Hepatol Int (2009) 3:468–479
DOI 10.1007/s12072-009-9134-1

ORIGINAL ARTICLE

A randomized trial of 24 versus 48 weeks of peginterferon α-2a in patients infected with chronic hepatitis C virus genotype 2 or low viral load genotype 1: a multicenter national study in Japan

Yoshiaki Iwasaki · Yasushi Shiratori · Shuhei Hige · Shuhei Nishiguchi · Hitoshi Takagi · Morikazu Onji · Haruhiko Yoshida · Namiki Izumi · Yutaka Kohgo · Kyosuke Yamamoto · Nobuhiro Sato · Akitaka Shibuya · Hidetsugu Saito · Michio Sata · Kazuyuki Suzuki · Shuichi Kaneko · Mitsuhiko Moriyama · Masao Omata

Received: 16 December 2008 / Accepted: 20 April 2009 / Published online: 22 May 2009

Abstract In a country such as Japan with the average age of patients with chronic hepatitis C treated with antivirals sometimes well above 60 years, the standard combination therapy is not well tolerated. In this randomized, prospective, controlled trial, we investigated the efficacy of 24-week peginterferon α monotherapy for easy-to-treat patients. A total of 132 patients chronically infected with hepatitis C virus (HCV) genotype 2 ($n = 115$) or low viral load HCV genotype 1 (<100 kIU/ml, $n = 17$) were treated with peginterferon α-2a (180 μg/week). Patients with a rapid virological response (RVR, HCV RNA negative or <500 IU/ml at week 4) were randomized for a total treatment duration of 24 (group A) or 48 (group B) weeks. Patients who did not show RVR (group C) were treated for 48 weeks. Sustained virological response (SVR) was assessed by qualitative reverse-transcription polymerase chain reaction. One hundred eight of 132 (82%) patients with RVR were randomized. SVR rates were 60% (group A), 79% (group B), and 27% (group C), respectively. Similar SVR rates were achieved in patients infected with HCV genotype 2 with low pretreatment viral load (<1000 kIU/ml) in group A (81%) and group B (79%) ($P = 0.801$), whereas in those with higher viral load (≥1000 kIU/ml), a lower SVR rate was identified in group A (26%) than in group B (67%) ($P = 0.041$). In

This study is conducted on behalf of the Japanese Consortium for the Study of Liver Diseases.

Clinical Trial Registry: www.umin.ac.jp/ctr/index.htm; identifier: UMIN000001067.

Y. Iwasaki (✉) · Y. Shiratori
Department of Gastroenterology and Hepatology, Okayama University Graduate School of Medicine, Dentistry, and Pharmaceutical Sciences, 2-5-1, Shikata-cho, Okayama 700-8558, Japan
e-mail: yiwasaki@cc.okayama-u.ac.jp

S. Hige
Department of Internal Medicine, Gastroenterology and Hematology Section, Hokkaido University Graduate School of Medicine, Hokkaido, Japan

S. Nishiguchi
Division of Hepatobiliary and Pancreatic Diseases, Department of Internal Medicine, Hyogo College of Medicine, Hyogo, Japan

H. Takagi
Department of Medicine and Molecular Science, Gunma University Graduate School of Medicine, Gunma, Japan

M. Onji
Department of Gastroenterology and Metabiology, Ehime University Graduate School of Medicine, Ehime, Japan

H. Yoshida · M. Omata
Department of Gastroenterology, University of Tokyo Graduate School of Medicine, Tokyo, Japan

N. Izumi
Division of Gastroenterology and Hepatology, Musashino Red Cross Hospital, Tokyo, Japan

Y. Kohgo
Division of Gastroenterology and Hematology/Oncology, Department of Medicine, Asahikawa Medical College, Hokkaido, Japan

K. Yamamoto
Department of Internal Medicine, Saga Medical School, Saga, Japan

N. Sato
Department of Gastroenterology, Juntendo University School of Medicine, Tokyo, Japan

conclusion, in patients infected with HCV genotype 2 and pretreatment viral load below 1000 kIU/ml who achieve RVR, 24-week treatment with peginterferon α-2a alone is clinically sufficient. Those who show no RVR or have higher baseline viral load, require alternative therapies.

Keywords Randomized trial · Chronic hepatitis C · Peginterferon-α monotherapy · Rapid virological response · Genotype 2 · Pretreatment viral load

Introduction

Hepatitis C virus (HCV) infection may progress to chronic hepatitis, cirrhosis, and hepatocellular carcinoma [1–3]. Interferon (IFN)-based treatment of HCV-infected patients can achieve viral clearance and thereby improve histology and prognosis [4, 5]. Thus, the primary aim of antiviral therapy in patients with chronic hepatitis C is a sustained virological response (SVR), defined as undetectable serum HCV RNA by a sensitive molecular assay 24 weeks after the end of treatment.

A combination therapy of peginterferon and ribavirin is currently recognized as the standard treatment of chronic hepatitis C, resulting in 40–50% of SVR rate in patients infected with HCV genotype 1 and around 80% in those infected with HCV genotype 2 or 3 [6–8]. The combination therapy, however, tends to be associated with adverse events more frequently than those that occur with IFN monotherapy [9–14], resulting in dose reduction or discontinuation of therapy and thus impaired response rate particularly in elderly patients [15]. Furthermore, patients with renal failure, ischemic vascular disease, and congenital hemoglobin abnormalities never tolerate ribavirin treatment of their chronic hepatitis C.

In Japan, the Bureau of National Health Insurance provides reimbursement for 24-week interferon α-2b plus ribavirin combination therapy for patients with chronic hepatitis C with high viral load or re-treatment, irrespective of viral load, since December 2001 and for 48 weeks of peginterferon α-2a monotherapy for all patients with chronic hepatitis C since December 2003. The bureau started to provide reimbursement for 24-week peginterferon α-2b and ribavirin therapy for those infected with HCV genotype 2 and high viral load or re-treatment, irrespective of viral load, since December 2005. Thus, Japanese patients infected with HCV genotype 2 and high viral load or re-treatment irrespective of viral load have been able to receive either peginterferon α monotherapy or combination therapy with ribavirin since December 2003.

There are three major phase II/III or phase III clinical trials of peginterferon α monotherapy in patients with chronic hepatitis C [16–18]. All three studies indicate that the long-acting pegylated forms of IFN-α are more potent than standard IFN-α monotherapies. Factors independently associated with SVR to peginterferon α include viral genotype, low pretreatment viral load, age, no cirrhosis, and body surface area [18]. The reported SVR rate in patients with HCV genotype 2 infection and a baseline viral load of less than 2 million copies/ml is around 60% or more [16, 17]. A phase II study of 48-week peginterferon α-2a therapy conducted in Japan demonstrated an SVR rate as high as 71% in patients with HCV genotype 2 infection [19]. Furthermore, 85% of the patients, who had undetectable levels of HCV RNA after 4 weeks of therapy, had an SVR [19]. Thus, data on viral kinetics have led to the hypothesis that in these patients, 24 weeks of treatment may be as effective as the recommended course of 48 weeks. Therefore, 48-week therapy may lead to overtreatment in some patients who have a rapid virological response (RVR). Shorter treatment duration should also be associated with better tolerability and lower rate of premature discontinuation of therapy. This is particularly relevant to elderly patients with HCV genotype 2 infection who can less tolerate the combination therapy with ribavirin and/or a longer treatment period. However, whether the duration of treatment with peginterferon α alone can be reduced from 48 to 24 weeks in patients chronically infected with HCV genotype 2 or low viral load HCV genotype 1 without compromising antiviral efficacy is not clear at present.

Therefore, the aim of this study was to compare the efficacy and safety of peginterferon α-2a administered alone for 24 or 48 weeks in patients with chronic HCV genotype 2 infection or low viral load HCV genotype 1 and had a virological response at week 4.

A. Shibuya
Gastroenterology Division of Internal Medicine, Kitasato University Hospital, Kanagawa, Japan

H. Saito
Department of Internal Medicine, School of Medicine, Keio University, Tokyo, Japan

M. Sata
Division of Gastroenterology, Department of Medicine, Kurume University School of Medicine, Fukuoka, Japan

K. Suzuki
First Department of Internal Medicine, Iwate Medical University, Iwate, Japan

S. Kaneko
Department of Cancer Gene Therapy, Kanazawa University Graduate School of Medicine, Ishikawa, Japan

M. Moriyama
Division of Gastroenterology and Hepatology, Department of Medicine, Nihon University School of Medicine, Tokyo, Japan

Materials and methods

Patients

Adult patients with chronic HCV infection who had the following characteristics were eligible for the study: (1) a positive test for anti-HCV antibody, (2) HCV genotype 1 and an HCV RNA level less than 100,000 IU/ml or HCV genotype 2 irrespective of viral load, (3) entry neutrophil and platelet counts and hemoglobin level of at least 1500/μl, 90,000/μl, and 10 g/dL, respectively. Patients with the following criteria were excluded: other viral infections such as infection with hepatitis B virus or human immunodeficiency virus; any other cause of liver disease such as autoimmune hepatitis, primary biliary cirrhosis, drug-induced liver disease, and excessive daily intake of alcohol; relevant disorders including decompensated liver disease, hepatocellular carcinoma, and other malignant neoplastic disease; concomitant use of immunosuppressive or herbal medications such as Sho-saiko-to; current illicit drug use; neurological or psychiatric diseases; and allergic to peginterferon α-2a or other interferons and biological preparations including vaccines.

Study design

The current study was an investigator-initiated study. This multicenter, open-label, randomized, controlled trial was originally discussed and designed on 12 July 2003, by a committee composed of 36 staff members from 33 participating hospitals and universities (the Japanese Consortium for the Study of Liver Diseases). The diagnostic criteria for chronic hepatitis C, treatment regimens, and follow-up protocols were finalized by the committee on 9 November 2003. This study compared the efficacy and safety of 24 vs. 48 weeks of treatment with peginterferon α-2a in patients with chronic HCV genotype 2 infection or low viral load HCV genotype 1 and showed an RVR (serum HCV RNA negative [<50 IU/ml] or <500 IU/ml by HCV RNA test at week 4 of therapy).

Eligible patients were treated with peginterferon α-2a (PEGASYS; Chugai Pharmaceuticals Inc., Tokyo, Japan) at a dose of 180 μg once per week subcutaneously. Patients who showed RVR at 4 weeks of treatment were randomized into either total treatment duration of 24 (group A) or 48 (group B) weeks. Randomization was performed at Okayama University Graduate School, centrally accessed through fax. Patients were assigned upon a report of RVR to group A or B with a computer-based random allocation system by a researcher who was independent of the study, and the allocation system was not accessible by any of the investigators who enrolled patients for the study. Randomization was stratified according to genotype (genotype 1 or 2) and previous IFN treatment (naive or re-treated) and was not blocked. Patients who were still positive for HCV RNA (by qualitative or quantitative HCV RNA tests) at week 4 were treated for 48 weeks (group C, Fig. 1). After the end of treatment, all patients were followed for an additional 24-week period.

The study was approved by ethics committee of each center and carried out according to the Declaration of Helsinki and the guidelines of the International Conference

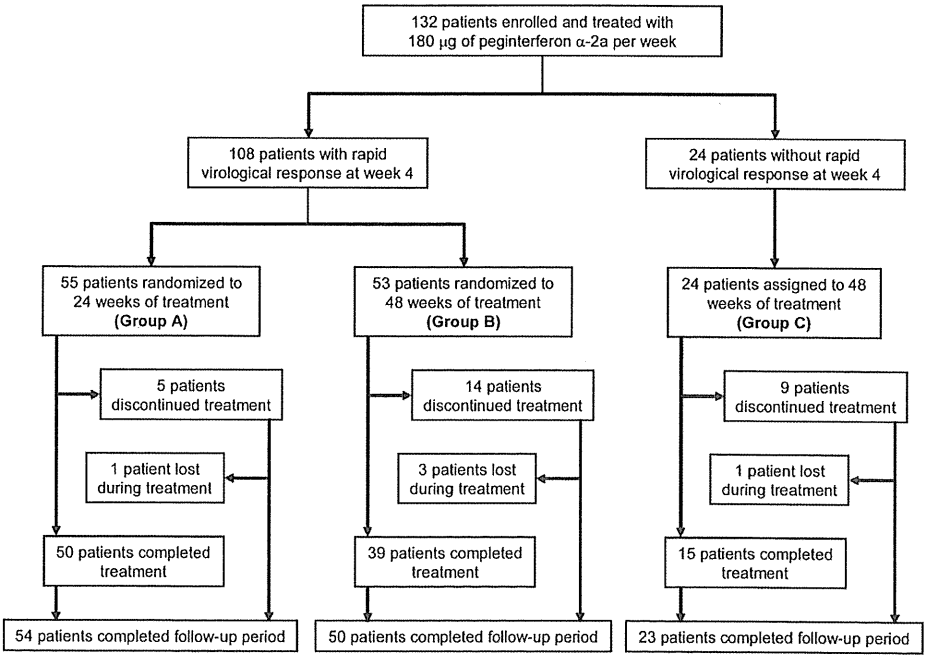

Fig. 1 Trial profile. Patients were randomized at week 8 for a total treatment duration of 24 (group A) or 48 (group B) weeks on the basis of the virological response at week 4. Patients who withdrew prematurely from treatment were encouraged to return for follow-up. For this reason, the number of patients who completed follow-up is higher than the number of patients who completed treatment

on Harmonization for Good Clinical Practice. All patients provided written informed consent before enrollment. Enrollment started in March 2004 and ended in December 2005.

Virological and histological evaluation

Serum HCV RNA was detected by qualitative reverse-transcription polymerase chain reaction (RT-PCR, Amplicor HCV, Roche Diagnostics Japan, Tokyo, Japan; low limit of detection 50 IU/ml). The serum HCV load was determined by quantitative RT-PCR (Amplicor HCV Monitor Test, Version 2.0, Roche Diagnostics Japan; low limit of detection 500 IU/ml). HCV RNA genotype was determined by RT-PCR with genotype-specific primers [20] or by serological grouping of serum antibodies determined by enzyme-linked immunosorbent assay (SRL Inc., Hachi-Oji, Tokyo) according to the method of Tanaka et al. [21] assuming that genotypes 1a and 1b corresponded to serological group 1 (genotype 1) and genotypes 2a and 2b corresponded to serological group 2 (genotype 2) [22].

Most patients underwent liver biopsy before therapy. In 40 patients, a liver biopsy was not available because the patients declined to have a biopsy specimen taken. Histopathological results were classified by local pathologists according to the METAVIR criteria reported previously [23, 24]. Treatment commenced within 12 months of liver biopsy.

Follow-up of patients

Patients were evaluated as outpatients for treatment safety, tolerance, and efficacy by each attending physician every week during treatment and every 4 weeks after the end of treatment for the rest of the study period.

Assessment of efficacy

During treatment, HCV RNA was quantified by PCR assay and was tested by qualitative test if HCV RNA became undetectable by the quantitative test. The end-of-treatment response (ETR) and SVR were assessed by qualitative PCR assay. ETR was defined as an undetectable serum HCV RNA level at the end of treatment. SVR was defined as an undetectable serum HCV RNA level by the end of treatment and throughout the follow-up period.

Safety analysis

Patients were assessed for safety and tolerance by the attending physician by monitoring adverse events and laboratory abnormalities. The study protocol permitted dose modification for patients who had clinically significant adverse events or important abnormalities in laboratory values. Adverse events were handled according to the instructions provided by the manufacturer for peginterferon α-2a, and therapy adjustments were applied. In general, dose reductions and discontinuation of therapy, if any, were made following the recommendations of the manufacturer. The dose was also reduced or the drug was discontinued at the discretion of the investigator at each of the participating clinical centers on the basis of the results of hematological, neuropsychiatric, and cutaneous or other adverse effects that were considered related to the medication. The dose of peginterferon could be restored to their original levels upon resolution of the event or abnormality.

Adherence to therapy was assessed as described previously [15], namely, by calculating the actual doses of IFN received as a percentage of the expected dose. Thus, patients who received 80% or more of their total IFN doses for 80% or more of the expected duration of therapy were considered to be 80% adherent. The dose of peginterferon received during the first 4 weeks was also assessed.

Sample size

The noninferiority margin was set at 10% between groups A and B. To obtain 80% statistical power with one-sided 5% significance level, a sample size of 81 patients per treatment group was necessary. With a dropout rate of 10% allowed, 90 patients per group were to be recruited. It was assumed that 70% of the patients would have undetectable HCV RNA at week 4. On the basis of this, the original plan specified enrollment of 270 patients to ensure randomization of an adequate number of patients at week 8. However, the Japanese Bureau of National Health Insurance started to provide reimbursement for peginterferon α-2b and ribavirin therapy for patients with HCV genotype 2 infection and high viral load or re-treatment irrespective of viral load since December 2005 and thus difficulty in new enrollment was anticipated; the enrollment was terminated by the end of the year.

Statistical analysis

Intention-to-treat analysis was used for all measures of efficacy. Patients who missed the examination at the end of the follow-up period were considered not to have had a response at that point. Patients who received at least one dose of study medication were included in the analysis of safety. The primary objective of the study was to establish the difference in SVR rates between treatment groups A and B.

Differences in baseline clinical characteristics, efficacy, and safety between the treatment groups were compared statistically by analysis of variance, χ^2 test, Fisher's exact test, Mann–Whitney U test, and Kruskal–Wallis test, where appropriate, using SAS, Version 9.1.3, software (SAS Institute, Inc., Cary, NC). Univariate and multivariate logistic regression analyses were used to establish those factors that contributed to the efficacy of peginterferon α-2a monotherapy. Variables with more than marginal statistical significance ($P < 0.10$) in univariate analysis were entered into multivariate analysis. A risk ratio with a 95% confidence interval was denoted for each analysis. Unless otherwise stated, P values below 0.05 were considered significant.

Results

Characteristics of patients

This study was performed between March 2004 and June 2007 at 33 centers in Japan. On the basis of the inclusion and exclusion criteria, 132 patients were enrolled (Fig. 1): 17 (13%) and 115 (87%) patients were infected with HCV genotypes 1 and 2, respectively. The baseline characteristics of the patients are summarized in Table 1.

Virological response

After 4 weeks of treatment with peginterferon α-2a, HCV RNA was below 500 IU/ml in 108 of 132 (82%) patients (Fig. 1). Among these 108 patients with RVR, HCV RNA was undetectable by qualitative test in 97 of 108 (90%) patients, whereas it was not tested by qualitative test in the rest of the patients. The RVR was achieved by 15 of 17 (88%) patients infected with HCV genotype 1 and low viral load and by 93 of 115 (81%) patients infected with HCV genotype 2 ($P = 0.737$). These patients were randomly assigned to group A ($n = 55$) and group B ($n = 53$). Patients with HCV RNA of 500 IU/ml or higher at week 4 were assigned to group C ($n = 24$) (Fig. 1). There were no significant differences in baseline parameters between groups A and B (Table 2).

An overall intention-to-treat virological response at the ETR was achieved in 122 of 132 (92%) patients and SVR in 81 of 132 (61%) patients. In groups A and B, 53 of 55 (96%) patients and 51 of 53 (96%) patients achieved ETR and 33 of 55 (60%) patients and 42 of 53 (79%) patients achieved SVR, respectively (Fig. 2). The SVR rate was

Table 1 Demographic, biochemical, molecular, and histological profiles of patients at baseline

	All patients	HCV RNA (kIU/ml) at week 4		P-value[†]
		<500	≥500	
Patients, n	132	108	24	
Gender, male/female (% male)	81/51 (61)	68/40 (63)	13/11 (54)	0.423[‡]
Age (years)[a]	56.4 ± 12.2	56.0 ± 12.2	57.8 ± 12.2	0.536[§]
Weight (kg)[a]	61.5 ± 12.6	62.0 ± 13.1	58.6 ± 9.5	0.257[§]
Naive/re-treatment	119/13	98/10	21/3	0.704[#]
Fibrosis staging, n (F1/F2/F3/F4)	57/26/8/1	48/22/6/1	9/4/2/0	0.881[‡]
Grading, n (A0-1/A2/A3)	52/38/2	44/31/2	8/7/0	0.868[‡]
Genotype, 1/2 (% genotype 1)	17/115 (13)	15/93 (14)	2/22 (8)	0.737[#]
HCV RNA (kIU/ml)[b]	285 (46–1620)	130 (37–1006)	1350 (360–3060)	<0.001[¶]
ALT (IU/l)[b] [7–42][c]	66 (35–117)	64 (36–119)	69 (31–109)	0.571[¶]
γ-GTP (IU/l)[b] [5–50][c]	45 (24–92)	54 (26–97)	33 (21–49)	0.014[¶]
Neutrophil count (/μl)[a] [1000–7500][c]	2844 ± 1036	2898 ± 1042	2615 ± 996	0.230[§]
Hemoglobin (g/dl)[a] [13.5–17.5][c]	14.0 ± 1.2	14.1 ± 1.2	14.0 ± 1.4	0.751[§]
Platelet count (10^3/μl)[a] [150–400][c]	175 ± 63	179 ± 66	155 ± 40	0.089[§]

ALT alanine aminotransferase, *γ-GTP* gamma glutamyl transpeptidase

[†] Comparison between groups according to HCV RNA at week 4

[‡] Chi-square test

[#] Fisher's exact test

[§] Unpaired-t test

[¶] Mann–Whitney U-test

Data are [a] mean ± SD or [b] median (interquartile range), [c] normal range

Table 2 Comparison of baseline profiles between groups A (24 weeks) and B (48 weeks)

	Group A	Group B	*P*-value[†]
Patients, *n*	55	53	
Gender, male/female (% male)	35/20 (64)	33/20 (62)	0.883[‡]
Age (years)[a]	56.9 ± 11.3	55.2 ± 13.1	0.473[§]
Weight (kg)[a]	59.9 ± 11.6	64.3 ± 14.3	0.087[§]
Naive/re-treatment	48/7	49/4	0.374[#]
Fibrosis staging, *n* (F1/F2/F3/F4)	26/12/2/0	22/10/4/1	0.558[‡]
Grading, *n* (A0-1/A2/A3)	28/11/1	16/20/1	0.097[‡]
Genotype, 1/2 (% genotype 1)	9/46 (16)	6/47 (11)	0.580[#]
HCV RNA (kIU/ml)[b]	190 (35–1660)	120 (38–580)	0.282[¶]
ALT (IU/l)[b] [7–42][c]	70 (37–117)	59 (36–120)	0.813[¶]
γ-GTP (IU/l)[b] [5–50][c]	43 (26–78)	63 (28–116)	0.242[¶]
Neutrophil count (/μl)[a] [1000–7500][c]	2936 ± 1047	2859 ± 1088	0.711[§]
Hemoglobin (g/dl)[a] [13.5–17.5][c]	14.1 ± 1.0	14.1 ± 1.3	0.943[§]
Platelet count (10^3/μL)[a] [150–400][c]	177 ± 47	181 ± 81	0.793[§]

ALT alanine aminotransferase, *γ-GTP* gamma glutamyl transpeptidase

[†] Comparison between groups A and B

[‡] Chi-square test

[#] Fisher's exact test

[§] Unpaired *t*-test

[¶] Mann–Whitney *U* test

Data are [a] mean ± SD or [b] median (interquartile range), [c] normal range

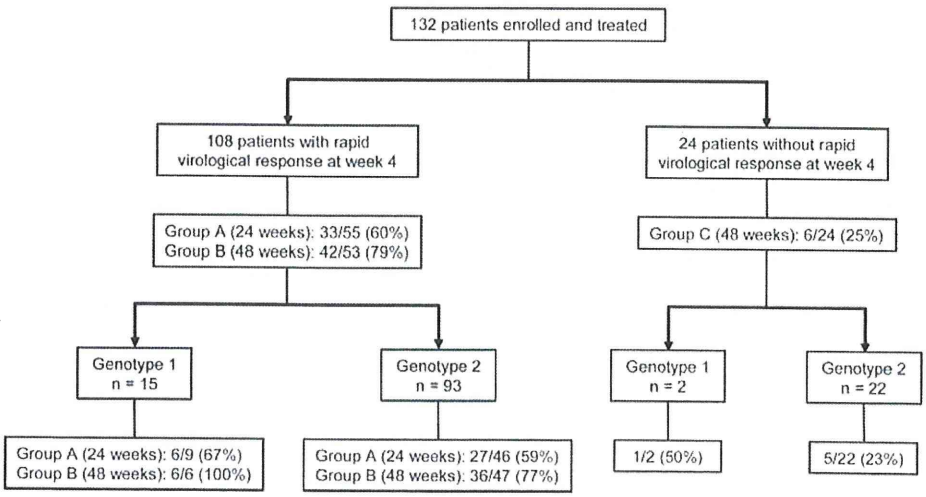

Fig. 2 Sustained virological response rate according to genotype in each treatment group

significantly higher in patients randomized to 48 weeks of therapy (group B) than in those randomized to 24 weeks of therapy (group A) ($P = 0.030$), namely, the relapse rate in group A was 40% (22/55), which was significantly higher than in group B (21%, 11/53, $P = 0.030$). Among patients with RVR confirmed by qualitative test (HCV RNA < 50 IU/ml), 29 of 48 (60%) patients achieved SVR in group A vs. 39 of 46 (85%) in group B ($P = 0.008$). The ETR and SVR rates in patients who did not show RVR and who were treated for 48 weeks (group C) were lower than in those who showed RVR (groups A and B) (75% vs. 96%, $P = 0.003$ for ETR and 25% vs. 69%, $P < 0.001$ for SVR, respectively) (Fig. 2).

Virological response according to HCV genotype and pretreatment viral load

The SVR rate in HCV genotype 1 and low viral load were not significantly different between treatment groups A and B (67% vs. 100%, respectively; $P = 0.229$) (Fig. 2),

although the number of patients of this subgroup was small for meaningful comparison.

The SVR rate in patients infected with HCV genotype 2 was higher in group B (77%) than in group A (59%, $P = 0.065$). There was an inverse correlation between SVR rate and baseline viral load (Fig. 3). This observation was significant in group A ($P < 0.001$) but not in group B ($P = 0.096$). On the basis of receiver operating characteristics analysis, 1,000,000 IU/ml was optimal for use as the cutoff point of baseline viral load to best discriminate patients who might achieve SVR in group A. The SVR rate of patients with HCV genotype 2 infection and a baseline viral load below 1,000,000 IU/ml was not compromised by 24-week treatment (group A) compared with 48-week treatment (group B) (81% and 79%, respectively), without significant difference between the two groups ($P = 0.801$). On the other hand, the SVR rate in those with a baseline viral load of 1,000,000 IU/ml or higher was significantly lower in group A than in group B (26% and 67%, respectively, $P = 0.041$) (Fig. 3).

Factors associated with RVR

Next, we analyzed the factors associated with RVR using data of all patients. The variables included were demographic features, baseline viral load, liver enzymes, and administered dose of peginterferon during the first 4 weeks (Table 3). Pretreatment HCV RNA level was lower and

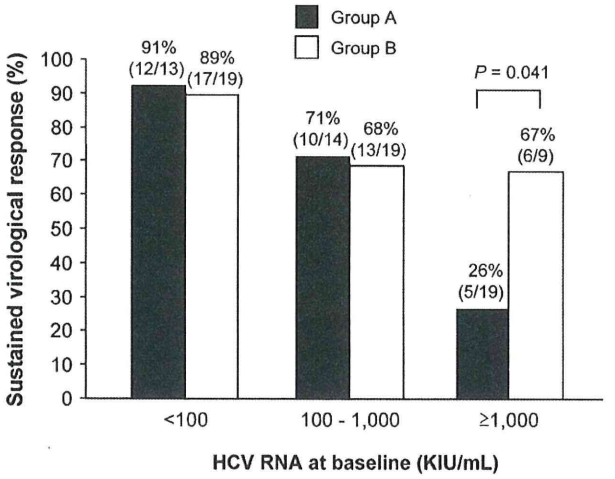

Fig. 3 Sustained virological response (SVR) rates stratified according to pretreatment HCV RNA level for patients of groups A (24 weeks) and B (48 weeks) infected with HCV genotype 2. The SVR rate was significantly lower in patients with higher baseline viral load in group A ($P < 0.001$) but not in group B ($P = 0.096$). The SVR rate in patients with a baseline viral load of less than 1,000,000 IU/ml was similar between group A (81%, 22/27) and group B (79%, 30/38) ($P = 0.801$). However, the SVR rate in those with a baseline viral load of 1,000,000 IU/ml or higher was lower in group A (26%, 5/19) than in group B (67%, 6/9) ($P = 0.041$)

γ-glutamyl transpeptidase (γ-GTP) level and platelet count were higher in patients with RVR (groups A and B) than in those without RVR (group C) (Table 1). On the basis of receiver operating characteristic analyses, 41 IU/l and 191 $\times$ 10^3/μl were optimal for use as the cutoff points of baseline γ-GTP level and platelet count, respectively. Multivariate analysis identified low baseline viral load (<1,000,000 IU/ml), high γ-GTP level (≥41 IU/l), and high platelet count (≥191 $\times$ 10^3/μl) were significant determinants of RVR (Table 3).

Factors associated with SVR

Next, the factors associated with SVR were analyzed using data of all patients. Univariate analysis indicated that grading score, pretreatment viral load, alanine aminotransferase (ALT) and γ-GTP levels, neutrophil count, RVR, and adherence to treatment were associated with SVR. On the basis of receiver operating characteristic analyses, 41 IU/l, 28 IU/l, and 3,155/μl were optimal for use as the cutoff points of baseline ALT level, γ-GTP level, and neutrophil count, respectively. Multivariate analysis was performed with the following variables: pretreatment viral load, ALT and γ-GTP levels, neutrophil count, RVR, and adherence to treatment but excluding grading score due to a significant association with ALT level and a substantial number of cases (40 cases) missing histological data. The analysis identified low viral load (<100,000 IU/ml), RVR, high ALT level (≥41 IU/l), and high γ-GTP level (≥28 IU/l) as independent determinants of SVR (Table 4).

Multivariate analysis for factors associated with SVR in patients with RVR identified low viral load (<100,000 IU/ml) and high ALT level (≥41 IU/l) as independent determinants of SVR. The SVR rates in patients with RVR and with high ALT levels (≥41 IU/l) were generally high except for group A patients with high viral load (≥1,000,000 IU/ml). On the other hand, the SVR rates for both groups A (black bars) and B (open bars) were entirely low in patients with low ALT levels (<41 IU/l) except those with low viral load (<100,000 IU/ml) (Fig. 4). In patients with high viral load (≥100,000 IU/ml) and low ALT levels (<41 IU/l), SVR was achieved only in group B patients, though at low rate.

Safety

Twenty-eight (21%) patients discontinued therapy and 14 of them discontinued because of adverse events, 4 because of laboratory abnormalities, 3 because of refusal of treatment, 2 because of insufficient response, and 5 because of failure to return (Table 5). Fatigue was the most common adverse event leading to discontinuation of therapy. The frequencies of discontinuation and discontinuation due to

Table 3 Logistic regression analysis of the factors associated with rapid virological response

Variable	RR (95% CI)	*P* value
Univariate analysis		
Pretreatment variables		
Gender (male vs. female)	1.438 (0.589–3.513)	0.425
Age (<55 vs. ≥55 years)	1.536 (0.583–4.049)	0.512
Weight (≥60 vs. <60 kg)	2.511 (0.938–6.723)	0.067
Treatment (naive vs. re-treatment)	1.400 (0.354–5.530)	0.631
Genotype (1 vs. 2)	1.773 (0.377–8.333	0.468
Fibrosis staging (F2-4 vs. F0-1)†	1.104 (0.356–3.425)	0.865
Grading (A2-3 vs. A0-1)†	1.167 (0.384–3.546)	0.786
HCV RNA (kIU/mL)		
<100	1	
100–1000	0.562 (0.140–2.251)	0.415
≥1000	0.159 (0.048–0.526)	0.003
ALT (≥60 vs. <60 IU/l)	1.437 (0.579–3.571)	0.435
γ-GTP (IU/l) (≥41 vs. <41 IU/l)	3.946 (1.504–10.352)	0.005
Neutrophil count (≥2500 vs. <2500/μL)	1.135 (0.465–2.771)	0.782
Hemoglobin (<14 vs. ≥14 g/dl)	1.427 (0.582–3.497)	0.437
Platelet count (≥191 vs. <191 × 10^3/μL)	6.567 (1.466–29.424)	0.014
Treatment-associated variables		
Adherence during 4 weeks of treatment (≥80% vs. <80%)	1.714 (0.419–7.011)	0.453
Stepwise multivariate analysis		
HCV RNA (kIU/ml)		
<100	1	
100–1000	0.399 (0.091–1.759)	0.225
≥1000	0.126 (0.034–0.464)	0.002
Platelet count (≥191 vs. <191 × 10^3/μl)	10.230 (2.056–50.902)	0.005
γ-GTP (IU/l) (≥41 vs. <41 IU/l)	3.989 (1.355–11.744)	0.012

ALT alanine aminotransferase, *γ-GTP* gamma glutamyl transpeptidase

† A biopsy was not available from 40 patients

adverse events were significantly lower in the 24-week treatment group (group A) than in the 48-week treatment group (groups B and C) (9% vs. 30%, $P = 0.005$ and 4% vs. 16%, $P = 0.042$, respectively).

Adherence to scheduled therapy (median and interquartile range) was 100% (63–100%), 77% (54–100%), and 85% (55–100%), respectively, for groups A, B, and C ($P = 0.012$ by Kruskal–Wallis test). The rate of adherence in group A was higher than in groups B and C ($P = 0.003$ and $P = 0.082$, respectively, by Mann–Whitney *U* test). There was no difference in adherence to therapy between groups B and C ($P = 0.597$). Thus, adherence to therapy in the longer treatment course (48 weeks) was lower than in the shorter treatment course (24 weeks).

Costs

Based on the current prices in the United States, spending on medication for 48 weeks of peginterferon α-2a monotherapy is $26,305 and that for 24 weeks treatment is $13,152. If we consider re-treatment for 48 weeks of the 40% of patients with RVR who relapse after 24 weeks of treatment, the mean cost of treating HCV genotype 2 infection or low viral load HCV genotype 1 patients with RVR would be $23,674 (Fig. 5). Thus, if all relapsers after 24 weeks of treatment receive re-treatment for 48 weeks, the mean saving per patient with this concept vs. 48 weeks to all would be $2,630 (10%).

Discussion

The key finding of this study is that in patients infected with HCV genotype 2 and low viral load (<1,000,000 IU/ml) who achieve RVR, 24-week treatment with peginterferon α-2a alone may be sufficient in terms of efficacy. Patients treated for 24 weeks also discontinued treatment less frequently and showed higher adherence than those

Table 4 Logistic regression analysis of the factors associated with sustained virological response

Variable	RR (95% CI)	*P*-value
Univariate analysis		
Pretreatment variables		
Gender (male vs. female)	1.190 (0.581–2.438)	0.635
Age (≥55 vs. <55 years)	1.273 (0.618–2.625)	0.512
Weight (<60 vs. ≥60 kg)	1.064 (0.520–2.175)	0.865
Treatment (re-treatment vs. naive)	1.468 (0.428–5.051)	0.541
Genotype (1 vs. 2)	2.247 (0.690–7.299)	0.179
Fibrosis staging (F2-4 vs. F0-1)†	1.964 (0.812–4.749)	0.134
Grading (A2-3 vs. A0-1)†	4.343 (1.727–10.922)	0.002
HCV RNA (kIU/mL) <100	1	
100–1000	0.367 (0.138–0.975)	0.044
≥1000	0.115 (0.044–0.298)	<0.001
ALT (≥41 vs. <41 IU/l)	4.570 (2.104–9.927)	<0.001
γ-GTP (IU/l) (≥28 vs. <28 IU/l)	6.182 (2.657–14.384)	<0.001
Neutrophil count (<3155 vs. ≥3155/μl)	3.135 (1.479–6.623)	0.003
Hemoglobin (≥14 vs. <14 g/dl)	1.125 (0.555–2.283)	0.744
Platelet count (<150 vs. ≥150 × 10^3/μl)	1.091 (0.507–2.347)	0.824
Treatment-associated variables		
RVR (yes vs. no)	6.818 (2.482–18.733)	<0.001
Adherence (≥80% vs. <80%)	1.940 (0.949–3.966)	0.070
Stepwise Multivariate Analysis‡		
HCV RNA (kIU/ml)		
<100	1	
100–1000	0.165 (0.046–0.589)	0.006
≥1000	0.102 (0.029–0.352)	<0.001
RVR (yes vs. no)	6.223 (1.821–21.305)	0.003
ALT (≥41 vs. <41 IU/l)	4.775 (1.373–16.601)	0.014
γ-GTP (IU/l) (≥28 vs. <28 IU/l)	3.466 (1.092–11.000)	0.035

ALT alanine aminotransferase, *γ-GTP* gamma glutamyl transpeptidase, *RVR* rapid virological response

† A biopsy was not available from 40 patients

‡ Grading was not included

treated for 48 weeks. Furthermore, the drug cost can be reduced by truncating treatment duration. Thus, by reducing the treatment period, these patients can avoid unnecessary treatment without compromising the chance for an SVR. In particular, the SVR rate in patients with HCV genotype 2 infection and low viral load (<1,000,000 IU/ml) who achieved RVR was as high as 81% by 24-week monotherapy. The SVR rate was comparable with that (84%, 81/96) reported previously in patients with HCV genotype 2 and 3 infection who received 24-week combination therapy of peginterferon α-2a plus ribavirin [7], although the latter included patients with HCV genotype 3 infection. On the other hand, the results of this study were not conclusive regarding patients with HCV genotype 1 infection and low viral load (<100,000 IU/ml). Further prospective controlled trial is warranted to confirm our findings in patients with HCV genotype 1 infection and low viral load or HCV genotype 2 infection and baseline viral loads of less than 1,000,000 IU/ml who achieve RVR.

In patients with HCV genotype 2 infection and high viral load (≥1,000,000 IU/ml), the SVR rate was lower for the 24-week treatment group than for the 48-week treatment group, even if the patients achieved RVR. Thus, a longer treatment (>24 weeks) with peginterferon is recommended for this group of patients. Furthermore, since the SVR rate was not more than 67% in this subgroup of patients, even if they were treated for 48 weeks, a combination with ribavirin or further extended treatment duration may be necessary as long as patients can tolerate the treatment.

The combination therapy of peginterferon and ribavirin is currently the therapeutic standard for chronic hepatitis C. However, the combination therapy tends to be associated with adverse events more frequently than those that occur with IFN monotherapy, resulting in dose reduction or discontinuation of therapy and thus impaired response rate [9–14]. This is true particularly in elderly patients. In a country such as Japan where the average age of patients with chronic hepatitis C to be treated by antivirals sometimes is well above 60, standard combination therapy is not well tolerated [15]. For example, in a phase III study of 48-week peginterferon α-2a plus ribavirin combination therapy

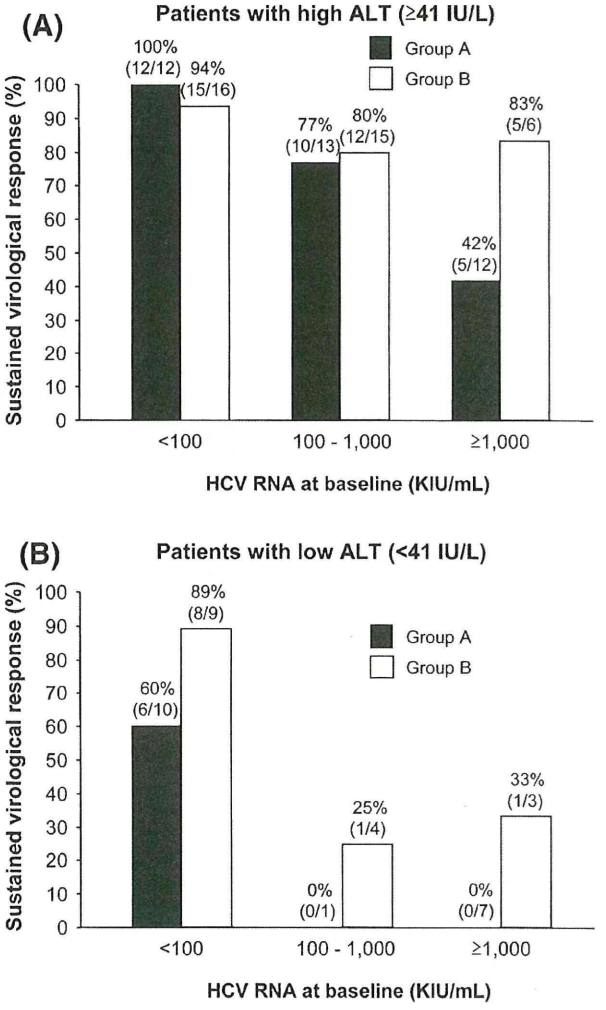

Fig. 4 Sustained virological response (SVR) rates stratified according to pretreatment HCV RNA and ALT levels in group A and B patients. The SVR rates in patients with high (≥41 IU/l) and low (<41 IU/l) ALT levels are shown in panels **a** and **b**, respectively. **a** The SVR rates in patients with high ALT levels (≥41 IU/l) were generally high except for group A patients with high viral load (≥1,000,000 IU/ml). **b** The SVR rates in both groups A and B were low in patients with low ALT levels (<41 IU/l) except those with low viral load (<100,000 IU/ml)

conducted in Japan [25], the SVR rate in the combination arm (78%, 18/23) was rather inferior to that of peginterferon α-2a monotherapy (placebo) arm (100%, 14/14) among patients with RVR (P = 0.061), although the difference did not reach statistical significance. In the same study, all of the patients who failed to achieve SVR in the combination arm discontinued treatment [25]. Thus, the combination therapy with ribavirin does not always lead to a better response than with monotherapy, at least in a subgroup of patients. It is noteworthy that most of the patients in the present trial were those who preferred peginterferon α monotherapy to combination therapy in spite

Table 5 Incidence and reason of discontinuation according to treatment group

Variable	All patients	Age (years)		
		A	B	C
n	132	55	53	24
Discontinuation	28 (21)	5 (9)	14 (26)	9 (38)
Adverse events	14 (11)	2 (4)	8 (15)	4 (17)
Fatigue	4	0	1	3
Depression	2	1	1	0
Arthralgia	2	0	2	0
Arrhythmia	2	0	1	1
Pyrexia	1	1	0	0
Headache	1	0	1	0
Hyperthyroidism	1	0	1	0
Colon cancer	1	0	1	0
Laboratory abnormality	4 (3)	1 (2)	1 (2)	2 (8)
High aminotransferase	2	1	0	1
Anemia	1	0	1	0
Neutropenia	1	0	0	1
Refusal of treatment	3 (2)	1 (2)	1 (2)	1 (4)
Insufficient response	2 (2)	0	1 (2)	1 (4)
Failure to return	5 (4)	1 (2)	3 (6)	1 (4)

Data are number of patients (percentage in each patient group)

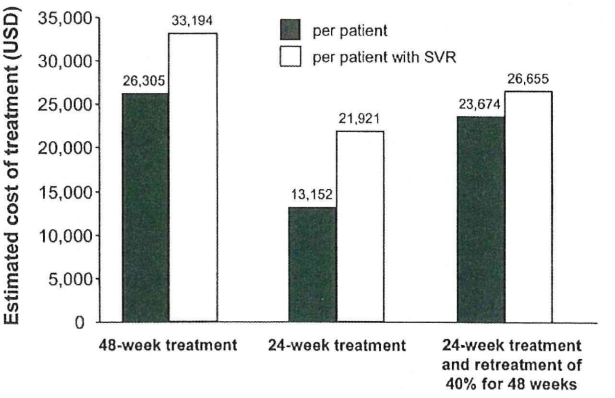

Fig. 5 The cost of treating patients infected with HCV genotype 2 or low viral load genotype 1 and RVR for 48, 28, or 24 weeks followed by 48 weeks of re-treatment of 40% of patients who relapse after the initial treatment

of the coverage for the latter therapy by the Bureau of National Health Insurance, as described previously.

In addition to elderly patients, those with renal failure, ischemic vascular diseases, and congenital hemoglobin abnormalities never tolerate ribavirin for chronic hepatitis C treatment [26]. Therefore, data on peginterferon α monotherapy are particularly relevant to these patients. The possibility of shorter combination therapy with peginterferon α and ribavirin in easy-to-treat patients such as those

chronically infected with HCV genotype 2 or 3 has been investigated in several trials [27–31]. However, to the best of our knowledge, there has been no randomized, controlled trial to identify optimal treatment duration of peginterferon α monotherapy. A further prospective randomized, controlled trial aiming at patients who cannot receive combination therapy with ribavirin is warranted.

A high ALT level has been identified as a significant factor for SVR [18]. The reason why patients with low or normal ALT levels do not respond well to peginterferon α monotherapy is currently unknown. The SVR rates were low in our patients with low ALT levels and HCV RNA levels of 100,000 IU/ml or higher in the two randomized groups (Fig. 4). Thus, these patients may not benefit by simply extending therapy from 24 to 48 weeks. Since a similar efficacy has been demonstrated in patients with persistently normal ALT levels compared with those with elevated ALT levels by combination therapy of peginterferon α-2a plus ribavirin [32], combination therapy should be considered for these patients.

A high γ-GTP level was unexpectedly identified as a factor for both RVR and SVR, independent of ALT levels. Again, the reason for this finding is unknown at present. It is well known that a low γ-GTP level is associated with SVR to combination therapy comprising peginterferon and ribavirin; the reason also being unexplained so far [33]. Thus, the present finding at least suggests that entirely different mechanisms may underlie these observations.

In this trial, RVR was defined as serum HCV RNA level below 500 IU/ml at week 4, although most of the patients who achieved RVR had HCV RNA levels below 50 IU/ml. The criterion of RVR used in this study was less strict than those reported recently, in which serum HCV RNA level below 50 IU/ml at week 4 has been utilized [30, 31]. This may result in a higher rate of achieving RVR and lower SVR rates, resulting in more relapsers, particularly in patients treated for a shorter duration of 24 weeks than a standard duration of 48 weeks. By using more strict criteria of serum HCV RNA level below the detection limit of qualitative PCR (≤50 IU/ml) at week 4 or negativity of HCV RNA at earlier time points during therapy, such as at week 2 [34], a subgroup of patients, who can be sufficiently treated with a shorter duration of therapy (such as 24 weeks) without compromising the chance for SVR, could be more specifically identified.

In conclusion, patients infected with HCV genotype 2 and have low baseline viral load (<1,000,000 kIU/ml), who can achieve RVR, can satisfactorily be treated for 24 weeks with peginterferon α-2a alone without compromising the SVR. We propose that these patients should first be treated with peginterferon α monotherapy for 24 weeks, as long as RVR is achieved, otherwise they should be switched to combination therapy with ribavirin at the time for another 24–48 weeks, depending on the response thereafter. However, the data of this study are less conclusive for patients with low viral load genotype 1 or 2 and viral load of more than 1,000,000 IU/ml. Additional trials are required to optimize treatment schedule in these patients.

Acknowledgments Investigators who participated in this study are as follows (listed in alphabetical order): K. Abe (Iwate Medical University), M. Ando (Mitoyo General Hospital), Y. Araki (Hiroshima City Hospital), A. Asagi (Kagawa Prefectural Central Hospital), N. Enomoto (Juntendo University), K. Hamamura (Shizuoka General Hospital), Y. Hiasa (Ehime University), S. Hige (Hokkaido University), T. Ide (Kurume University), J. Inoue (Hiroshima Teishin Hospital), Y. Ishii (Tottori City Hospital), Y. Iwasaki (Okayama University), N. Izumi (Musashino Red Cross Hospital), K. Joko (Matsuyama Red Cross Hospital), H. Jomura (Wakakoukai Hospital), S. Kakizaki (Gunma University), Y. Kamishima (Tomakomai-Nisshou Hospital), F. Kanai (University of Tokyo), S. Kaneko (Kanazawa University), J-H. Kang (Teine-Keijinkai Hospital), N. Kawada (Osaka City University), S. Kawano (Tottori City Hospital), S. Kawazoe (Saga Prefectural Hospital Kouseikan), K. Kita (Kagawa Prefectural Central Hospital), T. Kitamura (Musashino Red Cross Hospital), H. Kobashi (Tsuyama Central Hospital), Y. Kohgo (Asahikawa Medical College), H. Kokuryu (Shizuoka General Hospital), S. Konishi (Tomakomai-Nisshou Hospital), N. Masaki (International Medical Center of Japan), S. Matsumura (Mitoyo General Hospital), S. Minamitani (Aioi Hospital), T. Mizuta (Saga Medical School), M. Moriyama (Nihon University). J. Nishiguchi (Tokyo Dental College, Ichikawa General Hospital), S. Nishiguchi (Hyogo College of Medicine), S. Nishimura (Musashino Red Cross Hospital), K. Nouso (Hiroshima City Hospital), T. Ohtake (Asahikawa Medical College), R. Okamoto (Hiroshima City Hospital), H. Okushin (Himeji Red Cross Hospital), M. Omata (University of Tokyo), M. Onji (Ehime University), H. Saito (Keio University), M. Sata (Kurume University), N. Sato (Juntendo University), S. Sato (Teikyo University Hospital, Mizonokuchi), T. Senoh (Takamatsu Red Cross Hospital), A. Shibuya (Kitasato University Hospital), Y. Shiratori (Okayama University), N. Sohara (Gunma University), K. Suzuki (Iwate Medical University), Y. Suzuki (Sano Hospital), H. Takagi (Gunma University), K. Takaguchi (Kagawa Prefectural Central Hospital), Y. Tanaka (Matsuyama Red Cross Hospital), R. Terada (Okayama Saiseikai General Hospital), K. Ueda (Musashino Red Cross Hospital), K. Yamamoto (Saga Medical School), and H. Yoshida (University of Tokyo).

Conflict of interest statement None declared.

References

1. El Serag HB, Davila JA, Petersen NJ, McGlynn KA. The continuing increase in the incidence of hepatocellular carcinoma in the United States: an update. Ann Intern Med 2003;139:817–823.
2. Lauer GM, Walker BD. Hepatitis C virus infection. N Engl J Med 2001;345:41–52. doi:10.1056/NEJM200107053450107.
3. Seeff LB. Natural history of chronic hepatitis C. Hepatology 2002;36:S35–46. doi:10.1002/hep.1840360706.
4. Marcellin P, Boyer N, Gervais A, Martinot M, Pouteau M, Castelnau C, et al. Long-term histologic improvement and loss of detectable intrahepatic HCV RNA in patients with chronic hepatitis C and sustained response to interferon-alpha therapy. Ann Intern Med 1997;127:875–881.

5. Niederau C, Lange S, Heintges T, Erhardt A, Buschkamp M, Hurter D, et al. Prognosis of chronic hepatitis C: results of a large, prospective cohort study. Hepatology 1998;28:1687–1695. doi: 10.1002/hep.510280632.
6. Fried MW, Shiffman ML, Reddy KR, Smith C, Marinos G, Goncales FL Jr, et al. Peginterferon alfa-2a plus ribavirin for chronic hepatitis C virus infection. N Engl J Med 2002;347:975–982. doi:10.1056/NEJMoa020047.
7. Hadziyannis SJ, Sette H Jr, Morgan TR, Balan V, Diago M, Marcellin P, et al. Peginterferon-alpha2a and ribavirin combination therapy in chronic hepatitis C: a randomized study of treatment duration and ribavirin dose. Ann Intern Med 2004;140:346–355.
8. Manns MP, McHutchison JG, Gordon SC, Rustgi VK, Shiffman M, Reindollar R, et al. Peginterferon alfa-2b plus ribavirin compared with interferon alfa-2b plus ribavirin for initial treatment of chronic hepatitis C: a randomised trial. Lancet 2001;358:958–965. doi:10.1016/S0140-6736(01)06102-5.
9. Cummings KJ, Lee SM, West ES, Cid-Ruzafa J, Fein SG, Aoki Y, et al. Interferon and ribavirin vs interferon alone in the retreatment of chronic hepatitis C previously nonresponsive to interferon: a meta-analysis of randomized trials. JAMA 2001; 285:193–199. doi:10.1001/jama.285.2.193.
10. Davis GL, Esteban-Mur R, Rustgi V, Hoefs J, Gordon SC, Trepo C, et al. Interferon alfa-2b alone or in combination with ribavirin for the treatment of relapse of chronic hepatitis C. International Hepatitis Interventional Therapy Group. N Engl J Med 1998;339:1493–1499. doi:10.1056/NEJM199811193392102.
11. Kjaergard LL, Krogsgaard K, Gluud C. Interferon alfa with or without ribavirin for chronic hepatitis C: systematic review of randomised trials. Br Med J 2001;323:1151–1155. doi:10.1136/bmj.323.7322.1151.
12. Maddrey WC. Safety of combination interferon alfa-2b/ribavirin therapy in chronic hepatitis C-relapsed and treatment-naive patients. Semin Liver Dis 1999;19(Suppl 1):67–75.
13. McHutchison JG, Gordon SC, Schiff ER, Shiffman ML, Lee WM, Rustgi VK, et al. Interferon alfa-2b alone or in combination with ribavirin as initial treatment for chronic hepatitis C. Hepatitis Interventional Therapy Group. N Engl J Med 1998;339: 1485–1492. doi:10.1056/NEJM199811193392101.
14. Poynard T, Marcellin P, Lee SS, Niederau C, Minuk GS, Ideo G, et al. Randomised trial of interferon alpha2b plus ribavirin for 48 weeks or for 24 weeks versus interferon alpha2b plus placebo for 48 weeks for treatment of chronic infection with hepatitis C virus. International Hepatitis Interventional Therapy Group (IHIT). Lancet 1998;352:1426–1432. doi:10.1016/S0140-6736(98)07124-4.
15. Iwasaki Y, Ikeda H, Araki Y, Osawa T, Kita K, Ando M, et al. Limitation of combination therapy of interferon and ribavirin for older patients with chronic hepatitis C. Hepatology 2006;43:54–63. doi:10.1002/hep.20984.
16. Heathcote EJ, Shiffman ML, Cooksley WG, Dusheiko GM, Lee SS, Balart L, et al. Peginterferon alfa-2a in patients with chronic hepatitis C and cirrhosis. N Engl J Med 2000;343:1673–1680. doi:10.1056/NEJM200012073432302.
17. Lindsay KL, Trepo C, Heintges T, Shiffman ML, Gordon SC, Hoefs JC, et al. A randomized, double-blind trial comparing pegylated interferon alfa-2b to interferon alfa-2b as initial treatment for chronic hepatitis C. Hepatology 2001;34:395–403. doi: 10.1053/jhep.2001.26371.
18. Zeuzem S, Feinman SV, Rasenack J, Heathcote EJ, Lai MY, Gane E, et al. Peginterferon alfa-2a in patients with chronic hepatitis C. N Engl J Med 2000;343:1666–1672. doi:10.1056/NEJM200012073432301.
19. Data on file. PEGASYS. Tokyo (Japan): Chugai Pharmaceuticals Inc; 2002.
20. Okamoto H, Sugiyama Y, Okada S, Kurai K, Akahane Y, Sugai Y, et al. Typing hepatitis C virus by polymerase chain reaction with type-specific primers: application to clinical surveys and tracing infectious sources. J Gen Virol 1992;73:673–679. doi: 10.1099/0022-1317-73-3-673.
21. Tanaka T, Tsukiyama-Kohara K, Yamaguchi K, Yagi S, Tanaka S, Hasegawa A, et al. Significance of specific antibody assay for genotyping of hepatitis C virus. Hepatology 1994;19:1347–1353. doi:10.1002/hep.1840190605.
22. Shiratori Y, Kato N, Yokosuka O, Imazeki F, Hashimoto E, Hayashi N, et al. Predictors of the efficacy of interferon therapy in chronic hepatitis C virus infection. Tokyo-Chiba Hepatitis Research Group. Gastroenterology 1997;113:558–566. doi: 10.1053/gast.1997.v113.pm9247476.
23. Bedossa P, Poynard T. An algorithm for the grading of activity in chronic hepatitis C. The METAVIR Cooperative Study Group. Hepatology 1996;24:289–293. doi:10.1002/hep.510240201.
24. Desmet VJ, Gerber M, Hoofnagle JH, Manns M, Scheuer PJ. Classification of chronic hepatitis: diagnosis, grading and staging. Hepatology 1994;19:1513–1520. doi:10.1002/hep.1840190629.
25. Sakai T, Iino S, Okuno T, Omata M, Kiyosawa K, Kumada H, et al. High response rates with peginterferon alfa-2a (40 KD) (PEGASYS) plus ribavirin (COPEGUS) in treatment-naive Japanese chronic hepatitis C patients: a randomized, double-blind, multicenter phase III trial. Gastroenterology 2006;130(Suppl):A839. abstract.
26. Dienstag JL, McHutchison JG. American Gastroenterological Association technical review on the management of hepatitis C. Gastroenterology 2006;130:231–264. doi:10.1053/j.gastro.2005.11.010.
27. Lagging M, Langeland N, Pedersen C, Farkkila M, Buhl MR, Morch K, et al. Randomized comparison of 12 or 24 weeks of peginterferon alpha-2a and ribavirin in chronic hepatitis C virus genotype 2/3 infection. Hepatology 2008;47:1837–1845. doi: 10.1002/hep.22253.
28. Shiffman ML, Suter F, Bacon B, Nelson D, Harley H, Sola R, et al. Peginterferon alfa-2a and ribavirin for 16 or 24 weeks in HCV genotype 2 or 3. N Engl J Med 2007;357:124–134. doi: 10.1056/NEJMoa066403.
29. von Wagner M, Huber M, Berg T, Hinrichsen H, Rasenack J, Heintges T, et al. Peginterferon-alpha-2a (40 kD) and ribavirin for 16 or 24 weeks in patients with genotype 2 or 3 chronic hepatitis C. Gastroenterology 2005;129:522–527.
30. Yu ML, Dai CY, Huang JF, Hou NJ, Lee LP, Hsieh MY, et al. A randomised study of peginterferon and ribavirin for 16 versus 24 weeks in patients with genotype 2 chronic hepatitis C. Gut 2007;56:553–559. doi:10.1136/gut.2006.102558.
31. Dalgard O, Bjøro K, Ring-Larsen H, Bjornsson E, Holberg-Petersen M, Skovlund E, et al. Pegylated interferon alfa and ribavirin for 14 versus 24 weeks in patients with hepatitis C virus genotype 2 or 3 and rapid virological response. Hepatology 2008;47:35–42. doi:10.1002/hep.21975.
32. Zeuzem S, Diago M, Gane E, Reddy KR, Pockros P, Prati D, et al. Peginterferon alfa-2a (40 kilodaltons) and ribavirin in patients with chronic hepatitis C and normal aminotransferase levels. Gastroenterology 2004;127:1724–1732. doi:10.1053/j.gastro.2004.09.050.
33. Berg T, Sarrazin C, Herrmann E, Hinrichsen H, Gerlach T, Zachoval R, et al. Prediction of treatment outcome in patients with chronic hepatitis C: significance of baseline parameters and viral dynamics during therapy. Hepatology 2003;37:600–609. doi:10.1053/jhep.2003.50106.
34. Jeong S, Kawakami Y, Kitamoto M, Ishihara H, Tsuji K, Aimitsu S, et al. Prospective study of short-term peginterferon-alpha-2a monotherapy in patients who had a virological response at 2 weeks after initiation of interferon therapy. J Gastroenterol Hepatol 2008;23:541–545. doi:10.1111/j.1440-1746.2008.05356.x.

UICC
global cancer control

IJC
International Journal of Cancer

Identification of a secretory protein c19orf10 activated in hepatocellular carcinoma

Hajime Sunagozaka, Masao Honda, Taro Yamashita, Ryuhei Nishino, Hajime Takatori, Kuniaki Arai, Tatsuya Yamashita, Yoshio Sakai and Shuichi Kaneko

Department of Gastroenterology, Kanazawa University Hospital, Kanazawa, Ishikawa, Japan

The identification of genes involved in tumor growth is crucial for the development of inventive anticancer treatments. Here, we have cloned a 17-kDa secretory protein encoded by *c19orf10* from hepatocellular carcinoma (HCC) serial analysis of gene expression libraries. Gene expression analysis indicated that *c19orf10* was overexpressed in approximately two-thirds of HCC tissues compared to the adjacent noncancerous liver tissues, and its expression was significantly positively correlated with that of alpha-fetoprotein (AFP). Overexpression of *c19orf10* enhanced cell proliferation of AFP-negative HLE cells, whereas knockdown of *c19orf10* inhibited cell proliferation of AFP-positive Hep3B and HuH7 cells along with G1 cell cycle arrest. Supplementation of recombinant c19orf10 protein in culture media enhanced cell proliferation in HLE cells, and this effect was abolished by the addition of antibodies developed against c19orf10. Intriguingly, c19orf10 could regulate cell proliferation through the activation of Akt/mitogen-activated protein kinase pathways. Taken together, these data suggest that c19orf10 might be one of the growth factors and potential molecular targets activated in HCC.

Hepatocellular carcinoma (HCC) is one of the most common cancers with an estimated worldwide incidence of 1,000,000 cases per year.[1] Most HCCs develop as a consequence of chronic liver disease such as chronic viral hepatitis due to hepatitis C virus (HCV) or hepatitis B virus (HBV) infection.[2–7] Liver cirrhosis patients with any etiology are considered to be at an extremely high risk for HCC.[8–10] Indeed, ~7% of liver cirrhosis patients with HCV infection develop HCC annually,[8,11] and the advancement of reliable HCC screening methods for high-risk patients is crucial for the improvement of their overall survival.[12]

Currently, imaging diagnostic techniques such as ultrasonography, computed tomography, magnetic resonance image and angiography are the gold standards for the early detection of HCC.[13,14] In addition, tumor markers such as alpha-fetoprotein (AFP) and des-gamma carboxyl prothrombin (DCP) have been used for the screening of HCC,[15–18] although their sensitivity and specificity are not sufficiently high. Recently, a gene expression profiling approach shed new light on Glypican 3, a heparin sulfate proteoglycan anchored to the plasma membrane, as a potential HCC marker, and its clinical usefulness as a molecular target as well as a tumor marker is presently under investigation.[19]

There are several options available for the treatment of HCC, including surgical resection, liver transplantation, radiofrequency ablation, transcatheter arterial chemoembolization and chemotherapy, while taking the HCC stage and liver function into consideration. Recently, molecular therapy targeting the Raf kinase/vascular endothelial growth factor receptor (VEGFR) kinase inhibitor sorafenib improved the survival of patients with advanced HCC,[20,21] emphasizing the importance of deciphering the molecular pathogenesis of HCC for the development of effective treatment options.

Here, we investigated the gene expression profiles of HCC by serial analysis of gene expression (SAGE) to discover a novel gene activated in HCC.[22–25] We identified a gene, *c19orf10*, overexpressed in HCC and determined that the encoded 17-kDa protein (c19orf10) is a secretory protein. Murine *c19orf10* was originally discovered to encode a cytokine interleukin (IL)-25/stroma-derived growth factor (SF20) in 2001.[26] The gene *c19orf10* was mapped in the H2 complex region of mouse chromosome 17 between *C3* and *Ir5*, and the hypothetical protein was predicted as globular protein.[26] However, the subsequent study failed to reproduce its proliferative effect on lymphoid cells, and the paper was retracted by the authors in 2003.[26,27] Nevertheless, independent studies revealed that c19orf10 was indeed produced by synoviocytes, macrophages and adipocytes, although the function of c19orf10 remained elusive.[28,29] In our study, we identified that *c19orf10* was overexpressed in AFP-positive HCC samples. Our data imply that c19orf10 could activate the mitogen-activated protein kinase (MAPK)/Akt pathway and

Key words: hepatocellular carcinoma, serial analysis of gene expression, *c19orf10*
Additional Supporting Information may be found in the online version of this article
DOI: 10.1002/ijc.25830
History: Received 14 Mar 2010; Accepted 15 Nov 2010; Online 2 Dec 2010
Correspondence to: Shuichi Kaneko, Kanazawa University Hospital, 13-1 Takara-machi, Kanazawa, Ishikawa 920-8641, Japan,
Tel.: +81-76-265-2233, Fax: +81-76-234-4250,
E-mail: skaneko@m-kanazawa.jp

enhance cell proliferation in HCC cell lines, suggesting that c19orf10 may be a growth factor produced by tumor epithelial cells and/or stromal cells, and, therefore, would be a good target for the treatment of HCC.

Material and Methods

SAGE and HCC samples

HCC and normal liver SAGE libraries that we had constructed were reanalyzed using SAGE 2000 software. The size of each SAGE library was normalized to 300,000 transcripts per library. Monte Carlo simulation was used to select genes whose expression levels were significantly different between the two libraries. Each SAGE tag was annotated using the gene-mapping website SAGE Genie database (http://cgap.nci.nih.gov/SAGE/) and the SOURCE database (http://smd.stanford.edu/cgi-bin/source/sourceSearch) as previously described.[30] An additional 15 SAGE libraries of normal and cancerous tissues from various organs were retrieved using the National Center for Biotechnology Information SAGEmap (http://www.ncbi.nlm.nih.gov/SAGE/).

Fifteen HCC tissues (four HBV-related and 11 HCV-related) and the corresponding noncancerous liver tissues were obtained from HCC patients who received hepatectomy. Four normal liver tissues were obtained from patients undergoing surgical resection of the liver for the treatment of metastatic colon cancer. Additionally, 36 HCC tissues (17 HBV-related and 19 HCV-related) were obtained from HCC patients undergoing hepatectomy. These samples were snap frozen in liquid nitrogen immediately after resection and used for quantitative real-time detection PCR (RTD-PCR). Total RNA was extracted using a ToTALLY RNA™ kit (Ambion, Austin, TX).

The study protocol conformed to the ethical guidelines of the Declaration of Helsinki (1975) and was approved by the institutional ethical review board committee. All patients provided written informed consent for the analysis of the specimens.

Laser capture microdissection and RNA isolation

Laser capture microdissection (LCM) was performed as previously described.[31] Briefly, 20 HCV-related surgically resected HCC tissues were frozen in OCT compound (Sakura Finetech, Torrance, CA).[32] Inflammatory cells and cancerous cells in HCC tissues were separately excised by LCM using a Laser Scissors CRI-337 (Cell Robotics, Albuquerque, NM) under a microscope. Total RNA was isolated from these cells using a microRNA isolation kit (Stratagene, La Jolla, CA) in accordance with the supplied protocol, with slight modifications.[31]

Construction of C19ORF10 expression plasmid and recombinant adenovirus vector

PCR was performed on a Marathon cDNA library from Huh7 cells using the following primers: sense primers: 5′-GACCCTAGTCCAACATGGCGGCGCCC-3′ (the first PCR), 5′-ATGGCGGCGCCCAGCGGAGGGTGGAACGGC-3′ (the nested second PCR) and antisense primers: 5′-CACCGGA GATGAGAAGGTGCCACCCGC-3′ (the first PCR), 5′-CAG GGCTGCTGGTCACAGCTCAGTGCGCG-3′ (the nested second PCR). The 5′ and 3′ends of the cDNA were isolated using a SMART RACE cDNA Amplification kit (Clontech, Mountain View, CA) according to the manufacturer's recommendations. The PCR products were cloned into a TA vector (Invitrogen, Carlsbad, CA) to generate the pcDNA3.1-*c19orf10* expression plasmid. Using this plasmid, a C-terminally FLAG-tagged construct of *c19orf10* was generated and inserted in a pSI mammalian expression vector (Promega, Madison, WI), which was driven by the SV40 promoter (pSI-*c19orf10*).

The replication-incompetent recombinant adenovirus vector expressing FLAG-tagged *c19orf10* (Ad. *c19orf10-FLAG*) was generated by homologous recombination using the AdMax system (Microbix, Toronto, Canada) as previously described.[33] The generated recombinant adenovirus was purified by limiting dilution, and the titer of viral aliquots was determined by the 50% tissue culture infectious dose method as previously described.[34]

RTD-PCR

RTD-PCR was performed as previously described.[31] Briefly, template cDNA was synthesized from 1 μg of total RNA using SuperScript™ II RT (Invitrogen). RTD-PCR of *c19orf10* (Hs. 00384077_m1), *AFP* (Hs00173490_m1), *GPC3* (Hs01018938_m1), *KRT19* (Hs00761767_s1) and the *ACTB* internal control (Hs99999903_m1) was performed using a TaqMan® Gene Expression Assay kit (Applied Biosystems, Foster City, CA). The expression of selected genes was measured in triplicate by ΔΔCT method using the 7900 Sequence Detection System (Applied Biosystems).

Cell lines and transfection of plasmids

Human liver cancer cell lines HuH1, Huh7, Hep3B, HLE and HLF as well as HEK293 and NIH3T3 were cultured in Dulbecco's modified Eagle's medium (Invitrogen) supplemented with 10% heat-inactivated fetal bovine serum (Invitrogen) in 5% CO_2 at 37°C. Transfection of plasmids was performed using FuGENE™ 6 (Roche Diagnostics, Indianapolis, IN) according to the manufacturer's instruction. Briefly, 5×10^5 cells were seeded in a six-well plate 12 hr before transfection, and 3 μg of plasmid DNA was used for each transfection. All experiments were repeated at least twice.

Purification of c19orf10-FLAG fused protein and production of anti-c19orf10 antibody

Approximately 500 ml of culture supernatant obtained from HEK293 cells infected with Ad. *C19ORF10-FLAG* at a multiplicity of infection of 20 was applied to an anti-FLAG affinity gel column (Sigma-Aldrich, St. Louis, MO). The column was

Table 1. ESTs overexpressed in the HCC library

Tag sequence	*p* value	HCC	Normal liver	T/N ratio	Name	UniGene ID
TGGGCAGGTG	<0.00001	33	0	>33	Chromosome 5 open reading frame 13	Hs.483067
GCAAAATATC	<0.00001	31	2	15.5	Liver cancer-associated noncoding mRNA, partial sequence	Hs.214343
AGCCTGCAGA	0.0002	12	1	12	Chromosome 19 open reading frame 10	Hs.465645
TTGTGCACGT	0.000228	12	1	12	CDNA FLJ45284 fis, clone BRHIP3001964	Hs.514273
ACATTCTTGT	0.000042	12	0	>12	Transcribed locus, strongly similar to XP_496055.1	Hs.76704
ACAAGTACCC	0.001161	10	1	>10	Chromosome 5 open reading frame 13	Hs.483067
GAGGTGAAGG	0.000174	10	0	>10	KIAA1914	Hs.501106
GCTGGAGGAG	0.000114	10	0	>10	Transcribed locus	Hs.520115

subjected to elution by competition with FLAG peptide (5 μg/ml), and each 1 ml fraction of the eluted aliquot was collected to obtain the most concentrated c19orf10-FLAG protein in accordance with the manufacturer's protocol. The anti-c19orf10 antibodies were developed by immunizing rabbits with repeated intradermal injections of purified c19orf10-FLAG. Protein concentration was measured by the Bradford method.

Silencing gene expression by short interfering RNA

The selected short interfering RNA (siRNA) targeting *C19ORF10* (Si-*C19ORF10*; Silencer Select siRNAs s31855) and the irrelevant control sequence (Si-*Control*; Silencer Select siRNAs 4390843) was obtained from Applied Biosystems. Transfection of these siRNAs was performed using FuGENE™ 6 (Roche Diagnostics) as previously described.[30] Briefly, 2×10^5 cells were seeded in a six-well plate 12 hr before transfection. A total of 100 pmol/l of siRNA duplex was used for each transfection. The experiments were performed at least twice.

Cell proliferation assay

Cell proliferation was evaluated in quadruplicate using a Cell Titer 96 MTS Assay kit (Promega). Briefly, 2×10^3 HLE or HuH7 cells were harvested in a 96-well plate 12 hr before the transfection or addition of the recombinant proteins. Transfection of siRNAs or plasmids was performed using FuGENE™ 6 (Roche Diagnostics). After incubation with MTS/PMS solution at 37°C for 2 hr, the absorbance at 450 nm was measured. The experiments were performed at least twice.

Cell cycle analysis

Cells were fixed using 80% ice-cold ethanol and incubated with propidium iodide for 10 min. DNA content was analyzed using a FACS Caliber flow cytometer (BD Biosciences, San Jose, CA) counting 10,000 stained cells. The distribution of cells in each cell cycle phase was determined using FlowJo software (Tree Star, Ashland, OR).

Western blotting

Cells were lysed in radioimmunoprecipitation assay (RIPA) buffer, and the extracts were subsequently electrophoresed on sodium dodecyl sulfate–10% polyacrylamide gels and transferred onto protean nitrocellulose membranes. The blots were then incubated for 1 hr with an appropriate primary monoclonal antibody: phospho-PI3K (#4228), phospho-Akt (#4060), phospho-GSK-3β (#9323), phospho-c-Raf (#9427), phospho-MEK1/2 (#9154), phospho-p44/42 MAPK (Erk1/2) (#4370), Cdk4 (CDK4 (#2906)), Cdk6 (#3136), cyclinD1 (#2926), cyclinD3 (#2936), phospho-Rb (#9308), phospho-P53 (# 9286), phospho-cdc2 (#9111) and β-actin (#4970) (Cell Signaling Technology, Allschwil, Switzerland) and anti-FLAG antibodies (Sigma-Aldrich, St. Louis, MO). The blots were washed and exposed to peroxidase-conjugated secondary antibodies, such as anti-mouse or rabbit IgG antibodies, and visualized using the ECL™ kit (Amersham Biosciences, Piscataway, NJ). All experiments were performed at least twice.

Statistical analyses

Unpaired *t*-tests and Kruskal–Wallis tests were performed on the RTD-PCR and cell proliferation data using GraphPad Prism software (www.graphpad.com).

Results

Identification of *C19ORF10* overexpression in HCC by SAGE

To comprehensively explore the candidate novel genes activated in HCC, we reanalyzed two SAGE libraries derived from HCC tissues and normal liver tissues.[30] After normalization of each SAGE library size to 300,000 tags, we compared the HCC and normal liver libraries to obtain the list of genes overexpressed in HCC. We identified 79 genes significantly overexpressed in the HCC library by more than tenfold when compared to the normal liver library (Supporting Information Table 1). Among them, we explored expressed sequence tags (ESTs) as candidates for novel HCC-related genes to identify eight unique tags corresponding to seven ESTs (Table 1). We especially focused on the EST chromosome 19 open reading frame 10 (*c19orf10*) because the

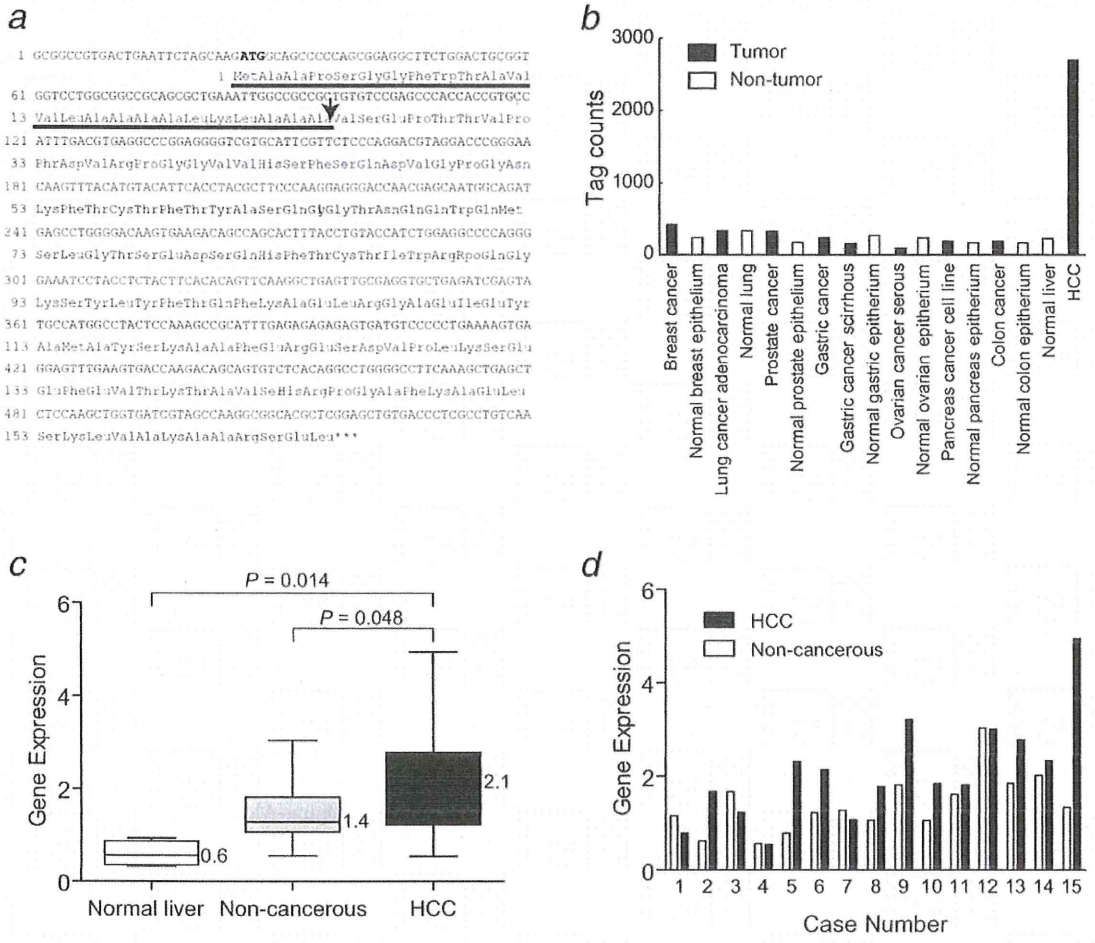

Figure 1. (*a*) Structure of a *c19orf10* gene and a c19orf10 protein. The DNA sequence of *c19orf10* and amino acid alignment of the encoded c19orf10 protein are shown. C19orf10 is predicted to have a molecular weight of 17 kDa and contain a signal peptide cleavage site (indicated as a black arrow). (*b*) *C19orf10* gene expression profiles in various tissues by SAGE. *Y*-axis indicates the number of tags corresponding to *c19orf10* in each tissue. (*c*, *d*) RTD-PCR analysis of *c19orf10*. RNA was isolated from 34 tissue samples: 15 HCC, 15 corresponding noncancerous liver samples and four normal liver samples. Differential expression of each gene among normal liver tissues, noncancerous liver tissues and HCC tissues was examined using the Kruskal–Wallis test and unpaired *t*-test. The mean value of gene expression data in each group is indicated (*c*). *C19orf10* was overexpressed in 10 of 15 examined HCC tissues compared to the noncancerous liver tissues (*d*).

sequence presumably encoded a secretory protein with a signal peptide sequence (Fig. 1*a*).

When we examined the expression profiles of *c19orf10* using retrieved SAGE data from various cancers and their normal counterparts, we identified that *c19orf10* was abundantly expressed in human HCC (Fig. 1*b*). We further examined the publicly available EST profiles of *c19orf10* (http://www.ncbi.nlm.nih.gov/unigene) and confirmed its tendency to be overexpressed in HCC compared to the normal liver (data not shown). We validated the overexpression of *c19orf10* in 15 independent HCC tissues and adjacent noncancerous liver tissues by RTD-PCR. Gene expression of *c19orf10* was significantly higher in the HCC tissues than in the normal liver tissues and adjacent noncancerous liver tissues ($p = 0.014$ and 0.048, respectively; Fig. 1*c*). *C19orf10* expression was elevated in HCC tissues compared to the adjacent noncancerous liver tissues in 10 of 15 patients (66.7%; Fig. 1*d*).

Overexpression of *C19ORF10* in AFP-positive HCC

As HCC is a heterogeneous mixture of cancer epithelial cells and stromal cells, and a previous report indicated that *c19orf10* is expressed in fibroblast-like synoviocytes. We, therefore, evaluated the expression of *c19orf10* in tumor epithelial cells and stromal cells separately using LCM and RTD-PCR in 20 HCC tissues (Fig. 2*a*). Although tumor

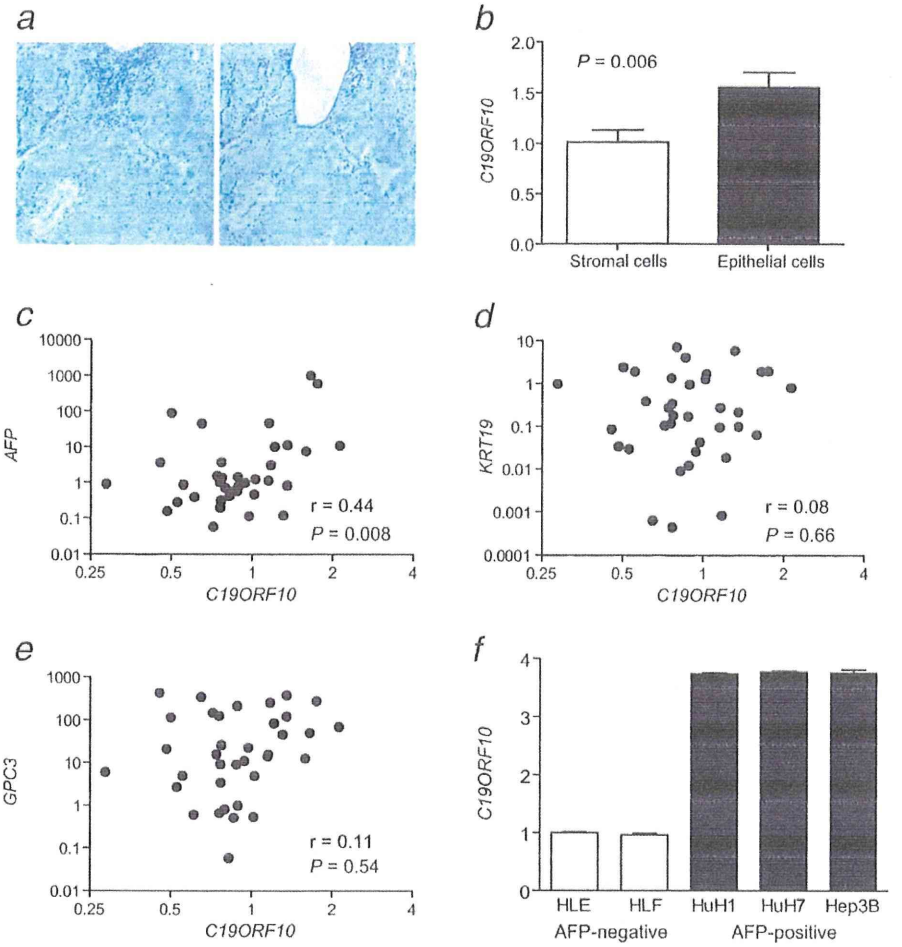

Figure 2. (*a*) Representative photomicrographs of an HCC tissue used for LCM (toluidine blue staining). Inflammatory mononuclear cells and stromal cells were separately captured (left: Pre-LCM, right: Post-LCM). (*b*) RTD-PCR analysis of *c19orf10* expression in inflammatory mononuclear cells and tumor epithelial cells in 20 HCV-related HCC tissues. Tumor-inflammatory mononuclear cells and stromal cells were isolated using LCM. RNAs were isolated from these cells as well as parenchymal tissues from the same liver, followed by RTD-PCR for *c19orf10* gene expression. Expression of the *c19orf10* gene was higher than that observed in HCC-infiltrating inflammatory mononuclear cells. $*p < 0.05$. (*c–e*) Scatter plot analysis of *c19orf10, AFP, KRT19* and *GPC3* expression in HCC. RNA was isolated from 17 HBV-related HCC and 19 HCV-related HCC. (*f*) RTD-PCR analysis of *c19orf10* in AFP-negative (HLE and HLF) and -positive (HuH1, HuH7 and Hep3B) liver cancer cell lines.

stromal cells expressed *c19orf10* at some level, the expression levels were significantly higher in tumor epithelial cells than in stromal cells ($p = 0.006$) (Fig. 2*b*).

To explore the relationship of *c19orf10* with other established HCC markers, we investigated the gene expression of *c19orf10*, *AFP* (alpha-fetoprotein), *KRT19* (cytokeratin 19) and *GPC3* (glypican 3). Because only 1 of 15 HCC tissues analyzed above (Fig. 1*d*) was AFP positive (data not shown), we further investigated the expression of *c19orf10* in an additional 36 HCC tissues using RTD-PCR. Interestingly, *c19orf10* expression was significantly positively correlated with *AFP* ($r = 0.44$, $p = 0.008$), but not with *KRT19* ($r = 0.08$, $p = 0.66$) nor *GPC3* ($r = 0.11$, $p = 0.54$) (Figs. 2*c–2e*). Furthermore, when we examined the expression of *c19orf10* in AFP-positive (HuH1, HuH7 and Hep3B) and -negative (HLE and HLF) HCC cell lines, we identified the overexpression of *c19orf10* in AFP-positive HCC cell lines (Fig. 2*f*). These data suggested that *c19orf10* is overexpressed and may play some role in AFP-positive HCCs.

C19orf10 regulates MAPK/Akt pathways and activates cell proliferation

To explore the functional role of c19orf10 in HCC, we performed *c19orf10* overexpression and knockdown studies using *c19orf10*-low HLE cells and *c19orf10*-high Hep3B and HuH7 cells, respectively. When we transfected HLE cells with

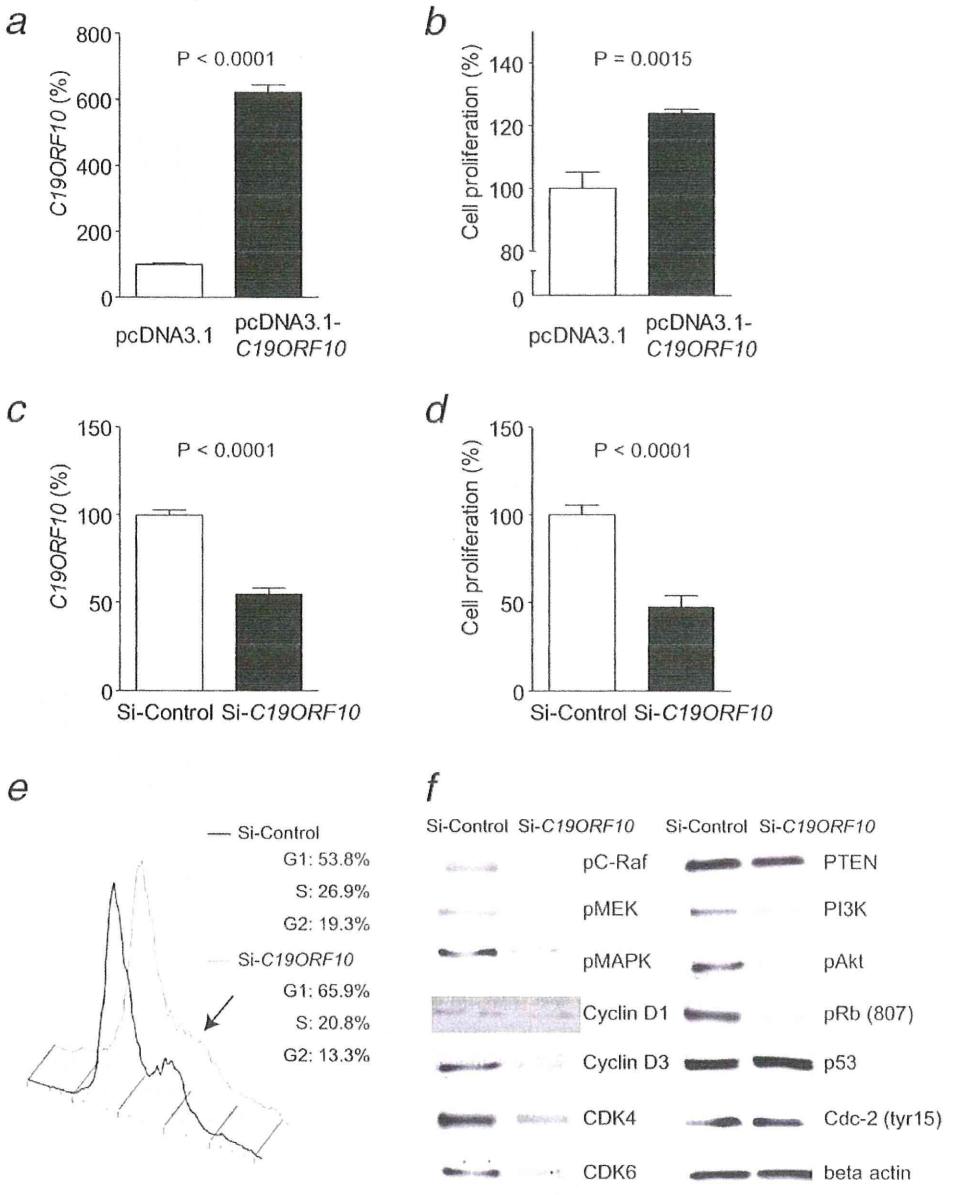

Figure 3. (*a*) RTD-PCR analysis of *c19orf10* expression in HLE cells transfected with pcDNA3.1 or pcDNA3.1-*c19orf10* plasmids. (*b*) Cell proliferation assay of HLE cells transfected with pcDNA3.1 or pcDNA3.1-*c19orf10* plasmids. Cell proliferation was evaluated 72 hr after each plasmid transfection. (*c*) RTD-PCR analysis of *c19orf10* expression in Hep3B cells transfected with Si-*Control* or Si-*c19orf10*. Gene expression was measured in triplicates 48 hr after transfection. (*d*) Cell proliferation assay of Hep3B cells transfected with Si-*Control* or Si-*c19orf10*. Cell proliferation was evaluated 72 hr after siRNA transfection. (*e*) Cell cycle analysis of HuH7 cells transfected with Si-*Control* or Si-*c19orf10*. Cell cycle was evaluated 72 hr after siRNA transfection. A black arrow indicates the G2 phase peak. (*f*) Western blotting analysis of Huh7 cells transfected with Si-*Control* or Si-*c19orf10*. Cells were lysed by RIPA buffer 72 hr after siRNA transfection.

pcDNA3.1 or pcDNA3.1-*c19orf10* plasmids, we identified an approximately sixfold overexpression of *c19orf10* when compared to the control 48 hr after transfection ($p < 0.0001$) (Fig. 3*a*). Interestingly, cell proliferation was modestly, but significantly, enhanced compared to the control 72 hr after transfection ($p = 0.0015$) (Fig. 3*b*).

We also transfected siRNAs targeting an irrelevant sequence (Si-*Control*) or *c19orf10* (Si-*c19orf10*) in Hep3B and

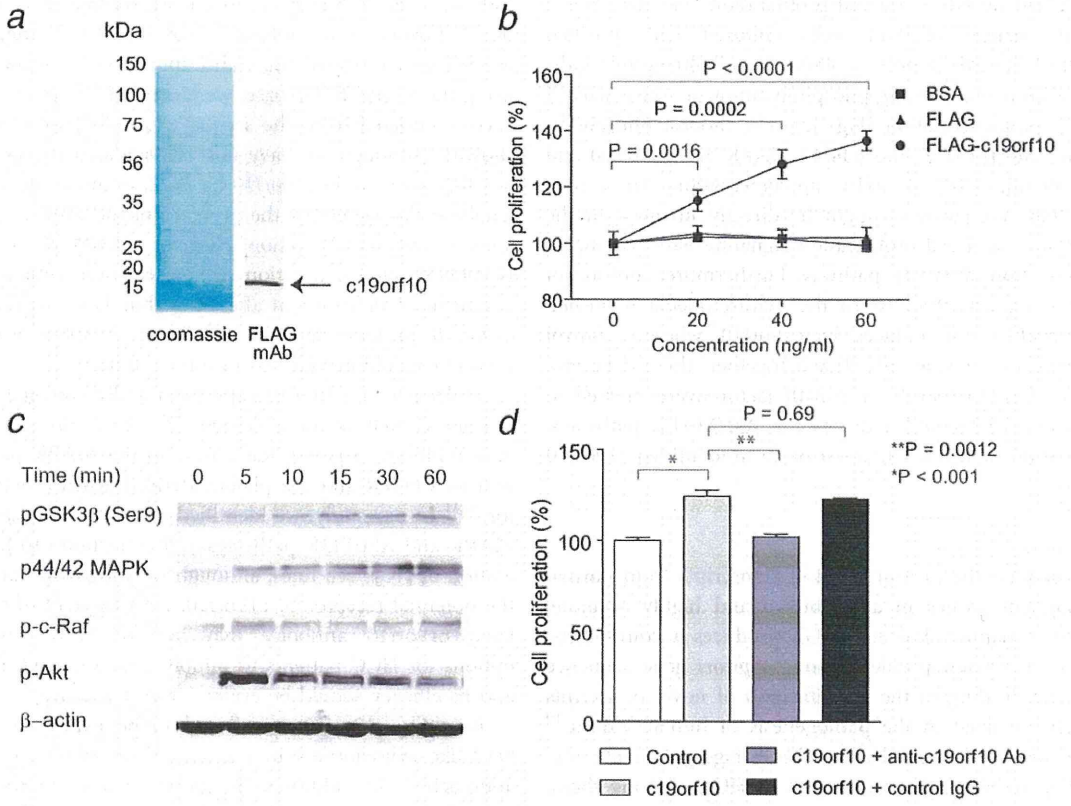

Figure 4. (*a*) Coomassie blue staining and Western blotting of culture supernatant of NIH3T3 cells transfected with pSI-*c19orf10-FLAG*. A black arrow indicates the 17-kDa c19orf10 protein. (*b*) Cell proliferation assay of HLE cells supplemented with recombinant c19orf10-FLAG, FLAG peptides or BSA. Cell proliferation was measured in quadruplicates 72 hr after supplementation. (*c*) Western blotting of HLE cells supplemented with c19orf10-FLAG (40 ng/ml). Cells were lysed at indicated time after c19orf10 supplementation. (*d*) Cell proliferation assay of HLE cells supplemented with control BSA (40 ng/ml) (white bar), c19orf10-FLAG (40 ng/ml) (light gray bar), c19orf10-FLAG (40 ng/ml) + anti-c19orf10 antibodies (gray bar) and c19orf10-FLAG (40 ng/ml) + control mouse IgG (black bar).

HuH7 cells. We observed an ~50% decrease in *c19orf10* expression in Hep3B cells transfected with Si- *c19orf10* compared to the control 48 hr after transfection with statistical significance ($p < 0.0001$). In this condition, cell proliferation was suppressed to 50% compared to the control 72 hr after transfection ($p < 0.0001$) (Figs. 3*c* and 3*d*). When we performed cell cycle analysis of HuH7 cells transfected with Si-*Control* or Si-*c19orf10*, we identified an increase of G1-phase cells and a decrease of S- and G2-phase cells by *c19orf10* knockdown, suggesting that the G1 cycle arrest was caused by the knockdown of *c19orf10* (Fig. 3*e*).

We examined the representative MAPK/Akt pathway-associated proteins and cell cycle regulators using Western blotting 72 hr after siRNAs transfection (Fig. 3*f*). Interestingly, phosphorylation of c-Raf, MEK, MAPK, PI3K and pAkt was inhibited by knockdown of *c19orf10*, suggesting the involvement of *c19orf10* in the MAPK/Akt pathways. Furthermore, phosphorylation of Rb, CDK4 and CDK6 was also inhibited by knockdown of *c19orf10*, consistent with the observation of G1 cell cycle arrest by *C19ORF10* knockdown. PTEN, p53 and phosphorylated CDC2 protein expression was not affected by knockdown of *c19orf10*.

C19orf10 encodes the secretory protein and stimulates cell proliferation

As the sequence of *c19orf10* suggested that it encodes a secretory protein, we transfected pSI-*c19orf10-FLAG* in NIH3T3 cells and examined the culture supernatant. Immunoprecipitation of the collected culture supernatant 48 hr after transfection using anti-FLAG antibodies indicated the existence of a 17-kDa protein (c19orf10), compatible with the molecular weight of the 142 amino acids protein encoded by *c19orf10* (Fig. 4*a*). We purified c19orf10-FLAG protein from the supernatant of HEK293 cells infected with Ad. *c19orf10-FLAG* using an anti-FLAG column. Supplementation of purified c19orf10-FLAG into the culture media for 72 hr enhanced the proliferation of HLE cells in a dose-dependent manner with statistical significance, whereas control FLAG peptides

and BSA had no effects on cell proliferation (Fig. 4*b*). Western blot analysis of HLE cells cultured with purified c19orf10-FLAG (40 ng/ml) or BSA control (40 ng/ml) indicated the immediate strong phospholyration of Akt peaked 5 min after supplementation (Fig. 4*c*). The modest phospholyration of GSK3β (Ser9) and p44/42 MAPK also followed and peaked 60 min after c19orf10 supplementation. These data suggest that Akt pathway might be directly involved in the c19orf10-mediated cell proliferation signaling with the subsequent activation of MAPK pathway. Furthermore, addition of antibodies against c19orf10 to the culture media abolished the cell proliferation induced by c19orf10, whereas control IgG had no effects (Fig. 4*d*). Taken together, these data suggest that c19orf10 may be a growth factor overexpressed in AFP-positive HCCs and activates the Akt/MAPK pathways, potentially through the activation of an unidentified c19orf10 receptor.

Discussion

SAGE facilitates the measurement of transcripts from normal and malignant tissues in a nonbiased and highly accurate, quantitative manner. Indeed, SAGE produces a comprehensive gene expression profile without *a priori* gene sequence information, leading to the identification of novel transcripts potentially involved in the pathogenesis of human cancer.[19] In our study, we identified seven SAGE tags potentially corresponding to novel genes activated in HCC. Among them, we identified the secretory protein c19orf10 activated in a subset of HCCs.

Several serum markers including AFP, DCP and Glypican 3 are currently used for the detection and/or the evaluation of the treatment for HCCs in the clinic.[15–18,35] These markers are known as oncofetal proteins, that is, expressed in the fetus, transcriptionally suppressed in the adult organ and reactivated in the tumor. We identified that the expression of *c19orf10* positively correlated with *AFP* expression but did not correlate with the expression of *GPC3* or the biliary marker *KRT19*. As *c19orf10* was rarely detected in the normal liver, it is possible that c19orf10 is also an oncofetal protein activated in HCC. We are currently developing a system to detect serum c19orf10 in HCC patients, and the significance of the serum c19orf10 value as an HCC marker should be clarified.

Recent advancement in molecular biology has revealed the considerable diversity of transcription initiation and/or termination of genes altered in the process of carcinogenesis. Indeed, using 5′ SAGE approach, we recently discovered the novel intronic transcripts activated in HCC.[36] Interestingly, when we investigated the transcription initiation of *c19orf10* using the 5′ SAGE database, we identified a potential 5′ splice variant initiated from the second exon of *c19orf10* (data not shown). Although we have not yet validated the presence of 5′ splice variants in *c19orf10* by PCR, examination of 5′ EST database also suggested the presence of the similar splice variants (GenBank Accession Number CR980295, BQ680744, BQ648461, *etc.*). Alteration of transcription initiation/termination in *c19orf10* might affect the abundance or function of c19orf10 protein, and the details of 5′ splice variants in *c19orf10* should be clarified in future studies.

Molecular targeting therapy has rapidly emerged for solid tumors as well as for leukemia.[37–39] Sorafenib is a multikinase inhibitor targeting Raf kinase in the MAPK pathway as well as VEGFR and the platelet-derived growth factor receptor.[40,41] In our study, we identified that c19orf10 activates the MAPK and Akt/PI3K pathways and contributes to the proliferation of HCC cell lines, although we still could not discover the potential receptor of c19orf10. Development of a neutralizing c19orf10 antibody may provide novel therapeutic options for HCC patients to inhibit these signaling pathways, and its efficacy should be evaluated in the future.

Recently, c19orf10 was found to be expressed in fibroblast-like synoviocytes in the synovium using a proteomics approach.[29] In addition, a recent article indicated that c19orf10 was expressed in preadipocyte cells and involved in adipogenesis using two-dimensional electrophoresis mass spectrometry analysis.[28] Thus, c19orf10 may have pleiotropic effects on various lineages of normal organs in various developmental stages, and the clarification of its distribution and biological properties in the whole body may provide more detailed information about the function of c19orf10.

In conclusion, we have identified the protein c19orf10 that regulates the Akt/MAPK pathways and cell cycle through an unidentified mechanism in HCC. Although further studies should be conducted to detect the potential c19orf10 receptor or signaling molecules binding to c19orf10, our study suggests that c19orf10 may be a novel growth factor, a potential tumor marker and also a potential target molecule for HCC treatment.

Acknowledgements

The authors thank Ms. Mikie Kakiuchi, Ms. Masayo Baba and Ms. Nami Nishiyama for their excellent technical assistance.

References

1. Befeler AS, Di Bisceglie AM. Hepatocellular carcinoma: diagnosis and treatment. *Gastroenterology* 2002;122:1609–19.
2. Tsukuma H, Hiyama T, Tanaka S, Nakao M, Yabuuchi T, Kitamura T, Nakanishi K, Fujimoto I, Inoue A, Yamazaki H, Kawashima T. Risk factors for hepatocellular carcinoma among patients with chronic liver disease. *N Engl J Med* 1993;328:1797–801.
3. Liang TJ, Jeffers LJ, Reddy KR, De Medina M, Parker IT, Cheinquer H, Idrovo V, Rabassa A, Schiff ER. Viral pathogenesis of hepatocellular carcinoma in the United States. *Hepatology* 1993;18:1326–33.
4. Mayans MV, Calvet X, Bruix J, Bruguera M, Costa J, Esteve J, Bosch FX, Bru C, Rodes J. Risk factors for hepatocellular carcinoma in Catalonia, Spain. *Int J Cancer* 1990;46:378–81.