Figure legends

Fig. 1. Comparison of *IFNλs* expression levels between chronic hepatitis C patients with rs12979860 CC or CT/TT. (a) Baseline mRNA levels of *IL29*, *IL28A,* and *IL28B* in PBMCs expressed relative to the internal control (/int.cont.). (b) Fold changes in *IL29*, *IL28A,* and *IL28B* expression in PBMCs stimulated for 8 h with poly(I:C) (10 μg/ml) after a 12-h pretreatment with IFNα-2b (100 IU/ml). Columns represent means ± SEM.

Fig. 2. Impact of *IFNλs* expression levels on therapy response in chronic hepatitis C patients. Fold changes in *IL29*, *IL28A*, and *IL28B* expression in PBMCs stimulated with IFNα-2b and poly(I:C). IFNλ induction levels were compared between (a) SVR (sustained virological responders), relapsers, and NR (non-virological responders) for peg-IFNα/ RBV (P/R) therapy. (b) VR (virological responders) and NR in patients with distinct IL28B genotypes (rs12979860 CC or CT/TT). (c) SVR for P/R, SVR for protease inhibitor (PI) plus P/ R triple therapy, and non-SVR for the triple therapy. Columns represent means ± SEM.

Fig. 3. Impact of *IFNλ4* on *IFNλs* expression and therapy response. Relationship of *IFNλ4* expression with (a) baseline expression of *IFNλs*, (b) *IFNλs* induction and (c) therapy response were compared in chronic hepatitis C patients with distinct *IL28B* genotypes (rs12979860 CC or CT/TT). The *IL28B*-unfavorable (CT/TT) group were subdivided into undetectable (−) or detectable (+) *IFNλ4* mRNA patients. (a) Baseline expressions of *IL29*, *IL28A*, and *IL28B* in PBMC. (b) Fold changes in *IL29*, *IL28A*, and *IL28B* expression in PBMCs stimulated f with IFNα-2b and poly(I:C). (c) Virological non-response rates for PEG-IFNα/ RBV therapy. Columns represent means ± SEM.

20141225

Fig. 4. Manipulating *IFNλ4* expression regulates *IL28B* induction and promoter activity. (a) Fold inductions of *IL28B* mRNA in BLCs transfected with *IFNλ4* and treated with IFNα (100U/ml). (b) Fold inductions of *IL28B* mRNA in HEK293T cells co-transfected with *IFNλ4* and IRF7 (control, 100ng, 500ng, 1000ng). Induction rates were expressed as fold change relative to control-transfected cells. (c) Fold inductions of *IL28B* promoter activity in HEK293/IL28B-Luc cells transfected with IFN*λ4* and treated with IFNα (0, 10, 100, 1000 IU/ml). (d, e) Fold inductions of *IL28B* promoter activity in HEK293/IL28B-Luc cells co-transfected with IFNλ4 and (d) IRF7 (control, 200ng, 500ng) or (e) p50:p65 (control, 200ng). Luciferase activities and cell viabilities were expressed as fold change relative to untreated or control-transfected cells. The error bars indicate standard deviation. *P<0.05.

20141225

Table 1. Characteristics of patients analyzed for IFNλ expression levels.

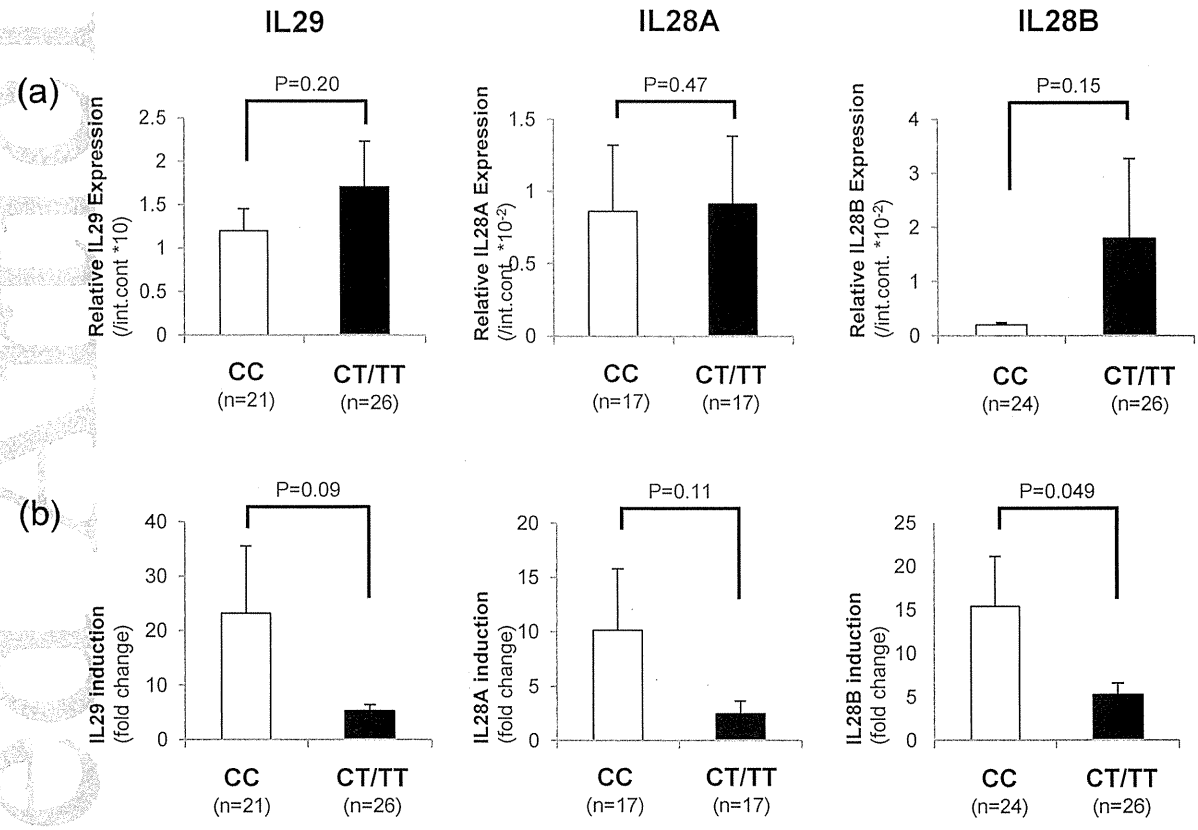
Characteristic	(n = 50)
Age median (range), year	64 (29-79)
Sex, n (%) male/female	19 (38) / 31 (62)
ALT median (range), IU/L	22 (5-157)
γGTP median (range), IU/L	23 (10-343)
LDL-C median (range), mg/dL	100 (38-169)
Hemoglobin median (range), g/dL	13.4 (9.3-16.8)
Platelet count median (range), $\times 10^4/\mu L$	15.5 (5.2-23.6)
Fibrosis stage, n (%)	
F1,2 / F3,4	28 (70) / 12 (30)
Viral load median (range), log IU/mL*	6.8 (4.8-7.6)
HCV core 70 a.a. n(%)†	
wild / mutant / ND	15 (30) / 21 (42) / 14 (28)
HCV core 91 a.a. n (%)	
wild / mutant / ND	18 (36) / 18 (36) / 14 (28)
ISDR substitutions, n (%)‡	
0,1 / 2≦ / ND	26 (52) / 6 (12) / 18 (36)
IL28B SNP (rs8099917), n (%)	
TT / TG, GG	27 (54) / 23 (46)
IL28B SNP (rs12979860), n (%)	
CC / CT, TT	24 (48) / 26 (52)
IL28B SNP (ss469415590), (%)	
TT / ΔG	24 (48) / 26 (52)
Effect of previous therapy, n (%)	
SVR / Relapse / NR	18 (36) / 14 (28) / 18 (36)

ALT, alanine aminotransferase; γ-GTP, γ-glutamyl transpeptidase; LDL-C, low-density lipoprotein cholesterol; HCV, Hepatitis C virus; ISDR, IFN sensitivity determining region; SVR, sustained virological responder; VR, virological responder; NR, non-responder; ND, not determined.

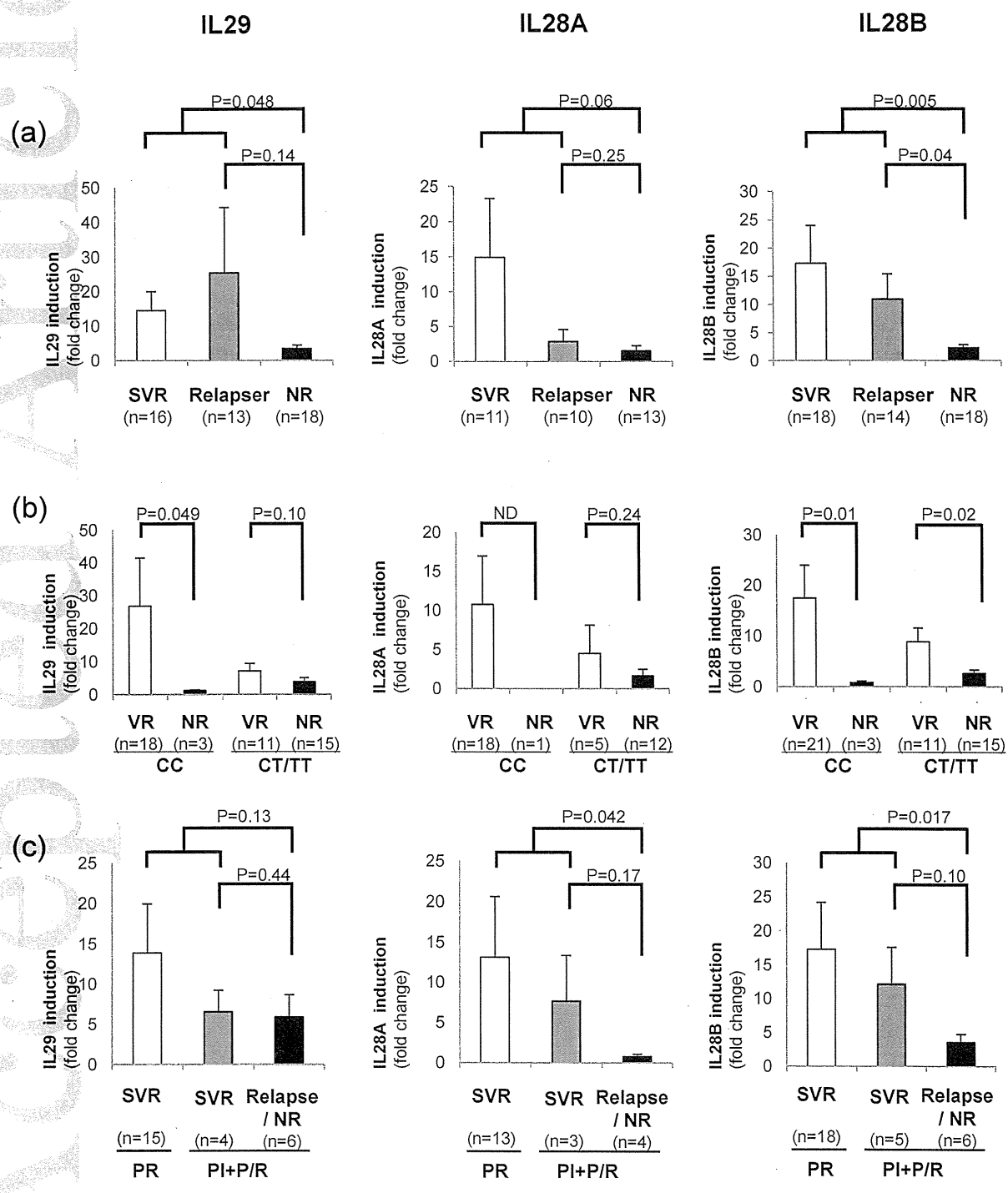
*HCV viral load was analyzed among Relapsers and Non-responders.

†HCV core amino acid (aa) 70R and 91L are considered wild type, while substituted amino acids are considered mutants.

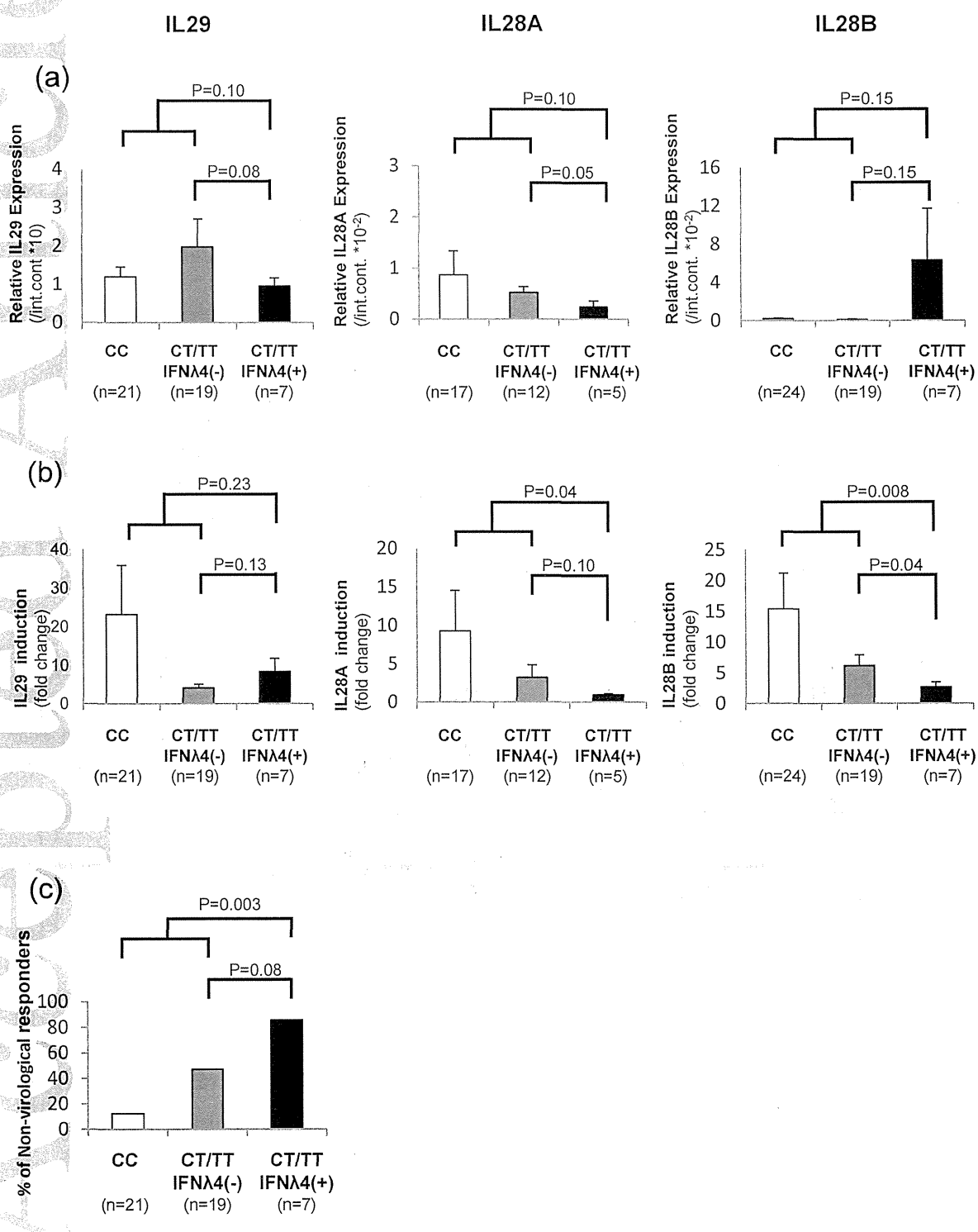
20141225



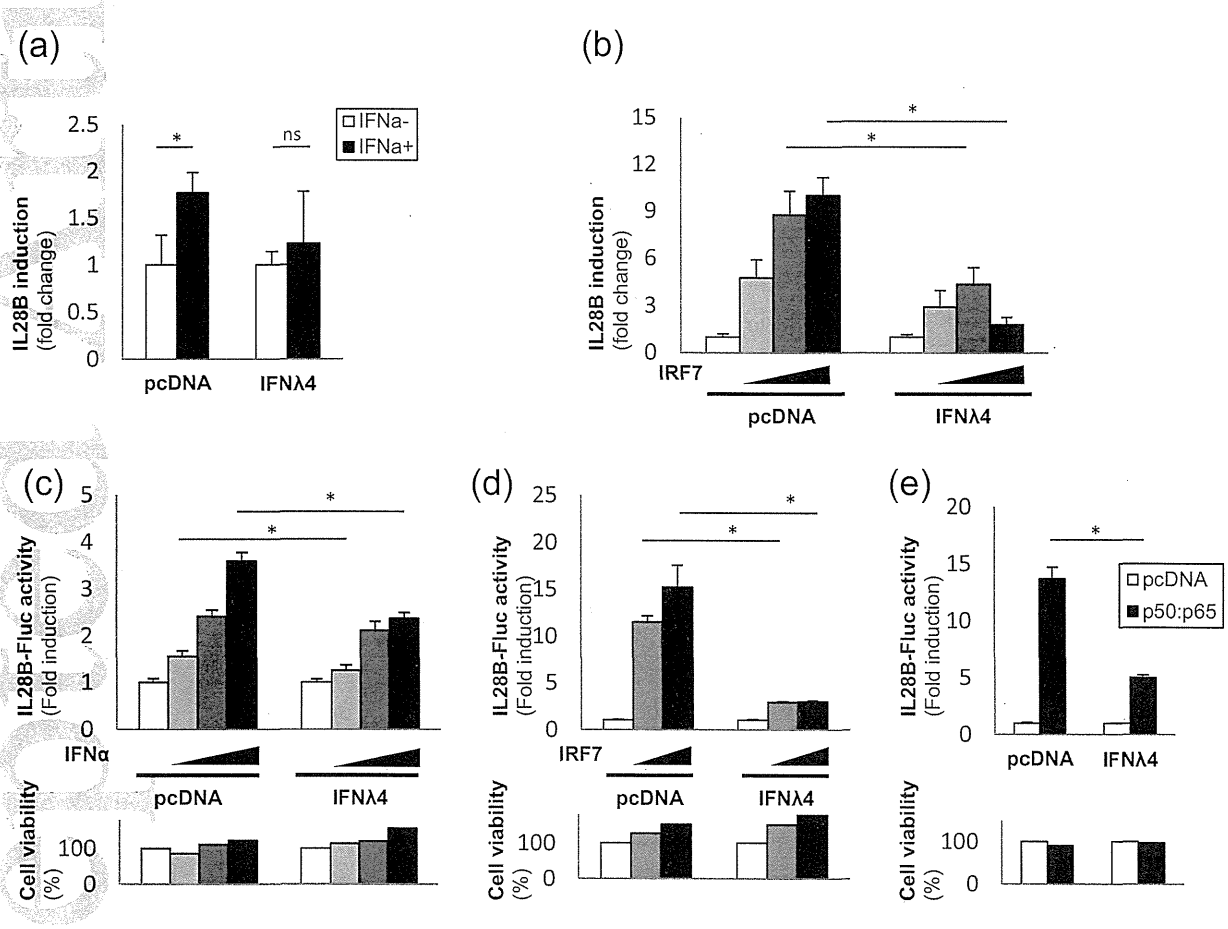
jgh_12902_f1.pdf



jgh_12902_f2.pdf



jgh_12902_f3.pdf



jgh_12902_f4.pdf

Original Article

Changes in Plasma Vascular Endothelial Growth Factor at 8 Weeks After Sorafenib Administration as Predictors of Survival for Advanced Hepatocellular Carcinoma

Kaoru Tsuchiya, MD, PhD[1]; Yasuhiro Asahina, MD, PhD[2,3]; Shuya Matsuda, MD[1]; Masaru Muraoka, MD[1]; Toru Nakata, MD[1]; Yuichiro Suzuki, MD[1]; Nobuharu Tamaki, MD[1]; Yutaka Yasui, MD[1]; Shoko Suzuki, MD[1]; Takanori Hosokawa, MD[1]; Takashi Nishimura, MD, PhD[1]; Ken Ueda, MD[1]; Teiji Kuzuya, MD, PhD[1]; Hiroyuki Nakanishi, MD, PhD[1]; Jun Itakura, MD, PhD[1]; Yuka Takahashi, MD, PhD[1]; Masayuki Kurosaki, MD, PhD[1]; Nobuyuki Enomoto, MD, PhD[4]; and Namiki Izumi, MD, PhD[1]

BACKGROUND: A new predictive biomarker for determining prognosis in patients with hepatocellular carcinoma (HCC) who receive sorafenib is required, because achieving a reduction in tumor size with sorafenib is rare, even in patients who have a favorable prognosis. Vascular endothelial growth factor (VEGF) receptor is a sorafenib target. In the current study, the authors examined changes in plasma VEGF concentrations during sorafenib treatment and determined the clinical significance of VEGF as a prognostic indicator in patients with HCC. **METHODS:** Plasma VEGF concentrations were serially measured in 63 patients with advanced HCC before and during sorafenib treatment. A plasma VEGF concentration that decreased >5% from the pretreatment level at 8 weeks was defined as a "VEGF decrease." An objective tumor response was determined using modified Response Evaluation Criteria in Solid Tumors 1 month after the initiation of therapy and every 3 months thereafter. **RESULTS:** Patients who had a VEGF decrease at week 8 (n = 14) had a longer median survival than those who did not have a VEGF decrease (n = 49; 30.9 months vs 14.4 months; $P = .038$). All patients who had a VEGF decrease survived for >6 months, and the patients who had both a VEGF decrease and an α-fetoprotein response (n = 6) survived during the observation period (median, 19.7 months; range, 6.5-31.0 months). In univariate analyses, a VEGF decrease, radiologic findings classified as progressive disease, and major vascular invasion were associated significantly with 1-year survival; and, in multivariate analysis, a VEGF decrease was identified as an independent factor associated significantly with survival. **CONCLUSIONS:** A plasma VEGF concentration decrease at 8 weeks after starting sorafenib treatment may predict favorable overall survival in patients with advanced HCC. ***Cancer* 2014;120:229-37.**

KEYWORDS: antiangiogenic therapy, biomarker, hepatocellular carcinoma, prognosis, α-fetoprotein.

INTRODUCTION

Hepatocellular carcinoma (HCC) is the most common primary malignancy of the liver (70%-85%) and a major cause of mortality. It is the fifth and seventh most frequent cancer and the second and sixth most frequent cause of cancer death in men and women, respectively.[1] At early stages or at Barcelona Clinic Liver Cancer stage A, a 5-year survival rate of 60% to 70% can be achieved in well selected patients with HCC who undergo surgical therapies (liver resection or transplantation) or locoregional procedures (ie, radiofrequency ablation).[2] However, treatment of advanced HCC that is not amenable to surgical or locoregional therapies remains a challenge in clinical practice.

Sorafenib is an oral, small-molecule tyrosine kinase inhibitor that blocks the synthesis of several intracellular proteins considered to be important for tumor progression, including the platelet-derived growth factor receptor beta, raf kinase, and the vascular endothelial growth factor (VEGF) receptor. VEGF is a homodimetric glycoprotein with a molecular weight of 45 kDa. The VEGF family includes VEGF-A, VEGF-B, VEGF-C, VEGF-D, and a structurally related molecule: placental growth factor. Three high-affinity VEGF tyrosine kinase receptors (VEGFRs) have been identified:

Corresponding author: Namiki Izumi, MD, PhD, Department of Gastroenterology and Hepatology, Musashino Red Cross Hospital, 1-26-1 Kyonan-cho, Musashino-shi, Tokyo 180-8610, Japan; Fax: (011) 81-422-32-9551; nizumi@musashino.jrc.or.jp

[1]Department of Gastroenterology and Hepatology, Musashino Red Cross Hospital, Tokyo, Japan; [2]Department of Gastroenterology and Hepatology, Tokyo Medical and Dental University, Tokyo, Japan; [3]Department of Liver Disease Control, Tokyo Medical and Dental University, Tokyo, Japan; [4]First Department of Internal Medicine, University of Yamanashi, Yamanashi, Japan

The first 2 authors contributed equally to this article.

DOI: 10.1002/cncr.28384, **Received:** April 2, 2013; **Revised:** August 10, 2013; **Accepted:** August 15, 2013, **Published online** October 7, 2013 in Wiley Online Library (wileyonlinelibrary.com)

VEGFR-1, VEGFR-2, and VEGFR-3. VEGFR-2 is the principal receptor that promotes the proangiogenic action of VEGF-A and has been the principal target of antiangiogenic therapies, although additional studies have underlined the importance of signaling through VEGFR-1. In 2 phase 3, placebo-controlled, randomized trials, sorafenib treatment significantly improved the time to tumor progression (TTP) and overall survival (OS) of patients with advanced HCC.[3,4] In those trials, however, no statistically significant pretreatment factors that predicted responses after patients started receiving sorafenib were identified.[5] Therefore, in clinical practice, it is extremely important to identify a predictive post-treatment biomarker that is associated with the treatment efficacy of sorafenib and the prognosis of patients after they start receiving sorafenib.

In general, the efficacy of treating solid tumors with systemic chemotherapy agents is assessed by radiologic findings. In 2010, Lencioni and Llovet published a modification of the Response Evaluation Criteria in Solid Tumors (RECIST).[6] However, the modified RECIST can be used only for typical HCC. Advanced HCCs often have atypical vascular patterns; therefore, evaluating tumor response to sorafenib is difficult with radiologic findings alone. Alternatively, α-fetoprotein (AFP) is the most popular tumor marker for HCC, and it has been reported that early AFP responses are a useful surrogate marker for predicting treatment response and prognosis in patients with advanced HCC who receive cytotoxic and antiangiogenic agents.[7-9] However, approximately 30% of patients with advanced HCC in the Sorafenib HCC Assessment Randomized Protocol (SHARP) trial had normal AFP concentrations.[10] Therefore, the identification of a new biomarker that can complementarily predict the efficacy of sorafenib and the prognosis of patients is necessary.

In a mouse model, an increase in hepatic VEGF levels was observed at 24 hours, 72 hours, and 120 hours after the administration of sorafenib,[11] suggesting that a change in VEGF levels may also occur during sorafenib therapy in humans. Therefore, we evaluated plasma VEGF changes during sorafenib treatment in patients with advanced HCC to determine whether VEGF has potential as a new biomarker for the prediction of treatment efficacy and prognosis after sorafenib administration.

MATERIALS AND METHODS

Patient Selection

Between December 2009 and August 2012, 95 consecutive patients with advanced, inoperable HCC received treatment with sorafenib at Musashino Red Cross Hospital. The diagnosis of HCC was based on guidelines established by the Liver Cancer Study Group of Japan[12] and the American Association for the Study of Liver Diseases[13] or by pathologic examination. According to these guidelines, a diagnosis of HCC is confirmed by histology or by characteristic radiologic findings, such as typical arterial enhancement of the tumor followed by a washout pattern in the images in the portal venous phase or the equilibrium phase on dynamic spiral computed tomography (CT) imaging or contrast-enhanced magnetic resonance imaging. Inclusion criteria were predefined as follows: 1) patients were alive 8 weeks after beginning treatment; and 2) patients had plasma VEGF and serum AFP concentrations evaluated at baseline, at 4 weeks, and at 8 weeks. Of 95 patients, 23 were unavailable for a week-8 VEGF measurement for the following reasons: 7 patients stopped sorafenib therapy because of erythema multiforme (grade 2-3) and started other therapies (radiation therapy or cytotoxic chemotherapy) within 1 month after starting sorafenib, 4 patients moved to another location before week 8, 5 patients refused to undergo a plasma VEGF measurement at week 8, and 7 patients were not available for obtaining VEGF concentration results. These 23 patients and 9 other patients who died within 8 weeks were excluded from the study. Hence, in total, 63 patients fulfilled the inclusion criteria. At enrollment, all patients had metastatic or locally advanced HCC that was not amenable to surgery or locoregional therapies, including transcatheter arterial chemoembolization (TACE) and local ablation. Written informed consent was obtained from all patients, and the ethics committee at Musashino Red Cross Hospital approved the study in accordance with the Declaration of Helsinki.

Sorafenib Treatment

The initial daily dose of sorafenib was 800 mg in 28 patients, 400 mg in 28 patients, and 200 mg in 7 patients. A reduced initial dose was allowed for patients who had the following factors: advanced age (≥80 years), gastrointestinal varices with a risk of bleeding, low body weight (<50 kg), and a poor performance status (≥2). In total, 60 patients underwent multiphase-multidetector CT imaging before starting sorafenib, 1 month after starting sorafenib, and every 3 months thereafter. Radiologic responses to therapy were evaluated according to modified RECIST. In all patients, serial measurements of plasma VEGF and serum AFP concentrations were performed before and after the receipt sorafenib and every month thereafter, with an allowance of ± 1 week. The endpoint of the current study was OS. In the follow-up visit after sorafenib administration, the medication was discontinued if progressive disease

(PD) was identified despite treatment, if intolerable adverse events occurred, or if inappropriate liver function was observed. Other palliative treatments or best supportive care were provided subsequently. An AFP response was defined as a decrease ≥20% in the serum AFP concentration during 8 weeks of treatment.

Plasma VEGF Measurements

Serial serum samples were collected prospectively from each patient. Venous blood samples were drawn into a serum separator tube and centrifuged at ×1800*g* for 10 minutes, and plasma samples were stored at −80°C until measurement. Plasma VEGF concentrations were measured quantitatively using an enzyme-linked immunosorbent assay kit (Quantikine Human VEGF Immunoassay; R&D Systems, Minneapolis, Minn) according to the manufacturer's instructions. We defined a decrease in the plasma VEGF level >5% from the pretreatment level at 8 weeks as a "VEGF decrease."

Statistical Analysis

Categorical variables were compared using the chi-square test, and continuous variables were compared using the Mann-Whitney test. All tests of significance were 2-tailed, and P values < .05 were considered statistically significant. OS curves were calculated using the Kaplan-Meier method, and differences between groups were assessed using the log-rank test. OS was determined as the interval between the date of treatment initiation and either death or the last visit. A Cox proportional-hazards model was used to determine the factors associated with OS. In univariate analyses, clinical and biologic parameters (sex, age, etiology, albumin, bilirubin concentrations, Child-Pugh class, plasma VEGF concentrations, and serum AFP concentrations) and tumor factors (vascular invasion and distant metastasis) were included. A logistic regression model was used to identify the factors associated with 1-year survival after the receipt of sorafenib. All statistical analyses were performed using StatView (version 5.0) software (Abacus Concepts, Berkeley, Calif).

RESULTS

Patient Characteristics

In total, 63 patients were enrolled in this study, and their characteristics are listed in Table 1. The diagnosis of HCC was confirmed by histology in 11 patients and by typical radiologic findings based on established guidelines in the remaining 52 patients. In all, 51 patients had previously received other therapeutic modalities, including 22 patients who previously received radiofrequency ablation, 22 who previously underwent TACE, 1 who previously received transcatheter arterial chemoinfusion, and 6 who previously underwent hepatic resection. Twelve patients had received sorafenib as initial therapy for HCC. Among the 63 enrolled patients, 33 were seropositive for hepatitis C virus antibody, 8 were seropositive for hepatitis B surface antigen, and 22 were seronegative for both hepatitis C virus antibody and hepatitis B surface antigen. Eighteen patients had evidence of extrahepatic metastasis, and 18 had major vascular invasion. No patient was lost to follow-up in this study.

TABLE 1. Characteristics of Study Patients With Advanced Hepatocellular Carcinoma (n = 63)

Characteristic	Median [Range]
Age, y	70 [40-85]
Sex: No. of men (%)	53 (84.1)
Baseline AFP, ng/mL	114 [2.0-98440]
Baseline plasma VEGF, pg/mL	288 [60-1580]
Treatment duration, mo	4.1 [0.1-28.3]
Overall survival, mo	9.3 [2.0-30.9]

Abbreviations: AFP, α-fetoprotein; VEGF: vascular endothelial growth factor.

Pretreatment Plasma VEGF Concentration and Prognosis and Extent of Hepatocellular Carcinoma

Pretreatment plasma VEGF concentrations in the 9 patients who died within 8 weeks were significantly higher than in the patients who survived beyond 8 weeks (813 ± 630 pg/mL vs 384 ± 18 pg/mL; P = .0024). Consistent with a previous study (the SHARP trial; Llovet et al[3]), our data suggested that the pretreatment plasma VEGF concentration is a useful prognostic factor for sorafenib therapy. However, there was no significant difference in OS between patients who had pretreatment plasma VEGF concentrations ≤450 pg/mL (n = 46) and those who had concentrations >450 pg/mL (n = 17; P = .731). The pretreatment plasma VEGF concentration could not predict prognosis for the patients who survived beyond 8 weeks.

We compared the size and extent of HCC between patients who had low plasma VEGF concentrations (≤450 pg/mL) and high plasma VEGF concentrations (>450 pg/mL). No difference was observed in the size or extent of HCC at baseline between patients with lower versus higher pretreatment plasma VEGF concentrations.

Association Between Changes in Plasma VEGF Concentrations and Overall Survival

The median OS assessed by the Kaplan-Meier method was 16.3 months for all 63 patients enrolled in the study

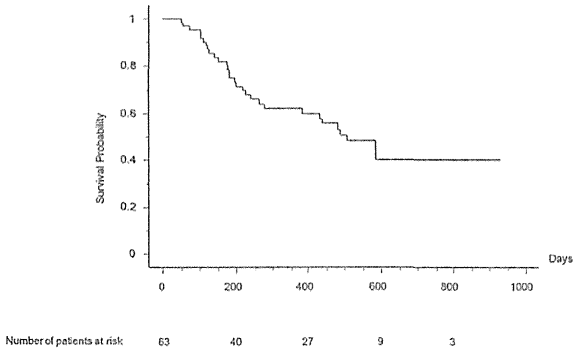


Figure 1. This Kaplan-Meier plot illustrates overall survival for all patients in the study.

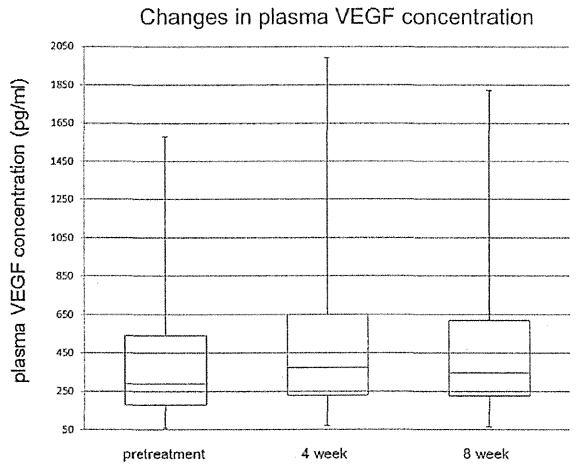


Figure 2. Changes in plasma vascular endothelial growth factor (VEGF) concentrations are illustrated.

(Fig. 1). Plasma VEGF concentrations at baseline, at 4 weeks, and at 8 weeks after the initiation of sorafenib treatment were 288 pg/mL (range, 60-1580 pg/mL), 372 pg/mL (range, 69-1990 pg/mL), and 347 pg/mL (range, 64-1840 pg/mL), respectively (Fig. 2). Plasma VEGF concentrations increased within 4 weeks after the administration of sorafenib in 47 of 63 patients (74.6%). The median survival of patients who had a decrease in their plasma VEGF concentration at week 4 (n = 16) and an increase in their plasma VEGF concentration at week 4 (n = 47) were 19.5 months and 16.8 months, respectively; and there was no significant difference in OS between changes in plasma VEGF at 4 weeks ($P = .645$). However, patients who had a VEGF decrease at week 8 (n = 14) had a longer median survival than those who did not have a VEGF decrease (n = 49; 30.9 months vs 14.4

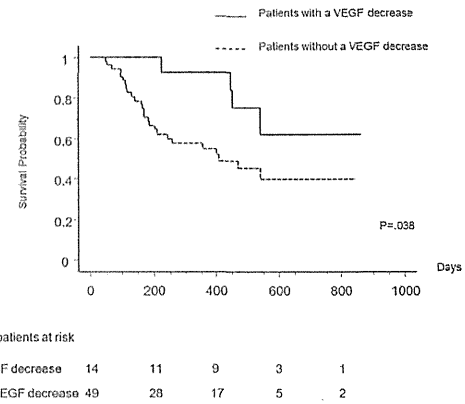


Figure 3. This Kaplan-Meier plot illustrates overall survival according to changes in vascular endothelial growth factor (VEGF) concentration.

months; $P = .038$) (Fig. 3), suggesting that a decrease in VEGF concentration 8 weeks after starting sorafenib treatment is closely associated with a favorable prognosis. The median percentage of decrease in the plasma VEGF concentration was 18.3% (range, 7%-41.7%). There were no differences in any pretreatment patient characteristics, including HCC stage and Child-Pugh score, between patients who did and did not have a VEGF decrease (Table 2).

Relation Between Radiologic Findings or Serum α-Fetoprotein Concentration and Overall Survival

The best radiologic responses to therapy assessed by modified RECIST were classified as a complete response (CR) (n = 4), a partial response (PR) (n = 16), stable disease (SD) (n = 34), and PD (n = 9). Fourteen patients had a VEGF decrease, and their best radiologic responses were a CR (n = 2), a PR (n = 2), SD (n = 9), and PD (n = 1). There was no significant difference in OS between the patients who had an objective response (CR + PR) and those with SD. The survival of patients who had PD was significantly worse than that of the patients without PD (median OS, 5.8 months and 19.4 months, respectively; $P = .0006$). There was no significant difference in OS between patients who had an AFP response and those who did not have an AFP response within the group that did not have PD (ie, those who attained a CR, a PR, or SD [the non-PD group]) (Fig. 4). There also was no significant difference ($P = .111$) between patients who did and did not have an AFP response among those in the non-PD group who had had an elevated AFP at baseline.

TABLE 2. Characteristics of Patients Categorized According to Variation in Vascular Endothelial Growth Factor Levels at 8 Weeks of Sorafenib Treatment

Characteristic	No. of Patients (%)		
	With VEGF Decrease, n = 14	Without VEGF Decrease, n = 49	*P*
Age, y	72	69	.325
Sex: Men	11 (78.6)	42 (85.7)	.679
Body weight, kg	58.3	62.3	.175
Cause of disease			.210
Hepatitis B	0 (0)	8 (16.3)	
Hepatitis C	9 (64.3)	24 (49)	
Other	5 (35.7)	17 (34.7)	
Prior treatment			.797
Yes	11 (78.6)	40 (81.6)	
No	3 (21.4)	9 (18.4)	
Baseline bilirubin, mg/dL	0.8	1.0	.375
Baseline albumin, g/dL	3.4	3.6	.190
Child-Pugh score			.178
5	7 (50)	30 (61.2)	
6	7 (50)	16 (32.7)	
7	0 (0)	3 (6.1)	
Maximum tumor size, cm			.892
≤5	8 (57.1)	22 (44.9)	
>5	6 (42.9)	27 (55.1)	
No. of tumors			.883
≤3	10 (71.4)	34 (69.4)	
>3	4 (28.6)	15 (30.6)	
Extrahepatic disease			.502
Yes	3 (21.4)	15 (30.6)	
No	11 (78.6)	34 (69.4)	
Site of metastatic disease			
Lung	1	7	
Bone	1	4	
Lymph node	1	3	
Lung and bone	0	1	
Major vascular invasion			.739
Yes	3 (21.4)	15 (30.6)	
No	11 (78.5)	34 (69.4)	

Abbreviations: VEGF: vascular endothelial growth factor.

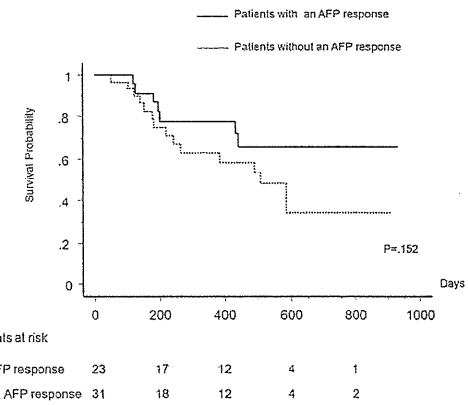


Figure 4. This Kaplan-Meier plot illustrates overall survival according to α-fetoprotein (AFP) response in patients without progressive disease (PD), classified as non-PD (ie, those who had a complete response, a partial response, or stable disease) according to modified Response Evaluation Criteria in Solid Tumors.

It is noteworthy that all patients who had a VEGF decrease and an AFP response survived during the observation period (median, 19.7 months; range, 6.5-31.0 months). In patients without a VEGF response (n = 49), there was no significant difference in OS between those who did and did not have an AFP response ($P = .147$). Of 49 patients who did not have a VEGF decrease at 8 weeks, 19 patients were able to survive beyond 1 year after starting sorafenib. Nine patients without a VEGF decrease at 8 weeks survived for >18 months.

Prognostic Factors After Sorafenib Administration

In univariate analysis, among all patients, a VEGF decease and an AFP response were associated significantly with OS after starting sorafenib. Major vascular invasion and PD, as evidenced by radiologic findings after sorafenib administration, also were significant prognostic factors. To predict which patients would have a highly favorable prognosis, the prognostic factors associated with 1-year survival after starting sorafenib were assessed in univariate and multivariate analyses. In the univariate analysis, a VEGF decrease, PD, and major vascular invasion were associated significantly with survival (Table 3). In the multivariate analysis, which was performed using those factors as covariates, a VEGF decrease was identified as an independent factor associated significantly with survival (Table 3). There was a significant difference in OS among the 3 groups (patients with a VEGF decrease and non-PD, patients without a VEGF decrease but non-PD, and patients without a VEGF decrease and PD; $P = .0013$) (Fig. 5). Only 1 patient who had a VEGF decrease was classified with PD. All 4 patients who had a VEGF decrease and an objective response (CR or PR) were able to survive during the observation period.

Adverse Events During Sorafenib Treatment

The overall incidence of treatment-related adverse events was 100%. The rate of discontinuation of sorafenib as a result of adverse events was 22.2%. Adverse events that led to the discontinuation of sorafenib treatment were liver dysfunction (63.6%), hand-foot skin reaction (18.2%), interstitial pneumonia (9.1%), and rash (9.1%). Dose reductions because of adverse events occurred in 62 patients. The most frequent adverse event leading to dose reductions was liver dysfunction (33.9%). In addition,

TABLE 3. Prognostic Factors Associated With 1-Year Survival After Sorafenib Administration

Risk Factor	OR (95% CI)[a]	*P*
Univariate analysis		
Age, by every 10 y	1.47 (0.75-2.87)	.266
Sex		
Women	1.00	
Men	0.26 (0.50-1.39)	.116
HBV infection		
Negative	1.00	
Positive	0.33 (0.06-2.02)	.231
HCV infection		
Negative	1.00	
Positive	1.23 (0.41-3.74)	.714
Albumin, by every 1 g/dL,	1.34 (0.45-3.99)	.604
Total bilirubin, by every 1 mg/dL	0.79 (0.28-2.25)	.656
Pre-AFP, by every 10 ng/mL	1.00 (1.00-1.00)	.161
Tumor size, cm		
<5	1.00	
≥5	0.42 (0.14-1.32)	.147
No. of tumors		
≤3	1.00	
≥4	0.26 (0.06-1.08)	.064
Major vascular invasion		
Yes	1.00	
No	4.00 (1.12-14.4)	.034
Extrahepatic metastasis		
Yes	1	
No	1.82 (0.56-5.90)	.320
5% VEGF decrease at wk 8		
No	1.00	
Yes	11.1 (1.29-94.6)	.028
PD		
No	1.00	
Yes	0.16 (0.29-0.86)	.033
Objective response: CR + PR		
No	1.00	
Yes	1.63 (0.49-5.42)	.426
AFP response		
No	1.00	
Yes	2.76 (0.80-9.52)	.107
Multivariate analysis[b]		
5% VEGF decrease at wk 8		
No	1.00	
Yes	10.0 (1.02-91.3)	.041
PD		
No	1.00	
Yes	0.20 (0.29-1.39)	.104
Major vascular invasion		
Yes	1.00	
No	3.03 (0.71-12.9)	.134

Abbreviations: AFP, α-fetoprotein; CI, confidence interval; CR, complete response; HBV, hepatitis B virus; HCV, hepatitis C virus; PD, progressive disease; PR, partial response; VEGF, vascular endothelial growth factor.
[a] The ORs for 1-year survival were calculated using logistic regression analysis.
[b] In the multivariate logistic analysis, a 5% VEGF decrease, PD, and portal invasion were included as covariates.

the incidence of adverse events was not related to plasma VEGF concentrations.

DISCUSSION

In the current study, we demonstrated that plasma VEGF concentrations change dynamically during sorafenib therapy, and changes in VEGF concentration are closely associated with OS in patients who receive treatment with sorafenib. VEGF is the major mediator of angiogenesis in HCC, and several studies have correlated VEGF concentrations with the prognosis of patients who have advanced HCC.[5,14-21]

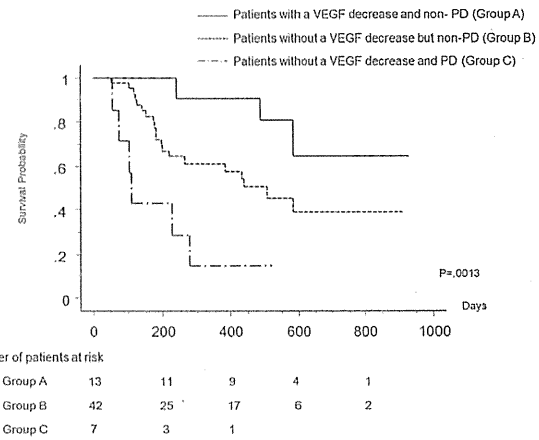


Figure 5. This Kaplan-Meier plot illustrates overall survival according to the combination of vascular endothelial growth factor (VEGF) changes and radiologic findings classified by modified Response Evaluation Criteria in Solid Tumors. Non-PD indicates patients who did not have progressive disease (PD) (ie, those who had a complete response, a partial response, or stable disease).

Recently, a new staging system was proposed that includes the plasma VEGF concentration along with the Cancer of the Liver Italian Program (CLIP) score; this new system—known as the V-CLIP score—classifies patients with advanced HCC more appropriately into a homogeneous prognostic group.[22] Therefore, the concentration of circulating VEGF is included as a candidate prognostic marker for HCC, especially in patients with advanced disease. The objective of our study was to elucidate the important question of whether an on-treatment change in VEGF is a potentially useful new biomarker for predicting prognosis in patients who survive beyond 8 weeks, because such an on-treatment predictor among patients who have relatively longer survival has not yet been elucidated. In this study, plasma VEGF concentrations increased from pretreatment levels within 4 weeks of starting sorafenib in 47 of 63 patients (74.6%). This was followed by a decrease in plasma VEGF levels at 8 weeks in 68.1% of patients. A possible mechanism of this transient increase in VEGF after starting sorafenib may be related to a reactive increase against the inhibition of VEGF activity or hypoxia induced by sorafenib. This

hypothesis is supported by the demonstration that plasma VEGF concentrations increased shortly after treatment with TACE.[24-26] It is believed that these increases in plasma VEGF concentration are related to the induction of tissue hypoxia.[27] However, the peak time point of VEGF elevation during sorafenib administration was different from that previously reported in TACE, in which a transient elevation of VEGF was observed within 7 days after TACE.[24-26] This observed difference may be related to the continuous induction of hypoxia by sorafenib administration.

It is noteworthy that, in our study, decreases in plasma VEGF observed within 8 weeks of sorafenib administration were associated with better OS. One possible reason for this association may be that the decrease in VEGF concentrations reflects a decrease in the number of tumor cells secreting VEGF. An association between changes in VEGF concentrations and disease progression was observed in a previous study of an anti-VEGF antibody, bevacizumab, in patients with advanced HCC.[23] In that study, plasma VEGF-A concentrations decreased from baseline in all patients after 8 weeks of bevacizumab therapy and increased to near baseline levels in 5 of 6 patients at the time of disease progression. Unfortunately, plasma VEGF-A levels after 8 weeks of bevacizumab in that study were available for only 8 of 46 patients who were enrolled the study, and plasma VEGF-A levels after 4 weeks were not evaluated. In our study, all patients were evaluated before and every 4 weeks after starting sorafenib. Moreover, we demonstrated the usefulness of plasma VEGF concentrations at 8 weeks and not at 4 weeks. Zhu et al[28] reported that plasma levels of VEGF and placental growth factor increased after cediranib, a pan-VEGFR tyrosine kinase inhibitor monotherapy for advanced HCC. In that study, progression-free survival was correlated inversely with baseline levels of VEGF, soluble VEGFR2 (sVEGFR2), and basic fibroblast growth factor and with on-treatment levels of basic fibroblast growth factor and insulin-like growth factor-1; and progression-free survival was directly associated with on-treatment levels of interferon-γ. Because changes of VEGF concentrations during therapy were not identified as a prognostic factor in the study by Zhu et al, biomarkers that predict prognosis may be different among different types of tyrosine kinase inhibitors. Jayson et al[29] reported that plasma VEGF-A in patients who received bevacizumab was potentially predictive and prognostic in metastatic breast, gastric, and pancreatic cancers; however, it was only prognostic (and not predictive) in metastatic colorectal cancer, nonsmall cell lung cancer, and renal cell carcinoma. In our study, we measured plasma VEGF concentrations and not plasma VEGF-A concentrations. Sorafenib is a multikinase inhibitor, whereas bevacizumab is a humanized monoclonal antibody that recognizes and blocks VEGF-A expression. Further studies to evaluate the clinical usefulness of determining VEGF and VEGF-A concentrations during sorafenib therapy are necessary in various cancers. Although the precise mechanism underlying the association between serial changes in VEGF and disease progression is unclear, the findings of the current study are extremely valuable for clinical practice in predicting the prognosis of patients who receive treatment with sorafenib.

Llovet et al[5] studied plasma biomarkers as predictors of outcome in patients with advanced HCC. They measured plasma biomarkers in 491 patients at baseline and in 305 patients after 12 weeks in a phase 3, randomized, controlled trial (the SHARP trial). Those authors concluded that angiopoietin-2 and VEGF were independent predictors of survival in patients with advanced HCC and that none of the tested biomarkers significantly predicted response to sorafenib. In our study, by measuring plasma VEGF monthly, we demonstrated that the changes 8 weeks after starting sorafenib were important for predicting OS.

It has been reported that modified RECIST guidelines are useful for predicting efficacy and prognosis after patients with advanced HCC receive treatment with sorafenib.[30] However, modified RECIST can only be used for typical hypervascular HCC, and not for atypical HCC, including poorly differentiated HCC and diffuse-type HCC. Moreover, the percentage of patients in our study who had PD was only 11.1% (9 of 63 patients), and the objective response rate (CR + PR vs SD) could not predict OS, suggesting that using only modified RECIST guidelines was insufficient for predicting OS in most patients who received sorafenib (non-PD patients). Therefore, it is important to identify a predictive biomarker for those patients who can expect long survival during sorafenib therapy, although their radiologic findings may not be categorized as objective responses.

From this point of view, decreases in VEGF observed in non-PD patients at week 8 may identify patients who have a favorable prognosis. According to our results, the median survival of patients who had a VEGF decrease was extremely good at 31.0 months, and we demonstrated that a VEGF decrease, but not modified RECIST or AFP, was the only significant post-therapeutic factor associated with favorable survival after sorafenib administration (Table 3). In our study, all

patients who had both a VEGF decrease and an AFP response survived during the observation period (median, 19.7 months). Taken together, the combination of a plasma VEGF decrease, an AFP response, and modified RECIST is useful for predicting an extremely favorable prognosis.

This study had a few limitations. The first was our subanalysis of consecutive patients. However, the median survival for the 23 excluded patients who were available for estimation was equivalent to that of the included patients (16.8 months); therefore, it is unlikely that selection bias affected our results. The second limitation is that we measured only plasma VEGF concentrations. In previous studies, many factors, including VEGF-A, short VEGF-A isoform, sVEGFR1, sVEGFR2, sVEGFR3, angiopoietin-2, and insulin-like growth factor-2, were evaluated as biomarkers. However, to our knowledge, this is the first clinical study to demonstrate the early dynamic changes in plasma VEGF concentrations in patients who received sorafenib. Finally, the number of patients in this study was relatively small to make recommendations to physicians. Our results indicated that patients who have decreased VEGF concentrations at 8 weeks have a favorable prognosis, regardless of their radiologic findings. However, further studies with a larger number of patients will be necessary to propose new recommendations.

In conclusion, changes in plasma VEGF concentrations during sorafenib treatment are dynamic in patients with advanced HCC, and an observed decrease in the plasma VEGF concentration 8 weeks after starting sorafenib is associated significantly with favorable OS. Today, because many clinical trials of new molecular-targeted agents for HCC are being conducted, it is necessary for hepatologists and oncologists to determine the time when alternative agents should be started as a second or third line of treatment. Our results have potentially important clinical implications for physicians and may influence their decisions regarding a treatment strategy for advanced HCC in individual patients.

FUNDING SUPPORT

This work was supported by grants from the Japanese Ministry of Welfare, Health, and Labor.

CONFLICT OF INTEREST DISCLOSURES

Yasuhiro Asahina received grants from the Japanese Ministry of Welfare, Health, and Labor and the Japanese Ministry of Education, Culture, Sports, and Science during the conduct of this study. Dr. Asahina has also received grants from Chugai Pharmaceutical Company, Ltd.; Toray Industries, Inc.; Bristol-Myers Squibb; Dainippon Sumitomo Pharma Company, Ltd.; Merck Sharp & Dohme (MSD); and Daiichi Sankyo Company, Ltd., and has received lecture fees from Chugai Pharmaceutical Company, Ltd. and MSD. Nobuyuki Enomoto has received grants and consulting fees from Bayer, Chugai-Roche, MDS, Bristol-Myers Squibb, and GlaxoSmithKline. Namiki Izumi has received lecture fees from MSD, Chugai Pharmaceutical Company, Ltd.; Daiichi-Sankyo Company, Ltd.; Bayer AG; and Bristol-Myers Squibb.

REFERENCES

1. World Health Organization, International Agency for Research on Cancer (IARC); Boyle P, Levin B, eds. World Cancer Report 2008. Lyon, France: IARC Press; 2008.
2. European Association for the Study of the Liver, European Organisation for Research and Treatment of Cancer. EASL-EORTC Clinical Practice Guidelines: management of hepatocellular carcinoma. *J Hepatol.* 2012;56:908-943.
3. Llovet JM, Ricci S, Mazzaferro V, et al. SHARP Investigators Study Group. Sorafenib in advanced hepatocellular carcinoma. *N Engl J Med.* 2008;359:378-390.
4. Cheng AL, Kang YK, Chen Z, et al. Efficacy and safety of sorafenib in patients in the Asia-Pacific region with advanced hepatocellular carcinoma: a phase III randomized, double-blind, placebo-controlled trial. *Lancet Oncol.* 2009;10:25-34.
5. Llovet JM, Pena CE, Lathia CD, Shan M, Meinhardt G, Bruix J. Plasma biomarkers as predictors of outcome in patients with advanced hepatocellular carcinoma. *Clin Cancer Res.* 2012;18: 2290-3000.
6. Lencioni R, Llovet JM. Modified RECIST (mRECIST) assessment for hepatocellular carcinoma. *Semin Liver Dis.* 2010;30:52-60.
7. Shao YY, Lin ZZ, Hsu C, Shen CH, Cheng AL. Early alpha-fetoprotein response predicts treatment efficacy of antiangiogenic systemic therapy in patients with advanced hepatocellular carcinoma. *Cancer.* 2010;116:4590-4596.
8. Kuzuya T, Asahina Y, Tsuchiya K, et al. Early decrease in α-fetoprotein, but not des-γ-carboxy prothrombin, predicts sorafenib efficacy in patients with advanced hepatocellular carcinoma. *Oncology.* 2011;81:251-258.
9. Personeni N, Bozzarelli S, Pressiani T, et al. Usefulness of alpha-fetoprotein response in patients treated with sorafenib for advanced hepatocellular carcinoma. *J Hepatol.* 2012;57:101-107.
10. Raoul JL, Bruix J, Greten TF, et al. Relationship between baseline hepatic status and outcome, and effect of sorafenib on liver function: SHARP trial subanalyses. *J Hepatol.* 2012;56:1080-1088.
11. Hora C, Romanque P, Dufour JF. Effect of sorafenib on murine liver regeneration. *Hepatology.* 2011;53:577-586.
12. Kudo M, Izumi N, Kokudo N, Matsui O, Sakamoto M, Makuuchi M. Management of hepatocellular carcinoma in Japan: consensus-based clinical practice guidelines proposed by the Japan Society of Hepatology (JSH) 2010 updated version. *Dig Dis.* 2011;29:339-364.
13. Bruix J, Sherman M; American Association for the Study of Liver Diseases. Management of hepatocellular carcinoma: an update. *Hepatology.* 2011;53:1020-1022.
14. El-Assal ON, Yamanoi A, Soda Y, et al. Clinical significance of microvessel density and vascular endothelial growth factor expression in hepatocellular carcinoma and surrounding liver: possible involvement of vascular endothelial growth factor in the angiogenesis of cirrhotic liver. *Hepatology.* 1998;27:1554-1562.
15. Yamaguchi R, Yano H, Iemura A, Ogasawara S, Haramaki M, Kojiro M. Expression of vascular endothelial growth factor in human hepatocellular carcinoma. *Hepatology.* 1998;28:68-77.
16. Yoshiji H, Kuriyama S, Yoshii J, et al. Synergistic effect of basic fibroblast growth factor and vascular endothelial growth factor in murine hepatocellular carcinoma. *Hepatology.* 2002;35:834-842.
17. Tamesa T, Iizuka N, Mori N, et al. High serum levels of vascular endothelial growth factor after hepatectomy are associated with poor prognosis in hepatocellular carcinoma. *Hepatogastroenterology.* 2009; 56:1122-1126.
18. Hu J, Xu Y, Shen ZZ, et al. High expressions of vascular endothelial growth factor and platelet-derived endothelial cell growth factor

predict poor prognosis in alpha-fetoprotein-negative hepatocellular carcinoma patients after curative resection. *J Cancer Res.* 2009;135: 1359-1367.
19. Poon RT, Lau C, Pang R, Ng KK, Yuen J, Fan ST. High serum vascular endothelial growth factor levels predict poor prognosis after radiofrequency ablation of hepatocellular carcinoma: importance of tumor biomarker in ablative therapies. *Ann Surg Oncol.* 2007;14: 1835-1845.
20. Schoenleber SJ, Kurtz DM, Talwalkar JA, Rober LR, Gores GJ. Prognostic role of vascular endothelial growth factor in hepatocellular carcinoma: systemic review and meta-analysis. *Br J Cancer.* 2009; 100:1385-1392.
21. Kaseb AO, Hanbali A, Cotant M, Hassan MM, Wollner I, Philip PA. Vascular endothelial growth factor in the management of hepatocellular carcinoma: a review of literature. *Cancer.* 2009;115: 4895-4906.
22. Kaseb A, Hassan M, Lin E, Xiao L, Kumar V, Morris J. V-CLIP: integrating plasma vascular endothelial growth factor into a new scoring system to stratify patients with advanced hepatocellular carcinoma for clinical trials. *Cancer.* 2011;117:2478-2488.
23. Siegel AB, Cohen EI, Ocean A, et al. Phase II trial evaluating the clinical and biologic effects of bevacizumab in unresectable hepatocellular carcinoma. *J Clin Oncol.* 2008;26:2992-2998.
24. Li X, Feng GS, Zheng CS, Zhuo CK, Liu X. Expression of plasma vascular endothelial growth factor in patients with hepatocellular carcinoma and effect of transcatheter arterial chemoembolization therapy on plasma vascular endothelial growth factor level. *World J Gastroenterol.* 2004;10:2878-2882.
25. Suzuki H, Mori M, Kawaguchi C, Adachi M, Miura S, Ishii H. Serum vascular endothelial growth factor in the course of transcatheter arterial embolization of hepatocellular carcinoma. *Int J Oncol.* 1999; 14:1087-1090.
26. Shim JH, Park JW, Kim JH, et al. Association between increment of serum VEGF and poor prognosis after transcatheter arterial chemoembolization in hepatocellular carcinoma patients. *Cancer Sci.* 2008;99:2037-2044.
27. von Marschall Z, Cramer T, Hocker M, Finkenzeller G, Wiedenmann B, Rosewicz S. Dual mechanism of vascular endothelial growth factor upregulation by hypoxia in human hepatocellular carcinoma. *Gut.* 2001;48:87-96.
28. Zhu AX, Ancukiewicz M, Duda DG, et al. Efficacy, safety, pharmacokinetics, and biomarkers of cediranib monotherapy in advanced hepatocellular carcinoma: a phase II study. *Clin Cancer Res.* 2013; 19:1557-1566.
29. Jayson GC, de Haas S, Delmar P, et al. Evaluation of plasma VEGF-A as a potential predictive pan-tumour biomarker for bevacizumab. Abstract 804. Paper presented at: 2011 European Multidisciplinary Cancer Congress; Stockholm, Sweden; September 23-27, 2011.
30. Edeline J, Boucher E, Rolland Y, et al. Comparison of tumor response by Response Evaluation Criteria in Solid Tumors (RECIST) and modified RECIST in patients treated with sorafenib for hepatocellular carcinoma. *Cancer.* 2012;118:147-156.

LIVER TRANSPLANTATION 20:291–297, 2014

ORIGINAL ARTICLE

Risk Factors for Exceeding the Milan Criteria After Successful Radiofrequency Ablation in Patients With Early-Stage Hepatocellular Carcinoma

Kaoru Tsuchiya,[1,2] Yasuhiro Asahina,[3,4] Nobuharu Tamaki,[1] Yutaka Yasui,[1] Takanori Hosokawa,[1] Ken Ueda,[1] Hiroyuki Nakanishi,[1,2] Jun Itakura,[1] Masayuki Kurosaki,[1] Nobuyuki Enomoto,[2] and Namiki Izumi[1]

[1]Department of Gastroenterology and Hepatology, Musashino Red Cross Hospital, Tokyo, Japan; [2]First Department of Internal Medicine, University of Yamanashi, Yamanashi, Japan; [3]Departments of Gastroenterology and Hepatology, Tokyo Medical and Dental University, Tokyo, Japan; and [4]Liver Disease Control, Tokyo Medical and Dental University, Tokyo, Japan

Radiofrequency ablation (RFA) is an effective and safe noninvasive treatment for hepatocellular carcinoma (HCC) and may be useful as a bridging therapy in liver transplantation. The prognosis after liver transplantation for patients within the Milan criteria is excellent. This study was aimed at identifying risk factors associated with exceeding the Milan criteria after initial locally curative RFA therapy. Among 554 primary HCC patients, 323 with early-stage HCC after RFA were analyzed (mean age = 66 years). Two hundred forty-eight patients had hepatitis C virus, 33 patients had hepatitis B virus, and 41 patients had neither hepatitis B nor hepatitis C; 256, 67, and 0 patients were classified as Child-Pugh A, B, and C, respectively. The rates of cumulative overall survival and recurrence exceeding the Milan criteria were analyzed with Kaplan-Meier analysis, and factors associated with overall survival were determined with Cox proportional hazards analysis. The cumulative overall survival rates at 1, 3, 5, and 10 years were 96.2%, 84.4%, 69.9%, and 40.6% respectively, without liver transplantation. The cumulative rates of recurrence exceeding the Milan criteria at 1, 3, and 5 years were 15.1%, 46.0%, and 61.1% respectively. An alpha-fetoprotein (AFP) level > 100 ng/mL and recurrence within 1 year after initial ablation were independently associated with earlier recurrence exceeding the Milan criteria and overall survival. The 3- and 5-year survival rates for patients with both risk factors were 33.5% and 22.6%, respectively, despite an early stage at initial ablation. In conclusion, a higher AFP level and HCC recurrence within 1 year of RFA are risk factors for exceeding the Milan criteria and for overall survival. Early liver transplantation or adjuvant therapy should be considered for patients with both risk factors. *Liver Transpl 20:291-297, 2014.* © 2013 AASLD.

Received August 1, 2013; accepted November 4, 2013.

Abbreviations: AFP, alpha-fetoprotein; CI, confidence interval; CLIP, Cancer of the Liver Italian Program; CT, computed tomography; HBV, hepatitis B virus; HCC, hepatocellular carcinoma; HCV, hepatitis C virus; HR, hazard ratio; PIVKA-II, protein induced by vitamin K absence or antagonist II; RFA, radiofrequency ablation; TACE, transcatheter arterial chemoembolization.

Kaoru Tsuchiya, Yasuhiro Asahina, Nobuyuki Enomoto, and Namiki Izumi designed the research. Kaoru Tsuchiya wrote the article. Nobuharu Tamaki, Yutaka Yasui, Takanori Hosokawa, Ken Ueda, Hiroyuki Nakanishi, Jun Itakura, and Masayuki Kurosaki conducted the research. Kaoru Tsuchiya, Yasuhiro Asahina, and Nobuyuki Enomoto analyzed the data.

Yasuhiro Asahina belongs to a department funded by donations from Chugai Pharmaceutical Co., Ltd., Toray Industries, Inc., Bristol-Myers Squibb, Dainippon Sumitomo Pharma Co., Ltd., and Merck Sharp & Dohme. The other authors of this article have no conflicts of interest to declare for this study.

This study was supported by grants from the Japanese Ministry of Education, Culture, Sports, Science, and Technology and the Japanese Ministry of Welfare, Health, and Labor.

Address reprint requests to Namiki Izumi, M.D., Ph.D., Department of Gastroenterology and Hepatology, Musashino Red Cross Hospital, 1-26-1 Kyonan-Cho, Musashino-Shi, Tokyo 180-8610, Japan. Telephone: +81-422-32-3111; FAX: +81-422-32-9551;
E-mail: nizumi@musashino.jrc.or.jp

DOI 10.1002/lt.23798
View this article online at wileyonlinelibrary.com.
LIVER TRANSPLANTATION.DOI 10.1002/lt. *Published on behalf of the American Association for the Study of Liver Diseases*

See Editorial on Page 257

Accounting for 70% to 85% of all cases, hepatocellular carcinoma (HCC) is the most common primary malignancy of the liver and a major cause of mortality; it is the fifth most frequently diagnosed cancer and the second most frequent cause of cancer death in men. In women, it is the seventh most commonly diagnosed cancer and the sixth leading cause of cancer death.[1,2] At present, the major curative treatments for HCC consist of hepatic resection, ablation therapy, and liver transplantation.[3] Although hepatic resection and ablation therapy often show excellent effects on HCC, they cannot prevent recurrence in the remnant liver or eliminate other complications caused by concurrent liver cirrhosis. On the other hand, liver transplantation has become a favored option for HCC treatment because it not only provides a local cure but also decreases the risks for recurrence and progressive liver disease. Liver transplantation for HCC patients with cirrhosis who meet the Milan criteria[4] (a solitary tumor $\leq$ 50 mm or 3 or fewer lesions, none > 30 mm) offers long-term survival similar to that observed for patients undergoing transplantation for nonmalignant liver disease.[5,6] Some recent studies[7-9] have reported that radiofrequency ablation (RFA) is an effective and safe noninvasive treatment for HCC, enables complete ablation of an area up to 3 cm in diameter, and is superior to microwave coagulation and percutaneous ethanol injection therapy. In a recent study,[10] the 1-, 3-, and 5-year tumor-free survival rates were all 60% for salvage liver transplantation for recurrent HCC within the Milan criteria; the corresponding rates were 70.2%, 48.0%, and 48.0% for hepatic resection and 41.0%, 20.3%, and 10.9% for RFA ($P = 0.004$). The patients in that study underwent either hepatic resection or RFA as an initial treatment for HCC within the Milan criteria. Therefore, it is very important to know when patients exceed the Milan criteria after initial RFA as a locally curative therapy for HCC. Hence, the aims of the present study were to identify the risk factors associated with recurrence exceeding the Milan criteria and to clarify prognostic factors for overall survival for early-stage HCC patients receiving RFA as an initial therapy.

PATIENTS AND METHODS

Patients

Between July 1999 and July 2005, 554 primary HCC patients were admitted to the Department of Gastroenterology and Hepatology at Musashino Red Cross Hospital (Tokyo, Japan). The patients received the following appropriate therapies according to the appropriate guidelines released during the study period by the Liver Cancer Study Group of Japan and according to the Barcelona Clinic Liver Cancer staging system[11]: 323 were treated with RFA, 35 were treated with surgical resection, 158 were treated with transcatheter arterial chemoembolization (TACE), 10 were treated with systemic cytotoxic chemotherapy, 2 were treated with percutaneous microwave coagulation, 4 were treated with percutaneous ethanol injection therapy, 2 were treated with radiation therapy, and 20 were treated with the best supportive care. There were no patients who underwent liver transplantation. Among these 554 patients, 323 were treated with RFA as an initial curative therapy for primary HCC, and they were included in the following analyses. The inclusion criteria for RFA were as follows: a solitary HCC tumor-$\leq$ 50 mm or 3 or fewer lesions, none > 30 mm; 3 or fewer lesions without major vascular or biliary invasion; a total bilirubin concentration < 2.5 mg/dL; a platelet count > $3 \times 10^4/mm^3$; and prothrombin activity > 50%. Some patients refused hepatic resection and chose RFA voluntarily because of concerns about complications or physician recommendations, which took into account impairment of liver function, HCC location, and cardiopulmonary dysfunction. Patients who had ascites uncontrolled by diuretics and/or had extrahepatic metastases were excluded. The reasons that the patients were selected for RFA instead of liver transplantation were as follows: a Child-Pugh classification of A (n = 256 or 79.3%), an age > 65 years (n = 198 or 61.3%), and heart or lung disease complications (n = 6 or 1.9%). The number of patients who were classified as Child-Pugh B and were younger than 66 years of age was 28 (8.7%). Among these patients, there was 1 patient who had severe heart disease; the remaining 27 patients did not have living donors. Written informed consent was obtained from all patients, and this study was approved by the ethics committee of Musashino Red Cross Hospital and was conducted in accordance with the Declaration of Helsinki.

HCC Diagnosis

The HCC diagnosis was confirmed by typical radiographic findings on dynamic computed tomography (CT) with or without hepatic arterial and portal angiography and magnetic resonance imaging or by needle biopsy. For triple-phase dynamic CT scans, arterial, portal, and equivalent phases were set at 35, 70, and 150 seconds, respectively, after the injection of the contrast agent. Spiral CT scans were obtained from 5-mm-thick sections. Board-certified radiologists diagnosed HCC on the basis of typical patterns, such as an early-phase hyperattenuation area or late-phase hypoattenuation on dynamic CT or magnetic resonance imaging. Liver biopsy was performed when a definitive diagnosis was not provided by imaging techniques, and the final diagnosis was confirmed by certified pathologists who were unaware of the patient's clinical data.

RFA Procedure

RFA was performed under local anesthesia with the percutaneous approach (n = 279) or under general anesthesia with the laparoscopic approach (n = 44);

both were used under real-time ultrasound guidance. The laparoscopic approach was selected for patients with HCC located on or near the liver surface.[12] We used an internally water-cooled 17-gauge cooled-tip electrode with an impedance-controlled generator (Cosman generator, Cool-Tip system, Radionics, Burlington, MA). Ultrasonography was performed with a 3.0- to 6.0-MHz convex probe and the Aloka SSD-5500 (Aloka, Tokyo, Japan), Sonoline Elegra (Siemens, Erlangen, Germany), and Aplio XV systems (Toshiba Medical Systems, Tokyo, Japan). When the target nodule was >20 mm in diameter, we performed multiple needle insertions and multiple ablations of 1 nodule.

Assessment of Treatment Efficacy and Follow-Up

A dynamic CT scan with a section thickness of 5 mm was performed to evaluate the efficacy of ablation 1 to 3 days after RFA. Complete HCC ablation was defined as hypo-attenuation of the entire tumor. Patients whose ablation was judged to be incomplete received additional therapy 1 week after the first ablation, which was continued until the treatment was judged to be completely effective. Blood was sampled every 2 to 3 months and tested for indicators of liver function and the markers alpha-fetoprotein (AFP) and protein induced by vitamin K absence or antagonist II (PIVKA-II). A dynamic CT scan was scheduled every 3 to 4 months, and chest CT or bone scintigraphy was performed if extrahepatic recurrence was suspected. HCC recurrence was defined as the detection of an early enhanced lesion by dynamic CT scanning concomitant with late washout. Local tumor progression was defined as the appearance of viable cancer tissue touching the initially treated tumor and distant recurrence separated from the primary site. When intrahepatic HCC recurrence was detected, RFA was performed if the recurrence met the initial inclusion criteria. If there was no indication for RFA, we chose TACE, percutaneous ethanol injection therapy, surgical resection, systemic chemotherapy, or symptomatic therapy according to the guidelines established by the Liver Cancer Study Group of Japan[11] and the American Association for the Study of Liver Diseases.[3] The end of follow-up was tumor progression beyond the Milan criteria, death, or latest medical attendance up to March 31, 2012.

Statistical Analysis

The primary endpoint of the present analysis was tumor progression beyond the Milan criteria, and the secondary endpoint was death. The cumulative incidences of recurrence exceeding the Milan criteria and survival after successful initial RFA were determined with the Kaplan-Meier method, and the risk factors associated with recurrence exceeding the Milan criteria and death were identified with a Cox proportional hazards regression model independently for tumor progression and death. The survival analysis was performed on a per-patient basis. The starting date for follow-up was defined as the completion date of the initial RFA session. Multivariate analysis was performed with a Cox proportional hazards model and included variables with a marginal P value < 0.05 according to univariate analysis. All statistical analyses were performed with StatView 5.0 (SAS, Inc., Cary, NC).

TABLE 1. Patient Characteristics (n = 323)

Characteristic	Value
Age (years)*	66 ± 9
Follow-up (years)†	4.0 (0.6-12.2)
Sex [n (%)]	
Male	191 (59.1)
Female	132 (40.9)
Clinical and laboratory data	
AFP (ng/mL)†	25.6 (1.2-76,600)
PIVKA-II level (mAU/mL)†	25.0 (7.0-10,600)
Child-Pugh score [n (%)]	
A	256 (79.3)
B	67 (20.7)
Pathology	
Maximum HCC diameter [n (%)]	
≤20 mm	117 (36.2)
21-30 mm	158 (48.9)
31-50 mm	48 (14.9)
Number of HCC nodules [n (%)]	
Single	226 (70.0)
Multiple	97 (30.0)
CLIP score [n (%)]	
0	174 (53.6)
1	114 (35.2)
2	32 (9.9)
3	3 (0.9)
Lymph node involvement (%)	0
Metastasis (%)	0
Major associated liver diseases [n (%)]	
HCV	248 (76.8)
HBV	31 (9.6)
HCV + HBV	3 (0.9)
Other	41 (12.7)

*The data are presented as the mean and standard deviation.
†The data are presented as the median and range.

RESULTS

The patient characteristics are shown in Table 1. The minimum follow-up period was 7 months, and the median follow-up period was 4.0 years (0.6–12.2 years). During follow-up, HCC recurred in 270 of the 323 patients (83.6%), and local tumor progression was observed in 47 patients (14.6%). Tumor progression beyond the Milan criteria was observed in 193 patients; 174 of these patients (90.2%) died because of tumor progression, and 19 (9.8%) died without tumor progression. The cumulative survival rates at 1, 3, 5, 7, and 10 years were 96.2%, 84.4%, 69.9%, 52.7%, and 40.6%, respectively. The cumulative rates of recurrence exceeding the Milan criteria at 1, 3, and

TABLE 2. Cox Proportional Hazards Analysis for Recurrence Exceeding the Milan Criteria (n = 323)

Factor	Univariate Analysis: *P* Value	Multivariate Analysis	
		P Value	HR (95% CI)
Age > 65 years	0.64		
Child-Pugh score: B versus A	0.10		
AFP level > 100 ng/mL	<0.001	0.006	1.59 (1.14-2.23)
PIVKA-II level > 100 mAU/mL	<0.001	0.21	1.26 (0.87-1.84)
Tumor size > 20 mm	0.003	0.01	1.54 (1.09-2.16)
Tumor number > 2	0.29		
Early recurrence*	<0.001	<0.001	2.76 (2.05-3.71)

*Within the first year after RFA.

TABLE 3. Cox Proportional Hazards Analysis for Overall Survival (n = 323)

Factor	Univariate Analysis: *P* Value	Multivariate Analysis	
		P Value	HR (95% CI)
Age > 65 years	0.64		
Child-Pugh score: B versus A	<0.001	<0.001	2.42 (1.61-3.64)
AFP level > 100 ng/mL	<0.001	<0.001	2.03 (1.37-3.00)
PIVKA-II level > 100 mAU/mL	0.14		
Tumor size > 20 mm	0.94		
Tumor number > 2	0.004	0.06	1.45 (0.99-2.13)
Early recurrence*	<0.001	<0.001	2.09 (1.43-3.03)

*Within the first year after RFA.

5 years were 15.1%, 46.0%, and 61.1%, respectively. Major complications were observed in only 2 cases (0.6%): one was gastric penetration after the ablation of segment 2, and the other was hemothorax after the ablation of segment 7. Both patients recovered without surgery.

Risk Factors for Exceeding the Milan Criteria and Overall Survival

A univariate analysis showed that a higher AFP level (>100 ng/mL), a higher PIVKA-II level (>100 mAU/mL), a larger tumor size (diameter > 20 mm), and an earlier recurrence of intrahepatic lesions (within 1 year of initial RFA) were significantly associated with the risk for recurrence exceeding the Milan criteria (Table 2). A multivariate analysis with a Cox proportional hazards model indicated that a higher AFP level [hazard ratio (HR) = 1.59, $P = 0.006$], a larger tumor size (HR = 1.54, $P = 0.012$), and early recurrence within 1 year of initial RFA (HR = 2.76, $P < 0.001$) were independent risk factors associated with recurrence exceeding the Milan criteria (Table 2). No association was observed between recurrence exceeding the Milan criteria and the Child-Pugh score. Risk factors associated with overall survival are shown in Table 3. A multivariate analysis with a Cox proportional hazards model indicated that a higher initial AFP level (HR = 2.03, $P < 0.001$), Child-Pugh class B (HR = 2.42, $P < 0.001$), and early recurrence within 1 year of initial RFA (HR = 2.09, $P < 0.001$) were independent risk factors associated with overall survival. There was no significant difference in overall survival or recurrence exceeding the Milan criteria between the patients whose imaging findings according to the modified Response Evaluation Criteria in Solid Tumors 3 months after RFA indicated an noncomplete response (n = 11) and the patients with a complete response (n = 312).

Predictability of the Long-Term Survival Rate and Recurrence Exceeding the Milan Criteria by Risk Group

To predict long-term survival and recurrence exceeding the Milan criteria, we formed risk groups on the basis of 2 relevant clinical predictors: the initial tumor marker (AFP level > 100 ng/mL) and the presence of earlier recurrence. The probability within the Milan criteria according to these predictors are shown in Fig. 1, and the cumulative survival rates are shown in Fig. 2. The 3- and 5-year survival rates for patients with both risk factors were 33.5% and 22.6%, respectively, although the patients were initially treated with