ing arterioportography (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 pentaacetic 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 hepatobilliary 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

Categorial 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 ± 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
Clinical and laboratory data			
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
Pathology			
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	
Major associated liver diseases			
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.

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	–

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 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-

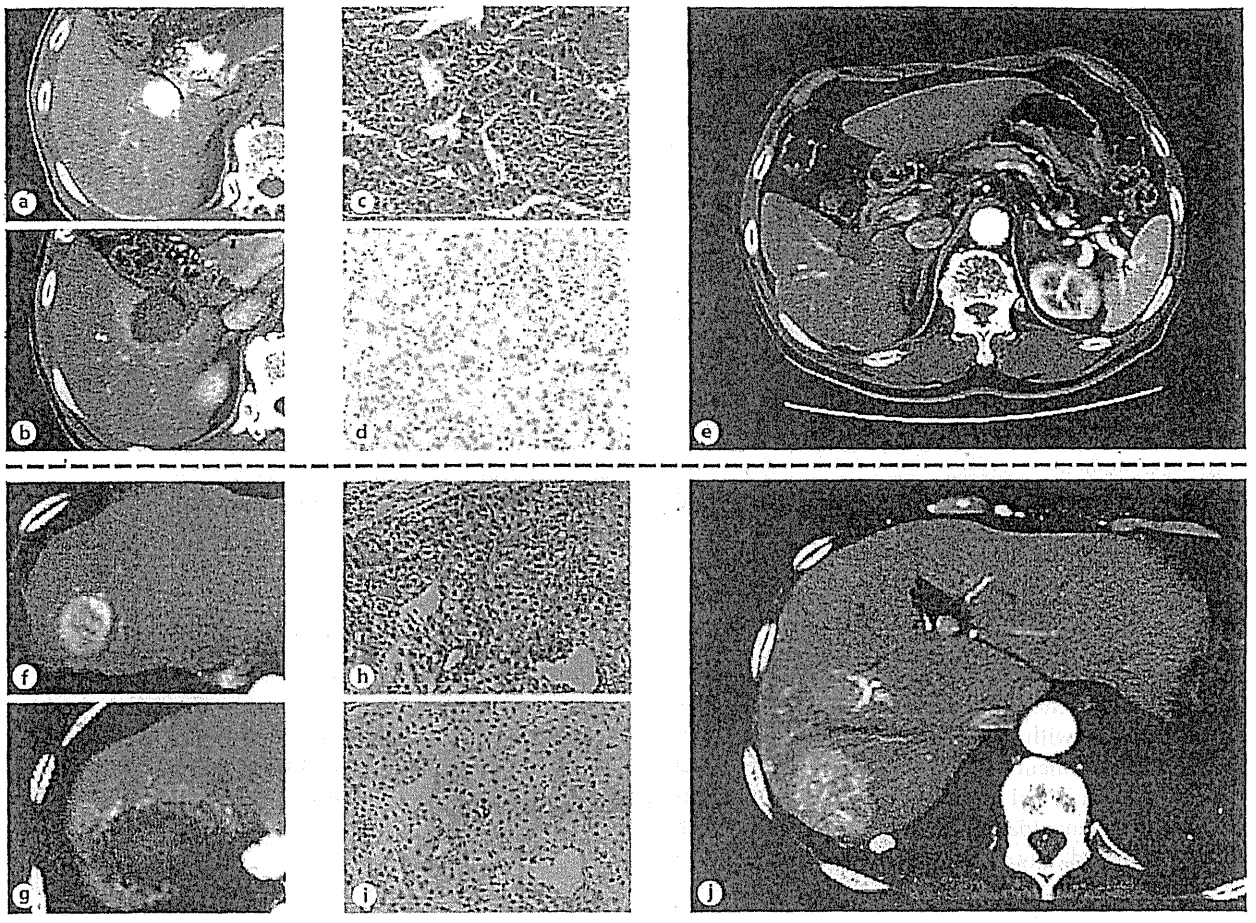

Fig. 2. a–e A patient with K19-negative HCC: a 70-year-old man with chronic hepatitis (anti-HCV positive). The HCC (25 mm in diameter, in segment 6) showed an early enhancement area by dynamic CT (a). Dynamic CT at 1 day after RFA (b). On histological investigation, the tumor showed moderately differentiated 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

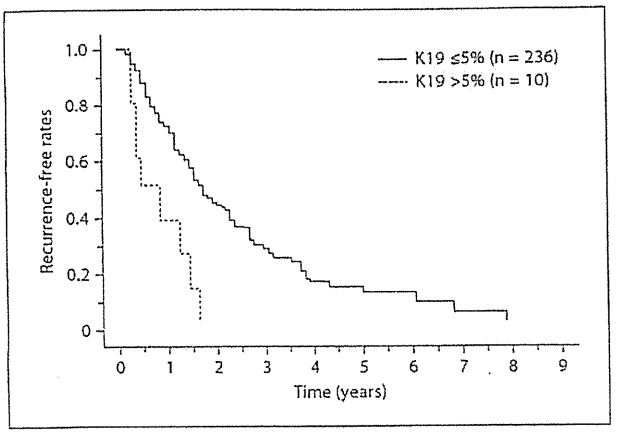

Fig. 3. The cumulative recurrence-free survival rate in patients with K19-positive (>5%) HCC was significantly lower than that in patients with K19-negative HCC (p = 0.0001).

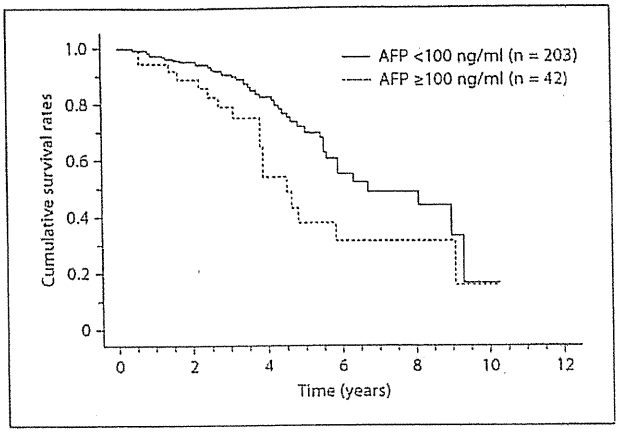

Fig. 4. The cumulative overall survival rate in patients with AFP ≥100 ng/ml was significantly lower than that in patients with AFP <100 ng/ml (p = 0.026).

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 (≤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/m	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.

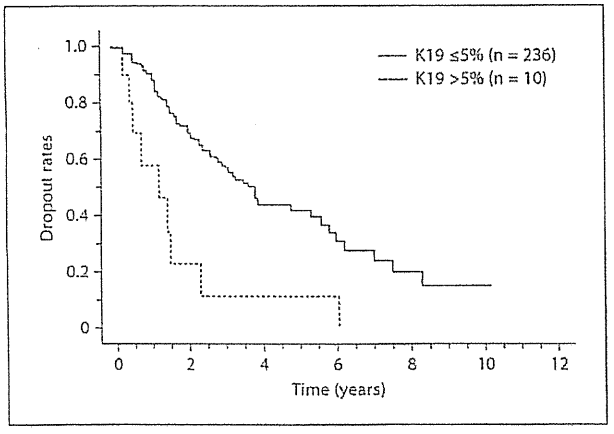

Fig. 5. The cumulative rate of exceeding the Milan criteria in patients with K19-positive HCC was significantly higher than that in patients with K19-negative HCC ($p < 0.0001$).

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 improving 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 predicting 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.

Acknowledgments

We thank Hiroshi Suzuki, Satoshi Kusakari and Yuko Hashimoto for their excellent technical assistance, which was indispensable to this study.

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

Disclosure Statement

The authors have nothing to disclose. They have no affiliation with the manufacturers of the drugs used in this study and have not received funding from the manufacturers to support this research.

References

1. Bruix J, Sherman M: Management of hepatocellular carcinoma. Hepatology 2005;42:1208–1236.
2. Tateishi R, Shiina S, Teratani T, Obi S, Sato S, Koike Y, Fujishima T, Yoshida H, Kawabe T, Omara M: Percutaneous radiofrequency ablation for hepatocellular carcinoma. An analysis of 1000 cases. Cancer 2005;103:1201–1209.
3. Shiina S, Teratani T, Obi S, Sato S, Tateishi R, Fujishima T, Ishikawa T, Koike Y, Yoshida H, Kawabe T, Omata M: A randomized controlled trial of radiofrequency ablation with ethanol injection for small hepatocellular carcinoma. Gastroenterology 2005;129:122–130.
4. Kudo M: Radiofrequency ablation for hepatocellular carcinoma: updated review in 2010. Oncology 2010;78:113–124.
5. Choi D, Lim HK, Rhim H, Kim YS, Lee WJ, Paik SW, Koh KC, Lee JH, Choi MS, Yoo BC: Percutaneous radiofrequency ablation for early-stage hepatocellular carcinoma as a first-line treatment: long-term results and prognostic factors in a large single-institution series. Eur Radiol 2007;17:684–692.
6. Lu DS, Yu NC, Raman SS, Lassman C, Tong MJ, Britten C, Durazo F, Saab S, Han S, Finn R, Hiatt JR, Busuttil RW: Percutaneous radiofrequency ablation of hepatocellular carcinoma as a bridge to liver transplantation. Hepatology 2005;41:1130–1137.
7. Mazzaferro V, Battiston C, Perrone S, Pulvirenti A, Regalia E, Romito R, Sarli D, Schavo M, Garbagnati F, Marchiano A, Spreafico C, Camerini T, Mariani L, Miceli R, Andreola S: Radiofrequency ablation of small hepatocellular carcinoma in cirrhotic patients awaiting liver transplantation: a prospective study. Ann Surg 2004;240:900–909.
8. Izumi N, Asahina Y, Noguchi O, Uchihara M, Kanazawa N, Itakura J, Himeno Y, Miyake S, Sakai T, Enomoto N: Risk factors for distant recurrence of hepatocellular carcinoma in the liver after complete coagulation by microwave or radiofrequency ablation. Cancer 2001;91:949–956.
9. Fernandes ML, Lin CC, Lin CJ, Chen WT, Lin SM: Risk of tumour progression in early-stage hepatocellular carcinoma after radiofrequency ablation. Br J Surg 2009;96:756–762.
10. Roskams T, De Vos R, Van Eyken P, Myazaki H, Van Damme B, Desmet V: Hepatic OV-6 expression in human liver disease and rat experiments: evidence for hepatic progenitor cells in man. J Hepatol 1998;29:455–463.
11. Yang XR, Xu Y, Shi GM, Fan J, Zhou J, Ji Y, Sun HC, Qiu SJ, Yu B, Gao Q, He YZ, Qin WZ, Chen RX, Yang GH, Wu B, Lu Q, Wu ZQ, Tang ZY: Cytokeratin 10 and cytokeratin 19: predictive markers for poor prognosis in hepatocellular carcinoma patients after curative resection. Clin Cancer Res 2008;14:3850–3859.
12. Zhuang PY, Zhang JB, Zhu XD, Zhang W, Wu WZ, Tan YS, Hou J, Tang ZY, Qin LX, Sun HC: Two pathologic types of hepatocellular carcinoma with lymph node metastasis with distinct prognosis on the basis of CK19 expression in tumor. Cancer 2008;112:2740–2748.
13. Uenishi T, Kubo S, Yamamoto T, Shuto T, Ogawa M, Tanaka H, Tanaka S, Kaneda K, Hirohashi K: Cytokeratin 19 expression in hepatocellular carcinoma predicts early postoperative recurrence. Cancer Sci 2003;94:851–857.
14. Durnez A, Verslype C, Nevens F, Fevery J, Aerts R, Pirenne J, Lesaffre E, Libbrecht L, Desmet V, Roskams T: The clinicopathological and prognostic relevance of cytokeratin 7 and 19 expression in hepatocellular carcinoma. A possible progenitor cell origin. Histopathology 2006;49:138–151.
15. Sobin LH, Fleming ID: TNM Classification of Malignant Tumors, fifth edition (1997). Union Internationale Contre le Cancer and the American Joint Committee on Cancer. Cancer 1997;80:1803–1804.
16. Kudo M, Okanoue T: Management of hepatocellular carcinoma in Japan: consensus-based clinical practice manual proposed by the Japan Society of Hepatology. Oncology 2007;72(suppl 1):2–15.
17. Hirohashi S, Ishak K, Kojiro M, Wanless I, Theise N, Tsukuma H: Tumours of the liver and intrahepatic bile ducts; in Hamilton SR, Aaltonen LA (eds): Pathology and Genetics of Tumours of the Digestive System. Lyon, IARC Press, 2000, pp 157–202.

18 De Baere T, Rehim MA, Teriitheau C, Deschamps F, Lapeyre M, Dromain C, Boige V, Dromain C, Boige V, Ducreux M, Elias D: Usefulness of guiding needles for radiofrequency ablative treatment of liver tumors. Cardiovasc Intervent Radiol 2006;29:650–654.
19 Lorentzen T: A cooled needle electrode for radiofrequency tissue ablation: thermodynamic aspects of improved performance compared with conventional needle design. Acad Radiol 1996;3:556–563.
20 Goldberg SN, Gazelle GS, Solbiati L, Rittman WJ, Mueller PR: Radiofrequency tissue ablation: increased lesion diameter with a perfusion electrode. Acad Radiol 1996;3:636–644.
21 Komorizono Y, Oketani M, Sako K, Yamasaki N, Shibatou T, Maeda M, Kohara K, Shigenobu S, Ishibashi K, Arima T: Risk factors for local recurrence of small hepatocellular carcinoma tumors after a single session, single application of percutaneous radiofrequency ablation. Cancer 2003;97:1253–1262.
22 Stigliano R, Marelli L, Yu D, Davies N, Patch D, Burroughs AK: Seeding following percutaneous diagnostic and therapeutic approaches for hepatocellular carcinoma. What is the risk and the outcome? Seeding risk for percutaneous approach of HCC. Cancer Treat Rev 2007;33:437–447.
23 Brillet PY, Paradis V, Brancatelli G, Rangheard AS, Consigny Y, Plessier A, Durand F, Belghiti J, Sommacale D, Vilgrain V: Percutaneous radiofrequency ablation for hepatocellular carcinoma before liver transplantation: a prospective study with histopathologic comparison. AJR Am J Roentgenol 2006;186:S296–S305.
24 Pawlik TM, Gleisner AL, Anders RA, Assumpcao L, Maley W, Choti MA: Preoperative assessment of hepatocellular carcinoma tumor grade using needle biopsy: implications for transplant eligibility. Ann Surg 2007;245:435–442.
25 Bruix J, Sherman M, Llovet JM, Beaugrand M, Lencioni R, Burroughs AK, Christensen E, Pagliaro L, Colombo M, Rodes J, EASL Panel of Experts on HCC: Clinical management of hepatocellular carcinoma. Conclusions of the Barcelona-2000 EASL conference. European Association for the Study of the Liver. J Hepatol 2001;35:421–430.
26 Rockey DC, Caldwell SH, Goodman ZD, Nelson RC, Smith AD: Liver biopsy. Hepatology 2009;49:1017–1044.
27 van Sprundel RG, van den Ingh TS, Spee B: Keratin 19 marks poor differentiation and a more aggressive behavior in canine and human hepatocellular tumours. Comp Hepatol 2010;9:4.

Clinical Study

Oncology

Oncology 2011;81:251–258
DOI: 10.1159/000334454

Received: July 3, 2011
Accepted after revision: September 30, 2011
Published online: November 23, 2011

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

Teiji Kuzuya Yasuhiro Asahina Kaoru Tsuchiya Keisuke Tanaka Yuichiro Suzuki Takahide Hoshioka Shinji Tamaki Tomoji Kato Yutaka Yasui Takahiro Hosokawa Ken Ueda Hiroyuki Nakanishi Jun Itakura Yuka Takahashi Masayuki Kurosaki Namiki Izumi

Division of Gastroenterology and Hepatology, Musashino Red Cross Hospital, Musashino, Japan

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.

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,

KARGER

Fax +41 61 306 12 34
E-Mail karger@karger.ch
www.karger.com

0030–2414/11/0814–0251$38.00/0

Accessible online at:
www.karger.com/ocl

Namiki Izumi
Division of Gastroenterology and Hepatology
Musashino Red Cross Hospital
1-26-1 Kyonan-cho, Musashino-shi, Tokyo 180-8610 (Japan)
Tel. +81 422 32 3111, E-Mail nizumi@musashino.jrc.or.jp

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

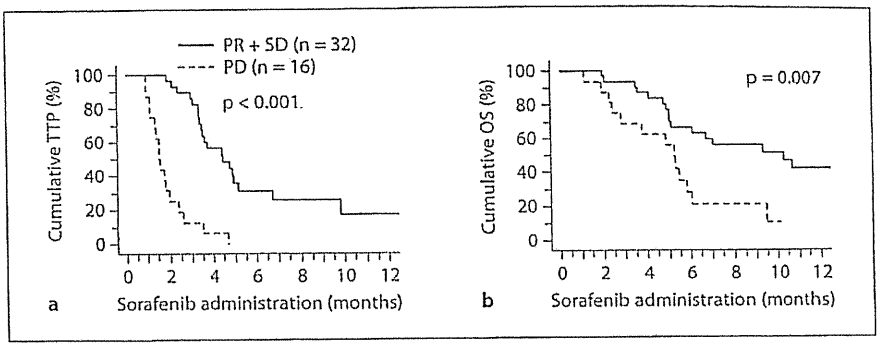

Fig. 1. Comparison of cumulative TTP (a) and OS (b) in the PR + SD and PD groups according to RECIST.

(n = 8); (b) body weight ≤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 ± 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 ± 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 ± 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-

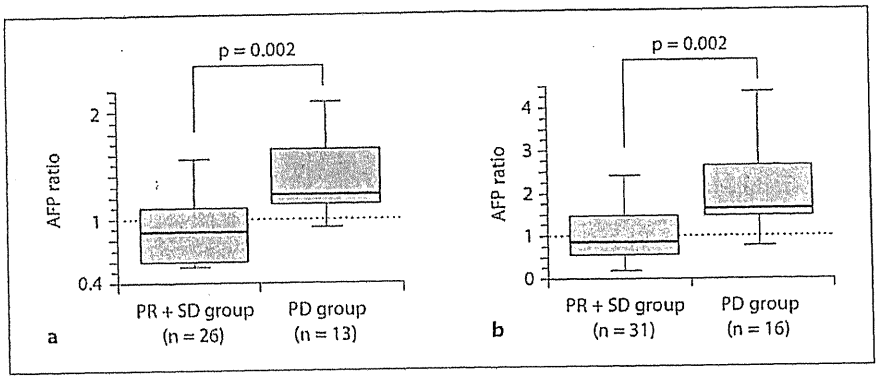

Fig. 2. AFP ratios 2 (a) and 4 weeks (b) after the start of sorafenib treatment in the PR + SD and PD groups according to RECIST.

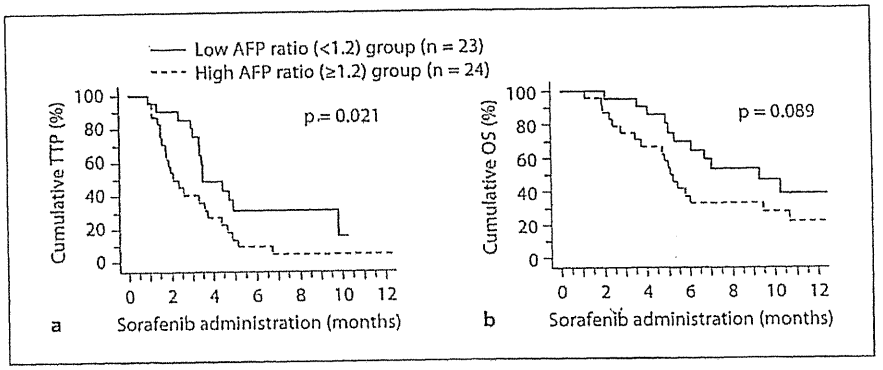

Fig. 3. Comparison of cumulative TTP (a) and OS (b) in the groups with low (<1.2) and high AFP ratio (≥1.2) 4 weeks after starting sorafenib therapy.

Table 2. Comparison of actual AFP levels (ng/ml) at baseline, and 2 and 4 weeks after the start of sorafenib therapy (stratified by antitumor 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 antitumor 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 (≥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

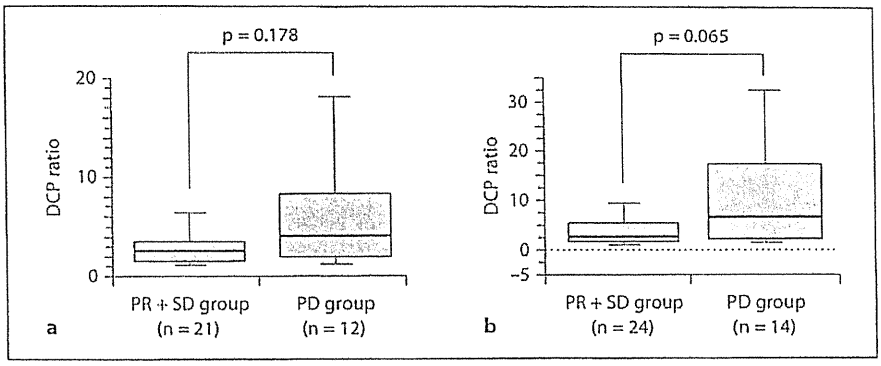

Fig. 4. DCP ratios 2 (a) and 4 weeks (b) after the start of sorafenib treatment in the PR + SD and PD groups according to RECIST.

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 (≥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 thera-

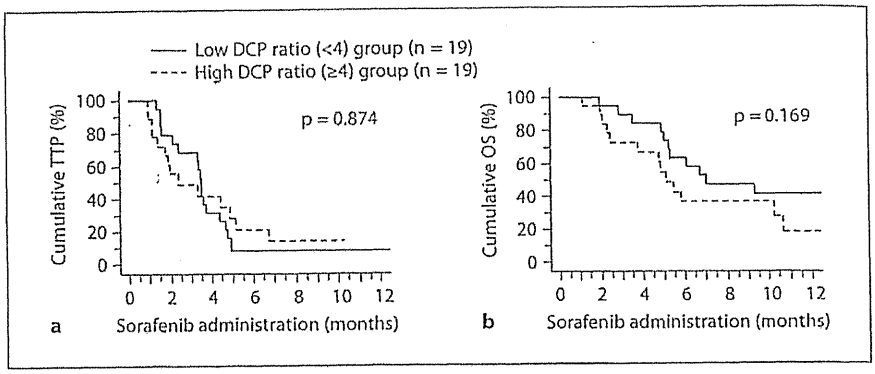

Fig. 5. Comparison of cumulative TTP (**a**) and OS (**b**) in the groups with low (<4) and high DCP ratio (≥4) 4 weeks after starting sorafenib therapy.

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

weeks after starting sorafenib administration in the PD group revealed that the median value after 4 weeks was significantly higher than that after 2 weeks. Even in the PR + SD group, the median value after 4 weeks was higher than that after 2 weeks. There were no significant differences between AFP levels after 2 and 4 weeks; thus, one of the reasons for this phenomenon was unevenness of AFP levels owing to the small sample size in this study.

With regard to DCP, there have been numerous reports that the time course change in DCP following treatment for HCC reflects therapeutic efficacy [17–19]. However, in the present study, we found that both the actual and relative levels of DCP were elevated in >90% of the patients, not only in the PD group but also in the PR + SD group, both 2 and 4 weeks after starting sorafenib therapy. To our knowledge, there have been no comprehensive clinical reports regarding the time course changes in DCP following sorafenib treatment. In a case report by Nakazawa et al. [27], DCP levels were markedly increased following treatment, even in patients who achieved a complete response on the basis of image analysis. From basic research, Murata et al. [28] reported that culturing a liver cancer cell line (HepG2) under hypoxic conditions resulted in increased DCP production by the cells. One possible mechanism for the increased DCP levels following sorafenib administration is that sorafenib-mediated inhibition of angiogenesis places tumor cells under hypoxic conditions, subsequently leading to increased DCP production. Thus, the increase in DCP levels following sorafenib administration may reflect HCC cell ischemia. Based on our results, increases in DCP soon after the start of sorafenib administration, regardless of antitumor effect, are not useful for assessing the antitumor responses, as DCP may increase in response to the ischemia caused by sorafenib.

Assessment by image analysis is the gold standard for evaluating antitumor responses of anticancer drugs [4, 22, 23]. However, such image analysis can be difficult in patients with multiple HCC lesions, vascular invasion, extrahepatic metastases, or ischemic tumors. In particular, patients in whom therapy using sorafenib is indicated are often in advanced stages of disease. There are limitations in using only radiological criteria to evaluate sorafenib treatment.

Our results suggest that the determination of early changes in AFP is useful for evaluating both antitumor response and prognostic efficacy of sorafenib, as assessed by TTP and OS, in patients with advanced HCC. In patients with advanced HCC treated with sorafenib, it is important to evaluate therapeutic efficacy as early as possible, as appropriate and early evaluation of sorafenib therapy can avoid unnecessary adverse events and allow second-line therapy when sorafenib therapy is not effective. In addition, determination of early changes in AFP is useful for evaluating the efficacy of new molecularly targeted agents currently under development. At present, there is no effective second-line treatment and we could not confirm whether continuing sorafenib administration would prolong the survival of patients with elevated AFP. Therefore, we cannot conclude that sorafenib therapy should be stopped in the case of elevated AFP ratio after 2 or 4 weeks of treatment. However, when an effective second-line treatment becomes available, an elevated AFP ratio may be a good indicator for switching to second-line therapy.

On the other hand, with regard to early changes in DCP, caution is required when assessing the antitumor response of sorafenib, as DCP elevation can occur irrespective of therapeutic effects.

In conclusion, our results suggest that early evaluation of AFP after starting sorafenib therapy is useful for predicting antitumor response. In contrast, early elevation of DCP does not necessarily suggest treatment failure of sorafenib. Appropriate and early evaluation of efficacy of sorafenib by AFP determination can provide valuable information that may influence subsequent decisions regarding patient management, thus avoiding unnecessary adverse events and allowing the opportunity for second-line therapy.

Acknowledgment

This study was supported by a grant-in-aid from the Ministry of Health, Labor and Welfare in Japan.

Disclosure Statement

The authors declare that they have no financial conflicts of interest.

References

1 Wilhelm SM, Carter C, Tang L, Wilkie D, McNabola A, Rong H, Chen C, Zhang X, Vincent P, McHugh M, Cao Y, Shujath J, Gawlak S, Eveleigh D, Rowley B, Liu L, Adnane L, Lynch M, Auclair D, Taylor I, Gedrich R, Voznesensky A, Riedl B, Post LE, Bollag G, Trail PA: BAY 43-9006 exhibits broad spectrum oral antitumor activity and targets the RAF/MEK/ERK pathway and receptor tyrosine kinases involved in tumor progression and angiogenesis. Cancer Res 2004;64:7099–7109.

2 Llovet JM, Ricci S, Mazzaferro V, Hilgard P, Gane E, Blanc JF, de Oliveira AC, Santoro A, Raoul JL, Forner A, Schwartz M, Porta C, Zeuzem S, Bolondi L, Greten TF, Galle PR, Seitz JF, Borbath I, Häussinger D, Giannaris T, Shan M, Moscovici M, Voliotis D, Bruix J, SHARP Investigators Study Group: Sorafenib in advanced hepatocellular carcinoma. N Engl J Med 2008;359:378–390.
3 Cheng AL, Kang YK, Chen Z, Tsao CJ, Qin S, Kim JS, Luo R, Feng J, Ye S, Yang TS, Xu J, Sun Y, Liang H, Liu J, Wang J, Tak WY, Pan H, Burock K, Zou J, Voliotis D, Guan Z: Efficacy and safety of sorafenib in patients in the Asia-Pacific region with advanced hepatocellular carcinoma: a phase III randomised, double-blind, placebo-controlled trial. Lancet Oncol 2009;10:25–34.
4 Llovet JM, Di Bisceglie AM, Bruix J, Kramer BS, Lencioni R, Zhu AX, Sherman M, Schwartz M, Lotze M, Talwalkar J, Gores GJ, Panel of Experts in HCC-Design Clinical Trials: Design and endpoints of clinical trials in hepatocellular carcinoma. J Natl Cancer Inst 2008;100:698–711.
5 Arii S, Sata M, Sakamoto M, Shimada M, Kumada T, Shiina S, Yamashita T, Kokudo N, Tanaka M, Takayama T, Kudo M: Management of hepatocellular carcinoma: report of Consensus Meeting in the 45th Annual Meeting of the Japan Society of Hepatology (2009). Hepatol Res 2010;40:667–685.
6 Kudo M, Ueshima K: Positioning of a molecular-targeted agent, sorafenib, in the treatment algorithm for hepatocellular carcinoma and implication of many complete remission cases in Japan. Oncology 2010;78:154–166.
7 Taketa K: Alpha-fetoprotein: reevaluation in hepatology. Hepatology 1990;12:1420–1432.
8 Oka H, Tamori A, Kuroki T, Kobayashi K, Yamamoto S: Prospective study of alpha-fetoprotein in cirrhotic patients monitored for development of hepatocellular carcinoma. Hepatology 1994;19:61–66.
9 Liebman HA, Furie BC, Tong MJ, Blanchard RA, Lo KJ, Lee SD, Coleman MS, Furie B: Des-gamma-carboxy (abnormal) prothrombin as a serum marker of primary hepatocellular carcinoma. N Engl J Med 1984;310:1427–1431.
10 Fujiyama S, Morishita T, Hashiguchi O, Sato T: Plasma abnormal prothrombin (des-gamma-carboxy prothrombin) as a marker of hepatocellular carcinoma. Cancer 1988;61:1621–1628.
11 Mita Y, Aoyagi Y, Yanagi M, Suda T, Suzuki Y, Asakura H: The usefulness of determining des-gamma-carboxy prothrombin by sensitive enzyme immunoassay in the early diagnosis of patients with hepatocellular carcinoma. Cancer 1998;82:1643–1648.
12 Toyoda H, Kumada T, Kaneoka Y, Osaki Y, Kimura T, Arimoto A, Oka H, Yamazaki O, Manabe T, Urano F, Chung H, Kudo M, Matsunaga T: Prognostic value of pretreatment levels of tumor markers for hepatocellular carcinoma on survival after curative treatment of patients with HCC. J Hepatol 2008;49:223–232.
13 Riaz A, Ryu RK, Kulik LM, Mulcahy MF, Lewandowski RJ, Minocha J, Ibrahim SM, Sato KT, Baker T, Miller FH, Newman S, Omary R, Abecassis M, Benson AB 3rd, Salem R: Alpha-fetoprotein response after locoregional therapy for hepatocellular carcinoma: oncologic marker of radiologic response, progression, and survival. J Clin Oncol 2009;27:5734–5742.
14 Chan SL, Mo FK, Johnson PJ, Hui EP, Ma BB, Ho WM, Lam KC, Chan AT, Mok TS, Yeo W: New utility of an old marker: serial alpha-fetoprotein measurement in predicting radiologic response and survival of patients with hepatocellular carcinoma undergoing systemic chemotherapy. J Clin Oncol 2009;27:446–452.
15 Kim BK, Ahn SH, Seong JS, Park JY, Kim do Y, Kim JK, Lee do Y, Lee KH, Han KH: Early α-fetoprotein response as a predictor for clinical outcome after localized concurrent chemoradiotherapy for advanced hepatocellular carcinoma. Liver Int 2011;31:369–376.
16 Vora SR, Zheng H, Stadler ZK, Fuchs CS, Zhu AX: Serum alpha-fetoprotein response as a surrogate for clinical outcome in patients receiving systemic therapy for advanced hepatocellular carcinoma. Oncologist 2009;14:717–725.
17 Shimada M, Takenaka K, Fujiwara Y, Gion T, Kajiyama K, Maeda T, Shirabe K, Sugimachi K: Des-gamma-carboxy prothrombin and alpha-fetoprotein positive status as a new prognostic indicator after hepatic resection for hepatocellular carcinoma. Cancer 1996;78:2094–2100.
18 Koike Y, Shiratori Y, Sato S, Obi S, Teratani T, Imamura M, Yoshida H, Shiina S, Omata M: Des-gamma-carboxy prothrombin as a useful predisposing factor for the development of portal venous invasion in patients with hepatocellular carcinoma: a prospective analysis of 227 patients. Cancer 2001;91:561–569.
19 Iwadou S, Nouso K, Kuwaki K, Kobayashi Y, Nakamura S, Tanaka H, Miyoshi K, Ohnishi H, Miyake Y, Shiraha H, Iwasaki Y, Shiratori Y, Yamamoto K: Time-dependent analysis of predisposing factors for the recurrence of hepatocellular carcinoma. Liver Int 2010;30:1027–1032.
20 Bruix J, Sherman M: American Association for the Study of Liver Diseases. Management of hepatocellular carcinoma: an update. Hepatology 2011;53:1020–1022.
21 Liver Cancer Study Group of Japan: General Rules for the Clinical and Pathological Study of Primary Liver Cancer, ed 2. Tokyo, Kanehara, 2003.
22 Eisenhauer EA, Therasse P, Bogaerts J, Schwartz LH, Sargent D, Ford R, Dancey J, Arbuck S, Gwyther S, Mooney M, Rubinstein L, Shankar L, Dodd L, Kaplan R, Lacombe D, Verweij J: New response evaluation criteria in solid tumours: revised RECIST guideline (version 1.1). Eur J Cancer 2009;45:228–247.
23 Furuse J, Ishii H, Nakachi K, Suzuki E, Shimizu S, Nakajima K: Phase I study of sorafenib in Japanese patients with hepatocellular carcinoma. Cancer Sci 2008;99:159–165.
24 Prete SD, Montella L, Caraglia M, Maiorino L, Cennamo G, Montesarchio V, Piai G, Febbraro A, Tarantino L, Capasso E, Palmieri G, Guarrasi R, Bianco M, Mamone R, Savastano C, Pisano A, Vincenzi B, Sabia A, D'Agostino A, Faiola V, Addeo R: Sorafenib plus octreotide is an effective and safe treatment in advanced hepatocellular carcinoma: multicenter phase II So.LAR. study. Cancer Chemother Pharmacol 2010;66:837–844.
25 Lencioni R, Llovet JM: Modified RECIST (mRECIST) assessment for hepatocellular carcinoma. Semin Liver Dis 2010;30:52–60.
26 Shao YY, Lin ZZ, Hsu C, Shen YC, Hsu CH, Cheng AL: Early alpha-fetoprotein response predicts treatment efficacy of antiangiogenic systemic therapy in patients with advanced hepatocellular carcinoma. Cancer 2010;116:4590–4596.
27 Nakazawa T, Hidaka H, Shibuya A, Koizumi W: Rapid regression of advanced hepatocellular carcinoma associated with elevation of des-gamma-carboxy prothrombin after short-term treatment with sorafenib – a report of two cases. Case Rep Oncol 2010;3:298–303.
28 Murata K, Suzuki H, Okano H, Oyamada T, Yasuda Y, Sakamoto A: Cytoskeletal changes during epithelial-to-fibroblastoid conversion as a crucial mechanism of des-gamma-carboxy prothrombin production in hepatocellular carcinoma. Int J Oncol 2009;35:1005–1014.

Oncology

Oncology 2011;81(suppl 1):11–17
DOI: 10.1159/000333253

Published online: December 22, 2011

Hepatocarcinogenesis in Hepatitis C: HCV Shrewdly Exacerbates Oxidative Stress by Modulating both Production and Scavenging of Reactive Oxygen Species

Hidetake Fujinaga Takeya Tsutsumi Hiroshi Yotsuyanagi Kyoji Moriya
Kazuhiko Koike

Department of Internal Medicine, Graduate School of Medicine, The University of Tokyo, Tokyo, Japan

Key Words
Hepatitis C · Hepatocellular carcinoma · Oxidative stress · Transgenic mouse · Core protein

Abstract
Persistent infection with hepatitis C virus (HCV) is a major risk for the development of hepatocellular carcinoma (HCC). One of the characteristics of HCV infection is the unusual augmentation of oxidative stress, which is exacerbated by iron accumulation in the liver, as observed frequently in hepatitis C patients. Using a transgenic mouse model, in which HCC develops late in life after the preneoplastic steatosis stage, the core protein of HCV was shown to induce the overproduction of reactive oxygen species (ROS) in the liver. In excessive generation of ROS, HCV affects the steady-state levels of a mitochondrial protein chaperone, i.e. prohibitin, leading to an impaired function of the mitochondrial respiratory chain with the overproduction of ROS. Insulin resistance and hepatic steatosis, which frequently accompany HCV infection, exacerbate ROS production. On the other hand, HCV compromises some of the antioxidant systems, including heme oxygenase-1 and NADH dehydrogenase quinone 1, resulting in the provocation of oxidative stress, together with ROS overproduction, in the liver with HCV infection. Thus, HCV infection not only induces ROS but also hampers the antioxidant system in the liver, thereby exacerbating oxidative stress that would facilitate hepatocarcinogenesis. Combination with the other activated pathway, including an alteration in the intracellular signaling cascade of MAP kinase, along with HCV-associated disturbances in lipid and glucose metabolism would lead to the unusual mode of hepatocarcinogenesis, i.e. very frequent and multicentric development of HCC, in persistent HCV infection.

Introduction

Approximately 200 million people are infected with hepatitis C virus (HCV) worldwide. More than two thirds of those with acute HCV infection suffer from persistent infection causing active or inactive chronic hepatitis, and approximately 30% of patients with chronic hepatitis are assumed to develop cirrhosis within their lifetime. Once HCV infection develops into cirrhosis, hepatocellular carcinoma (HCC) develops at an annual rate of 7% [1]. The strong association of oxidative stress with HCV infection has been demonstrated and can explain at least part of the clinical progression of the disease. The patho-

KARGER
Fax +41 61 306 12 34
E-Mail karger@karger.ch
www.karger.com

0030–2414/11/0817–0011$38.00/0

Accessible online at:
www.karger.com/ocl

Kazuhiko Koike, MD, PhD
Department of Gastroenterology, Graduate School of Medicine
The University of Tokyo
7-3-1 Hongo, Bunkyo-ku, Tokyo 113-8655 (Japan)
Tel. +81 3 5800 8800, E-Mail kkoike-tky@umin.ac.jp

genesis of chronic hepatitis C is not merely ascribed to inflammation caused by viral infection; the role of viral proteins in the pathogenesis has also been reported [2]. Of the proteins constituting HCV, the core protein in particular has various functions with respect to host cells and is closely related to oxidative stress. In this article, the relationship between HCV infection and oxidative stress is analyzed focusing on the pathological effect of the core protein of HCV, and the significance of oxidative stress in the pathogenesis of liver disease is discussed.

HCV Infection and Hepatocarcinogenesis

The mechanism underlying hepatocarcinogenesis in HCV infection is not fully understood yet. Inflammation induced by an immune response to HCV should be considered, of course, in a study on hepatocarcinogenesis in hepatitis viral infection: necrosis of hepatocytes due to chronic inflammation followed by regeneration enhances genetic aberrations in host cells, the accumulation of which culminates in HCC. This theory presupposes an indirect involvement of hepatitis viruses in HCC via hepatic inflammation. However, this context leaves us with a serious question: can inflammation alone result in the development of HCC in HCV infection with such a high incidence (90% in 15 years) or in a multicentric fashion? The other role of HCV would have to be weighed against a rare occurrence of HCC, even after the development of cirrhosis, in patients with autoimmune hepatitis in which severe inflammation in the liver persists. These backgrounds and reasonings lead to a possible activity of viral proteins for inducing neoplasia. This possibility has been evaluated by introducing genes of HCV into hepatocytes in culture with little success. One of the difficulties in using cultured cells is the carcinogenic capacity of HCV, if any, which would be weak and would take a long time to manifest itself. Actually, it takes 30–40 years for HCC to develop in individuals infected with HCV. On the basis of these viewpoints, we started to investigate carcinogenesis in chronic hepatitis C in vivo using transgenic mouse technology.

Transgenic Mouse Model for HCV-Related HCC

One of the major issues regarding the pathogenesis of HCV-associated liver lesions is whether the HCV proteins have direct effects on pathological phenotypes. For this purpose, several lines of mice have been established which are transgenic for the HCV cDNA. We have engineered transgenic mouse lines carrying the HCV genome by introducing the genes from the cDNA of the HCV genome of genotype 1b [3, 4]. Four different kinds of transgenic mouse lines are established, and they carry the core gene, envelope genes, the entire nonstructural (NS) genes, or the NS5A gene, respectively, under the same transcriptional regulatory element. Among these mouse lines, only the transgenic mice carrying the core gene developed HCC in two independent lineages [4]. The envelope gene transgenic mice did not develop HCC despite high expression levels of both E1 and E2 proteins [5], and the transgenic mice carrying the entire NS or NS5A gene developed no HCC.

Early in life, core gene transgenic mice develop hepatic steatosis, which is one of the histologic characteristics of chronic hepatitis C, along with lymphoid follicle formation and bile duct damages [6]. Thus, the core gene transgenic mouse model well reproduces the feature of chronic hepatitis C. It is important to note that no significant inflammation is observed in the liver of this animal model. Late in life, these transgenic mice develop HCC. Notably, the development of steatosis and HCC has been reproduced by other HCV transgenic mouse lines, which harbor the structural genes including the core gene [4, 7, 8]. These outcomes indicate that the core protein per se of HCV has an oncogenic potential when expressed in vivo.

Augmentation of Oxidative Stress in Hepatitis C

There is a notable feature in the localization of the core protein in hepatocytes; while the core protein predominantly exists in the cytoplasm associated with lipid droplets, it is also present in the mitochondria and nuclei [4]. On the basis of this finding, the pathways related to these two organelles, the mitochondria and nuclei, were thoroughly investigated.

One effect of the core protein is an increased production of oxidative stress in the liver. We would like to draw particular attention to the fact that the production of oxidative stress is increased in the core gene transgenic mouse model in the absence of inflammation in the liver [4]. The overproduction of oxidative stress results in the generation of deletions in the mitochondrial and nuclear DNA, an indicator of genetic damage [2].

Augmentation of oxidative stress is implicated in the pathogenesis of liver disease in HCV infection as shown by a number of clinical and basic studies [2, 9]. Reactive

oxygen species (ROS) are endogenous oxygen-containing molecules formed as normal products during aerobic metabolism. ROS can induce genetic mutations as well as chromosomal alterations and thus contribute to cancer development in multistep carcinogenesis [10, 11]. Recent studies have shown that oxidative stress is more augmented in hepatitis C than in other types of hepatitis such as hepatitis B [9].

Thus, a major role in the pathogenesis of HCV-associated liver disease has been attributed to oxidative stress augmentation, but little is known regarding the mechanism of increased oxidative stress in HCV infection. Hence, it is important to understand the mechanism of oxidative stress augmentation, in terms of both generation and scavenging of ROS, which may allow us to develop new tools of therapies for chronic hepatitis C.

Oxidative Stress and the Liver

Oxidative Stress and Reactive Oxygen

The main source of ROS in hepatocytes is the mitochondria. Outside of hepatocytes, ROS also originate from nicotinamide adenine dinucleotide phosphate (NADPH) oxidase and xanthine oxidase in Kupffer cells and inflammatory cells. A large percentage of consumed oxygen is constantly converted into ROS in the mitochondria accompanied by oxygen consumption in the electron transport system (ETS). Hepatocytes contain many mitochondria and therefore have a high ROS production. Generated ROS are very unstable and highly reactive and attack biomolecules such as DNA, lipids, and proteins. The liver not only produces much ROS but is also the center of the antioxidative effect in the form of protein synthesis. Oxidative stress refers to the oxidation-reaction-dominant state of the living body induced by an imbalance between the oxidation reaction caused by ROS and the antioxidation reaction. Main ROS include superoxide ($\cdot O_2^-$), hydrogen peroxide (H_2O_2), and the hydroxyl radical (HO$\cdot$). ROS are mainly produced from $\cdot O_2^-$ and converted into stable H_2O_2 through a dismutation reaction. H_2O_2 is converted into highly reactive HO$\cdot$ in the presence of a transition metal.

The Antioxidant System and Oxidative Stress Markers

Antioxidants include glutathione (GSH), thioredoxin (TRX), vitamin E, vitamin C, and β-carotene. Reactive oxygen elimination enzymes include superoxide dismutase (SOD), GSH peroxidase, heme oxygenase (HO)-1, and catalase. SOD is induced by oxidative stress and dismutates $\cdot O_2^-$ to H_2O_2 and oxygen. Catalase in peroxisomes also decomposes H_2O_2 to water and oxygen. TRX is also a protein induced by oxidative stress and is reduced via S-S binding of the substrate protein by two SH groups in TRX and acts on the H_2O_2 elimination system via peroxiredoxins. HO-1 is an inducible cytoprotective enzyme that catalyzes the initial and rate-limiting reaction in heme catabolism and cleaves prooxidant heme to form biliverdin with the release of carbon monoxide. Biliverdin is converted into bilirubin in mammals; both of these have been known to have very strong antioxidant activities.

ROS cause various forms of cellular damage. 4-hydroxy-2-nonenal (HNE) and malondialdehyde (MDA) are the peroxidation reaction products of lipids, and 8-hydroxydeoxyguanosine (8-OHdG) is the product of DNA base modification. These products serve as oxidative stress markers.

The Origin of ROS Production in HCV Infection

Then, where is the place for oxidative stress overproduction in the liver of hepatitis C patients? The core protein is mostly localized to the endoplasmic reticulum, but we and other groups have shown its localization to the mitochondria in cultured cells and transgenic mice [12]. In addition, the double structure of mitochondrial membranes is disrupted in hepatocytes of core gene transgenic mice. Evidence suggests that the core protein modulates some mitochondrial functions, including fatty acid β-oxidation, the impairment of which may induce lipid abnormalities and hepatic steatosis. In addition, the mitochondrion is an important source of ROS. In livers of transgenic mice harboring the core gene, increased ROS production has been observed [2]. A recent study found, via proteomic profiling of biopsy specimens, that impairment of key mitochondrial processes including fatty acid oxidation and oxidative phosphorylation and of the response to oxidative stress occurs in HCV-infected human liver with advanced fibrosis [13]. Therefore, it is probable that the HCV core protein affects mitochondrial functions since such pathogenesis is observed in both HCV core-transgenic mice and HCV-infected patients.

The recent progress in proteomics has opened new avenues for disease-related biomarker discovery. We performed a two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) of mitochondria isolated from HepG2 cells stably expressing the HCV core protein and