

Table 1. Characteristics of Patients Enrolled in the Present Study

Characteristics	Total	<65 year	≥65 year	P Value*
Patients, n	2166	1614	552	
Sex, n (%)				<0.001†
Male	1080 (49.9)	840 (52.0)	240 (43.6)	
Female	1086 (50.1)	774 (48.0)	312 (56.4)	
Age (SD), year	55.4 (12.1)	51.1 (10.8)	68.4 (2.9)	<0.001‡
BMI (SD), kg/m ²	23.3 (3.1)	23.4 (3.0)	23.3 (3.1)	0.9‡
Fibrosis stage, n (%)				<0.001†
F0	27 (1.3)	24 (1.5)	3 (0.5)	
F1	860 (39.7)	704 (43.6)	156 (28.2)	
F2	733 (33.8)	515 (31.9)	218 (39.5)	
F3	444 (20.5)	301 (18.6)	143 (25.9)	
F4	102 (4.7)	70 (4.3)	32 (5.8)	
%Severe steatosis (≥10%)	27.6	27.1	29.3	0.4†
ALT level (SD), IU/L	95 (18)	101 (119)	76 (58)	<0.001‡
HCV load (SD), KIU/mL	880 (1046)	861 (1016)	924 (1116)	0.2‡
HCV genotype, n (%)				<0.001†
1a	7 (0.3)	5 (0.3)	2 (0.4)	
1b	1414 (69.6)	1036 (68.9)	378 (71.3)	
2a	373 (18.3)	273 (18.2)	100 (18.9)	
2b	211 (10.4)	164 (10.9)	47 (8.9)	
Others	28 (1.4)	25 (1.7)	3 (0.6)	
Duration (SD), year	7.5 (4.4)	8.1 (4.4)	5.8 (3.7)	<0.001‡
IFN regimen, n (%)				<0.001†
IFN mono	1062 (49.0)	833 (51.6)	229 (41.5)	
PEG-IFN mono	306 (14.1)	200 (12.4)	106 (19.2)	
IFN + RBV	386 (17.8)	291 (18.0)	95 (17.2)	
PEG-IFN + RBV	412 (19.0)	290 (18.0)	122 (22.1)	
SVR, n (%)	686 (33.6)§	565 (36.6)¶	121 (24.3)¶¶	<0.001‡

Unless otherwise indicated, data are given as the mean (SD).
ALT, alanine aminotransferase; BMI, body mass index; HCV, hepatitis C virus; IFN, interferon; N/A, not applicable; PEG, pegylated; RBV, ribavirin; SVR, sustained virological response.
*Comparison between <65 years and ≥65 years.
†Chi-squared test.
‡Student t test.
§Virological responses were determined in 2042 patients.
¶Virological responses were determined in 1545 patients.
¶¶Virological responses were determined in 497 patients.

As shown in Fig. 2C-E, even when stratified by stage of fibrosis the cumulative incidences among patients at stages F0/F1, F2, and F3 were significantly greater in older patients than in younger patients (log-rank test, $P < 0.001$). These differences were not significant among patients with cirrhosis (Fig. 2F, log-rank test, $P = 0.7$).

The annual incidence of HCC after interferon treatment was calculated by the person-years method (Table 2); it increased with the degree of liver fibrosis from 0.2% (F0 or F1) to 4.6% (F4) and was higher among older patients at the same stage of liver fibrosis.

Among the 177 patients with HCC, 92 showed evidence of a single blood transfusion. We analyzed the relationship between duration of infection and age in these 92 patients. A significant and strong negative correlation was found between the interval from blood transfusion to development of HCC and the age of the patients at the time of blood transfusion ($r = -0.74$, $P < 0.001$) (Fig. 3A). The mean duration of chronic infection was 22.0 years in patients who had received blood transfusion at >40 years of age, which was significantly shorter than that in patients who received it at ≤ 40 years of age (40.6 years, $P < 0.001$).

The presence of cirrhosis at the time of development of HCC, which was defined as having any of the following criteria, was evaluated: (1) histological evidence for cirrhosis, (2) findings of cirrhosis in any radiological study, or (3) presence of marked portal hypertension (i.e., presence of esophagogastric varices). Following this, 142 of the 177 with HCC (80.2%) were diagnosed as having cirrhosis, of which 42 were diagnosed histologically, 69 radiologically, and 31 based on the presence of marked portal hypertension. No significant difference was found in the proportion of patients with cirrhosis between older and younger patients, at the rate of 78.3% (94/120) in older

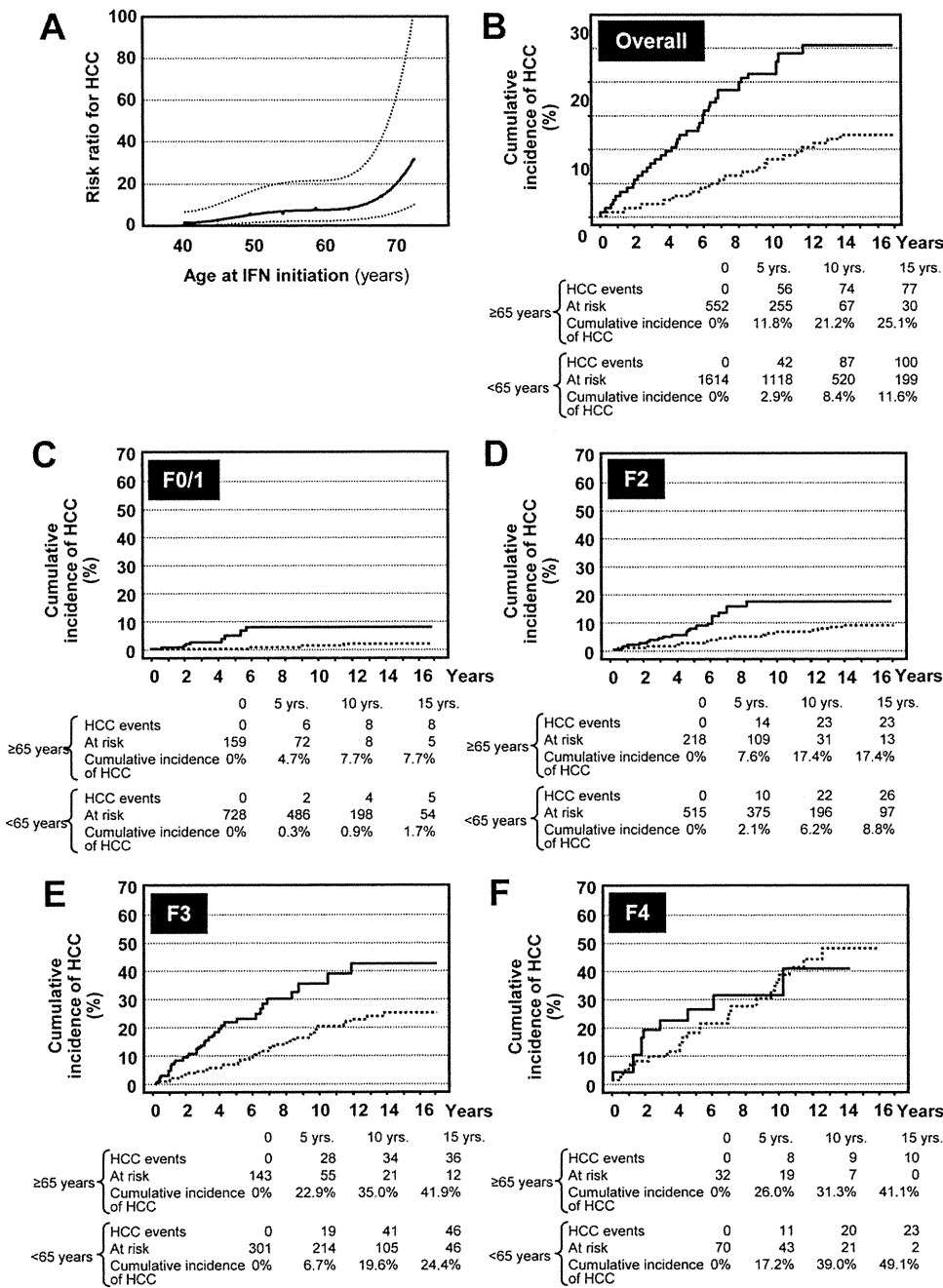


Fig. 2. Effect of aging on the risk for HCC. (A) Risk ratio (solid line) and 95% CI (dotted lines) for the risk of HCC according to age. To show the age-dependent relationship, a multivariate Cox proportional hazard model was used after adjustment for gender, stage of liver fibrosis, body mass index, and virological response to interferon therapy. Curves were fitted using polynomial regression. (B-F) Cumulative incidence of HCC after interferon therapy among younger (<65 years, n = 552, dotted line) and older patients (≥65 years, n = 1614, solid line). (B) Overall data, $P < 0.001$. (C) Patients with stage F0 or F1 liver fibrosis (no or mild fibrosis with portal expansion), $P < 0.001$. (D) Patients with stage F2 liver fibrosis (bridging fibrosis without architectural distortion), $P < 0.001$. (E) Patients with stage F3 liver fibrosis (bridging fibrosis with architectural distortion), $P < 0.001$. (F) Patients with stage F4 liver fibrosis (cirrhosis), $P = 0.7$. All P values were obtained by the log-rank test. The numbers of HCC events and patients at risk at each timepoint are shown below the graphs.

patients and 84.2% (48/57) in younger patients ($P = 0.36$, comparison at the age of HCC development).

Influence of Aging on Progression in Fibrosis Staging Over Time. In 271 patients who underwent paired biopsies, fibrosis staging progressed in 69 patients (25.5%), remained unchanged in 154 (56.8%), and regressed in 48 patients (17.7%). The overall rate of progression of fibrosis in these patients was 0.06 ± 0.02 fibrosis stages per year. Progression of fibrosis over time was significantly accelerated in older patients than in younger patients (0.21 ± 0.10 versus 0.03 ± 0.21 fibrosis stages per year, $P = 0.03$, Mann-Whitney U test) (Fig. 3B).

Effect of Viral Eradication on Risk for HCC in Older Patients. As shown in Fig. 4, the effect of viral eradication on the prevention of HCC was less significant in older patients than in younger patients. The annual incidence was higher among older patients than among younger patients with the same virological response (Table 2).

Influence of Liver Steatosis on Risk for HCC. The cumulative incidence of HCC after interferon therapy was significantly higher in patients with severe steatosis (≥10%) than in those with milder steatosis (at 5, 10, and 15 years: 8.6%, 19.1%, 32.0% versus 1.8%, 4.8%, 7.0%, respectively, log-rank test, $P < 0.001$).

Table 2. Annual Incidence of HCC After IFN Treatment

Factors	Total	<65 Years	≥65 Years
Fibrosis stage			
F0/F1	0.2%	0.1%	0.9%
F2	0.8%	0.6%	1.7%
F3	2.5%	1.8%	4.6%
F4	4.6%	4.4%	5.1%
Total	1.1%	0.8%	2.4%
Degree of liver steatosis			
<10%	0.5%	0.2%	1.4%
≥10%	2.0%	1.8%	3.0%
Virological response			
SVR	0.4%	0.2%	1.3%
Non-SVR	1.4%	1.0%	2.9%

Data were calculated by the person-years method. IFN, interferon; SVR, sustained virological response.

The annual incidence was higher in older patients than in younger patients with the same degree of liver steatosis (Table 2). In patients with severe steatosis (≥10%), superimposed NASH was diagnosed in 6.0% (26/435). Overall, superimposed NASH was significantly associated with hepatocarcinogenesis on univariate analysis (risk ratio, 4.1; 95% confidence interval [CI], 1.8-9.4; *P* < 0.001), but not on multivariate analysis. Superimposed NASH was significantly associated with high body mass index (27.2 ± 4.6 kg/m² versus 23.0 ± 3.1 kg/m², *P* < 0.001), hyperglycemia (186 ± 67 mg/dL versus 115 ± 39 mg/dL, *P* < 0.001), and advanced fibrosis (F3) (risk ratio, 2.9; 95% CI, 1.4-6.0; *P* = 0.005).

Factors Associated with Hepatocarcinogenesis After Interferon Therapy. Univariate analysis demonstrated factors that increase the risk ratio for the development of HCC (Table 3). Multivariate analysis using Cox proportional hazards regression confirmed that aging was one of the most significant independent factors associated with the development of HCC after interferon therapy. In this analysis, advanced fibrosis, presence of steatosis, male gender, lower total cholesterol level, higher fasting blood sugar level, higher baseline AFP level, insignificant improvement of mean AFP level after interferon therapy, and nonresponse to interferon therapy were also significantly associated with risk for HCC (Table 3).

We identified 22 patients in whom HCC developed even after achieving SVR. Univariate and multivariate logistic regression analyses indicated that both liver steatosis and aging were independently associated with the development of HCC among patients who achieved SVR (*n* = 686) (Table 4). Anti-HBc was detected in only 4 out of 22 patients and the age distribution was similar among anti-HBc-positive and anti-HBc-negative patients.

Response to Interferon Therapy in Older Patients.

Multivariate logistic regression analysis confirmed that aging, female gender, severe liver fibrosis, extremely severe liver steatosis, genotype-1, high HCV load, and nonuse of pegylated interferon and ribavirin were independent risk factors for non-SVR (Supporting Table 1). The odds ratio, determined by multivariate logistic regression analysis after adjustment for these factors, demonstrated that the risk for non-SVR was age-dependent (Supporting Fig. 1). It was also ≈2.5 times higher in patients aged ≥65 years than in those aged <35 years.

In patients with genotype-1b and a high viral load who were treated with pegylated interferon and ribavirin combination therapy, the SVR rate was significantly lower in older patients than in younger patients (<49 years, 59.3%; 50-59 years, 50.5%; 60-65 years, 27.3%; ≥65 years, 25.2%; intention-to-treat analysis). Multivariate logistic regression analysis showed that

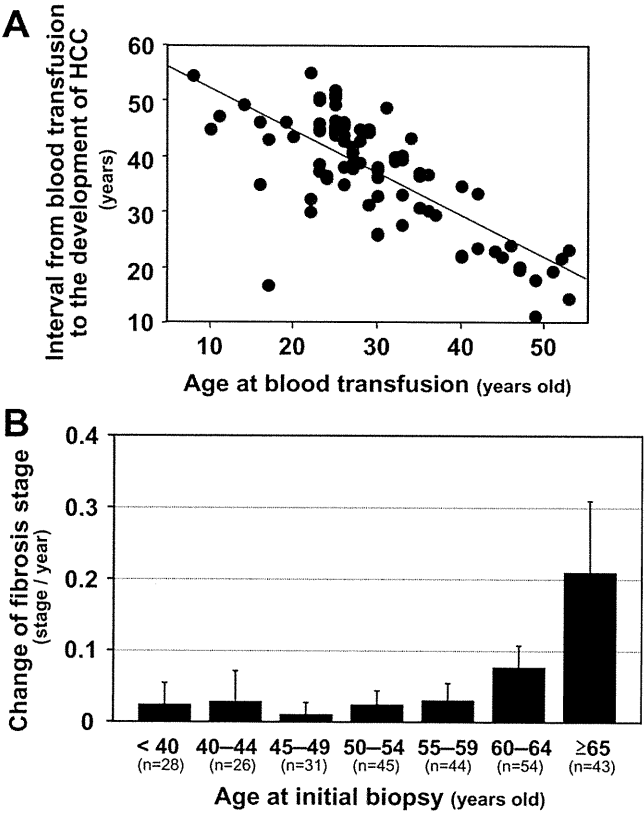


Fig. 3. (A) Relationship between the interval from blood transfusion to development of HCC and the age at blood transfusion (*n* = 92). A significant and strong negative correlation was observed (*r* = −0.74, *P* < 0.001). (B) Change in fibrosis staging over time. A total of 271 patients who had not achieved SVR by interferon therapy underwent a sequential biopsy after the initial biopsy. The yearly rate of progression of fibrosis was calculated as the change in fibrosis stage divided by the time between the paired biopsies. The yearly rate of progression of fibrosis was significantly higher in older patients (≥65 years) than in younger patients (<65 years) (*P* = 0.03, Mann-Whitney *U* test).

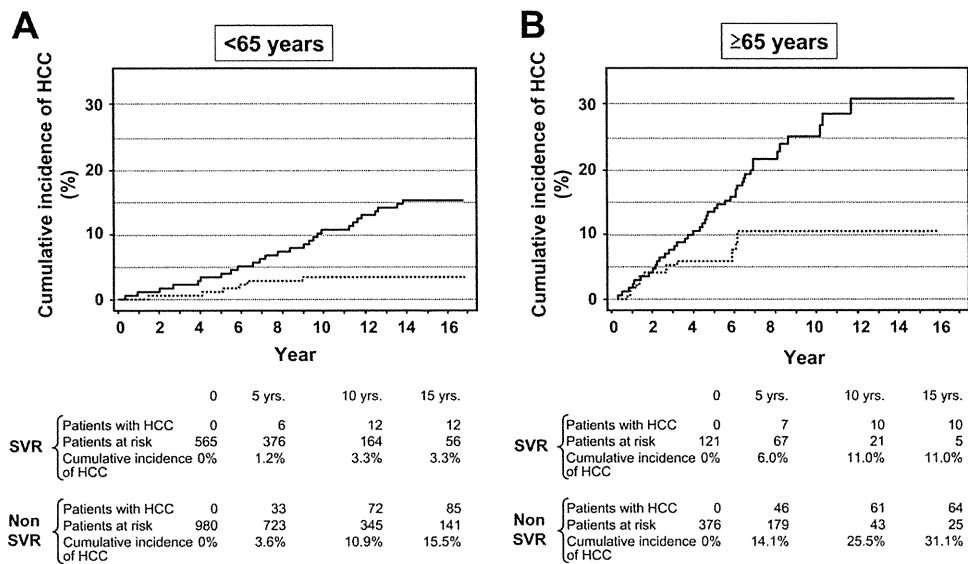


Fig. 4. Cumulative incidence of HCC after interferon therapy among SVRs (dotted lines) and non-SVRs (solid lines) according to age. (A) Younger patients (<65 years). The cumulative incidence of HCC was significantly higher in SVR than in non-SVR (log-rank test, $P < 0.001$). (B) Older patients (≥ 65 years). The cumulative incidence of HCC was significantly higher in SVR than in non-SVR (log-rank test, $P = 0.02$). However, the difference between SVR and non-SVR was less in older patients than in younger patients. The number of HCC events and patients at risk at each timepoint are shown below the graphs.

aging was the strongest independent factor contributing to SVR in these patients (data not shown). The odds ratio for the risk of non-SVR was 1.8 for each additional 10 years of age (95% CI, 1.5-2.3, $P < 0.001$).

Discussion

In this large cohort study we demonstrated that aging is significantly associated with the development of HCC in patients treated with interferon. The risk ratio increased predominantly in patients older than 65 years, which was more than 15 times that in patients in their 20s. Aging is becoming the most critical risk factor for the development of HCC. Although liver fibrosis was also an important risk factor, we clearly demonstrated that the risk for hepatocarcinogenesis after interferon treatment was significantly higher in older patients at each stage of liver fibrosis except for cirrhosis. Hence, physicians should be aware that older patients can develop HCC regardless of the stage of fibrosis.

Because the present study included a large cohort, it was difficult to determine the duration of infection in all patients, and this might have affected the risk determination for HCC development. Therefore, we analyzed the relationship between duration of chronic infection and HCC development in patients who underwent a single blood transfusion. We found a significant and strong negative correlation between the

interval from blood transfusion to development of HCC and the age of the patients at the time of blood transfusion. Consistent with our results, a previous report with posttransfusion HCV demonstrated that the age of patients, rather than the duration of HCV infection, was more significant for HCC development.¹⁴⁻¹⁶ Therefore, older age and not duration of infection is more likely to influence hepatocarcinogenesis. Moreover, our analysis of sequential biopsy specimens demonstrated that the progression rate of liver fibrosis significantly accelerated in patients aged >65 years. Hence, the progression of fibrosis along with aging may also contribute to the increased risk for hepatocarcinogenesis in older patients.

We further demonstrated that liver steatosis was an independent risk factor for the development of HCC, which was not mentioned in previous reports.⁸⁻¹¹ The presence of steatosis is related to both viral (genotype-3 or HCV core protein) and host metabolic factors.^{17,18} In our cohort, most superimposed NASH was associated with host metabolic factors such as high body mass index and hyperglycemia, whereas infection of genotype-3 was only noted in two patients. In vitro experiments have suggested an association between liver steatosis induced by HCV core protein and hepatocarcinogenesis,¹⁹ and have proposed virus-associated steatohepatitis as a new aspect of chronic hepatitis C.^{20,21} Because steatosis was likely to be related to hepatocarcinogenesis, patients with chronic hepatitis C, whose liver histology shows superimposed NASH,

Table 3. Factors Associated with HCC After IFN Therapy

Risk Factor Value	Univariate Analysis		Multivariate Analysis	
	Risk Ratio (95% CI)	P Value	Risk Ratio (95% CI)	P Value
Age (by every 10 year)	2.2 (1.8-2.7)	<0.001	3.0 (1.9-4.8)	<0.001
Sex				
Female	1		1	
Male	1.2 (0.9-1.6)	0.2	2.0 (1.0-3.8)	0.04
BMI (by every 10 kg/m ²)	2.0 (1.2-1.3)	0.005	1.1 (0.4-3.5)	0.8
Fibrosis stage				
F0/F1/F2	1		1	
F3/F4	5.4 (3.9-7.5)	<0.001	2.5 (1.2-4.9)	0.01
Degree of steatosis				
<10%	1		1	
≥10%	4.5 (3.0-6.9)	<0.001	3.5 (1.9-6.4)	<0.001
Esophagogastric varices				
No	1		1	
Yes	3.3 (2.0-5.3)	<0.001	1.6 (0.6-4.4)	0.3
Virological response				
SVR	1		1	
Non-SVR	3.3 (2.1-5.2)	<0.001	2.6 (1.2-5.5)	0.001
Genotype				
Non-1	1		1	
1	1.7 (1.2-2.5)	0.006	1.0 (0.5-2.3)	0.9
Albumin (by every 1 g/dL)	0.2 (0.1-0.3)	<0.001	0.6 (0.2-2.2)	0.3
ALT (by every 100 IU/L)	1.0 (0.9-1.0)	0.8	0.4 (0.1-1.8)	0.6
AST (by every 100 IU/L)	1.2 (1.1-1.3)	0.001	1.1 (0.6-1.8)	0.8
γ-GTP (by every 100 IU/L)	1.3 (1.1-1.6)	0.009	0.6 (0.3-1.6)	0.3
ALP (by every 100 IU/L)	1.3 (1.2-1.5)	<0.001	0.6 (0.3-1.2)	0.2
Total bilirubin (by every 1 mg/dL)	1.6 (1.3-2.1)	<0.001	1.2 (0.6-2.7)	0.6
Total cholesterol (by every 100 mg/dL)	0.3 (0.2-0.6)	<0.001	0.2 (0.1-0.6)	0.006
Triglyceride (by every 100 mg/dL)	0.8 (0.5-1.1)	0.2	0.1 (0.02-1.1)	0.08
Fasting blood sugar (by every 100 mg/dL)	1.8 (1.5-2.2)	<0.001	1.1 (1.0-1.1)	0.04
WBC (by every 100/μL)	0.1 (0.03-0.3)	<0.001	0.1 (0.01-2.2)	0.2
RBC (by every 10 ⁶ /μL)	0.5 (0.4-0.7)	<0.001	1.8 (0.7-4.4)	0.2
Platelet counts (by every 10 ⁶ /μL)	0.3 (0.2-0.4)	<0.001	0.6 (0.3-1.5)	0.3
Baseline AFP (by every 10 ng/mL)	1.0 (0.9-1.1)	0.2	1.3 (1.0-1.7)	0.04
Post IFN AFP (by every 10 ng/mL)	1.2 (1.1-1.3)	<0.001	1.9 (1.5-2.4)	<0.001
HCV load (by every 100 KIU/mL)	1.0 (0.9-1.0)	0.4	1.0 (1.0-1.1)	0.06
IFN regimen				
IFN monotherapy	1		1	
IFN + RBV (24 W)	1.2 (0.8-1.8)	0.4	1.5 (0.7-3.2)	0.3
PEG-IFN monotherapy (48 W)	1.1 (0.6-1.9)	0.8	1.5 (0.4-5.5)	0.6
PEG-IFN + RBV	0.4 (0.2-0.9)	0.03	1.0 (0.3-3.1)	0.9

Risk ratios for development of HCC were calculated by Cox proportional hazards regression analysis. AFP, alpha fetoprotein; ALP, alkaline phosphatase; ALT, alanine aminotransferase; AST, aspartate aminotransferase; BMI, body mass index; γ-GTP, gamma-glutamyltranspeptidase; HCC, hepatocellular carcinoma; IFN, interferon; PEG, pegylated; RBC, red blood cell counts; RBV, ribavirin; SVR, sustained virological response; WBC, white blood cell count.

may be at a higher risk of developing HCC. Further study is necessary to confirm this association in a clinical situation. Because several developed countries are in the midst of a growing obesity epidemic, the risk related to obesity cannot be ignored in patients with chronic hepatitis C who are treated with interferon.

Several retrospective cohort studies have been conducted to evaluate the effect of interferon on the incidence of HCC among patients with chronic hepatitis C.⁸⁻¹¹ Our results, obtained from one of the largest cohort studies, confirm the efficacy of viral eradication in preventing HCC. In one study conducted in a Western population, no statistically significant reduc-

tion was found in the development of HCC among patients with SVR compared with those without SVR (adjusted hazard ratio, 0.46; 95% CI, 0.12-1.70; *P* = 0.25).¹² Because relatively few occurrences of HCC were observed in this cohort, and the duration of follow-up was shorter, the differences in HCC development between patients with and without SVR might be less pronounced.

Interestingly, our results demonstrated that the risk for HCC remains even after achieving SVR in older patients, confirming the findings of previous studies conducted with a smaller number of patients.^{22,23} The cumulative incidence of HCC during the first 5 years

Table 4. Factors Associated with Development of HCC After Achieving SVR		
Risk Factor	Odds Ratio (95% CI)	P-value
Univariate analysis		
Age (by every 10 year)	3.2 (1.8-5.5)	<0.001
Sex		
Female	1	
Male	3.0 (1.0-8.8)	0.04
Fibrosis stage		
F0/F1/F2	1	
F3/F4	5.9 (2.5-14.0)	<0.001
Degree of steatosis		
<10%	1	
≥10%	5.5 (2.0-15.2)	0.001
BMI (by every 10 kg/m ²)	3.2 (0.8-12.6)	0.09
ALT (by every 10 IU/L)	0.9 (0.7-1.3)	0.7
AST (by every 10 IU/L)	1.1 (0.9-1.4)	0.3
Genotype		
Non-1	1	
1	1.2 (0.6-3.0)	0.5
HCV load (by every 100 KIU/mL)	0.9 (0.8-1.0)	0.2
IFN regimen		
IFN monotherapy	1	
IFN + RBV (24 W)	0.7 (0.2-2.3)	0.5
PEG-IFN monotherapy (48 W)	0.8 (0.2-3.6)	0.8
PEG-IFN + RBV	0.3 (0.03-2.0)	0.2
Multivariate analysis		
Age (by every 10 year)	2.7 (1.5-5.1)	0.002
Sex		
Female	1	
Male	4.1 (0.9-18.9)	0.06
Fibrosis stage		
F0/F1/F2	1	
F3/F4	2.6 (0.9-7.5)	0.08
Degree of steatosis		
<10%	1	
≥10%	5.6 (1.9-16.5)	0.002

Odds ratios for SVR were calculated by logistic regression analysis.
ALT, alanine aminotransferase; AST, aspartate aminotransferase; BMI, body mass index; HCV, hepatitis C virus; IFN, interferon; HCC, hepatocellular carcinoma; PEG, pegylated; RBV, ribavirin; SVR, sustained virological response.

after completion of interferon therapy was similar between SVR and non-SVR patients in the older age group, and the risk for HCC remained for 9 years after eradication of HCV in our patients. Therefore, HCC patients with SVR who have a risk factor should be screened for at least 5-10 years after the completion of interferon therapy.

It has been reported that coffee consumption has a protective effect against hepatocarcinogenesis^{24,25} and liver disease progression in patients with chronic HCV infection.²⁶ Because we could not review coffee consumption in all the patients and fewer data were available in the previous literature as to whether a habitual change of reducing coffee consumption occurs in older patients, it is unclear whether increased risk for HCC in older patients is an effect of this habitual change in older patients. However, the majority (68%) of Japa-

nese patients who have HCV (n = 1058) drink less than 1 cup of coffee per day, and only 7.6% consume more than 3 cups of coffee per day.²⁷ Therefore, it is unlikely that a habitual change in older patients affects the increased risk for hepatocarcinogenesis in older patients.

Recently, it was reported that interferon therapy might be less effective in preventing HCC among patients with chronic hepatitis C who are positive for anti-HBc antibody,²⁸ but this finding is still controversial.^{29,30} In the present study, anti-HBc was only detected in 4 of 22 patients in whom HCC developed after viral eradication, and age distribution was similar among anti-HBc-positive and anti-HBc-negative patients. Because no significant difference in mean age was found between anti-HBc-positive and anti-HBc-negative patients in the recent study conducted in Japan,²⁸ it is unlikely that previous exposure to hepatitis B virus or occult hepatitis B virus infection is responsible for the difference in risk for HCC between younger and elderly patients found in the present study.

In conclusion, aging has become one of the most important risk factors for HCC. Even after stratification by stage of fibrosis, the risk for HCC after antiviral treatment was significantly higher in older patients, and HCV eradication had a smaller effect on HCC-free survival in older patients. Patients with HCV should therefore be identified at an earlier age and antiviral treatment should be initiated. The present results have potentially important clinical implications for physicians that may influence their decisions about the treatment strategy in individual patients.

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Expression of Keratin 19 Is Related to High Recurrence of Hepatocellular Carcinoma after Radiofrequency Ablation

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Key Words

Hepatocellular carcinoma · Radiofrequency ablation · Recurrence · Keratin · Carcinogenesis · Needle biopsy · Hepatic progenitor cell

Abstract

Objective: Keratin (K) 19 positivity has been reported to be a useful predictive marker for recurrence in patients with hepatocellular carcinoma (HCC) who have undergone hepatic resection. We investigated the clinical usefulness of K19 positivity in patients who had received curative radiofrequency ablation (RFA). **Methods:** We retrospectively evaluated the clinicopathological features, including imaging and K19 expression, in 246 patients with HCC who were within the Milan criteria and had received curative RFA. Using a two-step insertion method, tumor biopsies were obtained just prior to RFA and were evaluated histologically. **Results:** Tumor seeding due to liver biopsy and RFA was not observed. Ten patients (4.1%) had K19-positive HCC. Imaging findings were similar between K19-positive and -negative HCC ($p = 0.187$). Nine out of 10 patients (90%) who had K19-positive HCC had

recurrence of HCC after RFA, and intrahepatic recurrences were observed within 12 months in 6 out of 10 (60.0%). K19 positivity was a significant risk factor for recurrence ($p < 0.0001$) and early recurrence (<1 year after RFA; $p = 0.012$). K19 expression ($p = 0.016$) was an independent risk factor for tumor status exceeding the Milan criteria after RFA. **Conclusion:** Expression of K19 is related to high recurrence of HCC after curative RFA.

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Introduction

Radiofrequency ablation (RFA) is regarded as an important treatment modality for hepatocellular carcinoma (HCC) [1–4], and its efficacy, especially for tumors <2 cm in diameter, is better than that of ethanol and nearly comparable to that of surgical resection [5]. In addition, RFA

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is considered to be a bridge to liver transplantation because the prolonged waiting time for cadaveric livers leads to dropouts from the waiting list [6]. Tumor recurrence after curative RFA has been a problem, as it is after hepatic resection. Tumor size (>3 cm in diameter) [7], time after treatment (>1 year) [7], the number of HCC nodules [8] and hepatitis C virus (HCV) infection [8] have been reported to be risk factors for intrahepatic tumor recurrence after curative RFA. Moreover, primary technical failure is reported to be a risk factor for tumor progression beyond the Milan criteria after RFA [9].

Keratin (K) 19, which is considered to be a biliary/hepatic progenitor cell marker [10], has attracted attention as a useful predictive marker for detecting the more aggressive HCCs after curative resection, because tumors with K19 expression have a poorer prognosis [11, 12] and higher rates of recurrence [13, 14] and lymph node metastasis [12] than K19-negative HCC. In these previous studies, surgical specimens were investigated and K19 positivity was defined as expression in >5% of tumor cells [11–14].

As a result, one would expect that K19 expression might be a useful predictive marker for detecting HCC with a worse outcome after RFA, especially regarding tumor recurrence. To the best of our knowledge, the correlation between clinicopathological features and K19 expression has not been investigated in HCC patients treated by RFA. Therefore, we performed a clinicopathological study on 246 HCC cases treated with RFA and investigated the relationship between the K19 expression and recurrence and prognosis after treatment.

Methods

Patients

Between April 1999 and February 2010, 1,284 patients were admitted to the Musashino Red Cross Hospital for the first treatment of HCC. A total of 684 patients were treated with RFA as the initial therapy for HCC. Ablation therapy was chosen either because the patients were considered not to be suitable for resection (n = 323), when considering impairment of liver function, number and distribution of the tumors as well as cardiopulmonary dysfunction, or because they preferred ablation and provided informed consent (n = 361), despite surgery also being feasible. From the outset, 172 patients were excluded because RFA was performed without tumor biopsy. Therefore, 512 consecutive patients, on whom tumor biopsies had been performed before RFA, were included and we evaluated these specimens retrospectively. The result of retrospective analysis was that there were 57 patients with no residual samples, 119 patients with no tumorous lesion and 9 patients with no definitive histological diagnosis because of a small and/or fragmented specimen. The remaining specimens

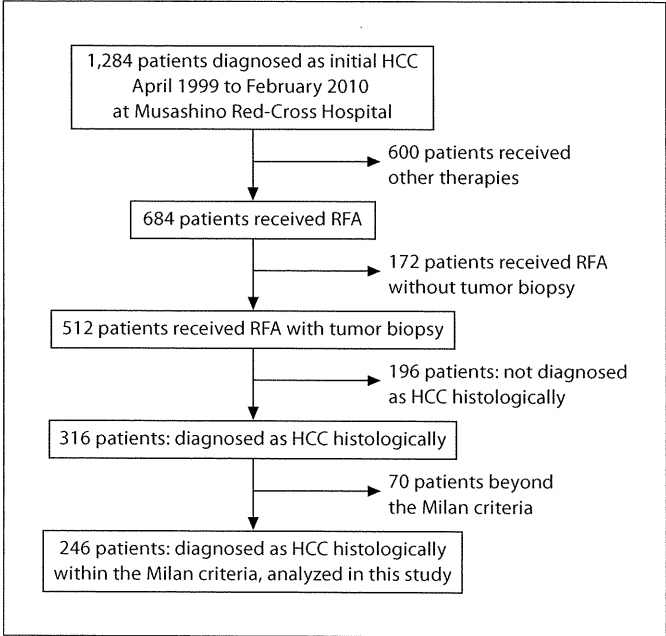


Fig. 1. Flow chart summarizing the patient selection for the study.

were diagnosed as HCC in 316 patients, as dysplastic nodule in 6 patients, as adenocarcinoma in 4 patients and as neuroendocrine tumor in 1 patient. Seventy patients were excluded, because their states of HCC were beyond the Milan criteria (≤ 3 cm and up to 3 nodules, or ≤ 5 cm and a single nodule). Therefore, 246 consecutive patients, on whom tumor biopsies had been performed before RFA and diagnosed as HCC retrospectively, were included in the study (fig. 1). The inclusion criteria for receiving RFA were as follows: total bilirubin concentration <3.0 mg/dl, platelet count $>3 \times 10^5/\text{mm}^3$, prothrombin activity $>50\%$ (approximately equal to an international normalized ratio of 1.5) and Child-Pugh score <8 points. Ascites were controlled by administration of diuretics before RFA. Patients with macroscopic vascular invasion or extrahepatic metastases were excluded. The criteria of the International Union against Cancer were used for TNM classification [15]. Written informed consent was obtained from all patients, and the study was approved by the ethics committee at Musashino Red Cross Hospital, in accordance with the Declaration of Helsinki.

Diagnosis of HCC

All the patients were diagnosed as having HCC on the basis of tumor markers and a combination of typical imaging findings on ultrasonography (US) and dynamic computed tomography (CT), according to the American Association for the Study of Liver Diseases and the Japan Society of Hepatology guidelines [1, 16]. When patients had 2 or 3 HCC nodules, a needle biopsy was taken from the main nodule. The histological diagnosis of HCC was based on the World Health Organization criteria [17].

For the evaluation of vascularity and Kupffer cell activity of the target nodule, CT during arteriography (CTHA) and CT dur-

ing arteriportography (CTAP) were performed in 188 (76.4%) patients, superparamagnetic iron oxide-enhanced magnetic resonance imaging (SPIO-MRI) was performed in 194 (78.8%) patients and gadolinium-ethoxybenzyl-diethylenetriamine penta-acetic acid magnetic resonance imaging (Gd-EOB-DTPA) was performed in 47 patients (19.1%), from March 2008. For triple-phase dynamic CT scans, arterial, portal and equivalent phases were 35, 70 and 150 s, respectively, after injection of contrast agent. Spiral CT scans were obtained from 3- to 5-mm-thick sections. Board-certified radiologists diagnosed HCC on the basis of typical patterns, such as an early-phase hyperattenuation area and late-phase hypoattenuation on dynamic CT. According to previous studies, the sensitivity of the diagnosis of HCC in CTHA/CTAP is higher than that of spiral CT. The diagnosis of HCC in CTHA/CTAP is hyperattenuation area in CTHA and hypoattenuation area in CTAP. It has been reported that the presence of Kupffer cells could be evaluated, and this was defined by a hyper-intensity area in the T2* image of SPIO-MRI as a typical imaging finding of HCC. Gd-EOB-DTPA MRI is a liver-specific contrast-enhanced agent, and hypointensity in the hepatobiliary phase is a typical imaging finding. We started to perform Gd-EOB-DTPA MRI instead of SPIO-MRI from March 2008, because it was reported that the sensitivity of Gd-EOB-DTPA MRI was superior to SPIO-MRI for the diagnosis of HCC.

Tumor Biopsy and RFA

There are 24 operators who participated in this study. They are specialized liver physicians who have great experiences in performing percutaneous ethanol injection for HCC, percutaneous tumor biopsy for liver tumor, percutaneous liver biopsy for hepatitis, percutaneous hepatobiliary drainage for obstructive jaundice, or percutaneous liver abscess drainage. A needle-guiding technique was used, consisting of an initial guided needle and a secondary outer needle (two-step insertion method). This method was reported by another center previously [18] and involves the initial insertion of a 21-gauge needle (Silux, Saitama, Japan) just adjacent to the tumor under real-time US guidance, and using this to insert a 14-gauge Daimon outer needle (Silux), also just adjacent to the tumor. After removal of the inner needle, an 18-gauge biopsy needle was inserted to obtain the tumor tissue sample. After removal of the biopsy needle, a 17-gauge cooled-tip electrode was inserted into the targeted tumor. The electrode, with a 2- or 3-cm exposed tip, was connected to a 480-kHz RF Generator (Radionics, Burlington, Mass., USA), which produces 200 W at 50 Ω of impedance [19, 20]. The equipment also allows the measurement of power output, tissue impedance and electrode tip temperature. A tip temperature of 10–20°C was maintained by infusion of chilled water through a peristaltic pump. After insertion of the electrode into the tumor, ablation was performed at 60 W for the 3-cm exposed tip and 40 W for the 2-cm exposed tip. The power was increased to 140 W at a rate of 10–20 W/min. When a rapid increase in impedance was observed during thermal ablation, the output was reduced. The duration of a single ablation was 12 min. After RF exposure, the pump was stopped and the temperature of the needle tip was measured. When the temperature of the electrode tip was >60°C, ablation was defined as being sufficient. When the target nodule was >2 cm in diameter, multiple needle insertions and ablations were performed in 1 nodule to achieve complete necrosis. A session was defined as a single intervention consisting of ≥ 1 ablations performed on ≥ 1 tumors at

the same time. After completion of nodule ablation, the intrahepatic needle track was treated by thermocoagulation to avoid needle track seeding. Finally, a mixture of gelatin sponge particles (Gelfoam®; Upjohn, Kalamazoo, Mich., USA) was injected into the puncture route. All procedures were completed within 15–20 min. After each session of RFA, a dynamic CT scan (section thickness 5 mm) was performed to evaluate the efficacy of ablation. Complete ablation of HCC was defined as non-enhancement of the lesion, including the whole surrounding liver parenchyma. The ablative margin was shown as the boundary between the low density area as ablated area and the isodensity area as surrounding normal liver parenchyma. The residual portion of the tumor was treated by additional RFA within a few days of the post-treatment CT scan. Follow-up consisted of monthly serial measurements of tumor markers [α -fetoprotein (AFP) and des- γ -carboxy prothrombin (DCP)], US examination every 2 months and dynamic CT every 3 months. We checked various complications of RFA with conventional contrast-enhanced CT and blood examination at day 1 after RFA.

Tumor Recurrence

Recurrence of HCC was defined as an early enhancement area on dynamic CT, concomitant with late wash out. Two types of recurrence, local tumor progression and distant intrahepatic recurrence, were identified. Local tumor progression was defined as an enhancing area located adjacent to the ablated area [21], while distant intrahepatic recurrence referred to the appearance of a new tumor in the liver, distant from the ablated area. Early recurrence was defined as a recurrence within 12 months of the initial RFA.

Immunohistochemistry

Immunohistochemistry using antibodies against K19 (1:100, BA17, Dakocytomation, Glostrup, Denmark) was performed on paraffin-embedded sections from 246 needle biopsy specimens. The slides were reviewed by 2 independent pathologists (M. Komuta and M. Sakamoto). Expression of K19 was considered positive if >5% of tumor cells were stained according to the expected pattern of reactivity.

Statistical Analysis

Categorical variables were compared with the χ^2 test and continuous variables with the Mann-Whitney test; a p value <0.05 was considered statically significant. Continuous variables were expressed as the mean $\pm$ standard deviation. The imaging findings were compared with the χ^2 test between K19-positive and -negative patients. Overall survival was defined as the interval between treatment and death or the date of the last follow-up or the date of the most recent follow-up visit. Probability of recurrence-free survival was defined as the interval between treatment and the date of HCC recurrence.

Univariate analysis was performed to identify clinical and biological parameters (sex, age, etiology, prothorombin activity, albumin, bilirubin levels, Child-Pugh class, serum AFP level, serum DCP level) and tumor factors (size, number, tumor stage, tumor differentiation, K19 expression) predicting overall survival, recurrence-free survival and the interval beyond the Milan criteria.

Survival curves were computed according to the Kaplan-Meier method and compared by the log-rank test. All variables with a p value <0.05 were subjected to multivariate analysis by Cox's

Table 1. Comparison of clinicopathological features of patients (n = 246) with HCC with and without K19 expression

Features	K19 >5% (n = 10)	K19 ≤5% (n = 236)	p value
Mean age ± SD, years	70 ± 8	68 ± 8	0.541
Sex, male/female	2/8	146/90	0.016
<i>Clinical and laboratory data</i>			
Mean AFP, ng/ml	489 [52.1]	12 [16.2]	0.062
Mean DCP, mAU/ml	42 [25]	321 [22]	0.773
Child-Pugh score A/B	8/2	200/36	0.655
Total bilirubin, mg/dl	0.9 ± 0.5	0.8 ± 0.4	0.480
Albumin, g/dl	3.4 ± 0.7	3.6 ± 0.5	0.137
PT, %	97 ± 12	92 ± 15	0.375
<i>Pathology</i>			
Tumor size, mm	24 ± 7	22 ± 8	0.392
Tumor number	1.3 ± 0.7	1.2 ± 0.6	0.891
Vascular invasion, yes/no	0/10	0/236	
Tumor differentiation			
well/moderate/poor	0/8/2	108/126/2	<0.0001
TNM stage I/II	8/2	183/53	0.855
Lymph node involvement			
yes/no	0/10	0/236	
Metastasis, yes/no	0/10	0/236	
<i>Major associated liver diseases</i>			
HBsAg+	1 (10)	24 (10.1)	0.895
HCV Ab+	9 (90)	189 (80.1)	
ALD	0	8 (3.4)	
NASH	0	2 (0.8)	
Unknown etiology	0	13 (5.6)	

Figures in parentheses are percentages; figures in brackets are medians. PT = Prothrombin time; HBsAg = hepatitis B surface antigen; HCV Ab = HCV antibody; ALD = alcoholic liver disease; NASH = non-alcoholic steatohepatitis.

proportional hazards model to assess their value as independent predictors.

All statistical analyses were performed using StatView (version 5.0) software (Abacus Concepts, Berkeley, Calif., USA).

Results

Proportion of HCCs Expressing K19

The biopsy number was 272, and the median length of our biopsy specimens was 8.2 ± 4.0 mm. In 117 cases, the specimens were <1 cm, and ≥1 cm in 155 cases. Pathological diagnosis and K19 staining were practicable in all specimens <1 cm. Expression of K19 in >5% of tumor

Table 2. Comparison of the image findings of patients with HCC with and without K19 expression

	K19 positive >5% (n = 10)	K19 negative (n = 236)	p value
CECT arterial phase high density	10/10	200/235	0.187
CTHA high density	7/7	159/181	0.326
CTAP low density	7/7	179/181	0.779
SPIO-MRI T2*	10/10	175/184	0.473
EOB-MRI			
Hepatobiliary phase low intensity	–	46/47	–

cells was observed in HCCs from 10 of 246 patients (4.1%). Two of the 10 HCCs (20.0%) were poorly differentiated, and 8 (80.0%) were moderately differentiated. None of the well-differentiated HCCs showed K19 positivity. Among the 10 patients with K19-positive HCCs, 2 had a HCC nodule >3 cm and 8 had HCC nodules ≤3 cm in diameter. The 8 HCC nodules with K19 positivity ≤3 cm in diameter were moderately (n = 7) and poorly differentiated HCCs (n = 1).

Clinicopathological Characteristics of Patients with HCC in Relation to Expression of K19

The clinicopathological characteristics of the patients in relation to K19 expression in HCCs are shown in table 1. The proportion of well-differentiated HCCs was significantly lower among K19-positive HCC patients (p < 0.0001). K19 expression was more frequent among female than among male patients (p = 0.016). There were no significant differences in age, clinical laboratory data, tumor size, number of tumor nodules, tumor stage in TNM classification or etiology between K19-positive and -negative HCC patients. There was no significant difference in tumor location (near the major vessels, bile ducts and organs) between K19-positive and -negative patients. The number of RFA sessions did not differ significantly between K19-positive and -negative HCC patients. Serum AFP before initial RFA was not evaluated in 1 patient.

Imaging Characteristics of HCCs in Relation to Expression of K19

Comparison of the various imaging findings, according to vascular profiling, and in relation to K19 expres-

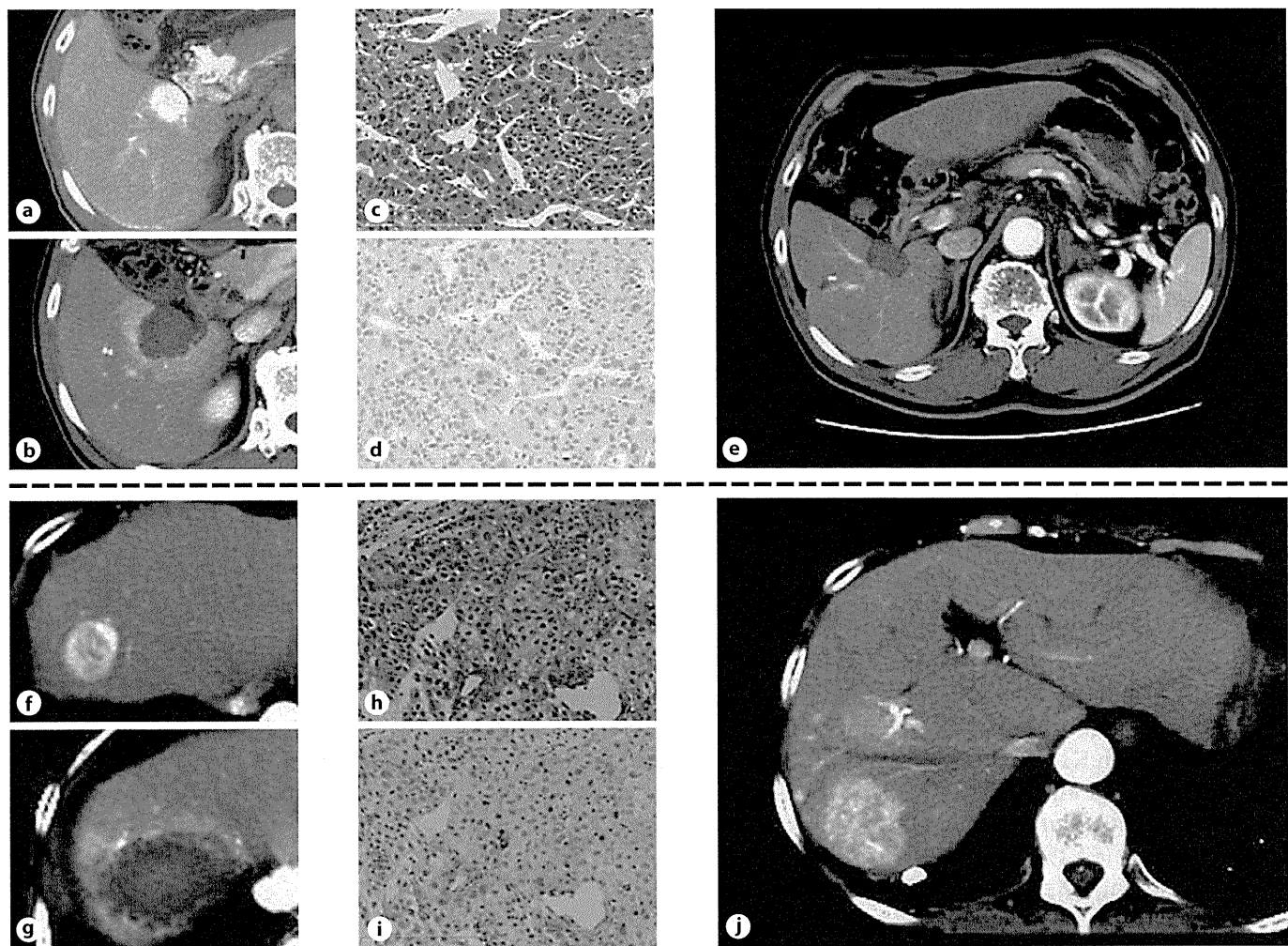


Fig. 2. a–e A patient with K19-negative HCC: a 70-year-old man with chronic hepatitis (anti-HCV positive). The HCC (25 mm in diameter, in segment 6) showed an early enhancement area by dynamic CT (**a**). Dynamic CT at 1 day after RFA (**b**). On histological investigation, the tumor showed moderately differentiated HCC on H&E staining (**c**), and K19 expression was negative in tumor cells (**d**). The HCC did not show early enhancement on dynamic CT 4 years and 10 months after curative RFA (**e**). **f–j** A patient with

K19-positive HCC: a 72-year-old female with chronic hepatitis (anti-HCV positive). The HCC (25 mm in diameter, in segment 8) showed an early enhancement area by dynamic CT (**f**). CT 1 day after RFA (**g**). On histological investigation, the tumor showed moderately differentiated HCC on H&E staining (**h**), and K19-positive cells were seen in the tumor (**i**). Five months after RFA, the HCC showed intrahepatic recurrence beyond the Milan criteria (**j**).

sion, is shown in table 2. These imaging findings were consistent with the histological diagnosis, as determined by pretreatment needle biopsy.

All K19-positive HCCs showed typical HCC images, such as hypervascularity at the arterial phase, hypovascularity at the portal and equilibrium phases in dynamic CT, and hyperintensity at the T2* image in SPIO-MRI. There was no significant difference between K19-positive and -negative patients in terms of the imaging findings.

Recurrence of HCC after RFA

The median follow-up period was 34.0 months (range 65 days to 10.3 years). A recurrence of HCC was diagnosed at least once during the follow-up period in 156 patients (63.4%). The cumulative recurrence-free survival at 1, 3 and 5 years was 69.9, 26.6 and 12.2%, respectively. Among the 156 patients with recurrent HCC, 14 (8.9%) had local tumor progression and 142 (91.1%) had distant intrahepatic recurrences. Five of 14 patients (35%) who had local tumor progression had K19-positive HCC and

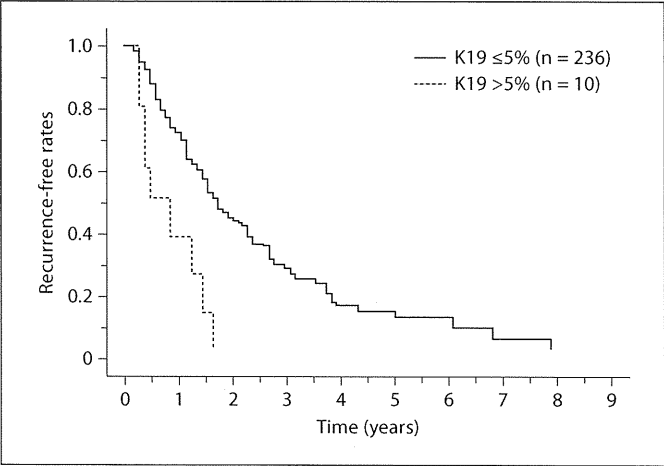


Fig. 3. The cumulative recurrence-free survival rate in patients with K19-positive (>5%) HCC was significantly lower than that in patients with K19-negative HCC ($p = 0.0001$).

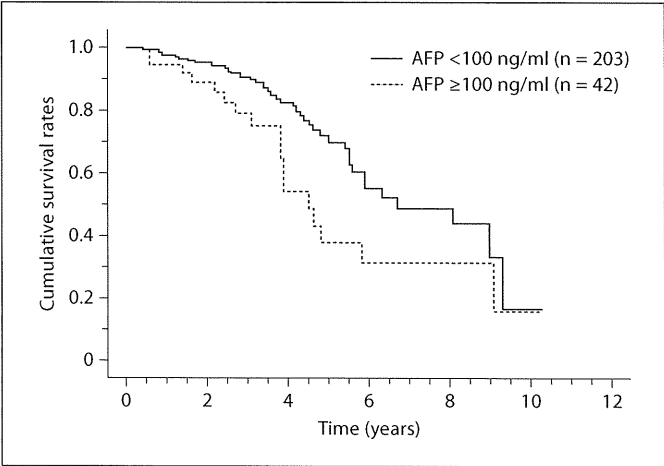


Fig. 4. The cumulative overall survival rate in patients with AFP ≥ 100 ng/ml was significantly lower than that in patients with AFP < 100 ng/ml ($p = 0.026$).

3 of 5 patients with K19-positive HCC (60%) showed vascular invasion at the local tumor progression. Nine of 10 patients (90.0%) with K19-positive HCC had recurrences after initial treatment and 6 of 10 (60.0%) were detected within 1 year of initial curative RFA. On the other hand, 147 of 236 patients (62.2%) with K19-negative HCC had recurrences, and only 58 patients (24.5%) had recurrences within 1 year after RFA. There were no patients with K19-negative HCC who showed vascular invasion at the local tumor progression. Patients with K19-positive HCC were more likely to have an early recurrence of HCC (< 1 year after RFA) than patients with K19-negative HCC ($p = 0.012$). The typical cases are shown in figure 2. The median recurrence-free survival in patients with K19-positive HCC was 194 days (range 93–635), while in patients with K19-negative HCC it was 446 days (range 65–2,978). Patients with K19-positive HCC had a significantly shorter recurrence-free survival than patients with K19-negative HCC ($p = 0.0001$) (fig. 3). The recurrence type, local tumor progression or distant intrahepatic recurrence differed between K19-positive and -negative patients. Local tumor progression was significantly higher in K19-positive patients than in K19-negative patients ($p < 0.0001$). Table 3 shows the results of univariate and multivariate analyses of prognostic factors for recurrence-free survival. In the multivariate analysis, K19 expression, the number of HCC nodules and total bilirubin ≥ 2 mg/dl were significant independent risk factors for HCC recurrence in all patients.

The percentage of distant metastasis and major portal invasion (VP3–4) was significantly higher in K19-positive than in K19-negative patients ($p < 0.0001$). Distal metastasis was detected in the lung (2 patients) and lymph node (1 patient), and major portal invasion was detected in 3 patients.

Risk Factors for Poor Prognosis

There was no patient who received liver transplantation in this study. Fifty-seven of 246 patients (23.1%) died during the follow-up period. The cause of death was progression of HCC in 37 patients, hepatic failure in 16 patients and causes unrelated to the liver in 4 patients. The overall survival rates for all patients were 97.2, 88.7 and 63.4% at 1, 3 and 5 years, respectively. A serum AFP level ≥ 100 ng/ml ($p = 0.034$), a total bilirubin level ≥ 2 mg/dl ($p < 0.0001$) and female sex ($p = 0.018$) were identified as risk factors for a poor prognosis in HCC in both univariate and multivariate analyses (table 4). Patients with high serum AFP levels (≥ 100 ng/ml) had significantly lower overall survival rates than patients with low serum AFP levels ($p = 0.026$) (fig. 4).

On the other hand, age (≥ 65 years), albumin concentration (≤ 3.5 g/dl), prothrombin time ($\leq 70\%$), DCP (≥ 100 mAU/ml), tumor size, the number of HCC nodules and K19 expression were not significant risk factors for poor prognosis in the univariate analysis (table 4).

Table 3. Risk factors associated with recurrence-free survival in 246 patients with HCC after complete ablation by RFA

Risk factor	Univariate			Multivariate		
	RR	95% CI	p	RR	95% CI	p
Age <65 years	1.43	1.02–2.02	0.037	1.28	0.90–1.81	0.163
Sex, female	1.24	0.90–1.71	0.162			
Total bilirubin ≥2 mg/dl	2.50	1.02–6.25	0.034	2.70	1.08–6.66	0.032
Albumin ≤3.5 g/dl	1.12	0.81–1.56	0.492			
PT ≤70%	1.28	0.73–2.22	0.394			
AFP ≥100 ng/ml	1.42	0.95–2.12	0.087			
DCP ≥100 mAU/ml	1.08	0.68–1.69	0.790			
Tumor size >3.0 cm	1.08	0.70–1.69	0.713			
2 or 3 tumor nodules	2.29	1.58–3.33	<0.0001	2.28	1.56–3.32	<0.0001
K19 positive (>5%)	3.57	1.75–7.14	0.0004	3.44	1.72–7.14	0.0005

RR = Risk ratio; CI = confidence interval; PT = prothrombin time.

Table 4. Risk factors associated with poor prognosis in 246 patients with HCC after complete ablation by RFA

Risk factor	Univariate			Multivariate		
	RR	95% CI	p	RR	95% CI	p
Age <65 years	1.19	0.68–2.09	0.527			
Sex, female	2.03	1.18–3.46	0.009	1.92	1.11–3.30	0.018
Total bilirubin ≥2 mg/dl	12.5	4.54–33.3	<0.0001	10.0	3.70–33.3	<0.0001
Albumin ≤3.5 g/dl	1.25	0.71–2.17	0.450			
PT ≤70%	1.49	0.59–3.84	0.674			
AFP ≥100 ng/ml	1.88	1.06–3.44	0.030	1.88	1.05–3.33	0.034
DCP ≥100 mAU/ml	1.06	0.53–2.12	0.880			
Tumor size >3.0 cm	1.12	0.44–1.78	0.730			
2 or 3 tumor nodules	1.23	0.67–2.26	0.492			
K19 positive (>5%)	1.29	0.46–3.57	0.632			

RR = Risk ratio; CI = confidence interval; PT = prothrombin time.

Risk Factors for Exceeding the Milan Criteria after RFA

Patients with K19-positive HCC exceeded the Milan criteria within 16.8 months. Multivariate analyses showed that K19 expression, high levels of DCP (≥100 mAU/ml), tumor number and total bilirubin ≥2 mg/dl were significant risk factors for tumor status exceeding the Milan criteria after curative RFA (table 5; fig. 5).

Complications

Most patients had mild pain or discomfort during RFA. Intraperitoneal hemorrhage and biloma were not

seen in any patient. None of the patients developed dissemination of HCC, or skin or peritoneal metastases. There was no fatal complication.

Percentage of K19 Stain

We also analyzed another percentage of K19 stain (>1%). Thirteen of 246 patients had K19-positive (>1%) HCC and 12 of 13 patients with K19-positive (>1%) HCC had recurrences beyond the Milan criteria. Nine of 12 (75.0%) were detected with recurrence of HCC within 1 year of initial curative RFA. The final results were the same for K19 positivity (>5 and >1%, respectively). The

Table 5. Risk factors associated with exceeding the Milan criteria in 246 patients with HCC after complete ablation by RFA

Risk factor	Univariate			Multivariate		
	RR	95% CI	p	RR	95% CI	p
Age <65 years	1.63	1.08–2.45	0.018	1.17	0.75–1.83	0.463
Sex, female	1.16	0.78–1.72	0.457			
Total bilirubin ≥2 mg/dl	2.94	1.05–8.33	0.039	3.57	1.25–10.0	0.017
Albumin ≤3.5 g/dl	0.97	0.64–1.47	0.857			
PT ≤70%	0.89	0.41–1.96	0.763			
AFP ≥100 ng/ml	2.17	1.38–3.44	0.0008	1.56	0.96–2.50	0.077
DCP ≥100 mAU/ml	2.32	1.42–3.70	0.0007	2.08	1.26–3.44	0.004
Tumor size >3.0 cm	1.03	0.61–1.72	0.914			
2 or 3 tumor nodules	2.98	1.91–4.64	<0.0001	3.05	1.91–4.88	<0.0001
K19 positive (>5%)	3.70	1.81–7.69	0.0003	2.47	1.19–5.18	0.016

RR = Risk ratio; CI = confidence interval; PT = prothrombin time.

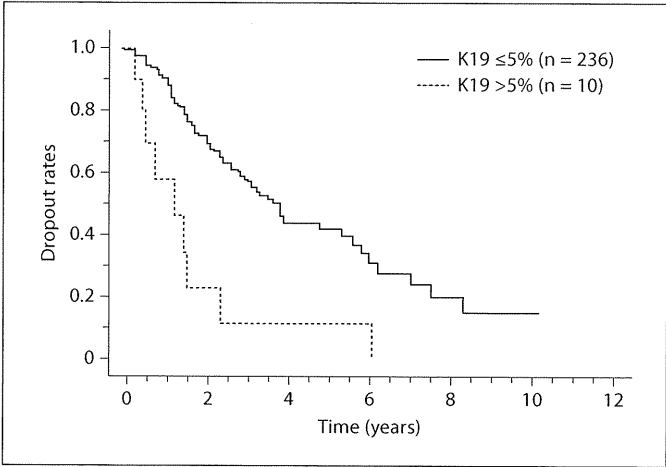


Fig. 5. The cumulative rate of exceeding the Milan criteria in patients with K19-positive HCC was significantly higher than that in patients with K19-negative HCC ($p < 0.0001$).

rate of recurrence and dropout from the Milan criteria were significantly higher in the patients with K19-positive (>1%) than in the patients with K19-negative HCC (data not shown).

Discussion

RFA therapy for HCC has been shown to achieve excellent results in appropriately selected patients [2–5]. However, recurrence of tumors is a serious impediment to im-

proving the prognosis for patients treated with curative RFA. Therefore, several factors have been investigated as potential predictive markers for recurrence after curative RFA [7–9]. Recently, K19 was proposed as an independent prognostic factor for HCC [11–14]. However, these investigations were performed on surgically resected cases only and not on tumor biopsies. Although tumor biopsy is controversial because of potential complications such as tumor seeding [22], it would be beneficial to clinicians and patients to predict the individual tumor characteristics from a biopsy. Until now, the relationship between K19 expression and tumor recurrence after RFA treatment has not been assessed. Therefore, we have investigated the relationship between K19 expression in tumor biopsies and the clinicopathological findings in HCC. In this study, we investigated K19 expression in biopsy specimens taken just prior to the RFA session, and K19 expression (>5%) was demonstrated in 10 of 246 patients (4.1%). Because most of our patients were in early stage (within the Milan criteria) and 108 of 246 patients (43.9%) had well-differentiated HCC, the positive rate of K19 stain in our study was lower than that in surgical specimens.

We also analyzed another percentage of K19 stain (>1%) and the final results were the same for K19 positivity (>5 and >1%, respectively). K19 expression (>1%) was a statistically significant independent predictor for recurrence of HCC after RFA. Although the amount of tissue obtained by tumor biopsy is small compared to resected material, present data suggest that even biopsy can provide meaningful data on tumor recurrence irrespective of the percentage of K19 positivity (1 or 5%) (online sup-

plementary tables 1 and 2; for supplementary material see www.karger.com/doi/10.1159/000328448).

K19 positivity was not an independent predictor of the overall rate of survival, and serum AFP (≥ 100 ng/ml), total bilirubin (≥ 2 mg/dl) and female sex were significant independent predictors of survival. It is suggested that the level of total bilirubin affects the liver function of the patient, and liver function is one of the most important prognostic factors for survival of HCC patients.

The average age of our patients in this study was 68 ± 8 years, and no patients received liver transplantation in this study. However, liver transplantation is the most desirable treatment for HCC worldwide. Because of the prolonged waiting time for liver transplantation, RFA has been considered a safe and effective bridging therapy to liver transplantation. In addition, pretransplant RFA in patients with HCC has been considered for downstaging of HCC, thus improving the patient's survival [6, 7, 23]. In this study, K19 expression of HCC was a significant independent predictor for exceeding the Milan criteria ($p = 0.016$). In fact, 9 of 10 patients with K19-positive HCC exceeded the Milan criteria within 16.8 months. Therefore, if RFA is considered as a bridging therapy session prior to liver transplantation, it would be useful to obtain information on K19 expression in tumor tissue by performing a tumor biopsy before RFA. Therefore, careful observation for early detection of recurrence should be considered if K19-positive HCC patients are awaiting liver transplantation.

Compared to surgical specimens, biopsies taken prior to RFA may present some difficulties with regard to histological investigation. Needle biopsies of the nodules are less often indicated when typical vascular imaging of HCC is obtained, compared to hypovascular nodules. Needle tract seeding should also be considered. Needle biopsy has played an important role in making a diagnosis in the past. Recently, more reliance has been placed on the vascular imaging profile, because of its sensitivity and specificity without the risk of tumor dissemination. In addition, in comparison to recent advances in imaging, the information obtained from liver biopsy is lacking, as these only provide simple histological characterization, such as tumor differentiation [24]. Moreover, the positive predictive value of the vascular profile on dynamic imaging for diagnosis of HCC exceeds 95% [25]. Therefore, the current tendency is to consider needle biopsy as non-essential for diagnosis. However, in this study, K19-positive HCC showed exactly the same imaging findings as K19-negative HCC, suggesting that it is difficult to distinguish between these tumor types by imaging profile alone. In

addition, K19-positive, moderately and/or poorly differentiated HCC showed similar cytological and structural abnormalities to K19-negative HCC, indicating that K19 positivity is unpredictable without staining. In figure 2, we present an impressive comparison of the features of K19-positive and -negative HCC, showing that, although the histology was similar, the prognosis for these patients was completely different. From these findings, it is clear that immunohistochemistry for K19 is the only way of demonstrating its positivity. Fortunately, staining for K19 on paraffin sections is common in diagnostic pathology, and it is not a problem to add this to routine hematoxylin and eosin (H&E) staining. Moreover, even for a general pathologist with no liver specialization, evaluating K19 expression should not be difficult, as long as care is taken not to count bile ducts, which may be associated with the remains of portal tracts. Taken together, these finding could indicate that it may be beneficial to check tumors for K19 positivity prior to RFA. Further research is warranted in larger groups to validate these findings and outweigh the potential additional clinical benefit compared to the potential risk of tract seeding during percutaneous biopsy.

Although biopsy has an important role in understanding the biological characteristics of HCC [26], tumor seeding by needle biopsy should be avoided. In practice, this is a major concern with needle biopsy of tumors. A review of tumor seeding following therapeutic procedures in HCC indicated that seeding occurred in 0–12.5% of cases (median 0.95%, mean 2.5%) [22]. As the time between biopsy and the treatment procedure was not specified, it is difficult to identify the factors that could have caused seeding. In the present study, tumor biopsies were performed just before RFA, using a needle-guiding technique, and tumor seeding was not observed. The same puncture line was used for both tumor biopsy and RFA, allowing complete ablation of the tumor using the tumor biopsy route. This may be one of the reasons it was possible in this study to biopsy the tumors without dissemination or bleeding. After treatment by RFA, the tumor cannot be investigated for histological features and K19 expression; therefore, we recommend taking a biopsy just before RFA for predicting tumor behavior using K19 expression. This would be valuable to both the clinician and the patient.

The mechanism of K19-positive HCC remains unclear. The facts that K19-positive cells are present in HCCs and that these positive cells form a spectrum suggest that K19-positive HCC may have originated from hepatic progenitor cells. These hepatic progenitor cells,

which are liver-specific adult stem cells, have potential stem cell features such as proliferation and differentiation. Once a tumor takes on these phenotypes, K19-positive HCC can still preserve these stem cell phenotypes. Therefore, this could be a possible reason why K19-positive HCC shows aggressive behavior in comparison with K19-negative HCC. In fact, previous publications and our study confirm these features [27].

In conclusion, we successfully evaluated the positivity of K19 in biopsy specimens. K19-positive HCCs showed significantly more frequent recurrence after curative RFA than K19-negative tumors and positive staining of K19 in the cytoplasm of HCC is closely associated with early intrahepatic recurrence (<1 year) and dropout from the Milan criteria. On imaging, K19-positive HCC showed only typical HCC findings and it was difficult to distinguish between K19-positive and -negative HCC. Taken together, these findings could indicate that >5% K19 positivity in tumor biopsy tissue is important for pre-

dicting tumor recurrence, which is not possible by imaging. Because of the high risk of tumor recurrence in K19-positive HCC, close observation for early detection of recurrence should be required.

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Disclosure Statement

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Early Decrease in α -Fetoprotein, but Not Des- γ -Carboxy Prothrombin, Predicts Sorafenib Efficacy in Patients with Advanced Hepatocellular Carcinoma

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Key Words

Antitumor response · Chemotherapy · Des- γ -carboxy prothrombin · α -Fetoprotein · Hepatocellular carcinoma · Sorafenib · Tumor markers

Abstract

Objectives: The aim of this study was to investigate the relationships between early changes in the tumor markers α -fetoprotein (AFP) and des- γ -carboxy prothrombin (DCP), and antitumor response in the early period following administration of sorafenib in patients with advanced hepatocellular carcinoma (HCC). **Methods:** Forty-eight advanced HCC patients were evaluated. AFP and DCP were measured at baseline, and after 2 and 4 weeks, and the antitumor responses were evaluated according to the RECIST criteria 4 weeks after starting sorafenib therapy. The ratios of each tumor marker were compared by stratifying the patients into the partial response (PR) + stable disease (SD) group or the progressive disease (PD) group. **Results:** Both 2 and 4 weeks after starting sorafenib therapy, the AFP ratio in the PR + SD group ($n = 32$) was significantly lower than in the PD group ($n = 16$; $p = 0.002$, $p = 0.002$). DCP was elevated in both the

PR + SD group and the PD group 2 weeks and 4 weeks after starting sorafenib therapy. **Conclusions:** Evaluation of AFP ratios 2 and 4 weeks after starting sorafenib therapy may be useful for predicting antitumor response. On the other hand, early elevation of DCP does not necessarily suggest treatment failure by sorafenib, as DCP elevation can occur despite therapeutic efficacy.

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Introduction

Sorafenib is a molecularly targeted multikinase inhibitor that suppresses both signal transduction of tumor growth and angiogenesis by inhibiting Raf kinase, and VEGF and PDGF receptor kinase [1]. The SHARP Study and the Asia-Pacific Study [2, 3], two large-scale, phase III, clinical studies, demonstrated that sorafenib significantly prolongs time to progression (TTP) and improves overall survival (OS) in patients with advanced hepatocellular carcinoma (HCC), and confirmed its efficacy in improving prognosis in these patients for the first time as a systemic chemotherapeutic agent. Accordingly,

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sorafenib has been recognized as the only standard systemic chemotherapeutic agent for patients with advanced HCC for whom resection and local therapy are not indicated [4–6].

α -Fetoprotein (AFP) and des- γ -carboxy prothrombin (DCP) are well-known and widely used serological tumor markers in the screening and diagnosis of HCC [7–11]. These tumor markers are also useful as indicators of the therapeutic effect by evaluating serial changes in these values before and after tumor resection and local ablation therapy. Although numerous studies have reported the relationships between the changes in tumor markers during treatment and antitumor response [12–19], there have been no comprehensive reports evaluating the relationship between prognosis and serial changes in AFP and DCP during treatment with sorafenib. Even in the SHARP Study and the Asia-Pacific Study, this relationship was not evaluated, despite the lack of systemic chemotherapeutic agents other than sorafenib that improve prognosis in advanced HCC.

Accordingly, we investigated cumulative TTP and OS stratified by antitumor effects based on image analysis, and assessed the relationship between antitumor effects and changes in AFP and DCP in the early period of sorafenib administration in patients with advanced HCC.

Patients and Methods

Patient Eligibility

Between July 2009 and December 2010, a total of 52 patients with advanced HCC were consecutively started on sorafenib (Nexavar®; Bayer Health Care Pharmaceuticals, West Haven, Conn., USA) therapy at the Division of Gastroenterology and Hepatology, Musashino Red Cross Hospital. Inclusion criteria for this study were as follows: HCC was diagnosed either by needle biopsy or by the combination of typical radiological findings on dynamic multidetector row computed tomography (MDCT) and elevated AFP serum levels, according to the American Association for the Study of Liver Diseases [20]; patients were classified as having advanced HCC if they were not eligible for or had disease progression after surgical or locoregional therapies; Eastern Cooperative Oncology Group performance status score of 0–1; Child-Pugh liver function class A or B (≤ 7); adequate hepatic function (albumin level >2.5 g/dl, total bilirubin level <3.0 mg/dl, and alanine and aspartate aminotransferase levels <5 times the upper limit of normal); dynamic MDCT was obtained at baseline and after 4 weeks of sorafenib treatment in order to assess the therapeutic effects.

Of 52 patients, 48 patients meeting the inclusion criteria were enrolled. HCC stage was diagnosed according to the criteria of the Liver Cancer Study Group of Japan [21]. This study was approved by the Ethics Committee of the Musashino Red Cross Hospital and was performed in compliance with the Helsinki Declaration.

Sorafenib Therapy

The starting dosage of sorafenib was 800 mg/day p.o. However, out of concern regarding the possibility of having to discontinue sorafenib treatment at an early stage due to adverse events, the initial dosage was set at 400 mg/day for patients aged ≥ 80 years, and those with a body weight ≤ 40 kg or a history of treatment for varices or ascites. Sorafenib therapy was continued until the occurrence of potentially fatal adverse events.

Image-Based Evaluation of Antitumor Effects

Dynamic MDCT images were taken at baseline and after 4 weeks of sorafenib treatment. Tumor responses were defined as the time point response [(in accordance with the Response Evaluation Criteria In Solid Tumors (RECIST; version 1.1)] [22] 4 weeks after sorafenib administration where the confirmation of response was not required. Patients in whom the effect was rated as partial response (PR) or stable disease (SD) were pooled in the PR + SD group, while patients showing progressive disease (PD) comprised the PD group. MDCT images were obtained every 2–6 weeks after the first MDCT image, which was obtained 4 weeks after the start of sorafenib administration.

Measurement and Evaluation of Serum AFP and DCP

The HCC tumor markers analyzed were serum AFP and DCP at baseline, and 2 and 4 weeks after starting sorafenib administration. Because DCP levels are influenced by vitamin K and warfarin, patients ingesting these agents were excluded from DCP analysis. For each patient, the baseline concentration of each tumor marker was assigned a value of 1, and the ratios for each tumor marker 2 and 4 weeks after the start of administration were calculated.

Statistics

Statistical analyses were performed using Stat View J software (version 5; SAS Institute, Cary, N.C., USA). TTP and OS after the start of sorafenib administration were analyzed by the Kaplan-Meier method, while comparisons between the two patient groups were performed by log-rank test. Tumor marker levels were analyzed by Wilcoxon signed-rank test, and comparisons of the ratios for the tumor markers between the two patient groups were performed by the Mann-Whitney U test. A value of $p < 0.05$ was considered to indicate a statistically significant difference.

Results

Patient Baseline Characteristics

Table 1 shows baseline characteristics of the 48 HCC patients enrolled in this study. The study cohort consisted of 38 males and 10 females, with a mean age of 69.9 ± 10.0 years. Six patients had never been treated for HCC, while the remaining 42 patients had previously undergone therapy. None of these previous treatments had involved molecularly targeted therapy. The starting dosage of sorafenib in this study was 800 mg/day in 26 patients and 400 mg/day in 22 patients. Criteria for starting sorafenib at 400 mg/day were as follows: (a) age ≥ 80 years