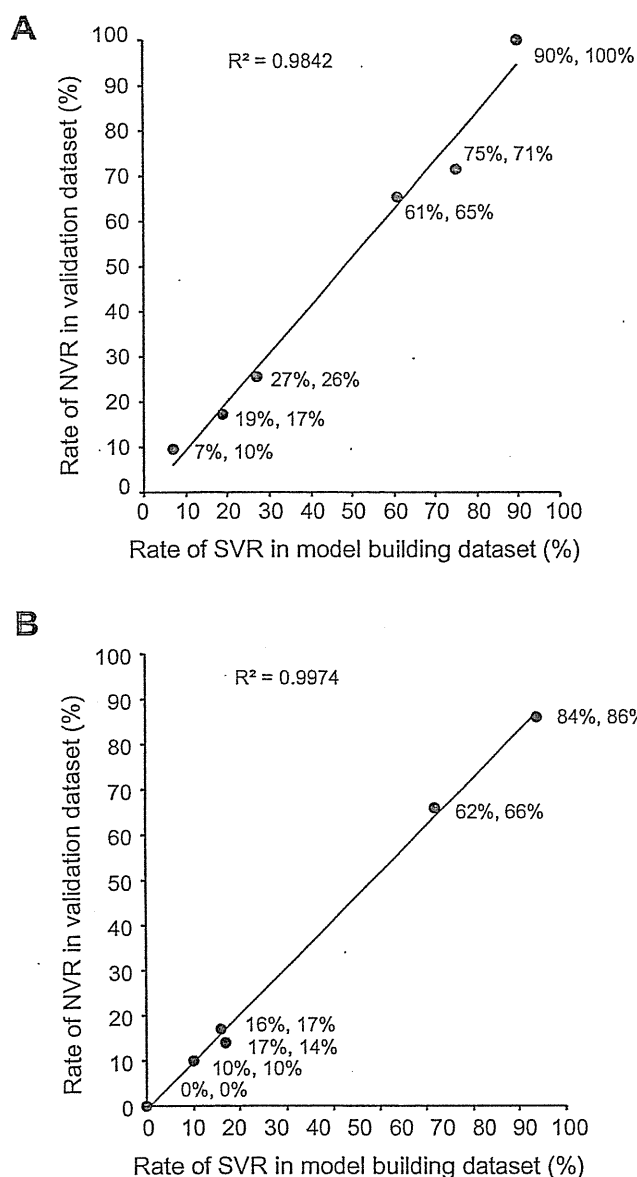


Fig. 5. Decision tree for the prediction of response to therapy. The boxes indicate the factors used for splitting. Pie charts indicate the rate of response for each group of patients after splitting. The rate of null virological response, relapse, and sustained virological response is shown.

estimate of the response before treatment, by simply allocating patients to subgroups by following the flow-chart form, which may facilitate clinical decision making. This is in contrast to the calculating formula, which was constructed by the traditional logistic regression model. This was not widely used in clinical practice as it is abstruse and inconvenient. These results support the evidence based approach of selecting the optimum treatment strategy for individual patients, such as treating patients with a low probability of NVR with current PEG-IFN/RBV combination therapy or advising those with a high probability of NVR to wait for more effective future therapies. Patients with a high probability of relapse may be treated for a longer duration to avoid a relapse. Decisions may be based on the possibility of a response against a potential risk of adverse events and the cost of the therapy, or disease progression while waiting for future therapy.

We have previously reported the predictive model of early virological response to PEG-IFN and RBV in chronic hepatitis C

[26]. The top factor selected as significant was the grade of steatosis, followed by serum level of LDL cholesterol, age, GGT, and blood sugar. The mechanism of association between these factors and treatment response was not clear at that time. To our interest, a recent study by Li et al. [47] has shown that high serum level of LDL cholesterol was linked to the *IL28B* major allele (CC in rs12979860). High serum level of LDL cholesterol was associated with SVR but it was no longer significant when analyzed together with the *IL28B* genotype in multivariate analysis. Thus, the association between treatment response and LDL cholesterol levels may reflect the underlining link of LDL cholesterol levels to *IL28B* genotype. Steatosis is reported to be correlated with low lipid levels [48] which suggest that *IL28B* genotypes may be also associated with steatosis. In fact, there were significant correlations between the *IL28B* genotype and the presence of steatosis in the present study (Table 4). In addition, the serum level of GGT, another predictive factor in our previous study, was signif-



**Fig. 6. Validation of the CART analysis.** Each patient in the validation group was allocated to one of the six subgroups by following the flow-chart form of the decision tree. The rate of (A) sustained virological response (SVR) and (B) null virological response (NVR) in each subgroup was calculated and plotted. The X-axis represents the rate of SVR or NVR in the model building patients and the Y-axis represents those in the validation patients. The rate of SVR and NVR in each subgroup of patients is closely correlated between the model building and the validation patients (correlation coefficient:  $r^2 = 0.98-0.99$ ).

icantly associated with *IL28B* genotype in the present study (Table 4). The serum level of GGT was significantly associated with NVR when examined independently but was no longer significant when analyzed together with the *IL28B* genotype. These observations indicate that some of the factors that we have previously identified may be associated with virological response to therapy through the underlining link to the *IL28B* genotype.

In conclusion, the present study highlighted the impact of the *IL28B* polymorphism and mutation in the ISDR on the pre-treatment prediction of response to PEG-IFN/RBV therapy. A decision model including these host and viral factors has the potential to

support selection of the optimum treatment strategy for individual patients, which may enable personalized treatment.

**Conflict of interest**

The authors who have taken part in this study declare that they do not have anything to disclose regarding funding or conflict of interest with respect to this manuscript.

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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

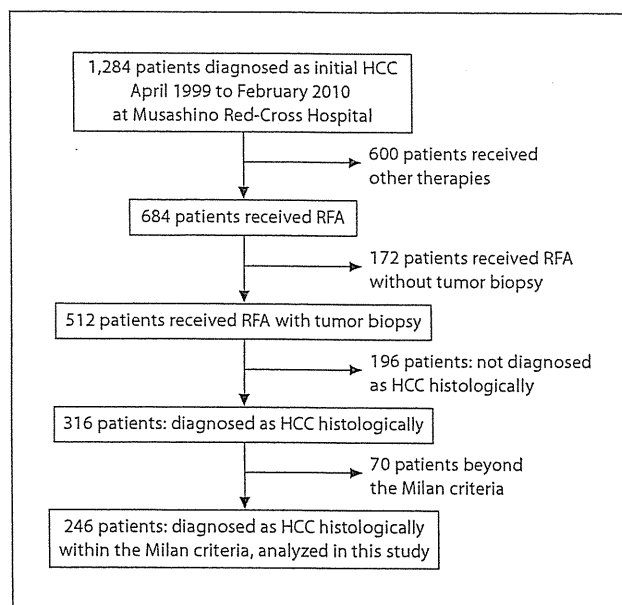


Fig. 1. Flow chart summarizing the patient selection for the study.

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 ( $\leq 3$  cm and up to 3 nodules, or  $\leq 5$  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 arteriography (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  $\Omega$  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  $\geq 1$  ablations performed on  $\geq 1$  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 [ $\alpha$ -fetoprotein (AFP) and des- $\gamma$ -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  $\chi^2$  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  $\chi^2$  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	-

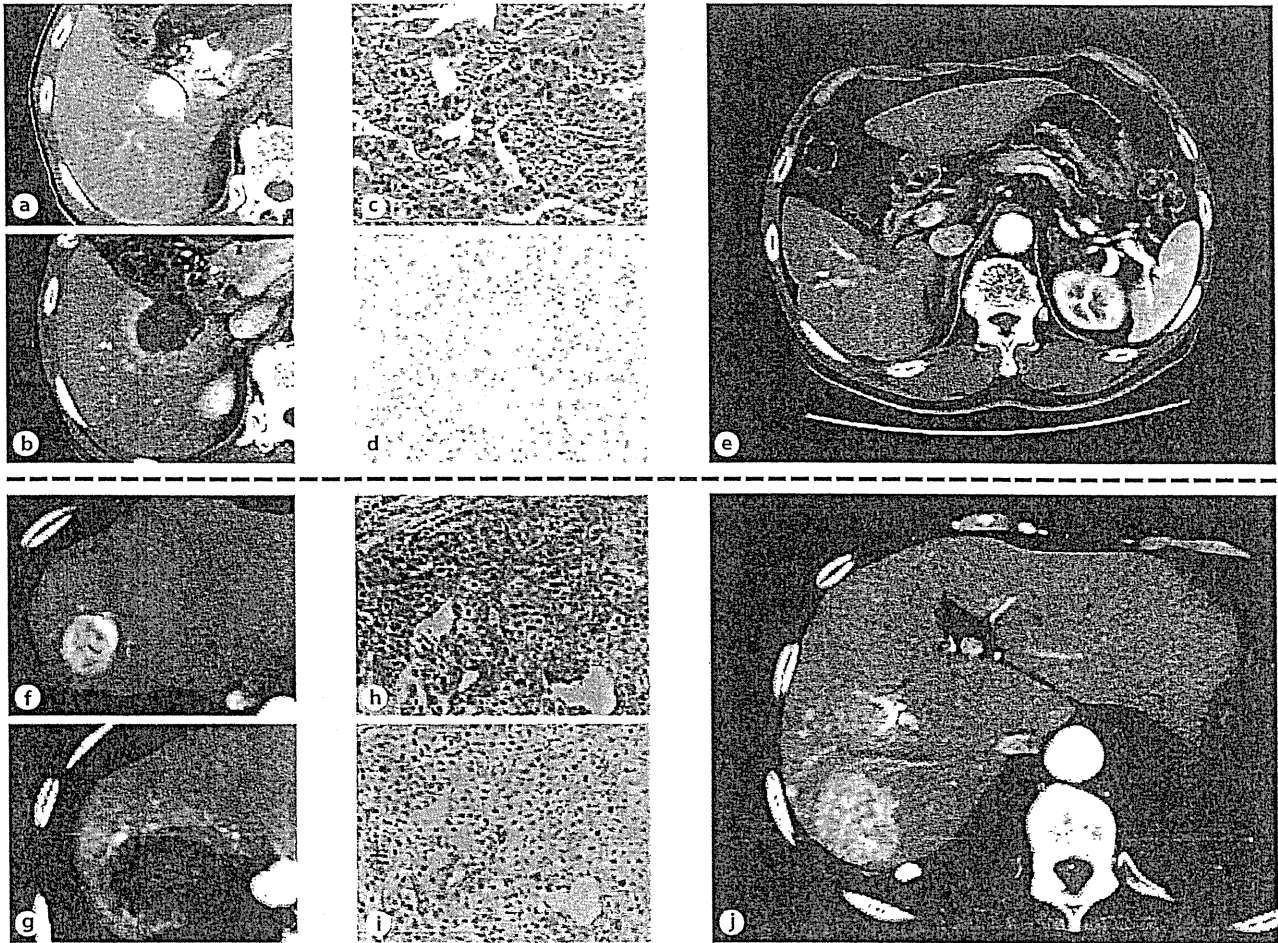
cells was observed in HCCs from 10 of 246 patients (4.1%). Two of the 10 HCCs (20.0%) were poorly differentiated, and 8 (80.0%) were moderately differentiated. None of the well-differentiated HCCs showed K19 positivity. Among the 10 patients with K19-positive HCCs, 2 had a HCC nodule >3 cm and 8 had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

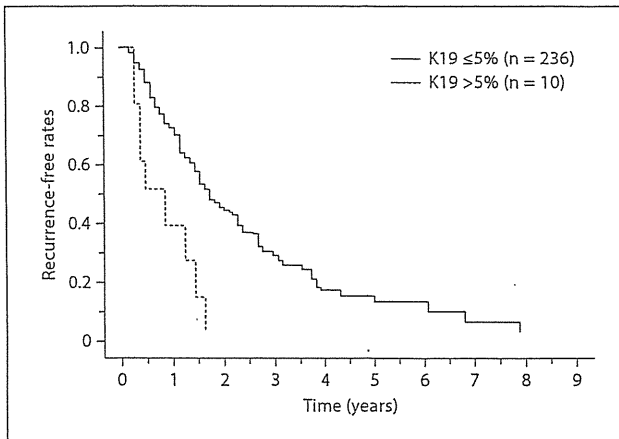
sion, is shown in table 2. These imaging findings were consistent with the histological diagnosis, as determined by pretreatment needle biopsy.

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*

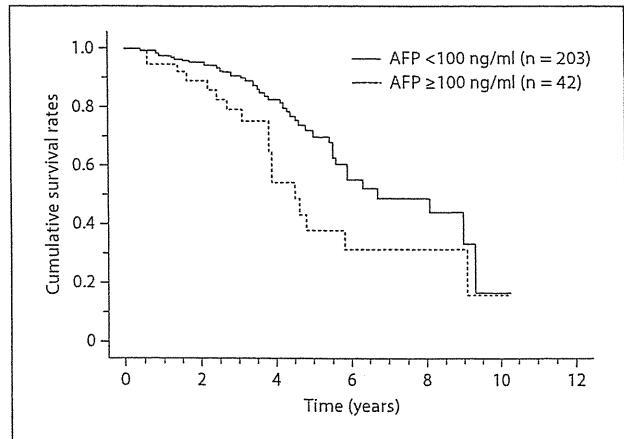
The median follow-up period was 34.0 months (range 65 days to 10.3 years). A recurrence of HCC was diagnosed at least once during the follow-up period in 156 patients (63.4%). The cumulative recurrence-free survival at 1, 3 and 5 years was 69.9, 26.6 and 12.2%, respectively. Among the 156 patients with 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 ( $p = 0.0001$ ).

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  $\geq 2$  mg/dl were significant independent risk factors for HCC recurrence in all patients.



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

The percentage of distant metastasis and major portal invasion (VP3–4) was significantly higher in K19-positive than in K19-negative patients ( $p < 0.0001$ ). Distant 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  $\geq 100$  ng/ml ( $p = 0.034$ ), a total bilirubin level  $\geq 2$  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 ( $\geq 100$  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 ( $\geq 65$  years), albumin concentration ( $\leq 3.5$  g/dl), prothrombin time ( $\leq 70\%$ ), DCP ( $\geq 100$  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 $\geq 2$ mg/dl	2.50	1.02–6.25	0.034	2.70	1.08–6.66	0.032
Albumin $\leq 3.5$ g/dl	1.12	0.81–1.56	0.492			
PT $\leq 70\%$	1.28	0.73–2.22	0.394			
AFP $\geq 100$ ng/ml	1.42	0.95–2.12	0.087			
DCP $\geq 100$ 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 $\geq 2$ mg/dl	12.5	4.54–33.3	$< 0.0001$	10.0	3.70–33.3	$< 0.0001$
Albumin $\leq 3.5$ g/dl	1.25	0.71–2.17	0.450			
PT $\leq 70\%$	1.49	0.59–3.84	0.674			
AFP $\geq 100$ ng/ml	1.88	1.06–3.44	0.030	1.88	1.05–3.33	0.034
DCP $\geq 100$ 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 ( $\geq 100$  mAU/ml), tumor number and total bilirubin  $\geq 2$  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.

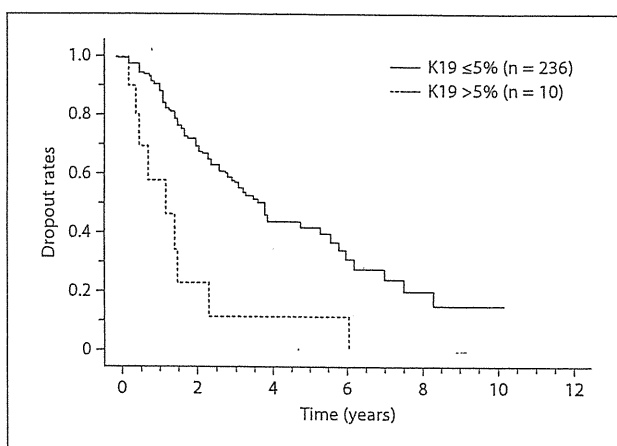
#### *Percentage of K19 Stain*

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 $\geq 2$ mg/dl	2.94	1.05–8.33	0.039	3.57	1.25–10.0	0.017
Albumin $\leq 3.5$ g/dl	0.97	0.64–1.47	0.857			
PT $\leq 70\%$	0.89	0.41–1.96	0.763			
AFP $\geq 100$ ng/ml	2.17	1.38–3.44	0.0008	1.56	0.96–2.50	0.077
DCP $\geq 100$ 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](http://www.karger.com/doi/10.1159/000328448)).

K19 positivity was not an independent predictor of the overall rate of survival, and serum AFP ( $\geq 100$  ng/ml), total bilirubin ( $\geq 2$  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  $68 \pm 8$  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 findings 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 $\alpha$ -Fetoprotein, but Not Des- $\gamma$ -Carboxy Prothrombin, Predicts Sorafenib Efficacy in Patients with Advanced Hepatocellular Carcinoma

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### Key Words

Antitumor response · Chemotherapy · Des- $\gamma$ -carboxy prothrombin ·  $\alpha$ -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  $\alpha$ -fetoprotein (AFP) and des- $\gamma$ -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].

$\alpha$ -Fetoprotein (AFP) and des- $\gamma$ -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<sup>®</sup>; 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 ( $\leq 7$ ); 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  $\geq 80$  years, and those with a body weight  $\leq 40$  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.

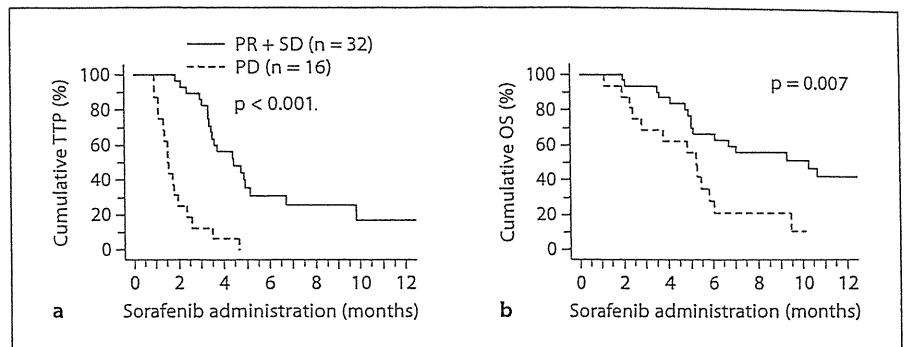
## Results

### 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  $69.9 \pm 10.0$  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  $\geq 80$  years



**Fig. 1.** Comparison of cumulative TTP (a) and OS (b) in the PR + SD and PD groups according to RECIST.



(n = 8); (b) body weight  $\leq 40$  kg (n = 2), and (c) history of treatment for varices or ascites (n = 12). The median baseline AFP level was 572 ng/ml (range, 2.3–148,000), and the median baseline DCP level was 424 mAU/ml (range, 15–305,000). The mean observation period was  $7.2 \pm 4.5$  months.

#### *Antitumor Responses 4 Weeks after the Start of Sorafenib Therapy*

According to RECIST, 4 weeks after the start of sorafenib therapy, there were no complete responses, 2 PR, 30 SD, and 16 PD. The response rate was 4.2%, and the disease control rate was 66.7%.

#### *Cumulative TTP and OS in the PR + SD and PD Groups*

Cumulative TTP in the two groups according to RECIST is shown in figure 1a. The median observation period was 3.2 months. The median TTP was significantly longer in the PR + SD group than in the PD group (4.4 vs. 1.5 months; hazard ratio, 0.14; 95% CI, 0.06–0.29;  $p < 0.001$ ).

Cumulative OS in the two groups according to RECIST is shown in figure 1b. The median observation period was 5.7 months. The median OS was significantly longer in the PR + SD group than in the PD group (10.3 vs. 5.2 months; hazard ratio, 0.36; 95% CI, 0.17–0.78;  $p = 0.007$ ).

#### *Comparison of Actual and Relative Levels of AFP at Baseline, and 2 and 4 Weeks after the Start of Sorafenib Therapy (Stratified by Antitumor Response)*

AFP was not measured in 9 and 1 patients 2 and 4 weeks after starting sorafenib administration, respectively. Accordingly, AFP was analyzed in 39 and 47 patients 2 and 4 weeks after starting sorafenib administration, respectively.

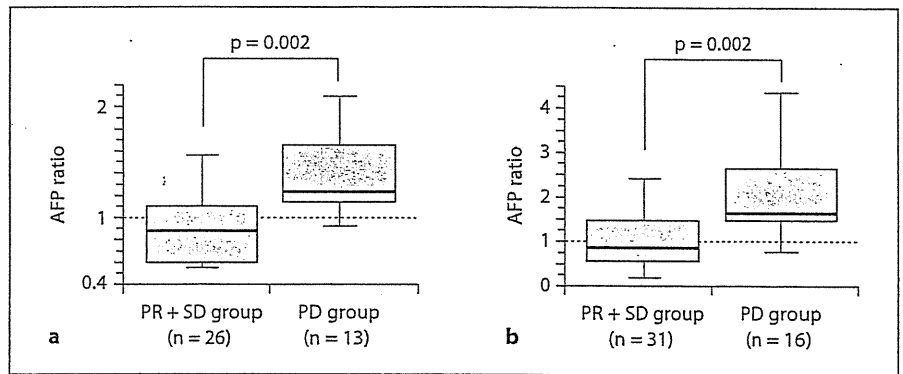
**Table 1.** Baseline characteristics of the 48 HCC patients enrolled in this study

Mean age, years	$69.9 \pm 10.0$
Male/female	38/10
HBV/HCV/NBNC	6/30/12
ECOG PS (0/1)	29/19
Child-Pugh score (5/6/7)	24/21/3
HCC stage (III/IVA/IVB)	11/18/19
Initial therapy/therapy for recurrence	6/42
Sorafenib starting dosage (800/400 mg)	26/22
Median serum AFP level, ng/ml	572
Range	2.3–148,000
Median serum DCP level, mAU/ml	424
Range	15–305,000
Mean observation period, months	$7.2 \pm 4.5$

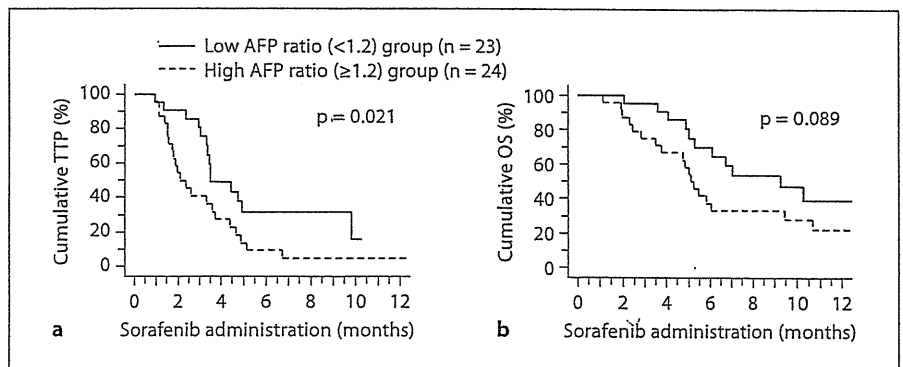
Numbers of patients are shown unless indicated otherwise. HBV/HCV = Hepatitis B/C virus; NBNC = non-HBV, non-HCV; ECOG = Eastern Cooperative Oncology Group; PS = performance status.

Data comparing actual AFP levels at baseline, and 2 and 4 weeks after starting sorafenib administration, both for the total patients and when stratified by antitumor response according to RECIST, are shown in table 2. Among the total number of patients, AFP showed no statistically significant differences between baseline and 2-week treatment levels, but in the PD group, AFP levels after 2 weeks of treatment were significantly elevated versus baseline levels ( $p = 0.013$ ). Similarly, in the total number of patients, AFP showed no statistically significant differences between baseline and 4-week treatment levels, but in the PD group, AFP was significantly higher after 4 weeks of treatment compared with baseline levels ( $p = 0.002$ ). In the PR + SD group, the median actual AFP level 4 weeks after starting sorafenib administration was higher than that at 2 weeks; however, there were no sig-

**Fig. 2.** AFP ratios 2 (a) and 4 weeks (b) after the start of sorafenib treatment in the PR + SD and PD groups according to RECIST.



**Fig. 3.** Comparison of cumulative TTP (a) and OS (b) in the groups with low (<1.2) and high AFP ratio ( $\geq 1.2$ ) 4 weeks after starting sorafenib therapy.



**Table 2.** Comparison of actual AFP levels (ng/ml) at baseline, and 2 and 4 weeks after the start of sorafenib therapy (stratified by anti-tumor response)

Groups	Baseline	After 2 weeks	p value	After 4 weeks	p value
Total	572 (2.3–148,000)	481 (2.2–163,300)	0.155	676 (1.1–281,700)	0.077
PR + SD	245.5 (2.3–148,000)	198 (2.2–163,300)	0.657	311 (1.1–281,700)	0.518
PD	2,321 (8.6–62,400)	3,303 (6.4–52,840)	0.013	6,258.5 (6.4–237,000)	0.002

nificant differences between AFP levels after 2 and 4 weeks ( $p = 0.423$ ). On the other hand, in the PD group, the median actual AFP level 4 weeks after starting sorafenib administration was significantly higher than that after 2 weeks ( $p = 0.003$ ).

Figure 2 compares the AFP ratios stratified by anti-tumor effects according to RECIST after 2 and 4 weeks of sorafenib treatment. AFP ratios 2 and 4 weeks after the start of sorafenib administration were 0.88 (range, 0.28–1.79) and 0.88 (range, 0.07–3.17) in the PR + SD group, and 1.24 (range, 0.74–2.12) and 1.63 (range, 0.64–7.35) in the PD group. At both time points, the ratio in the PR + SD group was significantly lower than in the PD group ( $p = 0.002$ ,  $p = 0.002$ ).

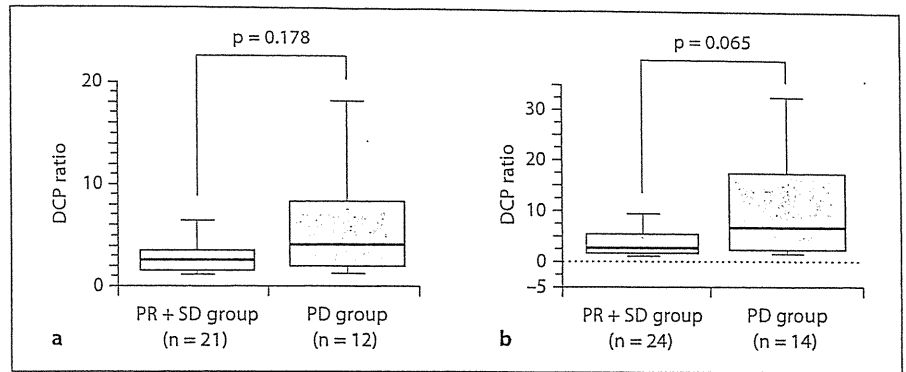
#### Cumulative TTP and OS in the Groups with Low and High AFP Ratio 4 Weeks after the Start of Sorafenib Therapy

The median AFP ratio 4 weeks after the start of sorafenib therapy was 1.2 (0.1–7.4).

Cumulative TTP (according to RECIST) in the groups with low (<1.2) and high AFP ratio ( $\geq 1.2$ ) 4 weeks after the start of sorafenib therapy is shown in figure 3a. The median TTP was significantly longer in the low AFP ( $n = 23$ ) ratio group than in the high AFP ratio group ( $n = 24$ ; 3.5 vs. 2.1 months; hazard ratio, 0.46; 95% CI, 0.23–0.91;  $p = 0.021$ ).

Cumulative OS in the low ( $n = 23$ ) and high AFP ratio groups ( $n = 24$ ) 4 weeks after the start of sorafenib therapy

**Fig. 4.** DCP ratios 2 (a) and 4 weeks (b) after the start of sorafenib treatment in the PR + SD and PD groups according to RECIST.



**Table 3.** Comparison of actual DCP levels (mAU/ml) at baseline, and 2 and 4 weeks after the start of sorafenib therapy (stratified by antitumor response)

Patients	Baseline	After 2 weeks	p value	After 4 weeks	p value
Total	424.5 (15–305,000)	741 (26–798,000)	<0.001	2,025 (78–1,020,000)	<0.001
PR + SD	425.5 (15–216,000)	741 (30–323,000)	<0.001	1,715 (81–524,000)	<0.001
PD	575.5 (19–305,000)	6,186.5 (26–798,000)	0.002	20,550 (78–1,020,000)	0.001

is shown in figure 3b. The median OS tended to be higher in the low than in the high AFP ratio group (9.3 vs. 5.1 months; hazard ratio, 0.53; 95% CI, 0.25–1.12;  $p = 0.089$ ).

#### Comparison of Actual and Relative Levels of DCP at Baseline, and 2 and 4 Weeks after the Start of Sorafenib Therapy (Stratified by Antitumor Response)

In the analysis of DCP, 7 patients who were taking vitamin K and 1 patient who was on warfarin were excluded. In addition, DCP was not determined in 7 and 2 patients 2 and 4 weeks after starting sorafenib administration, respectively. Accordingly, DCP was analyzed in 33 patients 2 weeks and in 38 patients 4 weeks after starting sorafenib administration.

Data comparing actual DCP levels at baseline, and 2 and 4 weeks after starting sorafenib administration, both for the total number of patients and patients stratified by antitumor response according to RECIST, are shown in table 3. Actual levels of DCP after 2 weeks of treatment were significantly higher than baseline levels in the total number of patients, the PR + SD group and the PD group. After 2 weeks of treatment, DCP was elevated in 97.0% (32/33) of the patients. Similarly, actual levels of DCP after 4 weeks of treatment were also significantly elevated from baseline levels in all patient groups; the total number of patients, the PR + SD group, and the PD group.

After 4 weeks of treatment, DCP was elevated in 92.1% (35/38) of the patients.

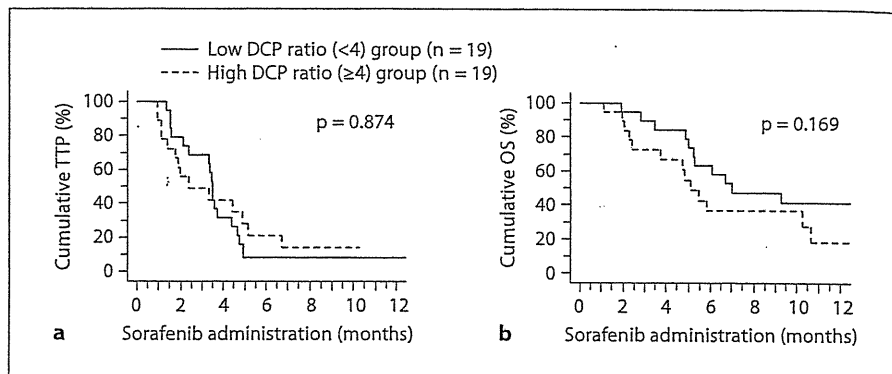
Figure 4 compares the DCP ratios between the PR + SD and PD groups according to RECIST after 2 and 4 weeks of sorafenib therapy. The DCP ratios 2 and 4 weeks after the start of sorafenib administration were 2.57 (range, 0.87–10.02) and 2.72 (range, 0.30–13.46) in the PR + SD group, and 4.02 (range, 1.12–35.03) and 6.73 (range, 1.25–45.08) in the PD group. There were no significant differences between the PR + SD and PD groups at either time point ( $p = 0.178$ ,  $p = 0.065$ ).

#### Cumulative TTP and OS in the Low and High DCP Ratio Groups 4 Weeks after the Start of Sorafenib Therapy

The median DCP ratio 4 weeks after the start of sorafenib therapy was 4.0 (0.3–45.1).

Cumulative TTP (according to RECIST) in the low (<4) and the high DCP ratio ( $\geq 4$ ) groups 4 weeks after the start of sorafenib therapy is shown in figure 5a. There were no significant differences in the median DCP ratio between the low ( $n = 19$ ) and the high DCP ratio group ( $n = 19$ ; 3.5 vs. 2.4 months; hazard ratio, 1.06; 95% CI, 0.51–2.19;  $p = 0.874$ ).

Cumulative OS in the low ( $n = 19$ ) and high DCP ratio groups ( $n = 19$ ) 4 weeks after the start of sorafenib therapy



**Fig. 5.** Comparison of cumulative TTP (a) and OS (b) in the groups with low (<4) and high DCP ratio ( $\geq 4$ ) 4 weeks after starting sorafenib therapy.

py is shown in figure 5b. There were no significant differences in median DCP ratio between the low and high DCP ratio groups (7.2 vs. 5.1 months; hazard ratio, 0.57; 95% CI, 0.25–2.55;  $p = 0.169$ ).

### Discussion

In the present study, we investigated the relationships between the changes in tumor markers, AFP and DCP, and antitumor responses in the early period following administration of sorafenib to patients with advanced HCC, and found that the relationship for AFP was different from that for DCP. With regard to AFP, both 2 and 4 weeks after starting sorafenib therapy, the ratio in the PR + SD group was significantly lower than in the PD group. On the other hand, DCP was elevated in both the PD group and the PR + SD group, and there were no statistically significant differences between the two groups either 2 or 4 weeks after starting sorafenib therapy. These results suggest that the time course changes for AFP in the early period after starting sorafenib administration are useful for predicting antitumor response assessed by image analysis.

Several studies have reported that the primary effect of sorafenib is inhibition of tumor growth rather than tumor shrinkage [2, 3, 23, 24]. In the SHARP Study, it was reported that the response rate in the sorafenib group based on RECIST criteria was only 2.3%, but both the cumulative TTP and cumulative survival duration were prolonged [2]. This can be considered an example of the limitations of using RECIST criteria, which focus on changes in the size of the entire tumor for evaluation of the therapeutic efficacy of molecularly targeted drugs. In the present study, cumulative TTP and OS were significantly better in the PR + SD group than in the PD group. In view of these findings, the primary clinical benefit of

sorafenib is disease stabilization. Accordingly, it is important to evaluate treatment response in patients treated with sorafenib. In the present study, we analyzed the tumor marker response according to radiological response using the RECIST criteria. On the other hand, modified RECIST criteria were recently proposed as a method to assess arterial involvement [25]. Further investigation using these modified RECIST criteria is thus necessary.

In order to evaluate tumor responses, the formal recommendation of the panel of experts in HCC-Design Clinical Trials was to conduct imaging surveillance every 6–8 weeks using CT or MRI [4]. In our hospital, dynamic MDCT was obtained after 4 weeks of sorafenib treatment in order to assess early therapeutic effects. We found that antitumor responses 4 weeks after sorafenib administration correlated with both TTP and OS. Therefore, the present results indicate that it may be beneficial to evaluate the time point response 4 weeks after sorafenib administration in patients receiving sorafenib.

In the present study, AFP was significantly elevated in the PD group both 2 and 4 weeks after the start of administration compared with baseline. There has only been one report on AFP response after sorafenib therapy [26]. Shao et al. [26] reported the AFP responder group as patients whose AFP levels decreased to less than 0.8-fold of baseline levels within 1 month following sorafenib administration, while the non-responder group did not show this decrease. Consistent with our results, both the cumulative survival and TTP rates were significantly better in the AFP responder group than in the non-responder group. Hence, in the case of sorafenib therapy, changes in AFP levels may be correlated with the antitumor effects evaluated by image analysis, similarly to the course following other therapies for HCC, such as hepatic resection, radiofrequency ablation therapy, and transarterial chemoembolization. A comparison of the actual AFP levels 2 and 4