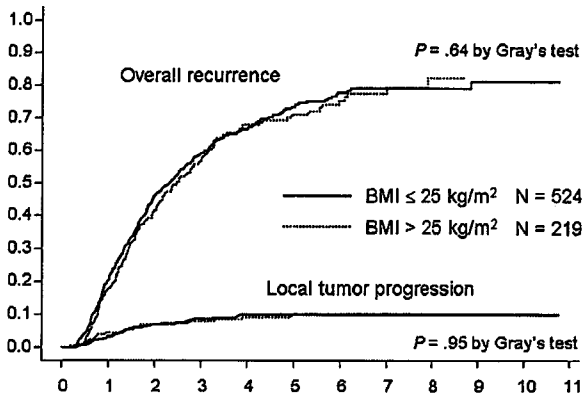

Fig. 2. Cumulative probability of survival was 95.4%, 87.2%, 75.7%, 64.0%, and 57.8% in the body mass index (BMI) $\leq 25\,kg/m^2$ group and 94.1%, 86.6%, 78.0%, 66.5%, and 58.8% in the $25\,kg/m^2 <$ BMI group at 1, 2, 3, 4, and 5 years, respectively.

Table 6. Multivariate analysis of survival

Variable	Hazard ratio (95%CI)	*P* Value
BMI > $25\,kg/m^2$	0.91 (0.72–1.16)	0.45
Age > 68	1.60 (1.27–2.01)	< 0.001
Platelet count > $100 \times 10^3/mm^3$	0.77 (0.60–0.99)	0.04
Child B/C	2.32 (1.81–2.97)	< 0.001
Tumor size > 3.0 cm	1.41 (1.10–1.82)	0.007
Multinodular	1.33 (1.06–1.67)	0.01
AFP > 400 ng/ml	1.93 (1.37–2.74)	< 0.001
DCP > 100 mAU/ml	1.74 (1.33–2.30)	< 0.001

AFP, α-fetoprotein; BMI, body mass index; DCP, des-γ-carboxyprothrombin; HCV, hepatitis C virus.

reported that in the age group of 66–69 years, 2.3% men and 4.4% women were BMI > $30\,kg/m^2$ and 30.1% men and 33.3% women were BMI > $25\,kg/m^2$ (31). These distributions were not different from those found in our study population ($P = 0.66$, 0.75, 0.75, and 0.76, respectively). Thus, the scarcity of obese patients in the current study was not exceptional and the proportion of these patients was actually larger than that in the Japanese general population, although the difference was not statistically significant.

In this study, we found no significant difference in cumulative HCC recurrence between the overweight group and control group ($P = 0.64$). Intrahepatic recurrence of HCC was considered to consist of two distinct types: multicentric carcinogenesis and intrahepatic metastasis (32). Theoretically, the former is dependent on factors responsible for HCC development such as advanced liver fibrosis, while the latter is dependent on the factors related to the primary tumor such as the size and number of tumor and the presence of vascular invasion. Obesity may enhance the risk of multicentric carcinogenesis through increased oxidative stress or may hinder the detection of intrahepatic metastatic lesions. Although our study does not exclude these possibilities, the effects of obesity on HCC recurrence were not as strong as the effects of the other factors listed in Table 4. Prospective studies on primary HCC development are required to investigate the effects of obesity on hepatocarcinogenesis.

Obesity is known to be a definite risk factor for cardiovascular diseases (33) and overall death (34). In this study, 223 patients had died during the observation period and the major causes of death were liver-related ones: HCC progression, hepatic failure, and upper gastro-intestinal bleeding were responsible for 193 (86.5%) cases. On the other hand, only 30 patients died of liver-unrelated causes. Even if some of them were related to obesity, the difference of survival between the overweight and control groups would have been obscured by the similarity of liver-related mortality, which constituted the majority of causes of death.

In conclusion, we have shown that overweight does not compromise the safety and efficacy of percutaneous ablation therapy for HCC. However, the number of RFA sessions was larger in the overweight group than in the control group, indicating surmountable technical difficulties in the former group. The larger number of ablation sessions potentially increases the risk of complications. We reported similar cases in ablating HCC nodules in high-risk locations (35). Caution should be exercised in performing ablation on such patients and expertise may be required.

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HEPATOLOGY

Recurrent hepatocellular carcinoma has an increased risk of subsequent recurrence after curative treatment

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hazard ratio, hepatocellular carcinoma, percutaneous ethanol injection, radiofrequency ablation, recurrence, risk factors.

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Abstract

Background and Aim: Local ablation therapy has been shown to be effective for small hepatocellular carcinoma (HCC); however, HCC recurrence is very frequent even after apparently curative treatment. In particular, recurrent HCC may be more prone to subsequent recurrence, although quantitative data are lacking. The aim of this study was to evaluate the difference in the risk for subsequent recurrence, if any, between primary and recurrent cases.

Methods: A retrospective analysis was conducted of 376 patients with HCC (uninodular and ≤5 cm, or 2–3 nodules each ≤3 cm) who underwent local ablation therapy. There were 207 primary cases (group I), 100 with first recurrence (group II), and 69 with second or later recurrence (group III). After confirming complete ablation, each patient was followed up for recurrence. Risk factors for recurrence-free survival were analyzed using proportional hazard regression.

Results: The median time to recurrence, as estimated by Kaplan–Meier method, was 30 months in group I, 23 months in group II, and 11 months in group III ($P < 0.001$). Multivariate proportional hazard regression analysis reveled that group (i.e. previous recurrence) was the strongest predictor of subsequent recurrence; compared to group I, group II showed a hazard ratio of 1.456 ($P = 0.015$) and group III, 3.011 ($P < 0.0001$). α-Fetoprotein level >100 ng/mL, treatment other than radiofrequency ablation, HCV antibody positivity, and tumor multinodularity also remained as significant predictors.

Conclusion: Hepatocellular carcinoma at second or later recurrence is three times as prone to subsequent recurrence as is primary HCC, when compared with adjustment for other tumor and hepatic factors.

Introduction

Hepatocellular carcinoma (HCC) is one of the most common malignancies worldwide, the incidence of which is still increasing in various regions.[1,2] With advances in imaging diagnostics, together with the understanding of high-risk patients, the cancer can be often detected at an early stage now.[2–4] Surgical resection has been regarded as the best curative treatment for HCC in early stages, with a 5-year survival rate of about 70%.[5,6] However, even after successful surgical resection, intrahepatic recurrence of HCC is as frequent as 80% in 5 years.[6–9] Moreover, surgery is often contraindicated by impaired liver function as HCC is prone to develop in cirrhotic patients. Thus, liver transplantation may be the preferred treatment for HCC, where recurrence rate is low, if the extension of primary tumor is limited within the Milan criteria (i.e. uninodular and ≤5 cm, or 2–3 nodules each ≤3 cm).[10–16] However, the availability of liver transplantation is seriously hindered by the scarcity of donor organs.

Emerging less-invasive modalities such as percutaneous ethanol injection (PEI) and radiofrequency ablation (RFA) are now considered to be effective treatments for small, localized HCC.[17–19] An advantage of ablation over surgical resection is the fact that it can be safely performed in patients with moderately impaired liver function. Subsequent local tumor progression may be a possible disadvantage but that can be minimized by careful RFA allowing a sufficient safety margin.[17] However, as in the case of surgical resection, intrahepatic recurrence does occur frequently in distant locations.

We have treated more than 2780 HCC cases by medical ablation therapy; 1230 with ethanol and 1374 with radiofrequency.[20] Although intrahepatic recurrences were not infrequent, they were usually found at a re-treatable stage as the patients were on a strict follow-up plan with regular imaging studies. We treated naïve and recurrent lesions similarly, repeating ablation until complete necrosis was confirmed in each lesion on contrast-enhanced computed tomography (CT), and the success rate did not differ.

Table 1 Baseline characteristics of patients

Factor	All patients ($n = 376$)	Group I ($n = 207$)	Group II ($n = 100$)	Group III ($n = 69$)	*P*-value
Age (years)	67 ± 8	67 ± 8	66 ± 9	67 ± 8	0.83
Sex (male/female)	245/131	125/82	67/33	53/16	0.042
HBsAg positive	43 (11%)	24 (11%)	11 (11%)	8 (11%)	0.987
HCV Ab positive	307 (81%)	164 (79%)	80 (80%)	63 (91%)	0.071
Child–Pugh class (A / B+C)	238/138	156/51	52/48	30/39	<0.0001
Serum albumin (mg/dL)	3.6 ± 0.5	3.6 ± 0.4	3.6 ± 0.5	3.5 ± 0.5	0.097
AST (IU/mL)	68 ± 37	67 ± 40	70 ± 35	71 ± 30	0.670
ALT (IU/mL)	63 ± 41	60 ± 42	66 ± 40	65 ± 39	0.466
AFP (ng/mL)	19 (1–16 709)	20 (1–16 709)	19 (2–878)	18.5 (2–3428)	0.153
AFP level (≥100 / <100 ng/mL)	71/301	44/163	15/82	12/56	0.462
DCP (mAU/mL)	18 (1–15 654)	19 (10–15 654)	18 (10–639)	21 (10–376)	0.527
Maximum tumor size (cm)	2.2 ± 0.7	2.3 ± 0.7	2.3 ± 0.8	2.0 ± 0.6	0.005
Number of tumors (1/2/3)	239/96/41	148/42/17	60/33/7	31/21/17	<0.0001
Time from initial diagnosis (months)	–	–	28.8 (4.3–96.6)	51.1 (8.8–142.0)	<0.001
Size of primary HCC (cm)	–	–	2.6 ± 1.4	2.5 ± 1.0	0.456
Number of primary HCC (1/2/3/4)			60/24/11/5	43/11/9/6	0.509
Treatment modality (RFA/other[†])	276/100	157/50	75/25	44/25	0.132

AFP, α-fetoprotein; ALT, alanine aminotransferase; AST, aspartate aminotransferase; DCP, des-γ-carboxy prothrombin; HBsAg, hepatitis B surface antigen; HCV Ab, hepatitis C virus antibody; IU/mL, international unit/mL; mAU, micro arbitrary unit; NS, not significant; NA, not applicable; RFA, radiofrequency ablation. [†]Other modalities included percutaneous ethanol injection or microwave coagulation therapy.
Values shown as mean ± SD, *n*/*n*, *n* (%) or median (range).

However, recurrence-free survival time appeared to be diminished after the treatment of recurrent lesions compared to primary ablation. In fact, diminishing disease-free survival was noted after the re-resection of recurrent HCC.[21–23] However, some patients with recurrent HCC become inappropriate for re-surgery because of bilobular multiple recurrence or deterioration in liver function. Those patients who can receive re-resection constitute only a small proportion of the total. Indication criteria are less strict for ablation therapy in terms of both lesion multiplicity and liver function. Thus, the analysis of HCC recurrence may be less biased when performed among patients who received ablation therapy.

The points of clinical interests will include whether and how much the risk of recurrence differs between primary and recurrent cases after controlling for other possible risk factors, such as tumor size or number. We sought to answer these questions by analyzing recurrence-free survival with a proportional hazard regression model.

Methods

Subjects

Between January 1999 and December 2001, 461 consecutive patients underwent local ablation therapy for HCC at Department of Gastroenterology, the University of Tokyo Hospital. Among them, patients with primary or recurrent small HCC meeting the Milan criteria, and no extrahepatic nodules or vascular invasion, were considered for inclusion. The diagnosis of HCC was based on histopathological confirmation and/or characteristic imaging features by dynamic-enhanced CT or magnetic resonance imaging (MRI) as described previously.[17,20] A total of 207 patients with primary HCC and 169 patients with recurrent HCC meeting the inclusion criteria were enrolled.

All patients with recurrent HCC had a history of curative surgical resection or local ablation therapy and those with a previous history of palliative treatment for HCC, for example hepatic arterial chemoembolization alone, were excluded. In 147 patients with recurrence, the original treatment for primary HCC had been local ablation at our program, mostly with PEI, with subsequent confirmation of total tumor necrosis performed before the observation period of this study. The remaining 22 patients had received surgical resection of primary HCC at other programs, and their surgical and pathological reports confirmed curative resection of primary HCC. There were 100 patients with the first recurrence after the initial treatment for HCC, 30 with second recurrence, 22 with third recurrence, and 17 patients with forth recurrence. To simplify the analysis, patients were grouped according to the number of prior treatments for HCC: group I ($n = 207$), primary cases; group II ($n = 100$), the first recurrence after curative primary therapy; and group III ($n = 69$), the second or later recurrence.

Baseline characteristics of patients are shown in Table 1. Each patient was enrolled into this analysis only once. In other words, only the first treatment episode during the study period was analyzed in each patient.

Local ablation therapy technique and follow-up

Before 1999, we used PEI or microwave coagulation therapy for local ablation of HCC. We started using RFA when the modality became available in Japan in 1999. Each ablation therapy was performed as described elsewhere.[20] The subjects of this study received one of these modalities, some as a randomized assignment.[17] We confirmed complete ablation of all visualized HCC nodules by contrast-enhanced CT or MRI after the last course of ablation protocol. After the treatment, each patient was followed up with blood tests for liver function and HCC biomarkers every 1–2 months, and abdominal ultrasound and contrast-enhanced CT

or MRI every 4 months. Recurrence of HCC was assessed radiographically. Both local tumor progression and extrahepatic metastasis were also considered as events.

Statistical analysis

Data are presented as mean ± SD for quantitative variables, unless otherwise specified. Differences between groups were analyzed by one-way analysis of variance (ANOVA) or Kruskall–Wallis test, considering the normality of distribution, for numerical variables and chi-squared test for categorical variables. Significances of individual differences were evaluated using Tukey's test if ANOVA was significant. Time to recurrence was estimated by Kaplan–Meier method and depicted graphically. Differences between groups were estimated by log-rank test. All patients were followed from the date of treatment until death or until 31 December 2004.

The Cox proportional hazard model was used to evaluate risk factors for recurrence including age, sex, tumor number and size, hepatitis virus seropositivity, tumor biomarkers, treatment modality, Child–Pugh classification, and the number of prior treatments. Tumor size and number at the initial presentation was also included for recurrence cases (group II or III). Time from the initial treatment was calculated for recurrence cases (group II or III), and was set as zero in primary cases (group I). The hazard ratio was calculated for group II (first recurrence of HCC) and group III (second or later recurrence of HCC) using group I (primary HCC) as the reference. Risk factors were analyzed in all patients and each subgroup. Those variables that showed *P*-value <0.1 in univariate analysis and those that had been reported as risk factors for recurrence, that is tumor size, tumor number, age, and sex, were entered into multivariate analysis. A factor with *P*-value <0.05 was considered as significant.

Results

Background characteristics and survival after treatment

The baseline characteristics of patients in each group are shown in Table 1. Patient age was similar in each group. The proportion of men was larger in group III than in group I. Patients positive for HCV antibody were more frequent in group III than in group I and II. The proportion of Child–Pugh B or C increased in order of the number of recurrences.

The median (range) follow-up period was 41 (6–71) months. During the period, 128 patients died. The causes of death were HCC in 73, liver failure in 31, varices rupture in 3, and liver-unrelated diseases in 21. The 1-, 3- and 5-year cumulative survival rates among overall patients were estimated by Kaplan–Meier analysis as 96.8%, 75.6% and 52.3%, respectively. Similarly, the 1- and 3-year survival rates were 96.6%, 82.5%, in group I, 96.0% and 74.6% in group II, and 98.5% and 55.9% in group III (overall $P < 0.0001$; group I *vs* II $P = 0.003$; group I *vs* III $P < 0.001$; group II *vs* III $P = 0.014$).

Probability of subsequent recurrence

Recurrence of HCC was diagnosed in 267 patients during follow-up. The cumulative incidence of recurrence was estimated by Kaplan–Meier method and depicted as 1 – Kaplan–Meier curve

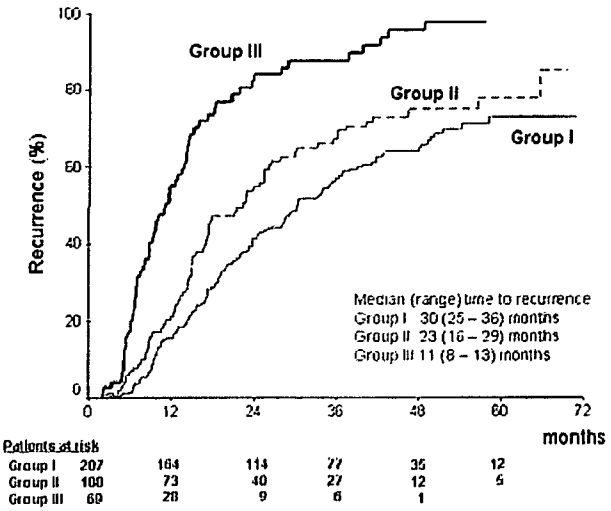

Figure 1 Probability of subsequent recurrence after local ablation therapy for hepatocellular carcinoma estimated by 1 – curve Kaplan–Meier analysis: (—) group I, primary HCC; (- - -) group II, first recurrence; and (—) group III, second or later recurrence. Probability of recurrence was significantly different between each pair of groups (group I *vs* II, $P = 0.025$; group I *vs* III, $P < 0.001$; and group II *vs* III, $P < 0.001$) and overall ($P < 0.0001$).

(Fig. 1). The probability of subsequent recurrence was higher when the patients had previous history of HCC treatment. Because the curve was similar between patients with second and later HCC recurrence, these patients were combined as group III, as defined in Methods section. Recurrence occurred in 132 patients (63%) in group I, 72 (72%) in group II, and 63 (91%) in group III during the observation period. The median (95% CI) time to recurrence was 30 (25–36) months in group I, 23 (16–29) months in group II, and 11 (8–13) months in group III (overall $P < 0.0001$; group I *vs* II $P = 0.025$; group I *vs* III $P < 0.001$; group II *vs* III $P < 0.001$). The 1- and 3-year cumulative recurrence rates were 15.6% and 56.1% in group I, 21.2% and 67.0% in group II, and 55.0% and 87.7% in group III.

Pattern of subsequent recurrence

The pattern of subsequent recurrence in 267 patients is summarized in Table 2. The mean size of recurrent HCC was 2.1 ± 0.9 cm in diameter. The recurrent HCC was uninodular and not larger than 5 cm in 118 (44.2%) and it was 2–3 nodules not larger than 3 cm in 85 (31.8%). Taken together, recurrence met the Milan criteria in 203 cases (76.0%). However, 40 (15.0%) failed the criteria due to more than three nodules, and 21 (7.9%) due to size. Extrahepatic metastasis and vascular or biliary invasion were relatively rare events. The frequency distribution of the patterns of HCC recurrence did not differ among the three groups based on the number of previous recurrences.

Risk factors for recurrence

Univariate analysis of risk factors for recurrence subsequent to the treatment was performed in each group using Cox proportional

Table 2 Patterns of recurrence at the time of subsequent recurrence

Factor	Group I ($n = 207$)	Group II ($n = 100$)	Group III ($n = 69$)
No. with recurrence	132	72	63
Time to recurrence (months median (95% CI)*	30 (25–36)	23 (16–29)	11 (8–13)
Tumor size, cm (mean ± SD)§	2.1 ± 1.0	2.3 ± 0.8	2.0 ± 1.1
Tumor number, intrahepatic (1/2/3/≥4)§	67/28/13/22	30/23/8/10	28/18/8/9
Within Milan criteria† (n (%))§	97 (73%)	57 (79%)	49 (77%)
Single, ≤5 cm	63	30	25
2–3 nodules, ≤3 cm	34	27	24
Outside Milan criteria			
Single, >5 cm	4	0	3
2–3 nodules, >3 cm	7	4	3
≥4 nodules	22	10	8
Extrahepatic lesion only	2	1	0
Extrahepatic + intrahepatic	0	0	3
Vascular or bile duct invasion	3	0	1
Location of extrahepatic recurrence			
Seeding	1	1	0
Lymph node	0	0	1
Lung	1	0	1
Bone	0	0	1

*$P < 0.0001$ by log rank test; §difference not significant. †Milan criteria: single nodule ≤5 cm or ≤3 nodules all ≤3 cm, no extrahepatic metastases, no vascular invasion. Group I, primary hepatocellular carcinoma; Group II, first recurrence; Group III, second or later recurrence.

hazard model. In group I, α-fetoprotein (AFP) level >100 ng/mL (hazard ratio [HR] = 1.572, $P = 0.024$), tumor size (HR = 1.022, $P = 0.048$), and multinodularity (HR = 1.367, $P = 0.095$) revealed a P-value less than 0.1. Similarly in group II, HCV antibody positivity (HR = 3.357, $P = 0.001$), AFP > 100 ng/mL (HR = 2.196, $P = 0.008$), multinodularity (HR = 1.994, $P = 0.003$), and treatment other than RFA (HR = 0.478, $P = 0.004$) had P-value less than 0.1. In group III, AFP > 100 ng/mL (HR = 1.917, $P = 0.065$) was the only factor with P-value less than 0.1. Time from the initial treatment and number and size of the initial HCC did not significantly affect subsequent recurrence in groups II and III. Other factors were not significantly related to recurrence.

Multivariate analysis of risk factors for recurrence subsequent to the treatment was performed in each group using the Cox proportional hazard model (Table 3). In group I, tumor size (HR = 1.030, 95% CI: 1.006–1.054, $P = 0.014$), AFP > 100 ng/mL (HR = 1.566, 1.031–2.379, $P = 0.035$), and multinodularity (HR = 1.514, 1.032–2.222, $P = 0.034$) were significant. In group II, AFP > 100 ng/mL (HR = 3.668, 1.678–8.016, $P = 0.001$) and multinodularity (HR = 2.192, 1.313–3.662, $P = 0.003$) remained significant. In group III, AFP > 100 ng/mL (HR = 2.559, 1.131–5.788, $P = 0.024$) was the only significant factor.

We then performed multivariable analysis on recurrence among overall patients, with the group as a candidate predictor, and the group was indeed revealed to be the strongest predictor. Compared to group I, group II showed a hazard ratio of 1.560 (95% CI: 1.070–2.274) and group III of 3.401 (2.020–4.826). The multivariate analysis also showed the followings as significant predictors: AFP > 100 ng/mL (HR = 1.910, 1.383–2.327), RFA (HR = 0.698, 0.526–0.925), HCV antibody positivity (HR = 1.608, 1.111–2.327), and multinodular HCC (HR = 1.404, 1.080–1.825).

Discussion

In this study, we demonstrated that the risk of subsequent recurrence was increased among patients with recurrent HCC as compared to primary ablation, even when adjusted for the background characteristics of tumor, hepatitis serology, and liver function. Among the patients with first-time recurrence, the risk of subsequent recurrence was 1.4-fold higher than in the primary cases. Among those with second or later recurrence, the risk increased to 3.0-fold. Reflecting the high risk, the median recurrence-free survival time was shorter among the patients with recurrent HCC as compared to primary cases. Among the patients with second or later recurrence, 50% of subsequent recurrence occurred within 1 year.

When liver function is well preserved, repeated resection for recurrent HCC is recommended in the non-transplantation setting. Nagasue *et al.* retrospectively analyzed 290 patients who had radical resection for primary HCC.[21] In their analysis, survival after the primary surgery was better among those who underwent second hepatectomy for recurrent HCC than among those without repeated surgery. Recurrence-free survival rate after the second hepatectomy was lower than that after the primary one, although the difference was not statistically significant. Minagawa *et al.* analyzed 334 patients who underwent radical hepatectomy.[22] Of 183 who had recurrence, 53 underwent repeated surgery. Survival after the second surgery was not different from that after primary surgery. They showed lower recurrence-free survival rate after the second resection but the difference was not statistically significant. Sun *et al.* analyzed 57 patients with hepatitis B who underwent second resection for recurrent HCC, and found that vascular invasion and time to recurrence were independent risk factors for further recurrence after second resection.[23] When the recurrent nodules were localized and liver function was preserved, survival after surgical resection was apparently better than that without surgical treatment.

In those analyses, the outcome of the second resection was compared with that of the first resection performed on the same individual patient. This is inevitably accompanied by a selection bias, where the recurrence group is a subset of the primary group containing only those who survived long enough to receive treatment for recurrence with preserved liver function. In our current study, we used a different cohort for each group; that is, one patient entered only once into analysis. The interval from the initial treatment was included into analysis as an explanatory factor but was not found to be significant for subsequent recurrence. The risk factors found to be significant in this study, either in group or overall analysis, namely, elevated AFP, anti-HCV seropositivity, multinodularity and large size of HCC nodules, were compatible with previous reports.[7–9,21–23] Patients who underwent RFA were at a lower risk of subsequent recurrence than those treated with PEI, as we have previously reported.[17] After controlling for these risk factors, we showed that the previous history of HCC treatment is the strongest risk factor of further recurrence.

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Table 3 Multivariate analysis using Cox regression hazard model

Factor	Group I ($n = 207$)		Group II ($n = 100$)		Group III ($n = 69$)		All patients ($n = 376$)	
	HR (95% CI)	*P*	HR (95% CI)	*P*	HR (95% CI)	*P*	HR (95% CI)	*P*
Event (number)	132		72		63		267	
Group								
Group 1 (indicator)							1	
Group 2							1.456 (1.077–1.968)	0.015
Group 3							3.011 (2.137–4.242)	<0.0001
Age (continuous)	1.003 (0.979–1.027)	0.807	1.025 (0.993–1.058)	0.122	1.017(0.986–1.049)	0.297	1.008 (0.993–1.024)	0.297
Sex male	1.064 (0.740–1.529)	0.738	1.370 (0.689–2.725)	0.370	1.438 (0.733–2.820)	0.291	1.210 (0.917–1.596)	0.177
HCV Ab positive	1.413 (0.874–2.285)	0.157	2.245 (0.955–5.275)	0.063	1.281 (0.468–3.503)	0.630	1.608 (1.111–2.327)	0.012
Child-Pugh class B+C (*vs* A)	1.327 (0.885–1.989)	0.171	1.402 (0.825–2.383)	0.211	0.958 (0.564–1.628)	0.875	1.270 (0.968–1.666)	0.085
AFP >100 ng/mL	1.566 (1.031–2.379)	0.035	3.668 (1.678–8.016)	0.001	2.558 (1.131–5.788)	0.024	1.910 (1.383–2.638)	0.0001
Max tumor size (continuous)	1.030 (1.006–1.054)	0.014	0.973 (0.936–1.013)	0.180	1.006 (0.963–1.052)	0.781	1.014 (0.998–1.032)	0.094
Tumor number 2+3 (*vs* 1)	1.514 (1.032–2.222)	0.034	2.192 (1.313–3.662)	0.003	0.838 (0.461–1.522)	0.562	1.404 (1.080–1.825)	0.011
Treatment RFA (*vs* other)	0.771 (0.510–1.166)	0.217	0.641 (0.357–1.151)	0.137	0.778 (0.415–1.456)	0.432	0.698 (0.526–0.925)	0.012

AFP, α-fetoprotein; CI, confidence interval; Group I, primary hepatocellular carcinoma; Group II, first recurrence; Group III, second or later recurrence; HCV Ab, hepatitis C virus antibody; HR, hazard ratio; RFA, radiofrequency ablation.

Current results may be applied to the selection of treatment strategy. In terms of surgery, some authors recommend primary resection with salvage transplantation, that is secondary transplantation in case of HCC recurrence.[24–26] The strategy of primary ablation and transplantation for recurrence may be considered for patients with impaired liver function contraindicating surgical resection.[27–29] At the time of recurrence, the size and number of recurrent HCC were within the criteria in 79% of cases.[24] In our current analysis, we found that the presentation of recurrent HCC was within the Milan criteria in 73% of cases. When liver transplantation is not readily feasible, primary local therapy, either surgical or medical, is at least the second best strategy. The patient will have a median of 2.5 years for subsequent recurrence to consider the option of transplantation together with financial and social affordability.

In conclusion, this analysis demonstrated that recurrent HCC is more prone to subsequent recurrence than is primary HCC, when compared adjusting for other tumor and hepatic factors. This should be considered when choosing treatment.

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ORIGINAL PAPER

Changes in hepatic functional reserve after percutaneous tumor ablation for hepatocellular carcinoma: long-term follow up for 227 consecutive patients with a single lesion

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Abstract
Background/Aims Percutaneous tumor ablation (PTA), such as ethanol injection, is currently accepted as a potentially curative treatment for hepatocellular carcinoma (HCC). Percutaneous tumor ablation is presumed to be relatively non-invasive, but there are few studies on long-term follow-up of liver function after tumor ablation.
Methods Changes in liver functions were monitored in 227 consecutive patients treated for a solitary HCC nodule by PTA between 1993 and 1997. The liver function evaluated based on Child-Turcotte classification prior to the initial treatment was Child A in 119 (52.4%) patients, B in 81(35.7%), and C in 27 (11.9%). The follow-up period was 46 ± 21 months.
Results The five-year survival rates of patients in Child A, B, and C group after treatment were respectively 76%, 45%, and 43%. Annual shift rate of Child A to Child B was 7%, and that of Child B to Child C was 14%. Tumor recurrence significantly affected aggravation of liver function in Child A ($P = 0.002$) but not in Child B patients ($P = 0.55$). Tumor size at initial treatment influenced changes of liver function in Child B group patients ($P = 0.009$).
Conclusions Preservation of liver function may be essential when treating HCC patients with impaired liver function.

Keywords Hepatocellular carcinoma · Hepatic functional reserve · Child Turcotte classification · Percutaneous tumor ablation · Percutaneous ethanol injection therapy · Percutaneous microwave coagulation therapy

Introduction

Hepatocellular carcinoma (HCC) is one of the most common malignancies worldwide with an increasing number of patients [1, 2]. Hepatectomy was once regarded as the only curative therapy for HCC [3], but surgery is not infrequently contraindicated by concomitant liver diseases, cirrhosis in particular. Fortunately, non-surgical percutaneous tumor ablation (PTA) therapies, namely, percutaneous ethanol injection therapy, percutaneous microwave coagulation therapy, and, most recently, percutaneous radio-frequency ablation (RFA), have been introduced and now play an important role in the treatment of HCC, especially in patients with impaired liver function [4, 5]. In general, PTA is preferred in the treatment of small HCC nodules [6]. However, complete tumor ablation is attainable also in large HCC nodules [7].

Percutaneous tumor ablation is feasible even in patients with relatively poor liver function. We previously showed

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that PTA, even when applied to large tumors, had little immediate adverse effects on liver function [8]. However, PTA may still predispose patients with poor functional reserve to liver failure by reducing functioning liver mass and worsen survival. Thus, information about long-term effects of PTA on liver function is indispensable, especially when PTA is considered in HCC patients with poor liver function. In this study, we analyzed changes in liver function of 227 patients who underwent curative PTA for HCC, placing special emphasis on liver function prior to PTA.

Patients and methods

Patients

Between January 1993 and December 1997, 516 patients with the first-time HCC development were treated at the Department of Gastroenterology, University of Tokyo Hospital, or its affiliates. Since one of the aims of this study was to assess changes in liver function after PTA with regard to the size of the HCC nodules, 277 patients having more than one nodule were excluded. Of the 239 patients with a solitary HCC, seven received hepatectomy and five received only palliative treatments. The remaining 227 patients who underwent curative PTA as described below constituted the subjects of this study. Informed consent was obtained from each patient before treatment in accordance with the Helsinki declaration.

Detection of HCC

HCC nodules were detected by abdominal ultrasonography, dynamic computed tomography, abdominal angiography, and/or magnetic resonance imaging. Diagnosis of HCC was confirmed by histologic examination of needle biopsy specimens in 180 patients (79%). In the remaining 47 cases, HCC was confirmed by hypervascularity of the tumor, typical of HCC, on angiography and/or contrast-enhanced computed tomography. Informed consent was obtained from each patient before treatment in accordance with the Helsinki declaration.

Treatment of HCC

Percutaneous ethanol injection was performed in 186 patients, 11 of them with transcatheter hepatic arterial embolization (THAE) prior to ethanol injection. Twenty-six patients received percutaneous microwave coagulation, which was introduced in 1996, and two of them received THAE before microwave coagulation. Fifteen patients underwent both ethanol injection and microwave coagulation for the same solitary nodule. Percutaneous ethanol injection was performed using a 21-gauge needle 15 cm in length (Daimon, Saitama, Japan), which was introduced percutaneously into the tumor nodule under ultrasonographic guidance [9]. During each session, 3–15 ml of ethanol (99.5 w/v) was injected into the tumor and the surrounding liver tissue. For percutaneous microwave coagulation, a 14-gauge guide needle with 15 cm in length was introduced percutaneously into the lesion under ultrasonic guidance. The microwave electrode was then placed in the tumor through the guide needle [10]. For both percutaneous ethanol injection and microwave coagulation, treatments were performed once or twice a week until complete necrosis of HCC was confirmed by dynamic computed tomography, where complete necrosis was indicated when the ablated lesion showed a low-density area in both arterial and portal venous phases with a diameter larger than the pretreatment tumor size. Ablation was determined incomplete if there was a contrast-enhanced region or if the area of necrosis was not larger than the pretreatment tumor size [11].

Follow-up

All patients were monitored at outpatient clinics with biochemical and other laboratory tests every 1–2 months. Development of ascites or hepatic encephalopathy, if any, was recorded. Liver function was evaluated according to Child-Turcotte classification [12]. HCC recurrence was examined by ultrasonography every 3 months and computed tomography every 6 months. Patients received additional treatment for recurrence, whenever possible, mainly by PTA. THAE was performed prior to PTA when there were more than three tumor foci. The follow-up period was defined as the interval between the date of initial treatment and the date of event occurrence (deterioration in liver function or death) or the end of December 2000. The mean follow-up period was 46 ± 21 months (range 2–96 months).

Statistics

The primary endpoint of this study was deterioration of liver function as evaluated by Child-Turcotte classification, which was based on five factors, serum levels of albumin and total bilirubin, presence of ascites and hepatic encephalopathy, and state of nutrition. Cumulative survival curves and deterioration rates of liver function were calculated by the Kaplan–Meier method and difference among groups were assessed by the log-rank test. Risk factors for aggravation of liver function were analyzed by Cox proportional hazards regression, where candidate predictive factors included demographic data prior to the initial PTA

(age, gender, HBs antigen, HCV antibody, alcohol consumption >50 g daily), history of interferon treatment, stage of fibrosis in the background liver, serum alanine transaminase (ALT) level, platelet count, prothrombin time, levels of tumor markers (α-fetoprotein and des-γ-carboxy prothrombin), tumor characteristics (size, pathologic grading), and the mode of treatment (percutaneous ethanol injection, percutaneous microwave coagulation, with or without THAE). Continuous variables were transformed into dichotomous data divided by the median value, except that AFP and DCP were represented by positivity. Association between HCC recurrence and degradation of liver function was evaluated by χ^2-test. A P value <0.05 was considered significant.

Results

Patient profile

The clinical profiles of 227 patients (158 men and 69 women, average age 64 years) on initial admission are shown in Table 1. As typical in Japan, 80% were positive for hepatitis C virus and 10% for hepatitis B virus. The size of the tumor was 26 ± 10 mm in diameter. Tumor cell differentiation was determined in 180 (79%) patients, as Edmondson I, II, III, and IV in 65, 85, 28, and 2, respectively. Adequate specimen could not be obtained in 27 patients and tumor biopsy was not performed in 20 patients. Stage of fibrosis in the background liver was evaluated in 202 (89%) patients, where 59% showed cirrhosis (F4) and 31% showed advanced fibrosis (F3). Seropositivity of tumor markers was 18% for AFP (cut off 100 ng/ml) and 12% for DCP (0.1 AU/L) Table 2.

Liver function was evaluated based on Child-Turcotte classification as Child A in 119, Child B in 81, and Child C in 27 patients. Age, gender, viral markers, and alcohol consumption did not differ among the three groups, while there was significant difference in the levels of albumin, bilirubin, ALT, platelet counts, prothrombin time, and stage of fibrosis. Tumor characteristics and the mode of treatment did not differ among the groups except that all patients in the Child C group received percutaneous ethanol injection Table 3.

Survival and changes in liver function

Cumulative survival rate among the overall 227 patients was 93, 76, and 61% at 1, 3, and 5 years, respectively. Compatible with previous reports, the 5-year survival rate was better in patients at Child A (76%) than in those at Child B (45%) or Child C (43%) ($P < 0.0001$, log-rank test, Fig. 1). Difference between Child B and Child C patients was not significant ($P = 0.4$). Among 119 patients who were initially at Child A, 44 showed deterioration in liver function to Child B. Cumulative deterioration was found in 9, 19, and 38% at 1, 3, and 5 years, respectively (Fig 2). Similarly, 49 of 81 patients who were initially at Child B were shifted to Child C, with a rate of 13, 44, and 70% at 1, 3, and 5 years, respectively (Fig 3) Table 4.

Factors predictive of deterioration in liver function

Predictive factors for deterioration in liver function after PTA were assessed using Cox proportional hazards regression for the change from Child A to Child B among 119 patients who were initially at Child A, and for that from Child B to Child C among 81 patients who were initially at Child B, separately.

Univariate analysis revealed that aging, fibrosis of background liver tissue, high grade of tumor pathology (Ed. III/IV), and serum AFP levels were significantly associated with the aggravation of liver function in Child A group patients. Serum AFP levels were only significant in stepwise multivariate analysis. In Child B group patients, aging, alcohol consumption (>50 g daily), and size of tumor foci were significant by univariate analysis, and size of tumor foci was the only significant factor by stepwise multivariate analysis.

Recurrence of HCC

Recurrence of HCC was detected in 143 of 227 (63%) patients, of which 22 were adjacent to the primary lesion, 99 were elsewhere, and 22 were both. Cumulative recurrence rates did not differ when compared based on Child-Turcotte classification of liver function before the initial PTA (data not shown). Seventy-nine of 119 (66%) patients who were initially at Child A function developed HCC recurrence, and liver function deteriorated to Child B in 40 of those 79 patients (51%). In contrast, only 4 of 40 patients (10%) who had no HCC recurrence showed deterioration in liver function ($P = 0.002$). In contrast, deterioration to Child C from Child B occurred in 31 of 47 patients (66%) with HCC recurrence, and in 18 of 34 patients (53%) without ($P = 0.554$).

Discussion

PTA has become accepted as a potentially curative treatment for HCC, showing survival rates compatible with that of hepatectomy [13]. In fact, the present study showed a 5-year cumulative survival of 76% among Child A patients and that of 43% among Child C patients. It is noteworthy that hepatectomy might have been contraindicated in the

Table 1 Patients profiles at the time of initial treatment for HCC

Variables	All 227 patients	Child A group (n = 119)	Child B group (n = 81)	Child C group (n = 27)
Host-related factors				
Sex (male/female)	158/69	89/30	52/29	17/10
Age (y.o.)	64 ± 6.5 (63)	63 ± 7.0 (63)	66 ± 5.2 (66)	63 ± 8.8 (61)
Patients grouped according to viral marker (N)				
HBs-Ag(+),HCV-Ab(–)	22	17	3	2
HBs-Ag(–),HCV-Ab(+)	182	93	66	23
HBs-Ag(+),HCV-Ab(+)	1	1	0	0
HBs-Ag(–),HCV-Ab(-)	22	8	12	2
Previous IFN therapy (Yes)	49	34	15	0
Alcohol consumption >50 g/day n/n (%)	76/196 (39%)	41/107 (38%)	27/66 (41%)	8/23 (35%)
Albumin (g/dl)	3.6 ± 0.4 (3.5)	4.0 ± 0.2 (3.9)	3.3 ± 0.2 (3.3)	2.8 ± 0.3 (2.8)
Total bilirubin (mg/dl)	0.9 ± 0.4 (1.1)	0.7 ± 0.2 (0.7)	1.0 ± 0.4 (0.9)	1.5 ± 0.8 (1.4)
ALT (IU/l)	76 ± 41 (62)	81 ± 43 (68)	73 ± 38 (60)	61 ± 45 (49)
Platelet count (104/mm3)	10.8 ± 4.0 (9.5)	12.9 ± 3.8 (12.8)	9.1 ± 3.1 (7.7)	6.9 ± 2.0 (7.2)
Prothrombin time (%)	71 ± 12 (70)	77 ± 10 (75)	68 ± 11 (66)	55 ± 14 (52)
Fibrosis staging of noncancerous tissue (F1/2/3/4)	3/17/62/120	3/11/42/51	0/6/16/49	0/0/4/20
Tumor-related factors				
Size of tumor (mm)	26 ± 10 (22)	26 ± 10 (21)	25 ± 10 (22)	27 ± 13 (24)
Pathological grading of tumors (Ed. I/II/III/IV)	65/85/28/2	39/40/17/2	19/30/10/0	7/15/1/0
Positivity of tumor markers n/n (%)				
AFP (>100 ng/ml)	38/216 (18%)	16/113 (14%)	17/79 (22%)	5/24 (21%)
DCP (>0.1 IU/l)	23/182 (12%)	9/93 (10%)	12/68 (18%)	2/20 (10%)
Treatment-related factors (n/n)				
THAE before PTA (With / Without)	11/216	7/112	3/78	1/26
Ethanol injection (with/without)[a]	201/26	105/14	69/12	27/0
Microwave coagulation (with/without)[a]	41/186	21/98	18/63	2/25
HCC recurrence				
Presence/Absence (n/n)	143/84	79/40	47/34	17/10

Note. The value represents mean ± SD (median)

Abbreviations: HCC, hepatocellular carcinoma; N, number of patients; ALT, alanin transaminase; Ed, Edmondson classification; AFP, α-fetoprotein; DCP, des-γ-carboxy prothrombin; THAE, transcatheter hepatic arterial embolization; PTA, percutaneous tumor ablation

[a] 15 patients received combined therapy of ethanol injection and microwave coagulation

Table 2 Occurrence rate of degradation in hepatic functional reserve with or without development of HCC recurrence

	HCC recurrence (+)	HCC recurrence (–)	P value
Child A group % (n/n)	50% (40/79)	10% (4/40)	0.002
Child B group % (n/n)	66% (31/47)	53% (18/34)	0.554

Abbreviations: HCC, hepatocellular carcinoma; HCC recurrence (+), patients who developed HCC recurrence during follow-up period; HCC recurrence (–), patients who did not develop HCC recurrence during follow-up period

latter group due to poor liver function. We have previously shown that PTA does not result in immediate deterioration in liver function. However, it was not known whether or not PTA affects liver function in a longer-term follow-up.

Since most patients with HCC have chronic liver diseases, usually chronic hepatitis and cirrhosis, changes in liver function are not easily attributed separately either to the progression of background liver disease or to the effect

Table 3 Risk factors contributing to degradation of functional reserve of the liver in Child A group

	Univariate analysis	
	Risk ratio (95% CI)	*P*
Host-related factors		
Age (>70 y.o.)	1.91 (0.96–3.82)	0.07
Sex (male)	0.88 (0.45–1.73)	0.71
HCV Ab positive	1.36 (0.60–3.05)	0.46
Previous interferon therapy (Yes)	0.77 (0.42–1.44)	0.42
Alcohol consumption (≥50 g/day)	1.30 (0.24–1.85)	0.40
ALT level (>80 IU/L)	1.47 (0.81–2.63)	0.20
Platelet count (>12.8 × 10^4)	0.84 (0.47–1.52)	0.57
Prothrombin time (>68%)	0.73 (0.39–1.36)	0.32
Fibrosis staging (F4 vs. F1-3)	2.03 (0.74–3.86)	0.31
Tumor-related factors		
Size of tumor (>21 mm)	1.26 (0.70–2.28)	0.44
Ed. Classification (ed. III/IV vs. I/II)	2.18 (1.08–4.42)	0.03*
Positivity of tumor markers		
AFP (>100 ng/ml)	3.36 (1.62–6.94)	0.001*
DCP (>1.0 IU/L)	1.54 (0.47–5.10)	0.48
Treatment-related factors		
THAE before PTA	0.83 (0.20–3.48)	0.81
Ethanol injection	0.78 (0.31–2.03)	0.62
Microwave coagulation	0.76 (0.42–1.43)	0.42
Multivariate analysis		
Fibrosis staging (F4 vs. F1-3)	0.76 (0.74–3.86)	0.21
Ed. Classification (ed. III/IV vs. I/II)	1.70 (0.76–3.13)	0.19
AFP (>100 ng/ml)	2.69 (1.16–6.21)	0.02*

Abbreviations: HCC, hepatocellular carcinoma; ALT, alanin transaminase; Ed, Edmondson classification; AFP, α-fetoprotein; DCP, des-γ-carboxy prothrombin; THAE, transcatheter hepatic arterial embolization; PTA, percutaneous tumor ablation

* $P < 0.05$

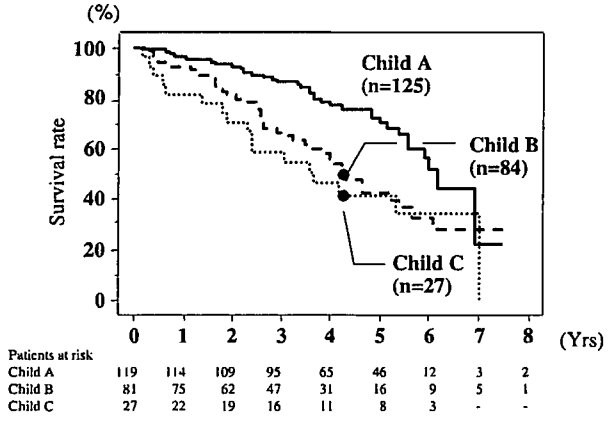

Fig. 1 Cumulative survival rates of patients after percutaneous tumor ablation (PTA) according to Child-Turcotte classification prior to initial PTA

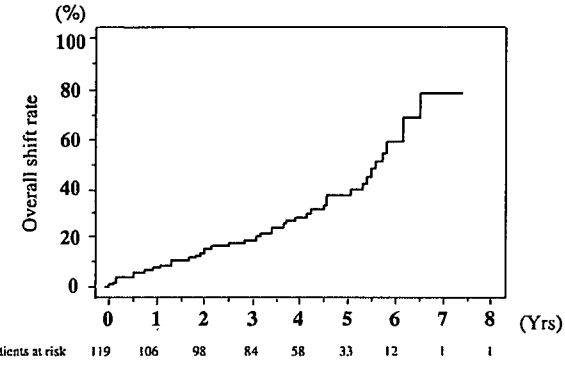

Fig. 2 Incidence of deterioration in liver function from Child A to Child B after percutaneous tumor ablation

of intervention. We presumed in this study that the degree of untoward effect of PTA, if any, is correlated with the size of the HCC nodules. The results showed that the size of tumor was associated with deterioration in liver function among patients at Child B but not among patients at Child A. It appears that damage to liver function caused by PTA can be fully recovered in patients with good liver functional reserve while it may not be negligible in patients with poorer-functional reserve.

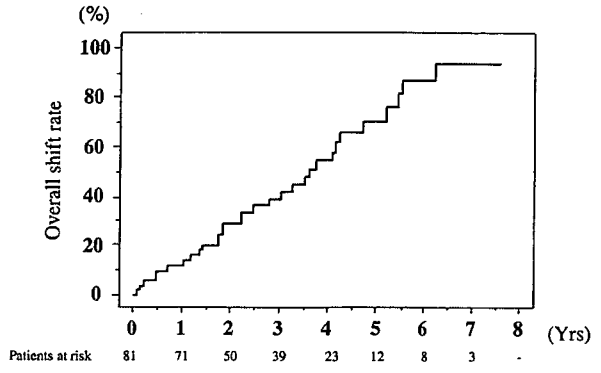

Fig. 3 Incidence of deterioration in liver function from Child B to Child C after percutaneous tumor ablation

Analysis of changes in liver function was further complicated by frequent recurrence of HCC. Recurrence of HCC is associated with deterioration in liver function in patients who were initially at Child A but not in those initially at Child B by χ^2-test. Cox proportional hazards regression using recurrence as a time-dependent covariate also showed the same results (data not shown). More precisely, liver function was relatively preserved in Child A patients in the absence of HCC recurrence while deterioration was common in Child B patients regardless of the presence or absence of HCC recurrence. In fact, HCC was still under control in 15 of 34 patients (44%) when they died. These data suggest that preservation of liver function is at least as important as ablation of HCC nodules in patients with already impaired liver function. Since the efficacy of antiviral therapies such as pegylated interferon plus ribavirin for HCV and entecavir etc. for HBV has been recently improved, they may be beneficial also in improving prognosis of hepatitis virus-related HCC patients by preventing further deterioration in liver function.

Multivariate analysis showed serum AFP levels significantly affected the liver function in Child A group patients. We have already showed that serum AFP levels prior to PTA were closely related to HCC recurrence [14]. There-

Table 4 Risk factors contributing to degradation of functional reserve of the liver in Child B group

	Univariate analysis	
	Risk ratio (95% CI)	*P*
Host-related factors		
Age (>70 y.o.)	1.94 (1.07–3.52)	0.03
Sex (male)	0.89 (0.49–1.62)	0.71
HCV Ab positive	1.10 (0.53–2.26)	0.81
Previous interreron therapy (Yes)	0.69 (0.31–1.53)	0.36
Alcohol consumption (≥50 g/day)	1.61 (0.86–2.99)	0.14
ALT level (>80 IU/L)	0.61 (0.33–1.11)	0.10
Platelet count (>7.7 × 10^4)	1.05 (0.60–1.83)	0.86
Prothrombin time (>68%)	1.07 (0.61–1.87)	0.81
Fibrosis staging (F4 vs. F1-3)	1.90 (0.94–3.79)	0.07
Tumor-related factors		
Size of tumor (>21 mm)	2.35 (1.31–4.21)	0.004
Ed. Classification (ed. III/IV vs. I/II)	1.56 (0.69–3.52)	0.29
Positivity of tumor markers		
AFP (>100 ng/ml)	1.12 (0.56–2.26)	0.74
DCP (>1.0 IU/L)	1.91 (0.91–4.07)	0.09
Treatment-related factors		
THAE before PTA	1.57 (0.48–5.15)	0.46
Ethanol injection	0.88 (0.39–2.00)	0.76
Microwave coagulation	1.51 (0.74–3.07)	0.25
Multivariate analysis		
Age (>70 y.o.)	1.25 (0.70–2.23)	0.44
Tumor size (>21 mm)	2.23 (1.23–4.07)	0.009*

Abbreviations: HCC, hepatocellular carcinoma; ALT, alanin transaminase; Ed, Edmondson classification; AFP, α-fetoprotein; DCP, des-γ-carboxy prothrombin; THAE, transcatheter hepatic arterial embolization; PTA, percutaneous tumor ablation

* $P < 0.05$

fore, it is possible that the significance of serum AFP levels should reflect the feasibility of HCC recurrence.

Since we did not assign patients to untreated controls, we cannot directly assess the long-term untoward effects of PTA on liver function. Nevertheless, the untoward effects were shown to be negligible in patients with relatively preserved liver function. On the other hand, the volume of ablation affected changes in liver function in patients with already impaired function. We have currently adopted strict criteria, based on dynamic computed tomography, regarding the complete ablation of HCC nodules. Prospective studies will be required in the future to clarify whether the complete ablation as such is beneficial in regard to the prognosis of patients with poor liver function.

To evaluate long-term prognosis of patients after ablation therapy, we did not include in this study patients treated with RFA, which has been recently shown to surpass ethanol injection as treatment of HCC. It will be important to assess long-term prognosis of patients treated with RFA especially in terms of preservation of liver function.

In conclusion, we showed that PTA did not affect long-term liver function in patients with preserved liver function regardless of the size of the HCC nodules. Recurrence of HCC determined their prognosis. In contrast, the size of the HCC was a significant risk factor for deterioration in liver function, while recurrence of HCC was not, in patients with already impaired liver function. Preservation of liver function may be essential when treating patients in the latter group.

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CLINICAL STUDIES

Expression of microsomal prostaglandin E synthase-1 in human hepatocelluar carcinoma

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Abstract

Background/Aims: The objective of this study was to evaluate the expression of microsomal prostaglandin E synthase-1 (mPGES-1) in hepatocellular carcinoma (HCC) tissues. *Methods:* Forty surgically resected HCC tissues with adjacent non-tumorous liver tissues and 14 surgically resected, histologically normal liver tissues were used. The immunohistochemical expressions of the mPGES-1 protein in these HCC tissues and normal control livers were analysed. mPGES-1 mRNA expression was also analysed by the real-time polymerase chain reaction method using the same tissues. *Results:* Microsomal prostaglandin E synthase-1 was not expressed in hepatocytes but instead in vascular endothelial cells and bile duct epithelial cells in normal liver tissues. The mPGES-1 expression in HCC tissues was significantly greater than its expression in the non-tumorous tissues. All types of HCC expressed more mPGES-1 than normal or hepatitis livers, and the levels of mPGES-1 expression in poorly differentiated HCC were similar to the levels in well-differentiated HCC. The mPGES-1 mRNA expression paralleled its protein expression in these tumorous and non-tumorous tissues. *Conclusions:* The present study is the first to demonstrate a high expression of mPGES-1 in well-differentiated HCC as well as in poorly differentiated HCC. These findings suggest that mPGES-1 may play a role in the advanced as well as early stage of hepatocarcinogenesis.

Hepatocellular carcinoma (HCC) is one of the most common malignancies worldwide (1). Although HCC occurs infrequently in developed countries, rising incidences of it have been reported in Japan (2). HCC is often diagnosed in its late stage, which makes curative therapies such as surgical resection unlikely (3). The limitation in the present modes of treatment emphasizes the importance of developing an effective method of chemoprevention (4). Clinical and biochemical evidence has suggested that prostaglandin (PG) E_2 plays a crucial role in the development of colorectal cancer and possibly other cancers (5). The major prostanoid produced by several types of cancer is PGE_2, which results from three biosynthetic reactions involving phospholipase A2, cyclooxygenase (COX), and terminal PGE_2 synthase (PGES) (6).

Overexpression of COX-2 has been associated with tumorigenesis of colon cancer (7, 8). Although the precise functional roles of COX-2 in carcinogenesis remains to be defined, accumulated data indicate that COX-2-derived PGE_2 participates in colon-cancer carcinogenesis and that selective COX-2 inhibitions create a potent suppression in the growth of colon cancer (9, 10). Overexpression of COX-2 has also been demonstrated in HCC tumorous tissues using immunohistochemical methods. The enhanced expression of COX-2 in well-differentiated HCC suggests that COX-2 is involved in the early stage of hepatocarcinogenesis (11, 12).

Prostaglandin E synthase catalyses the conversion of PGH_2 (which is produced from arachidonic acid by COX-1 or COX-2) to PGE_2. Recent advances have led to identification of at least three PGES: microsomal PG E synthase-1 (mPGES-1), mPGES-2 and cytosolic PGES (cPGES-1) (13). mPGES-1 is induced by proinflammatory stimuli and is down-regulated by anti-inflammatory glucocorticoids (14). By comparison,

cPGES-1 is constitutively and ubiquitously expressed (15). A possible linkage of mPGES-1 with tumorigenesis has been indicated by a recent observation that mPGES-1 is constitutively expressed in several cancers (16, 17). On the basis of these findings, it is reasonable to investigate whether mPGES-1 may be associated with the development of HCC. In this study, we analysed the expression of mPGES-1 in human HCC tissues and present its potential involvement in HCC.

Patients and methods

Patients

Patients with HCC who underwent hepatic resection between April 2003 and April 2005 at the Nagasaki Medical Center, who gave written informed consent, were enrolled in this study. Patient profiles are summarized in Table 1. Histologically normal livers free of hepatitis B or C viral infections were obtained from 14 patients with liver metastases from colorectal cancer. This study protocol was approved by the Ethics Committees National Nagasaki Medical Center.

Immunohistochemistry

For immunohistochemical analysis of mPGES-1, formalin-fixed paraffin-embedded tissue blocks of HCC tissues were cut into 4-μm-thick sections. The sections were deparaffinized in xylene and subsequently rehydrated in sequential ethanols (100–70%). After washing three times with 10 mM phosphate-buffered saline (PBS) (pH 7.4), antigen retrieval was performed by: heating in a microwave at 95 °C for 15 min, and then washing twice in PBS for 5 min. The sections were treated with peroxidase blocking solution (Dako Japan, Kyoto, Japan) for 5 min, and incubated with a primary antibody for 60 min at room temperature. The primary antibodies used were a 1:80 dilution of an anti-mPGES-1 polyclonal antibody (Cayman Chemical, Ann Arbor, MI, USA). A standardized two-step method with ENVISION PLUS (Dako) was used for detection. The reaction products were visualized using 3, 3-0-diaminobenzidine as a chromogen (Dako), and counterstained with Mayer's haematoxylin (Dako). Negative controls were prepared using control goat immunoglobulin G (IgG).

Hepatocytes in a non-tumorous region and HCC cells excluding mesenchymal cells in all stained specimens were evaluated as described previously (11). In brief, the intensity of staining for mPGES-1 was scored in each specimen on a scale of 0–3, in which 0, negative staining; 1, weakly positive staining; 2, moderately positive staining; 3, strongly positive staining (Fig. 1). The intensity of staining was evaluated for the maximum intensity among positive cells ('maximum intensity of staining' I) and the intensity level observed in the largest number of positive cells ('most extensive intensity level' II). The extent to which positive cells were seen in each specimen ('extent of distribution' of positive cells III) was estimated and scored on a scale of 0–4, in which 0, negative; 1, positive staining in 1–25% of cells; 2, in 26–50%; 3, in 51–75%; 4, in

Table 1. Patient characteristics and histological features of hepatocellular carcinoma

Patient No.	Age	Sex	LC	HBsAg	Anti-HCVAb	Tumor differentiation
1	70	F	+	–	+	WD
2	70	F	+	–	+	WD
3	64	M	+	–	+	WD
4	86	M	–	–	+	WD
5	67	F	+	+	–	WD
6	60	M	+	–	+	WD
7	66	F	+	–	+	WD
8	72	F	+	+	–	WD
9	66	M	–	–	+	WD
10	71	M	+	–	+	WD
11	63	M	–	–	+	WD+MD
12	53	M	+	+	–	MD
13	67	M	+	–	+	MD
14	69	M	+	–	+	MD
15	73	M	+	–	+	MD
16	64	M	+	–	+	MD
17	65	M	+	+	–	MD
18	64	M	+	–	+	MD
19	70	F	+	–	+	MD
20	51	M	+	–	+	MD
21	71	F	+	–	+	MD
22	48	M	+	+	–	MD
23	61	M	+	–	+	MD
24	51	M	+	+	–	MD
25	45	M	+	–	+	MD
26	56	M	+	–	+	MD
27	68	F	+	+	–	MD
28	37	M	–	+	–	MD
29	68	M	+	+	–	MD
30	75	M	–	–	+	MD
31	63	M	–	–	+	MD
32	66	F	+	–	+	MD
33	65	M	+	–	+	MD
34	66	F	+	+	–	MD+PD
35	74	M	+	–	+	PD
36	56	M	+	–	+	PD
37	57	M	+	–	+	PD
38	64	M	+	–	+	PD
39	58	M	+	+	–	PD
40	54	M	+	+	–	PD

LC, liver cirrhosis; WD, well differentiated; MD, moderately differentiated; PD, poorly differentiated.

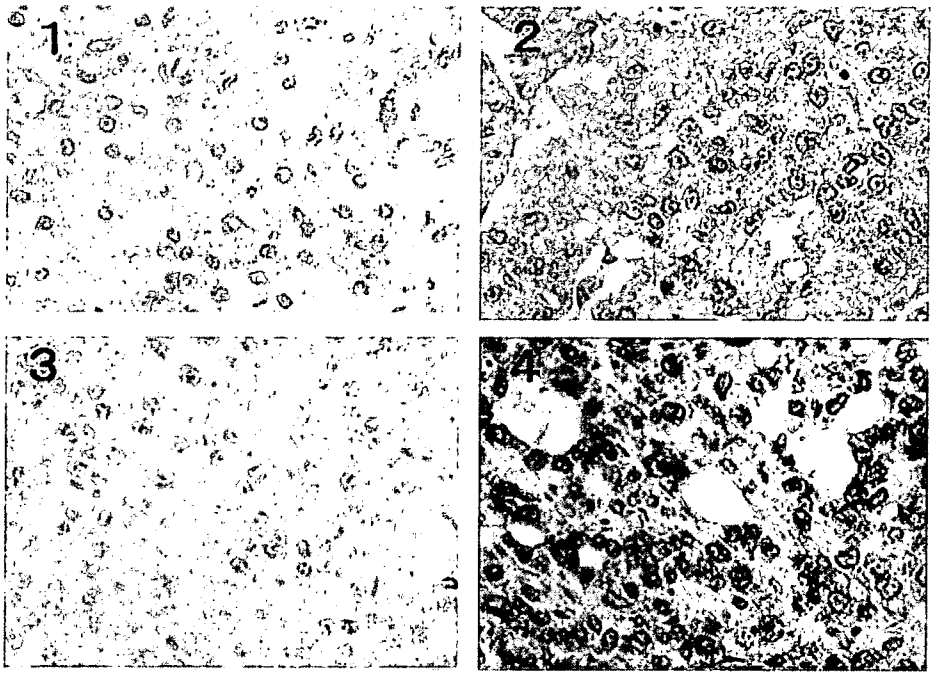

Fig. 1. Various patterns of immunohistochemical staining for microsomal prostaglandin E synthase-1 (mPGES-1) in liver tissues; 1, no immunoreactivity for mPGES-1 [grade 1, well-differentiated hepatocellular carcinoma (HCC) tissues]; 2, mPGES-1 is weakly positive in hepatocytes (grade 2, moderately differentiated HCC tissues); 3, mPGES-1 is moderately positive in hepatocytes (grade 3, moderately differentiated HCC tissues); 4, mPGES-1 is strongly positive in hepatocytes (grade 4, moderately differentiated HCC tissues) (original magnification × 200).

76–100%. Each section was evaluated for the sum of these three parameters (I+II+III). Immunoreactivity for mPGES-1 was compared statistically using the average of the sum in each histological category.

Protein extraction and Western blotting

Western blotting was performed on a representative sample of HCC tissues and non-tumorous liver tissues. The tissues were homogenized on ice in RIPA buffer (PBS, 1% NP-40, 0.5% sodium deoxycholate, and 0.1% sodium dodecyl sulphate) containing 100 ng/mL phenylmethylsulphonyl fluoride, 4 μg/mL aprotinin, 2 μg/mL leupeptin, 1 μg/mL pepstatin, 10 μg/mL antipain, 10 μg/mL soybean trypsin inhibitor, and 2 mmol/L ethylenediaminetetraacetic acid. The homogenates were clarified by centrifugation. Protein concentration was measured with a Bio-Rad protein assay kit (Bio-Rad, Hercules, CA, USA). After being boiled for 5 min in the presence of 2-mercaptoethanol, samples containing 50 μg tissue lysates were separated on 12.5% sodium dodecyl sulphate-polyacrylamide gels and then transferred onto equilibrated Hybond PVDF membranes (Amersham International, Buckinghamshire, UK). After skim-milk blocking, the membranes were incubated with the anti-mPGES-1 polyclonal antibody (a dilution of 1:500). The bound antibodies were detected with horseradish peroxidase – labelled rabbit anti-goat IgG (Southern Biotechnology Associates, Birmingham, AL, USA) using the enhanced chemiluminescence detection system (ECL kit; Amersham International).

Reverse transcriptase-polymerase chain reaction (RT-PCR) method

Total RNA was extracted from fresh-frozen tissues of both the tumorous area and the surrounding non-tumorous area using the Isogen RNA extraction kit (Nippon Gene, Toyama, Japan). First-strand cDNA was synthesized by reverse transcription at 45 °C for 45 min in a 50 μL reaction mixture containing 1 μg of total RNA and MuLV reverse transcriptase (Invitrogen, Carlsbad, CA, USA). After denaturing at 99 °C for 5 min, followed by cooling at 5 °C, the cDNA was amplified using PCR. PCR was performed in a 25 μL reaction mixture containing 1 μL of cDNA template, 1 × Perkin-Elmer PCR buffer, 1.5 mM $MgCl_2$, 0.8 mM deoxynucleotide triphosphates, 0.8 μM of each primer for mPGES-1 and 1 U of Taq DNA polymerase

(AmpliTaq Gold; Roche Molecular Systems Inc., Branchburg, NJ, USA). The PCR primers used for detection of mPGES-1 cDNAs were 5′-GAA-GAAGGCCTTTGCCAAC-3′ (sense) and 5′-GGAA-GACCAGGAAGTGCATC-3′ (antisense), and the amplified products were 406 bp respectively. The condition for multiplex PCR was set up as follows: one cycle of denaturing at 95 °C for 12 min, followed by 28 cycles of 95 °C for 1 min, 62 °C for 1 min and 72 °C for 1 min, before a final extension at 72 °C for 10 min.

Real-time PCR were measured by real-time quantitative RT-PCR using the Light Cycler system (Roche Diagnostics, Mannheim, Germany) with LightCycler-DNA master SYBRGreen I (Roche Diagnostics). Each PCR reaction mixture contained 10 μL of a 10-fold dilution of cDNA template in water, 2 μL of SYBR-Green I, 2 μL of LightCycler-Primer Set and 6 μL of water in a total volume of 20 μL. After an initial denaturation at 95 °C for 10 min, PCR was performed for 45 cycles (95 °C for 10 s, 68 °C for 10 s and 72 °C for 5 s). Both a melting curve analysis and gel migration analysis were included in the experiments to verify the absence of non-specific products such as primer–dimers. The LightCycler-primer sets for mPGES-1 were purchased from Roche Diagnostics (sense; 5′-CCA GTA TTG CAG GAG CGA CC-3′, antisense; 5′-CCA GAA AGG AGT AGA CGA AGC C-3′). Results are expressed as the ratio of mPGES-1 cDNA concentration relative to the concentration of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) cDNA in the same sample.

Statistical analysis

Statistical significances were assessed using Mann–Whitney's *U*-test. $P < 0.05$ was considered statistically significant.

Results

Immunohistochemical analysis for mPGES-1

Immunohistochemistry was used to assess the expression of the mPGES-1 protein in normal liver tissues and in HCC tissues. Cytoplasmic staining for mPGES-1 was observed in vascular endothelial cells of control liver tissues. By contrast, mPGES-1 expression was not detected in hepatocytes in normal liver tissues (Fig. 2). We next performed immunohistochemical staining for the 40 HCC liver tissues. The mPGES-1 staining was positive to various degrees in the HCC specimens. The marked expression of mPGES-1 was demonstrated in well-differentiated HCC tissues as well as in poorly differentiated HCC tissues (Fig. 3). In non-tumorous

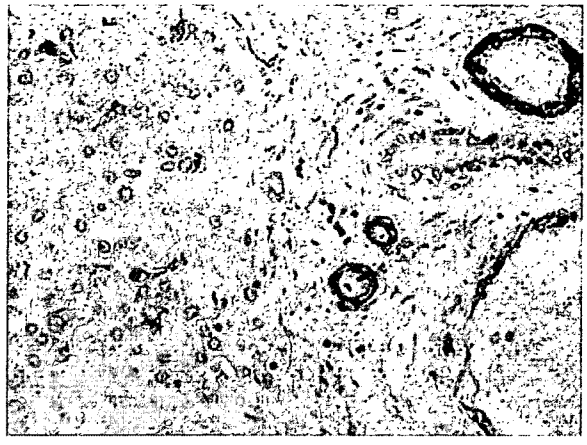

Fig. 2. Immunohistochemical localization of microsomal prostaglandin E synthase-1 (mPGES-I) in normal liver tissues; mPGES-1 was stained in vascular endothelial cells and mononuclear cells distributed in the sinusoidal space of normal liver tissues (original magnification, × 200).

liver tissues including those with cirrhosis and chronic hepatitis, some expression of mPGES-1 was observed in hepatocytes. However, its expression was markedly lower compared with that in HCC cells. Scores for the mPGES-1 expression in non-tumorous tissues (normal tissues, those with chronic hepatitis, and those with cirrhosis) and in HCC tissues are summarized in Fig. 4. The levels of the mPGES-1 expression were significantly increased in HCC tissues compared with that in normal liver tissues, chronic hepatitis, and cirrhosis. However, mPGES-1 expression levels were not significantly different among well, moderately and poorly differentiated HCC tissues. To evaluate whether background liver histology affects the levels of mPGES-1 protein expression in HCC, we divided HCC patients according to their background liver histology. As shown in Fig. 5, background liver histology did not affect the mPGES-1 expression in HCC tissues.

Western blot analysis for mPGES-1

To confirm the specificity of the mPGES-1 antibody, a single representative set of tissue samples (HCC tissues and non-tumorous tissues from a patient with HCC plus cirrhosis) were subjected to Western blot analysis. The HCC tissues yielded a single band with 16 kDa molecular weight, indicating the presence of the mPGES-1 protein, but the non-tumorous tissues provided no result. By contrast, the β-actin expression was not different between the HCC tissues and the non-tumorous tissues (Fig. 6).