patients had coagulation disorders was not associated with a sustained virological response. Age, especially younger than 40 years old, was a good predictive factor for a sustained virological response, as was reported previously [Poynard et al., 2000; Fried et al., 2002b].

TABLE V. Reasons for Discontinuing Combination Therapy

Reason	Number	Weeks after starting treatment
Patients with coagulation disorders		
Peritonitis due to appendicitis	1	16
Pneumoniae	1	18
No HCV eradication	3	24, 28, 29
IDDM	1	44
Patients without coagulation disorders		
Fatigue	5	1, 2, 4.9, 19
Bleeding from duodenal varies	1	8
Dizziness	1	12
Palpitation	1	13
Cholecystitis	1	16
Symptom of Parkinson's disease	1	16
Fundal hemorrhage	1	17
Hepatocellular carcinoma	2	19, 21
Suspicion of Interstitial pneumonia	1	20
Gastric cancer	2	21, 36
Self-discontinuation	1	24
Neutropenia	1	25
Eruption	1	25
No HCV eradication	7	24, 25, 25, 27, 28, 29, 29

These results suggest that male patients who are infected with HCV genotype 1 and have coagulation disorders will have a higher sustained virological response than patients without coagulation disorders, if the coagulation disorder patients do not discontinue treatment. However, these results do not account for the differences in age. Therefore, male, age-matched patients infected with HCV genotype 1 were evaluated. The characteristics that differed between patients with and without coagulation disorders were body weight, BMI and baseline Hb levels.

In male, age-matched patients infected with HCV genotype 1, the sustained virological response rate based on both intention-to-treat and per-protocol analyses was not different between patients with and without coagulation disorders.

Using a multivariate analysis, whether patients had coagulation disorders was not associated significantly with a sustained virological response. Only BMI and GGT were identified as factors associated with a sustained virological response to combination therapy in male, age-matched patients infected with HCV genotype 1. A previous report showed that GGT levels may represent a surrogate marker of tumor necrosis factor-alpha expression in the liver and explain the importance of serum analyses to in predict the treatment outcome [Taliani et al., 2002]. Several studies revealed that GGT is one predictor of a sustained virological response [Taliani et al., 2002, 2006; Villela-Nogueira et al., 2005]. In western countries, obesity and a high BMI are associated with the absence of a sustained virological response to combination therapy of pegIFN or IFN with ribavirin [Bressler et al., 2003; Camma et al., 2004]. However, in Japan, most of the patients who are treated with combination therapy are not obese and have lower BMIs than patients in western countries. In this population, the mean BMI was 22.7 ± 2.8. In this low BMI population, a higher BMI would be associated with a sustained virological response. However, the reason why a low BMI is associated with the absence of a sustained virological response has not elucidated.

Adverse effects are thought to increase in patients with coagulation disorders; however, there was not a significant difference in adverse effects necessitating discontinuation of pegIFN and ribavirin between patients with and without coagulation disorders (13.0% vs. 9.4%). In addition, severe adverse effects and bleeding adverse effects were not associated with coagulation disorders. A previous report showed that IFN and ribavirin combination therapy may reduce the use of clotting factors in hemophilia patients with chronic hepatitis C [Honda et al., 2005; Yamamoto et al., 2006]. Ribavirin may reduce the side effect of bleeding during combination therapy. In this study, patients with coagulation disorders did not experience an adverse effect of bleeding.

In conclusion, treatment of chronic hepatitis C with combination therapy was effective comparably between patients with and without coagulation disorders and there were no adverse effects of bleeding.

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Baseline Factors and Early Viral Response (Week 4) to Antiviral Therapy With Peginterferon and Ribavirin for Predicting Sustained Virologic Response in Patients Infected With Hepatitis C Virus Genotype 1: A Multicenter Study

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Both baseline predictive factors and viral response at week 4 of therapy are reported to have high predictive ability for sustained virologic response to peginterferon and ribavirin combination therapy in patients with hepatitis C virus (HCV) genotype 1. However, it is not clear how these baseline variables and week 4 response should be combined to predict sustained virologic response. In this multicenter study, the authors investigated the impact of baseline predictive factors on the predictive value of week 4 viral response. Receiver-operating characteristic curve analyses were performed to evaluate the ability of week 4 reduction in HCV RNA levels to predict sustained virologic response in 293 Japanese patients infected with HCV genotype 1b. Analyses were performed in all patients and in patient subgroups stratified according to baseline variables. Overall, week 4 viral reduction demonstrates a high predictive ability for sustained virologic response. The sensitivity, specificity, positive predictive value (PPV), negative predictive value, and accuracy were higher than those of viral reduction at week 12. However, the best cut-off levels differ depending on the baseline factors and they were lower in patients with unfavorable baseline predictors. When patients had the TG/GG rs8099917 genotype, the best cut-off was markedly low with low PPV. Week 4 viral response can be a predictor of sustained virologic response in patients with HCV genotype 1 and is better than week 12 viral response. However, the cut-off levels should be modified based on the baseline predictive variables. ***J. Med. Virol. 85:65–70, 2013.*** © 2012 Wiley Periodicals, Inc.

KEY WORDS: chronic hepatitis C; peginterferon and ribavirin; week 4 viral response; baseline predictive factors, genetic polymorphism near the *IL28B* gene

INTRODUCTION

Although the combination antiviral therapy with peginterferon (PEG-IFN) and ribavirin has increased markedly the rate of patients with a sustained virologic response, that is, the eradication of hepatitis C virus (HCV), only 50% of patients infected with HCV genotype 1 had achieved a sustained virologic response, approximately. Several studies reported that early HCV viral dynamics during therapy have a high

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predictive value for a sustained virologic response in HCV genotype 1-infected patients. Previous studies reported that the response of HCV during combination therapy, that is, the changes in serum HCV RNA levels after starting therapy, has been shown to be an important predictor of the treatment outcome [Zeuzem et al., 2001; Buti et al., 2002; Berg et al., 2006]. Several recent reports have emphasized the importance of evaluating the viral dynamics at 4 weeks after starting therapy to predict a sustained virologic response. A rapid virologic response, in which serum HCV RNA is undetectable at 4 weeks after starting therapy, has been a strong predictive factor of a sustained virologic response reportedly [Martinez-Bauer et al., 2006; Poordad et al., 2008; Martinot-Peignoux et al., 2009; de Segadas-Soares et al., 2009]. In addition to a rapid virologic response, reduced serum HCV RNA levels at 4 weeks after starting therapy has also been reported to have a strong predictive value for the likelihood of achieving sustained virologic response to PEG-IFN and ribavirin combination therapy in patients infected with HCV genotype 1 [Yu et al., 2007; Huang et al., 2010; Toyoda et al., 2011; Marcellin et al., 2012]. These studies suggested that a reduction in HCV RNA levels at week 4 is closely associated with the probability of achieving sustained virologic response.

Aside from early viral response to therapy, several baseline host and viral factors are associated with treatment outcome. Genetic polymorphism near the *IL28B* gene (rs12979860 or rs8099917) is the strongest baseline factor associated with treatment outcome in patients with HCV genotype 1 reportedly [Ge et al., 2009; Suppiah et al., 2009; Tanaka et al., 2009; McCarthy et al., 2010; Rauch et al., 2010]. In addition, studies from Japan have reported that amino acid substitutions at residue 70 of the HCV core region and amino acids 2,209–2,248 of the NS5A region of HCV (i.e., interferon sensitivity-determining region, ISDR) are viral factors associated with treatment outcome in patients with HCV genotype 1b [Enomoto et al., 1996; Akuta et al., 2005, 2007a; Donlin et al., 2007; Maekawa and Enomoto, 2009; Hayes et al., 2011]. Given these various predictors for a sustained virologic response, that is, week 4 viral response and baseline variables, how should they be combined to predict treatment outcome more precisely? In the present study, the authors investigated how to incorporate week 4 viral response to PEG-IFN and ribavirin combination therapy with baseline predictive factors to predict a sustained virologic response.

MATERIALS AND METHODS

Patients and Analyses

In this multicenter study, 682 patients who underwent PEG-IFN alpha-2b and ribavirin combination therapy in a standard treatment regimen at one of the participating institutions, (Musashino Red Cross Hospital, Kurume University Hospital, Shin-Matsudo Central General Hospital, Kagawa Prefectural Central Hospital, and Ogaki Municipal Hospital) between December 2004 and January 2010 were initially included into the retrospective analyses. All patients were infected with HCV genotype 1b; patients with HCV genotype 1a are usually not found in the Japanese general population. Pretreatment HCV RNA levels were ≥ 5.0 $\log_{10}$ IU/ml, based on a quantitative real-time PCR-based method (COBAS AmpliPrep/COBAS TaqMan HCV Test; Roche Molecular Systems, Pleasanton, CA; lower limit of quantification, 1.7 $\log_{10}$ IU/ml: lower limit of detection, 1.0 $\log_{10}$ IU/ml) [Colucci et al., 2007; Pittaluga et al., 2008], because the use of ribavirin along with PEG-IFN is not approved by Japanese National Medical Insurance System for patients with pretreatment HCV RNA levels <5.0 $\log_{10}$ IU/ml. No patients had co-infection with hepatitis B virus or human immunodeficiency virus. All patients had 100% medication adherence for both PEG-IFN and ribavirin during the initial 4 weeks of therapy and 80% or more throughout the treatment period. Among these 682 patients, three baseline factors, genetic polymorphism near the *IL28B* gene, amino acid substitution at residue 70 of the HCV core region, and ISDR sequence had been measured prior to treatment in 405 patients. We excluded 112 of these 405 patients with extended treatment duration up to 72 weeks because the extension of treatment duration might influence outcomes, leaving 293 patients who underwent 48-week standard regimen included in the final sample (Fig. 1).

Receiver-operating characteristic (ROC) analyses were performed to evaluate the value of week 4 reduction in HCV RNA levels in predicting sustained virologic response and an area under the ROC curve (AUROC) was generated. Best cut-off levels were determined based on the sensitivity and specificity. Sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV), and accuracy were also calculated using these cut-off levels. Analyses were performed for all patients and subgroups according to baseline variables. The same analyses were performed on the reduction in HCV RNA levels at week 12 after starting therapy.

The study protocol was in compliance with the Helsinki Declaration and was approved by the ethics committee of each participating institution.

Measurements of Serum HCV RNA Levels, Amino Acid Substitution at Residue 70 in the HCV Core, Amino Acid Sequence of ISDR, and Genetic Polymorphism Near the *IL28B* Gene

After each patient gave informed consent, serum samples were obtained during the patient's regular hospital visits just prior to beginning treatment, every 4 weeks during the treatment period, and during the 24-week follow-up period after treatment. Serum samples were stored at −80°C until they were analyzed. HCV RNA levels were measured using a quantitative

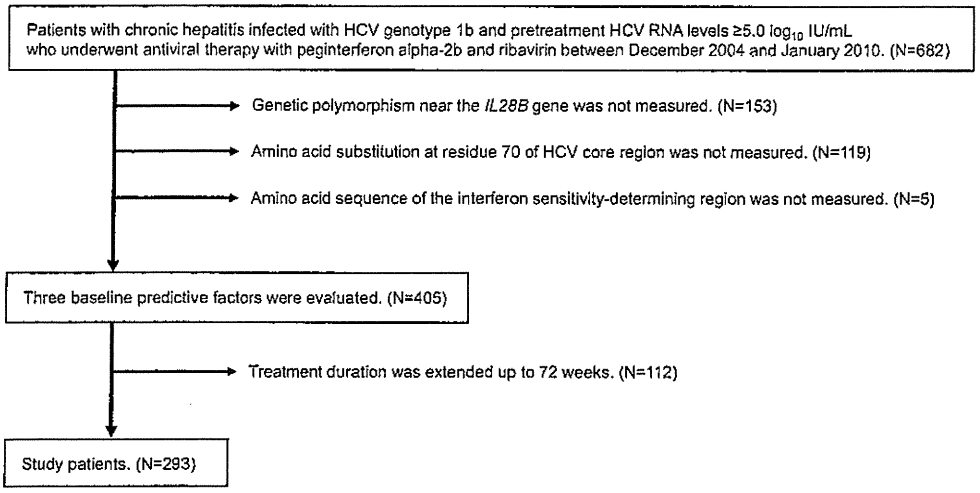

Fig. 1. Schematic representation of the study patients.

real-time PCR-based method (COBAS AmpliPrep/COBAS TaqMan HCV Test). The reductions in HCV RNA 4 and 12 weeks after starting therapy were calculated based on the pretreatment HCV RNA levels. When calculating the reduction in HCV RNA levels, HCV RNA concentration was defined as zero when HCV RNA was undetectable (i.e., rapid virologic response at week 4 and complete early virologic response at week 12).

Amino acid 70 of the HCV core region and the amino acid sequence of the ISDR were analyzed by direct nucleotide sequencing of each region as described previously [Enomoto et al., 1996; Akuta et al., 2007b]. The following PCR primer pairs were used for direct sequencing of the HCV core region:

5′-GCCATAGTGGTCTGCGGAAC-3′ (outer, sense primer), 5′-GGAGCAGTCCTTCGTGACATG-3′ (outer, antisense primer), 5′-GCTAGCCGAGTAGTGTT-3′ (inner, sense primer), and 5′-GGAGCAGTCCTTCGTGACATG-3′ (inner, antisense primer). The following PCR primers were used for direct sequencing of ISDR: 5′-TTCCACTACGTGACGGGCAT-3′ (outer, sense primer), 5′-CCCGTCCATGTGTAGGACAT-3′ (outer, antisense primer), 5′-GGGTCACAGCTCCCTGTGAGCC-3′ (inner, sense primer), and 5′-GAGGGTTGTAATCCGGGCGTGC-3′ (inner, antisense primer). When evaluating the ISDR, HCV was defined as wild-type when there were zero or one amino acid substitutions in residues 2,209–2,248 as compared with the HCV-J strain [Kato et al., 1990], and as non-wild-type when there was more than one substitution.

Genotyping of rs8099917 polymorphisms near the *IL28B* gene was performed using the TaqMan SNP assay (Applied Biosystems, Carlsbad, CA) according to the manufacturer's guidelines. A pre-designed and functionally tested probe was used for rs8099917 (C_11710096_10, Applied Biosystems). Genetic polymorphism of rs8099917 reportedly corresponds to rs12979860 in more than 99% of individuals of Japanese ethnicity [Tanaka et al., 2010]. The TT genotype of rs8099917 corresponds to the CC genotype of rs12979860, the GG genotype of rs8099917 corresponds to the TT genotype of rs12979860, and the TG heterozygous genotype of rs8099917 corresponds to the CT of rs12979860.

RESULTS

Patients Characteristics and Baseline Variables

Table I summarizes patient characteristics. The polymorphism of rs8099917 was TT genotype in 204 patients (69.6%). Amino acid substitution at residue 70 was arginine in 200 patients (68.3%). HCV-ISDR was non-wild-type in 78 patients (26.6%). All these variables (TT genotype of rs8099917, arginine at residue 70, and non-wild-type ISDR) were reportedly associated with favorable response to therapy.

As a final outcome, 113 patients (38.6%) achieved sustained virologic response. Sensitivity, specificity, PPV, NPV, and accuracy were 97%, 48%, 54%, 97%, and 67%, respectively, according to genotypes of rs8099917 near the *IL28B* gene. They were 85%, 42%, 48%, 82%, and 59%, respectively, according to amino acid substitutions at residue 70 in the HCV core region, and 43%, 84%, 63%, 70%, and 78%, respectively, according to ISDR of HCV NS5A region.

Association Between Week 4 Viral Reduction and Treatment Outcome Based on Baseline Predictive Factors

Table II shows the predictive value of a reduction in serum HCV RNA levels at week 4 of therapy in all patients and based on each baseline predictive variable. Week 4 viral reduction demonstrates a high predictive ability for a sustained virologic response with

TABLE I. Characteristics of Study Patients

Age (years), median (range)	60 (20–80)
Sex (male/female) (%)	150 (51.2)/143 (48.8)
BMI, median (range)	22.6 (15.8–33.3)
Prior treatment for HCV (no/yes) (%)	201 (68.6)/92 (31.4)
Initial dose of PEG-IFN (μg), median (range)	80.0 (40.0–150.0)
Initial dose of ribavirin (mg), median (range)	600 (200–1,000)
Pretreatment HCV RNA levels (log_{10} IU/ml), median (range)	6.1 (5.0–7.4)
Platelet count ($\times 10^3/\mu l$)	159 (43–373)
Hemoglobin (g/dl)	13.9 (8.6–18.1)
Neutrophil count (μl^{-1})	2,430 (4,670–7,480)
Alanine aminotransferase (IU/L)	49 (10–485)
Genetic polymorphisms of rs8099917 (TT/TG or GG) (%)	204 (69.6)/89 (30.4)
Amino acid at residue 70 of HCV core (arginine/glutamine or histidine) (%)	200 (68.3)/93 (31.7)
Amino acid sequence of ISDR (non-wild-type/wild-type) (%)	78 (26.6)/215 (73.4)

(N = 293).
BMI, body mass index; HCV, hepatitis C virus; PEG-IFN, peginterferon; ISDR, interferon sensitivity-determining region.

a high AUROC in all patients, in which sensitivity, specificity, PPV, NPV, and accuracy were more than 80%. The best cut-off for the prediction was 3.1-log_{10} reduction. When patients were stratified according to baseline predictive factors, AUROC remained above 0.85, indicating retention of high predictive ability. However, the best cut-off levels differ depending on baseline factors, and they were lower in patients with unfavorable baseline predictors (TG/GG genotype of rs8099917 near the *IL28B* gene, glutamine/histidine at residue 70 of the HCV core region, and wild-type of ISDR). Especially, when patients had the TG/GG rs8099917 genotype, the calculated best cut-off level was markedly lower than that of patients with the TT genotype. Sensitivity, specificity, PPV, NPV, and accuracy were more than 70% in all patient subgroups, except for patients with the TG/GG genotype in whom PPV was only 10%.

Association Between Week 12 Viral Reduction and Treatment Outcome Based on Baseline Predictive Factors

Table III shows the predictive value of a reduction in serum HCV RNA levels at week 12 of therapy in all patients and based on each baseline predictive variable. The predictive ability of week 12 viral reduction for sustained virologic response was decreased in comparison to that of week 4 with a low AUROC in all patients. The specificity, PPV, and accuracy of the prediction at week 12 were also lower than those at week 4. The best cut-off levels increased to 5.0-log_{10} reduction. When patients were stratified according to the genetic polymorphisms of rs8099917 near the *IL28B* gene and according to amino acid substitutions at residue 70 of the HCV core region, the differences of the best cut-off levels based on these baseline factors were less marked than those at week 4, although the best cut-off levels remained lower in patients with unfavorable baseline predictors. The difference of best cut-off levels between patients with TT genotype and with TG/GG genotype of rs8099917 also decreased, but PPV in patients with TG/GG genotype remained low (21%). In contrast, the difference in the best cut-off levels increased when patients were stratified according to amino acid sequences in ISDR. The best cut-off level of the reduction in HCV RNA levels at week 12 for predicting sustained virologic response was higher in patients with HCV of wild-type ISDR, an unfavorable baseline variable, than in patients with HCV of favorable non-wild-type ISDR, which was inverse to the evaluation with week 4 viral reduction in which the cut-off level was higher in patients with HCV of non-wild-type ISDR.

TABLE II. AUROC, Best Cut-Off Level, Sensitivity, Specificity, PPV, NPV, and Accuracy of the Reduction in Serum HCV RNA Levels 4 Weeks After Starting PEG-IFN and Ribavirin Combination Therapy From Pretreatment Levels for Predicting Sustained Virologic Response

	N	AUROC	Best cut-off	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)	Accuracy (%)
Overall	293	0.92746	3.1 log_{10}	88	87	81	92	87
IL28B-TT	204	0.88353	3.2 log_{10}	87	78	82	84	83
IL28B-TG or GG	89	0.84302	1.1 log_{10}	100	69	10	100	70
Core 70-R	200	0.91023	3.2 log_{10}	86	83	82	87	85
Core 70-Q or H	93	0.94350	2.8 log_{10}	88	93	75	97	92
ISDR-non-wild type	78	0.93455	3.0 log_{10}	90	90	94	84	90
ISDR-wild type	215	0.92654	2.9 log_{10}	92	84	71	96	87

AUROC, area under the receiver-operating characteristics curve; PPV, positive predictive value; NPV, negative predictive value; HCV, hepatitis C virus; PEG-IFN, peginterferon; R, arginine; Q, glutamine; H, histidine; ISDR, interferon sensitivity-determining region.

TABLE III. AUROC, Best Cut-Off Level, Sensitivity, Specificity, PPV, NPV, and Accuracy of the Reduction in Serum HCV RNA Levels 12 Weeks After Starting PEG-IFN and Ribavirin Combination Therapy From Pretreatment Levels for Predicting Sustained Virologic Response

	N	AUROC	Best cut-off	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)	Accuracy (%)
Overall	293	0.86907	5.0 $\log_{10}$	88	73	67	91	79
IL28B-TT	204	0.79216	5.11 $\log_{10}$	81	61	70	73	71
IL28B-TG or GG	89	0.92829	4.6 $\log_{10}$	100	87	21	100	88
Core 70-R	200	0.81791	5.0 $\log_{10}$	88	63	69	86	75
Core 70-Q or H	93	0.94272	4.9 $\log_{10}$	100	84	59	100	87
ISDR-non-wild type	78	0.87298	5.0 $\log_{10}$	88	79	88	79	85
ISDR-wild type	215	0.89572	5.4 $\log_{10}$	84	79	63	92	81

AUROC, area under the receiver-operating characteristics curve; PPV, positive predictive value; NPV, negative predictive value; HCV, hepatitis C virus; PEG-IFN, peginterferon; R, arginine; Q, glutamine; H, histidine; ISDR, interferon sensitivity-determining region.

DISCUSSION

This study was conducted to confirm the predictive value of week 4 viral dynamics of HCV for predicting sustained virologic response to the combination therapy with PEG-IFN and ribavirin in patients infected with HCV genotype 1 and with pretreatment HCV RNA levels of $\geq$5.0 $\log_{10}$ IU/ml in a large multicenter study of Japan. The comparison of the predictability for sustained virologic response between week 4 and week 12 viral reductions revealed the higher predictive ability of week 4 viral response. In a recent study, Marcellin et al., [2012] suggested that a $\geq$3 $\log_{10}$ reduction in HCV RNA levels at week 4 of PEG-IFN and ribavirin combination therapy is a reliable factor for predicting sustained virologic response in patients with HCV genotype 1. Our current results are consistent with their analysis for patients with HCV genotype 1b and those with pretreatment HCV RNA levels $\geq$5.0 $\log_{10}$ IU/ml overall. The reduction in HCV RNA levels at week 4 appears to be a good and reliable predictor for a sustained virologic response. Although week 12 viral response (i.e., early virologic response) has been used as a pivotal decision criterion to extend treatment duration or to discontinue treatment, the predictive value is lower when the reduction in HCV RNA levels is compared to week 4 viral response.

When patients were stratified based on baseline predictive factors, however, the best cut-off levels for sustained virologic response were not constant. The cut-off levels decreased in patients with unfavorable baseline factors, that is, TG/GG genotype of rs8099917, glutamine/histidine at residue 70 of the HCV core region, and wild-type sequence of ISDR, indicating that the reduction in HCV RNA occurs slowly in patients with these unfavorable baseline variables. Conversely and paradoxically, the results may indicate that one can expect sustained virologic response in patients with a smaller reduction in HCV RNA levels at week 4 if they have unfavorable baseline variables.

When predictive value was evaluated using week 12 viral reduction, the best cut-off levels remained lower in patients with unfavorable TG/GG rs8099917 genotype and patients with HCV of unfavorable glutamine/histidine at residue 70 of the HCV core region. In contrast, the best cut-off level was higher in patients with HCV of unfavorable wild-type ISDR. Previous studies reported the association between the genetic polymorphisms near the *IL28B* gene (rs12979860 and rs8099917) and amino acid substitution at residue 70 of HCV core region [Abe et al., 2010; Kobayashi et al., 2010], whereas no associations were reported between these two variables and ISDR mutation. This might explain the difference in the relationship of early viral response during therapy between with two baseline predictive factors, *IL28B* genetic polymorphisms and amino acid substitution of HCV core region and with ISDR mutation.

The calculated PPV was markedly low in patients with the unfavorable TG/GG genotype of rs8099917 (CT/TT genotype of rs12979860) both by the evaluations at weeks 4 and 12 viral responses. Therefore, it appears to be difficult to identify patients in this subgroup who are likely to achieve a sustained virologic response by their week 4 viral response, although week 4 viral response can be a factor used to identify patients with a high likelihood of achieving sustained virologic response in other subgroups.

In conclusion, week 4 viral response can be a predictor of sustained virologic response in patients with HCV genotype 1. However, the cut-off levels should be modified based on baseline host and viral predictive variables. In addition, week 4 viral response is not predictive in patients with unfavorable genotype of genetic polymorphism near the *IL28B* gene.

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PLOS ONE

Direct Association of Heat Shock Protein 20 (HSPB6) with Phosphoinositide 3-kinase (PI3K) in Human Hepatocellular Carcinoma: Regulation of the PI3K Activity

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Abstract

HSP20 (HSPB6), one of small heat shock proteins (HSPs), is constitutively expressed in various tissues and has several functions. We previously reported that the expression levels of HSP20 in human hepatocellular carcinoma (HCC) cells inversely correlated with the progression of HCC, and that HSP20 suppresses the growth of HCC cells via the AKT and mitogen-activated protein kinase signaling pathways. However, the exact mechanism underlying the effect of HSP20 on the regulation of these signaling pathways remains to be elucidated. To clarify the details of this effect in HCC, we explored the direct targets of HSP20 in HCC using human HCC-derived HuH7 cells with HSP20 overexpression. HSP20 proteins in the HuH7 cells were coimmunoprecipitated with the p85 regulatory subunit and p110 catalytic subunit of phosphoinositide 3-kinase (PI3K), an upstream kinase of AKT. Although HSP20 overexpression in HCC cells failed to affect the expression levels of PI3K, the activity of PI3K in the unstimulated cells and even in the transforming growth factor-α stimulated cells were downregulated by HSP20 overexpression. The association of HSP20 with PI3K was also observed in human HCC tissues *in vivo*. These findings strongly suggest that HSP20 directly associates with PI3K and suppresses its activity in HCC, resulting in the inhibition of the AKT pathway, and subsequently decreasing the growth of HCC.

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Introduction

Heat shock proteins (HSPs) are induced by a variety of stresses, such as heat and chemical stress [1]. HSPs have recently been classified into seven groups, including HSPA (HSP70), HSPB (small HSPs), HSPC (HSP90) and HSPH (HSP110) [2,3]. High-molecular-weight HSPs, such as HSPA (HSP70), HSPC (HSP90) and HSPH (HSP110), have been well characterized and are recognized to act as molecular chaperones which prevent the aggregation of unfolded proteins, giving them a cytoprotective function [1,4]. On the other hand, the human genome also encodes at least 10 small HSPs [3,5]. Small HSPs (HSPB) with monomeric molecular masses ranging from 15 to 30 kDa, such as HSP27 (HSPB1), αB-crystallin (HSPB5) and HSP20 (HSPB6) are constitutively expressed in cells and tissues such as skeletal, smooth and cardiac muscles. The HSPB family is currently considered to play essential roles, such as in protein intracellular transport and in protecting the cytoskeletal architecture [3]. The small HSPs have significant similarities in terms of their amino acid sequences, known as the α-crystallin domain [3,6]. Among the ten known small HSPs, it has been shown that HSP20 (HSPB6) has particularly versatile functions, and is associated with processes ranging from insulin resistance, to the prevention of vasospasms, to airway smooth muscle relaxation, and also has been demonstrated to have a protective function in the heart [7–10]. We have previously shown that HSP20 acts extracellularly to inhibit the platelet aggregation induced by thrombin or botrocetin [11,12]. However, the exact roles of HSP20 have not yet been fully clarified.

Human hepatocellular carcinoma (HCC) is the fifth most common cancer and is the third leading cause of cancer-related death worldwide. Even after resection of the primary tumor, recurrence is common, and the survival rate is 30–40% at five-year post-surgery [13]. There is accumulating evidence that the growth factor receptor signaling pathways are dysregulated in human HCC [14,15]. Phosphoinositide 3-kinase (PI3K) phosphorylates phosphatidylinositol lipids in response to various growth factors. PI3K, which transmit signals from growth factor receptor tyrosine kinases (RTKs), consists of heterodimers of the p85 regulatory subunit and the p110 catalytic subunit [16–18]. The primary function of the p85 regulatory subunit is to bind, stabilize and inhibit the p110 catalytic subunit until RTK activation [19,20].

The p110 catalytic subunit of activated PI3K converts the plasma membrane lipid phosphatidylinositol-4,5-bisphosphate (PIP2) to phosphatidylinositol-3,4,5- triphosphate (PIP3).

The p110 catalytic subunit is encoded by three genes, α, β and δ. The p110α and p110β isoforms are ubiquitously expressed, while p110δ is largely leukocyte-specific [21]. Phosphatase and tensin homologue (PTEN), a phosphatase that catalyzes the dephosphorylation of the 3 position of PIP3, plays an important role as a negative regulator of the PI3K-AKT pathway [16,17]. Phosphoinositide-dependent kinase 1 (PDK1), which is associated with PIP3, stimulates the phosphorylation of AKT, resulting in its activation. The activation of the AKT pathway affects cell growth, the cell cycle, cell survival and cytoskeletal rearrangement [16,18]. Constitutive PI3K-AKT activation reportedly induces cancers of the endometrium, thyroid, prostate, breast, intestine and liver [18].

Studies on the pathogenesis of HCC have identified mutations in PI3K [13], and knockdown of the p85 regulatory subunit of PI3K in the mouse liver activates AKT and induces aggressive and high-grade HCC [22]. In our previous study [23,24], we reported that the HSP20 expression levels inversely correlate with the TNM stage of human HCC, and that the overexpression of HSP20 in human HCC-derived HuH7 cells represses cell proliferation. The activation of the AKT pathway and the mitogen-activated protein kinases pathways, including the extracellular signal-regulated kinase (ERK) and c-Jun N-terminal kinase pathways induced by either transforming growth factor-α (TGFα) or hepatocyte growth factor, are suppressed in HSP20-overexpressing cells [24]. However, the exact mechanisms behind the effects of HSP20 in HCC remain to be elucidated.

The aim of this study was to clarify the direct target of HSP20 involved in the inhibition of the AKT pathway in HCC. We herein demonstrate that HSP20 interacts with PI3K and downregulates its activity in HCC.

Materials and Methods

Antibodies, Chemicals and Plasmids

HSP20 antibodies were purchased from Enzo Life Sciences Inc. (Farmingdale, NY, USA) and EMD Millipore Corp. (Billerica, MA, USA). Antibodies against PI3K p85, PI3K p110α, PI3K p110β, ERK (p44/p42 mitogen-activated protein kinase), MEK, Ras, AKT, phospho-AKT (Ser-473), phospho-AKT (Thr-308), PTEN and rabbit-IgG (peroxidase-conjugated, conformation specific) were purchased from Cell Signaling Technology, Inc. (Danvers, MA, USA). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) antibodies and rabbit IgG were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA). Wild-type human HSP20 cDNA (clone ID 6074542), which was obtained from Open Biosystems, Inc. (Huntsville, AL, USA), was subcloned into the eukaryotic expression vector, pcDNA 3.1(+), as described previously [24]. The eukaryotic expression vector, pcDNA 3.1(+), Dynabeads protein A and Trizol reagent were purchased from Life Technologies Corp. (Carlsbad, CA, USA). Recombinant human TGFα was obtained from R&D systems Inc. (Minneapolis, MN, USA). LY294002 was purchased from ENZO Life Sciences Inc. The Omniscript Reverse Transcriptase kit was purchased from QIAGEN (Hilden, Germany). Fast-start DNA Master SYBR Green I was purchased from Roche Diagnostics K.K. (Basel, Switzerland). The BCA protein assay kit was obtained from Thermo Fisher Scientific Inc. (Waltham, MA, USA). The PI3K activity enzyme-linked immunosorbent assay (ELISA) kit (PI3-Kinase Activity ELISA: Pico) was purchased from Echelon Biosciences Inc., (Salt Lake City, UT, USA). All other materials and chemicals were obtained from commercial sources.

Cell Culture and Transient Transfections

Human HCC-derived HuH7 cells were obtained from the Health Science Research Resources Bank (Tokyo, Japan). The HuH7 cells were maintained in Roswell Park Memorial Institute (RPMI) 1640 (Sigma-Aldrich Corp., St. Louis, MO, USA) medium supplemented with 1% fetal calf serum (FCS) (Hyclone Corp., Logan, UT, USA). For transfections, the HuH7 cells were cultured in 90 mm diameter dishes (1×10^6 cells/dish) and transfected with 4 μg of the wild-type HSP20 plasmid or control (empty) pcDNA3.1(+) vector using the UniFector transfection reagent (B-Bridge International, Mountain View, CA, USA) in 4 ml of RPMI1640 medium without FCS. One day after transfection, the medium was changed to 6 ml of RPMI1640 medium with 1% FCS for coimmunoprecipitation and real-time RT-PCR experiments, or without FCS for the PI3K activity assay. The cells were then cultured for another 24 h.

Protein Preparation

For coimmunoprecipitation, the transfected cells and snap-frozen HCC tissues were lysed in ice-cold TNE lysis buffer (10 mM Tris-HCl, pH 7.8, 1% Nonidet P-40, 150 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol, 1 mM sodium fluoride, 1 mM sodium vanadate and protease inhibitor cocktail (Roche Diagnostics K.K.)). The lysates were then centrifuged at 10,000×*g* at 4°C for 30 min, and the supernatant was collected as TNE-soluble proteins. For the Western blot analysis of AKT and phospho-AKT, the transfected cells were pretreated with the indicated concentrations of LY294002 or vehicle for 60 min, and then stimulated with 20 ng/ml TGFα or vehicle for 10 min. After stimulation, the cells were lysed, homogenized and sonicated in lysis buffer, as described previously [23,24].

Coimmunoprecipitation

The indicated antibodies were added to the TNE-solubilized proteins, and the mixture was shaken gently overnight at 4°C, followed by the addition of 50 μl of Dynabeads protein A and incubation for a further 1 h with continuous mixing. Protein immunocomplexes were isolated with the use of a magnetic particle concentrator (6-tube magnetic separation rack, New England BioLabs Inc., Ipswich, MA, USA). The immunoprecipitated proteins and TNE-soluble proteins (for analysis total protein) were resuspended in the loading buffer for sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE), heated at 95°C for 5 min, and analyzed by a Western blot analysis using peroxidase-labeled rabbit IgG (conformation specific) (L27A9) monoclonal antibodies (Cell Signaling Technology, Inc.).

Western Blot Analysis

A Western blot analysis was performed as described previously [23]. Briefly, SDS-PAGE was performed by the method described by Laemmli [25]. The proteins in the gel were transferred onto polyvinylidene fluoride (PVDF) membranes, which were then blocked with 5% fat-free dry milk in phosphate-buffered saline (PBS) with 0.1% Tween20 for 1 h before incubation with the indicated primary antibodies. Peroxidase-labeled rabbit IgG antibodies were used as secondary antibodies. The peroxidase activity on the PVDF membranes was visualized on X-ray film by means of an ECL Western blotting detection system (GE Healthcare, Waukesha, WI, USA) as described in the manufacturer's protocol.

PI3K Activity Assay

The cultured HSP20-overexpressing cells were stimulated with or without 20 ng/ml TGFα for 10 min. After stimulation, the PI3K activity in the cells was determined using a PI3-Kinase Activity ELISA kit according to the manufacturer's instructions. The absorbance of samples was measured at 450 nm with an EL 340 Bio Kinetic Reader (Bio-Tek Instruments, Inc., Winooski, VT, USA).

Real-time RT-PCR

Total RNA was isolated and transcribed into complementary DNA using the Trizol reagent and Omniscript Reverse Transcriptase Kit, respectively. Real-time RT-PCR was performed using a Light Cycler system (Roche Diagnostics) in capillaries with the Fast-Start DNA Master SYBR Green I provided with the kit. Sense and antisense primers were synthesized based on the report by Biéche *et al.* for human PI3KR1 mRNA [26]. The sense and antisense primers for human GAPDH mRNA were purchased from Takara Bio Inc. (Tokyo, Japan) (primer set ID:HA067812). The PI3KR1 mRNA levels were normalized to those of GAPDH mRNA.

Tissue Specimens

HCC tissues were obtained by surgical resection from patients in the Department of Surgery, Ogaki Municipal Hospital (Gifu, Japan) according to a protocol approved by the committee for the conduct of human research at Ogaki Municipal Hospital and at Gifu University Graduate School of Medicine. Written informed consent was obtained from all of the patients.

Statistical Analysis

The data are expressed as the means ± SD. The statistical significance of the data from the cell culture experiments was analyzed using the Mann-Whitney U test. All P values were derived from two-tailed tests, and values of $P<0.05$ were considered to statistically significant. Each experiment was repeated three times with similar results.

Results

HSP20 does not Directly Interact with AKT or ERK in HCC Cells

In our previous study [23,24], we showed that HSP20 is expressed in the tumor tissues of human HCC. However, HCC cell lines do not express the HSP20 protein. Therefore, we transfected wild-type HSP20 cDNA into HuH7 cells, a HCC-derived cell line, to make them express the HSP20 protein, and then analyzed its function.

We also previously demonstrated, that HSP20 inhibits the activation of AKT and ERK via MEK, the upstream kinase of ERK, in human HCC tissues [24]. In cardiomyocytes, HSP20 reportedly interacts with phosphorylated AKT and maintains it in its phosphorylated state [27]. Therefore, we first investigated whether HSP20 directly interacts with these signal molecules, AKT, ERK and/or MEK, in the HuH7 cells. Although HSP20 was overexpressed in the transfected HuH7 cells (Figure 1A, lane 2 in comparison with lane 1), it was not coimmunoprecipitated with AKT, ERK or MEK (Figure 1A, lane 4).

We then examined whether HSP20 could interact with Ras, which is considered to function upstream of MEK [28]. Although the Ras protein was highly expressed in both the empty and HSP20 vector-transfected HuH7 cells (Figure 1B, lanes 1 and 2), it was not coimmunoprecipitated with HSP20 (Figure 1B, lane 4).

It is generally recognized that PI3K is an upstream kinase of AKT [16,17]. We therefore confirmed that PI3K acts upstream of AKT in the HCC cells. A PI3K inhibitor, LY294002 [29], dose-dependently suppressed the TGFα-induced AKT phosphorylation at both the serine and threonine residues in the HuH7 cells, suggesting that PI3K also regulates the AKT activity in HCC cells (Figure 2).

HSP20 Represses the PI3K Activity in HCC Cells

Next we examined whether HSP20 affects the PI3K activity in the HuH7 cells. We found that the basal activity level of PI3K in the HSP20-overexpressing HuH7 cells was significantly repressed compared with that in the empty vector-transfected cells (Figure 3A). Accumulating evidence suggests that the protumorigenic growth factor signaling pathways, such as the TGFα/epidermal growth factor (EGF) signaling pathway, are dysregulated in human HCC [14,15]. PI3K is activated by RTKs, including the receptor for TGFα [16,18,19]. We have previously reported that HSP20 inhibits the TGFα-stimulated AKT signaling pathway in HuH7 cells [24]. Therefore, we examined the PI3K activity in the HSP20-overexpressing HuH7 cell in the presence of TGFα stimulation. Compared with basal state, the PI3K activity in the empty vector-transfected HuH7 cells was enhanced by TGFα stimulation (Figure 3B, left column in comparison to Figure 3A, left column). The TGFα-induced PI3K activity was significantly repressed in the HSP20-overexpressing HuH7 cells (Figure 3B, right column).

HSP20 does not Affect the Expression of PI3K or PTEN in HCC Cells

To clarify the effect of HSP20 on PI3K in HCC, we examined the protein levels of PI3K in the HSP20-overexpressing HuH7 cells. The levels of both PI3K p85 and PI3K p110α were not affected by HSP20 overexpression (Figure 4A). In addition, the expression level of PI3K p85 mRNA also did not show any significant difference in the cells with HSP20 overexpression compared with that in the control cells (Figure 4B). These findings suggest that HSP20 does not affect the synthesis or stability of PI3K in HCC cells.

PTEN, a tumor suppressor protein, acts as a lipid phosphatase on PIP3, and prevents AKT activation [16,17]. We examined whether HSP20 affects PTEN in the HCC cells. As shown in Figure 4C, the protein levels of PTEN were not changed by HSP20 overexpression. Furthermore, the HSP20 in the HuH7 cells was not coimmunoprecipitated with PTEN (Figure 4D).

HSP20 Directly Interacts with PI3K in HCC Cells

Next, we examined whether HSP20 directly interacts with PI3K in HCC cells. As shown in Figure 5A, the HSP20 protein in the HSP20-overexpressing HuH7 cells was coimmunoprecipitated with PI3K p85, the regulatory subunit, and also with PI3K p110α and PI3K p110β, the catalytic subunits (Figure 5A, lane 2 in comparison with lane 1). In addition, HSP20 antibodies pulled down PI3K p85, PI3K p110α and PI3K p110β from the HSP20-overexpressing HuH7 extracts (Figure 5B, lane 2 in comparison with lane 1). We also demonstrated that the overexpressed HSP20 protein in the HuH7 cells was also immuoprecipitated by HSP20 antibodies (Figure 5B). Furthermore, we found that PI3K p85, PI3K p110α, PI3K p110β and HSP20 were not immunoprecipitated with normal rabbit IgG (Figure 5C). These results suggest that the HSP20 protein directly interacts with the PI3K protein in HCC cells.