blood sugar, and systolic blood pressure, and standardized beta coefficients showed that, of these parameters, serum GGT had the strongest influence on T-SOD activity (Table 3). From these results, we considered that the serum GGT level was an independent and important predictor of serum T-SOD activity, although the correlation was weak. Interestingly, in the ordered categorical analysis using sextiles, serum T-SOD activity declined in a stepwise fashion even within the normal range of the serum GGT level ($P < 0.0001$; Fig. 1a).

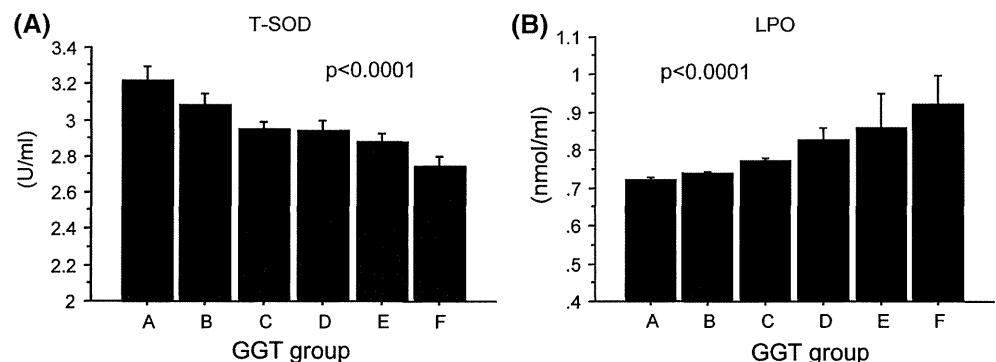### Correlation between serum LPO and GGT level

Next, we assessed the correlation between serum GGT and the serum LPO level, which was used as a representative marker for oxidative stress accumulation. In contrast to the serum T-SOD activity, the serum LPO level increased in a stepwise fashion even within the normal range of the serum GGT level ($P < 0.0001$; Fig. 1b). Thus, the serum GGT level closely reflected both anti-oxidative stress activity and the accumulation of oxidative stress.

Table 3 Multiple linear regression analysis using serum T-SOD activity as dependent variable

Variables	Standardized beta	P
Age	0.077	<0.0001
BMI		NS
SBP	−0.045	0.023
DBP		NS
TC		NS
HDL-C	0.096	<0.0001
TG		NS
Glucose	−0.057	0.003
Insulin		NS
GGT	−0.112	<0.0001
ALT	−0.057	0.003

NS Not significant

Correlation of serum GGT level with metabolic syndrome and IMT

To assess the clinical relevance of serum GGT levels, we investigated the correlation of the serum GGT level with the metabolic score and IMT. Similar to oxidative stress, the metabolic score also increased in a stepwise fashion, even within the normal range of the serum GGT level ($P < 0.0001$; Fig. 2). Furthermore, the serum GGT level was significantly and positively correlated with IMT ($P < 0.0001$; Fig. 3). Thus, an increased serum GGT level was associated with the metabolic syndrome and atherosclerosis.

Influence of drinking status on the relationship of the serum GGT level to serum SOD activity and metabolic syndrome

The serum GGT level is known to be influenced by the amount of alcohol consumed. To investigate the influence of drinking status on the relationship of the serum GGT level to SOD activity and the metabolic syndrome, we stratified subjects into four groups according to the amount of alcohol consumed: never ($n = 1053$), 1–30 g/day ($n = 886$), 31–60 g/day ($n = 631$), and >60 g/day ($n = 337$). As shown in Fig. 4a, the serum GGT level showed a significant positive graded association with the amount of alcohol consumed. T-SOD activity was negatively correlated with the amount of alcohol consumed and the metabolic score was positively correlated with this parameter (Fig. 4b, c). These findings suggest that drinking status may confound the relationship of the serum GGT level to T-SOD activity and the metabolic syndrome. Therefore, to rule out the influence of drinking status, we re-analyzed these relationships after stratifying the sample based on the amount of alcohol consumed. As shown in Fig. 4d, the serum GGT level showed a significant negative correlation with serum T-SOD activity in all subgroups stratified by drinking status. Furthermore, the serum GGT level showed
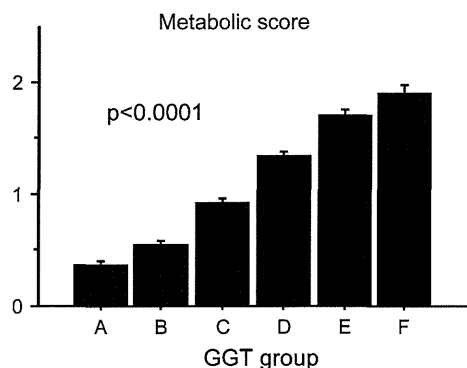
Fig. 1 Relationship of serum gamma-glutamyltransferase (*GGT*) level to serum total superoxide dismutase (*T-SOD*) activity and lipid peroxide level. *Bar graph* shows serum T-SOD activity (**a**) and lipid peroxide (*LPO*) level (**b**) according to sextiles of serum GGT level

Fig. 2 Correlation between serum GGT level and metabolic syndrome. *Bar graph* shows metabolic score according to sextiles of serum GGT level
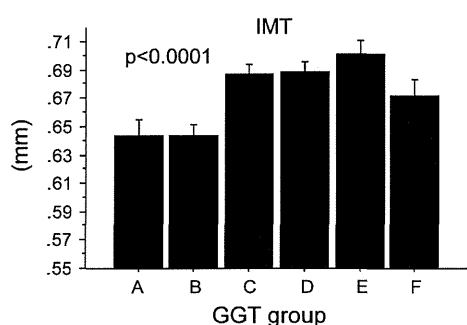

Fig. 3 Correlation between serum GGT level and atherosclerosis. *Bar graph* shows intima-media thickness (*IMT*) according to sextiles of serum GGT level

a clear positive correlation with the metabolic score in all subgroups (Fig. 4e). These results suggest that the serum GGT level was correlated with serum T-SOD activity and the metabolic syndrome, independent of drinking status.

Gender-separated analysis

Because there is a gender difference in serum GGT levels, we performed a gender-separated analysis to identify correlations among serum GGT level, oxidative stress, and the metabolic syndrome. The serum GGT level was more strongly correlated with oxidative stress in males than in females (Fig. 5a, b). In contrast, the metabolic score showed similar positive correlations with the serum GGT level in both genders (Fig. 5c).

Influence of fatty liver on the relationship between serum GGT and serum SOD activity

Because the serum GGT level is also affected by the presence of fatty liver [9], we assessed the influence of fatty liver on the relationship between serum GGT and serum SOD activity. Among the 2249 subjects who

underwent abdominal ultrasonography, a fatty liver was found in 652 (28.9%). The serum GGT level was significantly higher in subjects with a fatty liver (66.9 ± 76.2 IU/L) than in subjects without a fatty liver (46.3 ± 51.2 IU/L; $P < 0.0001$). However, serum T-SOD activity was significantly lower in subjects with a fatty liver (2.84 ± 1.08 U/mL) than in subjects without a fatty liver (3.00 ± 1.30 U/mL; $P = 0.007$), suggesting that the presence of fatty liver was associated with decreased serum T-SOD activity. Next, we conducted a subgroup analysis for the correlation between serum GGT and serum SOD activity, stratified according to the presence of fatty liver. Subjects without a fatty liver showed a significant negative correlation between the serum GGT level and serum SOD activity ($\rho = -0.158$, $P < 0.0001$), whereas subjects with a fatty liver showed a similar but statistically insignificant tendency toward a negative correlation ($\rho = -0.067$, $P = 0.08$). Thus, the presence of fatty liver may have partially confounded the relationship between the serum GGT level and serum T-SOD activity.

Discussion

In the present study, by analyzing a large dataset of subjects who underwent general health screening, we found that serum GGT levels, even within the normal range, closely reflected oxidative stress and metabolic syndrome. To our knowledge, this is the first report describing the relationship between serum GGT level and serum T-SOD activity.

GGT increases as an adaptive response upon exposure to oxidative stress, and GGT metabolizes extracellular GSH to provide component amino acids for intracellular GSH resynthesis [10–12]. GSH protects cells from oxidative stress by reacting with hydrogen peroxide, superoxide anions, singlet oxygen, and hydroxyl radicals [21]. GSH has been implicated in protection against ROS-mediated cell death in a variety of cell types [21, 22]. Thus, a higher serum GGT level may reflect chronic depletion of intracellular GSH due to the high accumulation of oxidative stress, which can lead to various diseases.

GGT is directly involved in ROS generation. This concept is based on the experimental findings that cysteinylglycine, a product of GGT action on GSH, has a strong ability to reduce Fe^{3+} to Fe^{2+}, which promotes ROS generation [13]. Furthermore, GGT-generated glutathione hydrolysis triggers iron-catalyzed LDL oxidation, which promotes plaque [23]. In fact, the serum GGT level was significantly correlated with carotid artery IMT in our study. However, IMT in group F, which had the highest serum GGT, was slightly decreased compared with results in groups C–E (Fig. 4). This finding may have been
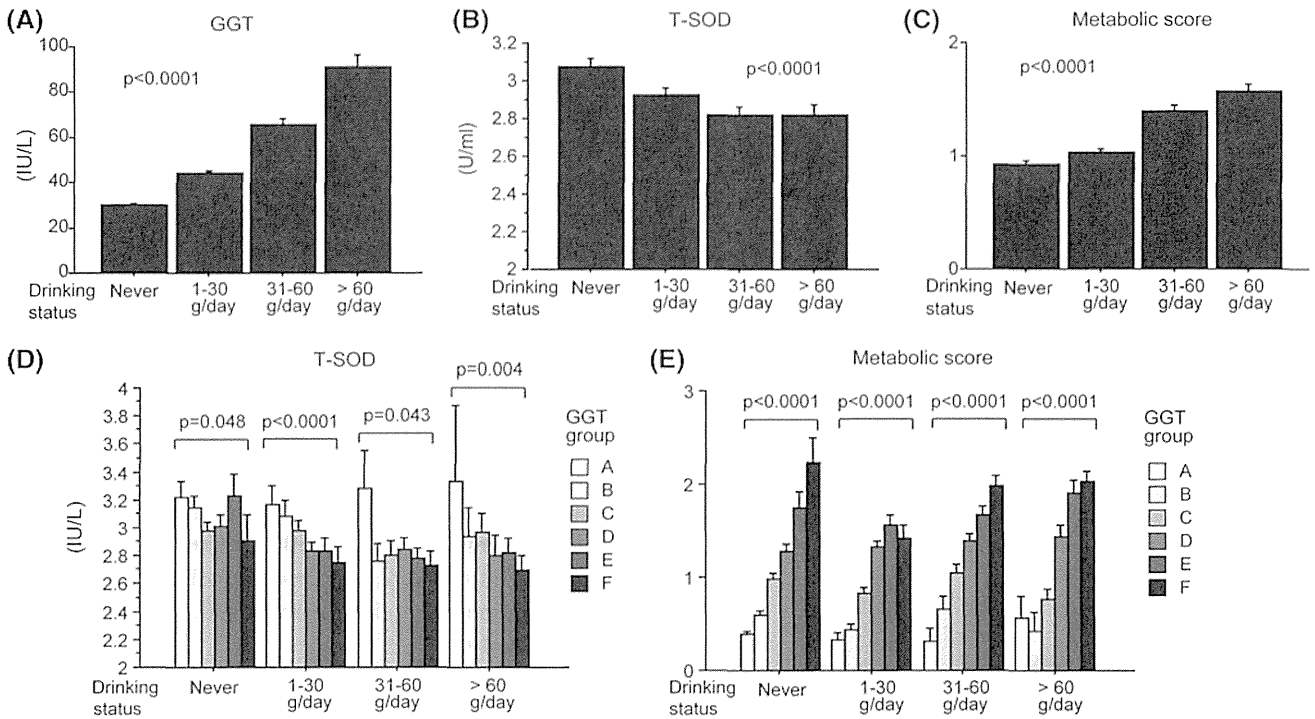
(A) GGT

(B) T-SOD

(C) Metabolic score

(D) T-SOD

(E) Metabolic score

Fig. 4 Influence of drinking status on the relationship of serum GGT level to serum T-SOD activity and metabolic syndrome. Influence of drinking status on serum GGT level, serum T-SOD activity, and metabolic score is shown. *Bar graph* shows serum GGT level (**a**),

serum T-SOD activity (**b**), and metabolic score (**c**) according to drinking status. *Bar graph* also shows the correlation between serum GGT and serum T-SOD activity (**d**), and the correlation between serum GGT and metabolic score (**e**) stratified by drinking status

Fig. 5 Gender separated analysis of serum GGT level as the marker of oxidative stress and metabolic syndrome. *Bar graph* shows (**a**) serum T-SOD level, (**b**) serum lipid peroxide level, and (**c**) metabolic score according to sextiles of serum GGT level

(A) T-SOD

(B) LPO

(C) Metabolic score
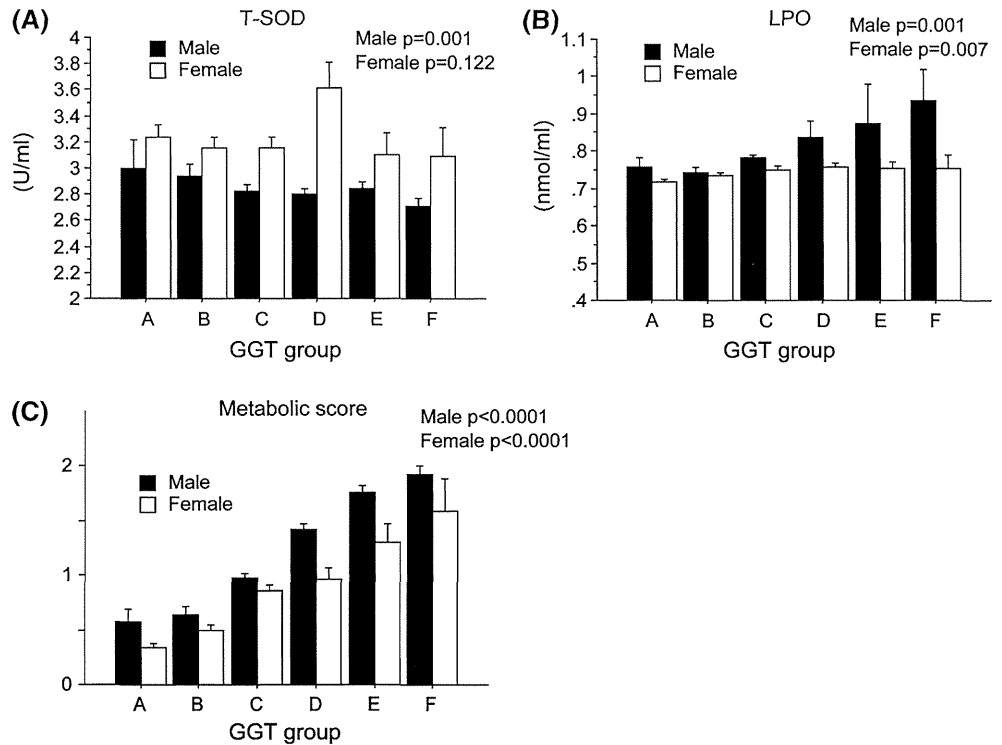
confounded by the protective effect of moderate alcohol consumption on atherosclerosis.

Our finding that serum GGT was negatively correlated with serum T-SOD activity suggests that decreased anti-oxidative stress activity may link the serum GGT level to the progression of various diseases. Serum SOD is the first-line anti-oxidant enzyme defense system, particularly for the endothelium against extracellular ROS, which initiate processes involved in atherogenesis [24, 25]. Thus, GGT may be involved not only in intracellular oxidative stress through GSH synthesis, but also in extracellular oxidative stress by modulating SOD expression. A previous report showed that CuZn-SOD mRNA was upregulated in GGT mutant mice [26], suggesting that GGT may be directly correlated with SOD activity. However, in our study, a subgroup analysis according to the presence of fatty liver indicated that a fatty liver may have partially confounded the relationship between the serum GGT level and serum T-SOD activity. CuZn-SOD-deficient mice have been shown to exhibit lipid accumulation in the liver, suggesting that decreased SOD activity may lead to fatty liver, which may induce an elevation of serum GGT levels [27]. From the present type of cross-sectional study, we cannot conclude whether there is a causal relationship between GGT and SOD activity, so further study is needed. We can at least say that decreased anti-oxidative stress activity may be linked to the serum GGT level and various diseases.

Our gender-separated analysis revealed that the serum GGT level was more strongly correlated with oxidative stress in males than in females. Although we cannot clarify the cause of this difference from this study, this finding may be interesting from the point of view of gender differences in the anti-oxidative stress defense system.

Our study has some limitations. First, we did not take into account the amount of coffee intake, which might affect GGT levels and oxidative stress [28]. Second, as mentioned above, our cross-sectional study design could not identify causal relationships among GGT, SOD activity, and metabolic syndrome. These relationships may be based on genetic background; for example, a single nucleotide polymorphism of SOD genes, or may simply represent a reactive phenomenon against ROS accumulation. Third, we did not exclude subjects who were taking antihypertensive and/or antidiabetic drugs, and this lack of exclusion could potentially affect the results of our study. In addition, we did not have sufficient information about the ingestion of anti-oxidative supplements that may have reduced the level of serum oxidative stress markers.

In conclusion, we showed that the serum GGT level, even within the normal range, was significantly associated with anti-oxidative stress activity, the accumulation of oxidative stress, the metabolic syndrome, and atherosclerosis. Measurement of the serum GGT level is simple and inexpensive, and it can be used as a sensitive marker of oxidative stress and metabolic syndrome. Furthermore, we should consider serum GGT levels during health examinations, even when these levels are within the normal range.

Conflict of interest None.

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Research Article

Increased activity of serum mitochondrial isoenzyme of creatine kinase in hepatocellular carcinoma patients predominantly with recurrence

Yoko Soroida[1,†], Ryunosuke Ohkawa[1,†], Hayato Nakagawa[1,2], Yumiko Satoh[1], Haruhiko Yoshida[2], Hiroto Kinoshita[2], Ryosuke Tateishi[2], Ryota Masuzaki[2], Kenichiro Enooku[1,2], Shuichiro Shiina[2], Takahisa Sato[3], Shuntaro Obi[3], Tadashi Hoshino[4], Ritsuko Nagatomo[1], Shigeo Okubo[1], Hiromitsu Yokota[1], Kazuhiko Koike[2], Yutaka Yatomi[1], Hitoshi Ikeda[1,2,*]

[1]Department of Clinical Laboratory Medicine, Graduate School of Medicine, The University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-8655, Japan; [2]Department of Gastroenterology, Graduate School of Medicine, The University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-8655, Japan; [3]Department of Hepatology, Kyoundo Hospital, 1-8 Kandasurugadai, Chiyoda-ku, Tokyo 101-0062, Japan; [4]Division of Laboratory Medicine, Department of Pathology and Microbiology, Nihon University School of Medicine, 30-1 Oyaguchi-kamimachi, Itabashi-ku, Tokyo 173-8610, Japan

Background & Aims: Mitochondrial isoenzyme of creatine kinase (MtCK) is reportedly highly expressed in hepatocellular carcinoma (HCC). Clinical relevance of serum MtCK activity in patients with HCC was assessed using a novel immuno-inhibition method.
Methods: Among patients with cirrhosis caused by hepatitis B or C virus, 147 patients with HCC (12 with the first occurrence and 135 with recurrence) and 92 patients without HCC were enrolled.
Results: Serum MtCK activity was higher in cirrhotic patients with HCC than in those without HCC or healthy subjects. Elevated serum MtCK activity in HCC patients decreased after radiofrequency ablation. In case of prediction of HCC, MtCK had a sensitivity of 62.6% and a specificity of 70.7% at a cut-off point of 8.0 U/L, with an area under the receiver operating curve of 0.722 vs. 0.713 for alpha-fetoprotein (AFP) and 0.764 for des-gamma-carboxy prothrombin (DCP). Among the HCC patients, serum MtCK activity was elevated in 52.9% individuals with serum AFP level <20 ng/ml and 63.2% individuals with serum DCP level <40 mAu/ml. Even in patients with a single HCC ⩽2 cm, the sensitivity of serum MtCK activity for the prediction of HCC was 64.4%, which was comparable to the overall sensitivity. This increased activity was due to an increase in ubiquitous MtCK,

not sarcomeric MtCK, and the enhanced mRNA expression of ubiquitous MtCK was observed in cell lines originating from HCCs in contrast to healthy liver tissues.
Conclusions: Serum MtCK activity merits consideration as a novel marker for HCC to be further tested as for its diagnostic and prognostic power.
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Introduction

Hepatocellular carcinoma (HCC) is a common malignancy worldwide [1]. Its incidence is currently increasing in many countries [2,3], and it usually develops in the setting of chronic liver injury [3]. Because liver cirrhosis is the strongest risk factor for HCC development, patients with cirrhosis require cancer surveillance. Given the improvements in the overall survival of patients with cirrhosis [4] and the increasing incidence of HCC in many countries, effective strategies for the early detection of HCC are urgently needed, since the prognosis of HCC is deemed poor unless the cancer can be detected and treated at an early stage [5].

Alpha-fetoprotein (AFP) has been the most widely used serum marker for HCC surveillance [5]. Prospective studies assessing AFP as a surveillance tool indicate a sensitivity of 39–64%, a specificity of 76–91%, and a positive predictive value of 9–32% for early HCC [6–8]. Des-gamma-carboxy prothrombin (DCP) is also a specific marker for HCC, but its sensitivity is not sufficiently high, even when combined with AFP [9–11]. Liver ultrasound reportedly has a sensitivity of 78%, a specificity of 91%, and a positive predictive value of 73% for the detection of early HCC [12]. However, the accuracy of ultrasound is operator-dependent, limiting its value as a surveillance test [13]. Thus, additional markers for HCC are still needed.

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Creatine kinase (CK) is a central controller of cellular energy homeostasis. By reversible interconversion of creatine into phosphocreatine, CK builds up a large pool of rapidly diffusing phosphocreatine for temporal and spatial buffering of ATP levels. Thus, CK plays a particularly important role in tissues with large and fluctuating energy demands, such as muscle and brain, and the mitochondrial isoenzyme of CK (MtCK) has been assumed to be important for the energetics of oxidative tissues [14], suggesting that MtCK also plays a pivotal role in malignant tissues. Indeed, overexpression of MtCK has been reported in malignant liver tissue [15], and the increased activity of serum MtCK has been reported in patients with malignant tumors including hepatic cancer, gastric cancer, and lung cancer [16]. Furthermore, the elevated activity of MtCK was recently determined in tissue samples of HCC [17]. These findings suggest that MtCK activity may be useful as a serum marker for HCC. However, Castaldo et al. reported that serum MtCK activity was detected in only 16% of HCC patients [18].

In these previous studies, serum MtCK level was measured using electrophoresis and densitometry [16,18]. On the other hand, a novel method for directly determining the enzymatic activity of MtCK has been recently established [19], and this method may have a better sensitivity and accuracy for the measurement of MtCK activity than the previous method. In the present study, we sought to examine the status of serum MtCK activity in patients with HCC using this novel method.

Patients and methods

Subjects

Consecutive HCC patients with cirrhosis caused by hepatitis B virus (HBV) or hepatitis C virus (HCV), who were treated at the Department of Gastroenterology, of the University of Tokyo Hospital, Tokyo, Japan, between January and April 2010, were enrolled (n = 147). Patients with cirrhosis caused by HBV or HCV but who did not have HCC (n = 92) were also enrolled. Diagnosis of cirrhosis was based on the presence of clinical and laboratory features indicating portal hypertension (the presence of esophageal varices and/or collateral circulation as observed using endoscopy, ultrasonography, CT or MRI). The diagnosis of HCC was made by dynamic CT or MRI [20], with hyperattenuation during the arterial phase and washout during the late phase regarded as definite signs of HCC [21]. The absence of HCC was determined by surveillance ultrasonography or by dynamic CT or MRI. Blood samples were drawn within one month after the diagnosis and prior to the initiation of treatment in HCC patients. In non-HCC patients, blood samples were obtained within one month since the last surveillance imaging, and the absence of HCC was confirmed at least 6 months after the analysis of blood samples. Whole blood specimens were also obtained from 61 healthy controls without liver damage.

In addition, HCC patients with cirrhosis and advanced lesions, i.e., a maximum diameter of 6 cm or larger, diffuse liver lesions, portal vein tumor thrombosis and/or extrahepatic metastasis, who visited the Department of Hepatology, Kyoundo Hospital, Tokyo, Japan, between December 2008 and September 2011, were also enrolled (n = 20). This study was carried out in accordance with the ethical guidelines of the 1975 Declaration of Helsinki and was approved by the Institutional Research Ethics Committees of the authors' institutions. Informed consent was obtained for the use of the samples in this study.

Measurement of MtCK activity

The MB fraction of creatine kinase (CK-MB), known as a serum marker for myocardial infarction, has been conventionally measured using an immuno-inhibition method against the MM fraction of creatine kinase (CK-M); however, the appearance of MtCK in serum can render this measurement inaccurate. To resolve this problem, a novel immuno-inhibition method has been recently developed using two types of anti-MtCK monoclonal antibodies in addition to an anti-CK-M antibody [19]. Using this new method, we were able to focus on the measurement of MtCK activity, adjusting the results according to the presence or absence of anti-MtCK monoclonal antibodies during CK-MB measurement. To measure

ubiquitous and sarcomeric MtCK, anti-ubiquitous MtCK antibody and anti-sarcomeric MtCK antibody (a kind gift from Shino-test Corporation and Sysmex Corporation) [19] were used, respectively. JCA-BM8040 (JEOL, Tokyo, Japan) was used as an automatic analyzer.

The regression line of this assay was linear up to at least 1800 U/L. The minimum detection limit was 1.9 U/L. The within-run coefficient variations were 3.1% and 0.8% at the mean MtCK activities of 25.7 and 64.4 U/L, respectively. The between-run coefficient variations were 2.3% for both the mean MtCK activities of 24.0 and 59.5 U/L.

Radiofrequency ablation (RFA)

Among 147 patients with HCC, 112 patients were treated using RFA with curative intent, the detailed procedure of which has been meticulously described elsewhere [22]. In some of these patients, serum MtCK activity was measured after RFA.

MtCK and other CK isoenzyme analyses using electrophoresis and immunoblotting

MtCK and other CK isoenzyme analyses were performed using electrophoresis according to a previously described method [19], where 30 μl of serum was analyzed with or without prior incubation with 1 μl of anti-CK-M antibody (a kind gift from Shino-test Corporation and Sysmex Corporation) for 5 min at room temperature.

The serum samples were also applied to sodium dodecyl sulfate polyacrylamide gel electrophoresis under reducing conditions, and then transferred to a polyvinylidene difluoride membrane (Invitrogen, Carlsbad, CA, USA). After blocking the membrane with the agent derived from skim milk (Block Ace; Dainippon Sumitomo Pharmaceutical Co., Ltd., Osaka, Japan), it was incubated with anti-ubiquitous MtCK antibody (dilution, 1:1000) or anti-CK-B antibody (dilution, 1:1000, Sigma–Aldrich, Inc., St. Louis, MO USA) overnight at 4 °C and then with horseradish peroxidase-conjugated secondary antibody (dilution, 1:1000) for 1 h at room temperature. Immunoreactive proteins were visualized using a chemiluminescence kit (GE Healthcare, Little Chalfont, Buckinghamshire, UK), and recorded using a LAS-4000 image analyzer (Fuji Film, Tokyo, Japan).

Quantitative real-time PCR

Total RNA of human HCC cell lines, JHH7, Alex, HuH7, and HepG2 (obtained from Health Science Research Resources Bank, Japan Health Science Foundation) was extracted using TRIZOL reagent (Invitrogen). Human liver RNA was purchased from Cell Applications Inc. (San Diego, CA, USA). One microgram of purified total RNA was transcribed using a SuperScriptTM First-Strand Synthesis System for RT-PCR (Invitrogen). A real-time PCR was performed with the same sets of ubiquitous MtCK primers (5'-CCTGCTAAGCAAAGATAGCC-3' and 5'-TAATGCTTGGTGTGGAT-GAC-3') and 18s rRNA primers (5'-GTAACCCGTTGAACCCCATT-3' and 5'-CCATC-CAATCGGTAGTAGCG-3'). The PCR reactions were performed in a Light Cycler 2.0 instrument (Roche Molecular Diagnostics, Mannheim, Germany) using the LightCycler FastStart DNA Master SYBR Green I kit (Roche Molecular Diagnostics). The samples were incubated initially for 10 min at 95 °C, followed by 45 cycles of 95 °C for 10 sec, 60 °C for 10 sec, and 72 °C for 10 sec. The relative amount of ubiquitous MtCK was determined from the respective standard curves and normalized to the signal of 18s rRNA.

Statistical analysis

Comparisons of the distributions of demographic and clinical variables among the groups were performed using Mann–Whitney U test or Chi-square test. Wilcoxon's signed rank test was used to compare the serum MtCK activities before and after RFA. A two-sided significance level of 5% was used for all the analyses. Data processing and analysis were performed using SPSS software version 17.0 or 19.0 (SPSS, Inc., Chicago, IL).

Results

Subjects

Clinical and laboratory variables of the cirrhotic patients with or without HCC are shown in Table 1. These variables did not differ

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Table 1. Characteristics of cirrhotic patients with and without HCC.

	Cirrhotic patients		p value
	without HCC	with HCC	
No. of patients	92	147	
Age (yr)	67.9 ± 10.8	71.0 ± 7.2	0.127
Gender (M:F)	34:58	92:55	<0.001
HBV:HCV	5:87	12:135	0.425
AST (IU/L)	55.1 ± 30.5	60.9 ± 37.2	0.319
ALT (IU/L)	46.3 ± 31.5	47.0 ± 32.2	0.900
Albumin (g/dl)	3.7 ± 0.6	3.5 ± 0.6	0.001
Bilirubin (mg/dl)	1.2 ± 1.1	1.1 ± 0.7	0.332
MtCK (U/L)	7.4 ± 6.2	14.3 ± 11.9	<0.001
AFP (ng/ml)	20.5 ± 38.5	289.6 ± 1066.3	<0.001
DCP (mAU/ml)	19.1 ± 12.3	318.7 ± 1065.2	<0.001
Platelet count (×10⁴/μl)	9.5 ± 4.4	9.6 ± 4.2	0.887
APRI	13.9 ± 10.6	14.1 ± 10.1	0.450
Number of lesions	n.a.	2.8 ± 3.1	
1		73	
2		25	
3		18	
4		9	
≥5		22	
Maximum tumor diameter (cm)	n.a.	1.9 ± 1.1	
≤2.0		107	
2.1-3.0		26	
>3.0		14	
Portal vein thrombosis	n.a.	7	
Metastasis	n.a.	0	

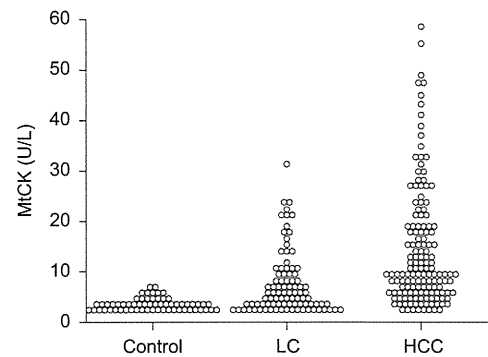Data provided are means ± SD.
n.a., not available.

Fig. 1. Scatter plots showing serum MtCK activity in control subjects (Control), cirrhotic patients without HCC (Cirrhosis), and cirrhotic patients with HCC (HCC). Mean serum MtCK activity in cirrhotic patients with HCC (14.3 U/L) was significantly higher than that in patients without HCC (7.3 U/L; $p < 0.001$) and control subjects (3.4 U/L; $p < 0.001$).

significantly between the two groups, except for sex, serum albumin level, serum MtCK activity, serum AFP level and serum DCP level. Serum MtCK activity did not differ between men and women in either the healthy subjects (3.2 ± 1.1 and 3.6 ± 1.4 U/L, respectively; $p = 0.535$) or the subjects overall (10.4 ± 10.9 and 9.4 ± 8.8 U/L, respectively; $p = 0.623$). A significant but small difference was seen in serum albumin levels between cirrhotic patients with HCC and those without HCC; however, other variables suggesting the grade of liver fibrosis, such as serum bilirubin level, platelet count, or the aspartate aminotransferase-to-platelet ratio index (APRI; calculated as aspartate aminotransferase [U/L]/upper normal × 100/platelet count [10^9/L]) were not significantly different between the two groups (Table 1).

Increased serum MtCK activity in patients with HCC

Serum MtCK activity was significantly elevated in cirrhotic patients with HCC, compared with healthy subjects, as shown in Fig. 1 ($p < 0.001$): the mean serum MtCK activity was 14.3 U/L in the former group and 3.4 U/L in the latter group. Serum MtCK activity in the cirrhotic patients without HCC was 7.4 U/L, which

was also significantly higher than that in the healthy subjects ($p < 0.001$). However, serum MtCK activity was significantly higher in the cirrhotic patients with HCC than in those without HCC, as depicted in Fig. 1 ($p < 0.001$). In addition, serum MtCK activity did not differ between patients with HBV and those with HCV (14.3 ± 11.1 and 11.3 ± 10.5 U/L, respectively; $p = 0.163$). Serum MtCK activity in the HCC patients according to BCLC stage is shown in Table 2, in which the significant correlation between serum MtCK activity and BCLC stage was not observed. Serum MtCK activity in patients with a single HCC ≤2 cm was increased and not different from that in patients with HCC with multiple lesions and/or larger than 2 cm (Table 2).

Because consecutive HCC patients with cirrhosis, who visited our department between January and April 2010, were enrolled in this study, there were 12 patients, who developed HCC for the first time, while recurrences had occurred in 135 patients. Serum MtCK activity was not significantly different between the two groups; 10.0 ± 5.2 U/L in the former, and 14.7 ± 12.2 U/L in the latter ($p = 0.430$).

We could measure serum MtCK activity in 14 patients, who had higher levels of serum MtCK activity prior to the treatment and underwent RFA with curative intent, at 2 to 12 weeks following the treatment. In these patients, although its number was small, serum MtCK activity was decreased significantly after RFA (n = 14, $p = 0.001$).

Sensitivity and specificity of MtCK, AFP, and DCP for differentiating HCC from cirrhosis without HCC

To examine a potential predictability of serum MtCK activity for HCC, receiver operating curves (ROCs) were plotted to define the optimal cut-off values and to identify the sensitivity and specificity of MtCK, AFP, and DCP for differentiating cirrhotic patients with HCC from those without HCC (Fig. 2 and Table 3). The area under the receiver operating curve (AUROC) for serum MtCK activity was 0.722 (95%CI: 0.658 – 0.786), with a sensitivity of 62.6%, a specificity of 70.7%, a positive predictive value of 77.3%, a negative predictive value of 54.2%, and a cut-off point of 8.0 U/L; the AUROC for serum AFP level was 0.713 (95%CI: 0.649 – 0.777), with a sensitivity of 52.4%, a specificity of 76.8%, and a cut-off of 20 ng/ml (recommended cut-off for AFP); and the AUROC for serum DCP level was 0.764 (95%CI: 0.705 –

Table 2. HCC stage and serum MtCK activity.

	With the 1st occurrence vs. recurrence[b]	
	Number of patients	MtCK (U/L)
BCLC[a] stage		
0	3 vs. 32	14.6 ± 4.6[c] vs. 9.9 ± 8.3
A	3 vs. 53	8.5 ± 3.0 vs. 15.3 ± 12.6
B	6 vs. 27	8.5 ± 5.4 vs. 16.3 ± 14.2
C	0 vs. 7	n.a.[d] vs. 13.9 ± 16.1
D	0 vs. 16	n.a. vs. 19.7 ± 10.1
Single lesion ≤2 cm	59	14.2 ± 10.9
Others	88	14.4 ± 12.5

[a]Barcelona Clinic Liver Cancer.
[b]BCLC stage at the first diagnosis of patients with recurrence was BCLC 0, 54; A, 58; B, 28; C, 5; D, 1; unknown, 1. Treatment at the first diagnosis of all patients was surgery, 19; RFA, 76; percutaneous ethanol injection therapy, 8; percutaneous microwave coagulation therapy, 1; transcatheter arterial chemoembolization, 42; unknown, 1.
[c]Data provided are means ± SD.
[d]Not applicable.

Table 3. The area under the receiver operating curve (AUROC) for MtCK, AFP, and DCP predicting HCC predominantly with recurrence.

Parameters	AUROC (95% CI)
All HCC	
MtCK	0.722 (0.658-0.786)
AFP	0.713 (0.649-0.777)
DCP	0.764 (0.705-0.822)
HCC with a single lesion ≤2 cm	
MtCK	0.729 (0.648-0.809)
AFP	0.672 (0.581-0.762)
DCP	0.694 (0.608-0.780)

Fig. 3. **Number of HCC patients with positive or negative MtCK, AFP, and DCP.** The cut-off for serum MtCK activity for the prediction of HCC was defined as 8.0 U/L. The cut-offs for AFP and DCP for the prediction of HCC were 20 ng/ml and 40 mAu/ml, respectively.
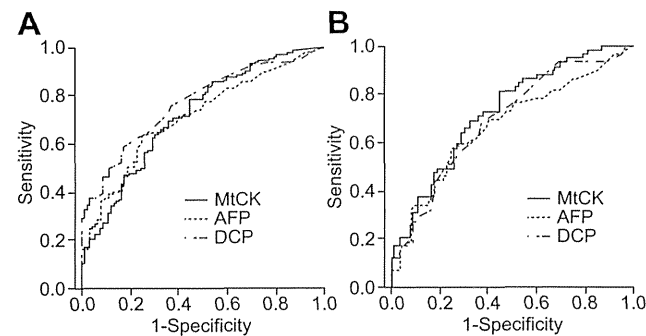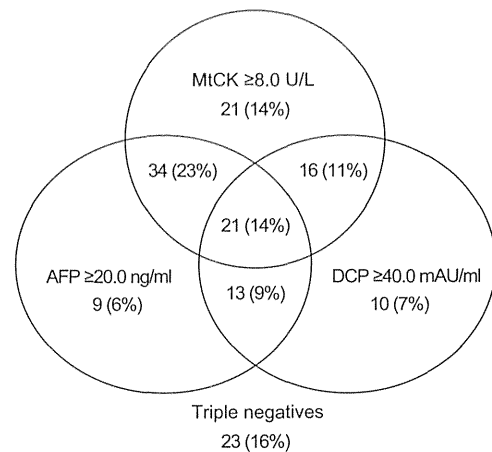

Fig. 2. **ROC curves comparing MtCK, AFP, and DCP in patients with cirrhosis without HCC and with HCC.** The ROC statistical analyses were performed to compare the diagnostic accuracy of MtCK, AFP, and DCP for (A) HCC in cirrhotic patients and for (B) HCC of a single lesion and smaller than 2 cm.

0.822), with a sensitivity of 40.1%, a specificity of 91.9%, and a cut-off of 40 mAu/ml (recommended cut-off for DCP; Fig. 2). Thus, MtCK had an AUROC between that of AFP and DCP.

Using a cut-off value of 8.0 U/L, serum MtCK activity was elevated in 37 of 70 HCC patients with an AFP <20 ng/ml, in 55 of 87 HCC patients with a DCP <40 mAu/ml, and in 21 of 44 patients with an AFP <20 ng/ml and a DCP <40 mAu/ml (Fig. 3). When AFP and MtCK were combined for the diagnosis of HCC, the sensitivity was increased to 77.6%; when DCP and MtCK were combined, the sensitivity was increased to 78.2%. On the other hand, 23 HCC patients (16%) were not diagnosed even with MtCK, AFP, and DCP, in which serum MtCK activity was 4.7 ± 1.8 U/L and HCC had a maximum diameter of 1.7 ± 0.9 cm with 1.8 ± 1.8 lesions.

Because ultrasonography plays an important role in HCC surveillance in Japan, especially in patients with cirrhosis, we wondered whether MtCK could support the diagnostic capability of ultrasonography. In this study, ultrasonography was not capable of detecting HCC in 13 of 147 HCC patients. Among these patients, serum MtCK activity was higher than the cut-off value

of 8.0 U/L in 7 patients (53.8%), among whom only MtCK, but not AFP or DCP levels, were higher than the cut-off values in three patients (23.1%). These results suggest that MtCK activity may support ultrasonography findings for the diagnosis of HCC. In the population of ultrasound-detected HCC, HCC was predictable in 84.7% of those patients with combination of MtCK with a cut-off of 5.6 U/L and AFP with a cut-off of 20 ng/ml, and in 88.3% with combination of MtCK, AFP, and DCP with a cut-off of 40 mAu/ml. On the other hand, in the population of ultrasound-undetected HCC, HCC was predictable in 76.9% of those patients with combination of MtCK with a cut-off of 5.4 U/L and AFP with a cut-off of 20 ng/ml, and in 84.6% with combination of MtCK, AFP, and DCP with a cut-off of 40 mAu/ml.

In addition, serum MtCK activity was higher than 8.0 U/L in 66.7% patients with first HCC occurrence and in 62.2% patients with HCC recurrence. When analyzed among patients with a maximum HCC diameter ≤2 cm with single nodule, the AUROC for MtCK was higher than AFP or DCP (Fig. 2 and Table 3), and serum MtCK activity was higher than 8.0 U/L in 64.4% of those patients, whereas in 62.6% of whole patients. On the other hand, because patients in the current original cohort had mostly small

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HCCs (a maximum diameter of 3 cm or smaller), three or fewer lesions with no extrahepatic metastasis (Table 1), serum MtCK activity was additionally analyzed in more advanced HCC patients. Serum MtCK activity of those patients was 10.4 ± 9.2 U/L (n = 20), which was comparable to, not higher than, that in the HCC patients in the original cohort. Among them, nine patients responded to 5-fluorouracil and interferon-α [23] or transcatheter arterial chemoembolization. Among the responders, serum MtCK activity decreased to 31.7% of the value prior to the treatment in one patient, and overall, serum MtCK activity decreased significantly to 77.1% of the value prior to treatment (n = 9, p = 0.003).

High level of serum ubiquitous MtCK activity in HCC patients and of ubiquitous MtCK mRNA expression in HCC cell lines

Regarding MtCK, two tissue-specific isoenzymes are known, i.e., sarcomeric MtCK is found in striated muscles of vertebrates, while ubiquitous MtCK has been detected in most other tissues including brain, kidney, and sperm [24]. Thus, we examined which of the two isoenzymes was increased in the sera of HCC patients with high levels of MtCK activity. Specific antibodies to sarcomeric MtCK and ubiquitous MtCK were applied separately for the measurement of MtCK activity in 135 patients with HCC. Sarcomeric MtCK activity was under minimum detection limit of 1.9 U/L in 131 patients; in the remaining four patients, sarcomeric MtCK activity was 2.0, 2.2, 2.5, and 2.6 U/L, respectively. In the latter four patients, ubiquitous MtCK activity was 13.6, 5.2, 9.2, and 5.1 U/L, respectively. Thus, a small increase in sarcomeric MtCK activity was observed in only four out of 131 patients, which might be explained by a measurement error near the minimum detection limit. Collectively, the increase in serum MtCK activity in patients with HCC was mostly due to ubiquitous MtCK activity.

To examine other CK isoenzymes, the sera of HCC patients were analyzed using electrophoresis. As shown in Fig. 4A, octameric MtCK bands were found in the samples with high MtCK activities (>30 U/L; lanes 2–8), and dimeric MtCK bands were also found in these samples after incubation with anti-CK-M antibody because of close migration of the dimeric MtCK to the position of CK-MM [19]. Of note, no correlation was seen between serum MtCK activity and serum CK-MM activity or CK-MB activity. The sera of HCC patients were also examined using an immunoblot analysis. As demonstrated in Fig. 4B, serum CK-B did not correlate with serum ubiquitous MtCK, although CK-MB and CK-BB were not analyzed separately. Collectively, no correlation was observed between serum ubiquitous MtCK activity and other serum CK isoenzyme activities.

Finally, ubiquitous *MtCK* mRNA expressions in HCC cell lines, JHH7, Alex, HuH7, and HepG2 were determined using real-time PCR. The ratio of ubiquitous *MtCK* mRNA to *18s* rRNA was much higher in HCC cell lines than in the normal human liver, as depicted in Fig. 4C.

Discussion

Healthy liver tissue is one of the few tissues that, in general, do not express detectable amounts of MtCK or cytosolic CK isoforms [14]. Thus, their expression in the liver is assumed to be a sign of pathological development associated with, for example,
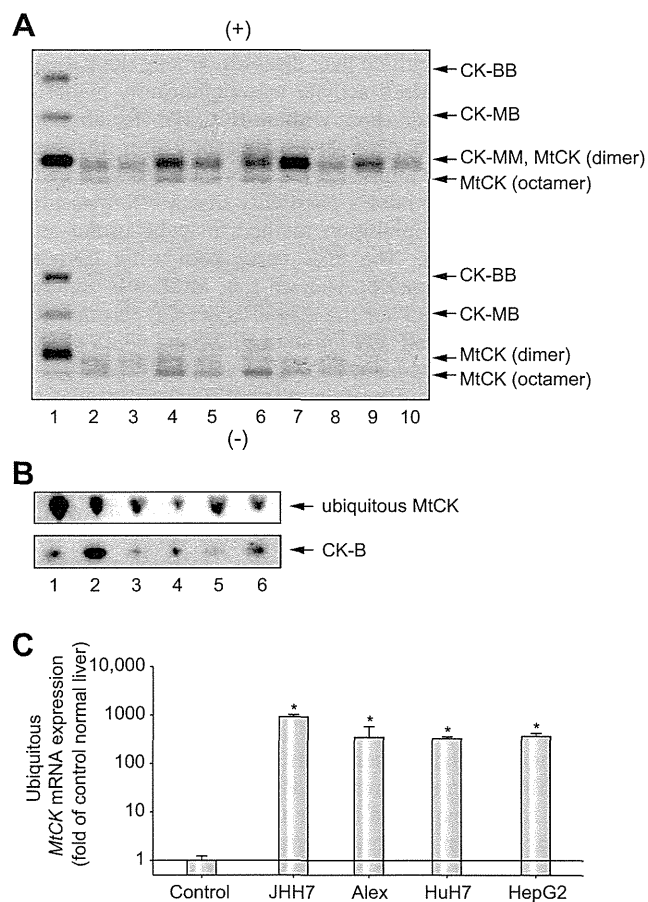

Fig. 4. CK and MtCK isoenzymes in the sera of HCC patients and in HCC cell lines. (A) MtCK and other CK isoenzymes in the sera of HCC patients. The sera of HCC patients with high MtCK activities (>30 U/L; lanes 2–8) and low MtCK activities (<8 U/L; lanes 9–10) were analyzed using electrophoresis with or without prior incubation with anti-CK-M antibody. Lane 1, CK isoenzyme controls. The octameric MtCK bands were found in the samples with high MtCK activities and the dimeric MtCK bands were also found in these samples after incubation with anti-CK-M antibody because of close migration of the dimeric MtCK to the position of CK-MM. (B) Ubiquitous MtCK and CK-B in the sera of HCC patients. The sera of HCC patients with high MtCK activities (>30 U/L; lanes 1–2), intermediate MtCK activities (8–9 U/L; lanes 3–4) and low MtCK activities (<3 U/L; lanes 5–6) were examined using an immunoblot analysis for ubiquitous MtCK and CK-B. (C) Ubiquitous *MtCK* mRNA expression in HCC cell lines and the control normal liver. Ubiquitous *MtCK* mRNA expression in human HCC cell lines, JHH7, Alex, HuH7, and HepG2, and the control normal liver was quantitated using real-time PCR, and the relative amount was normalized to the signal of *18s* rRNA. Columns and bars represent means ± SD of duplicate samples. The asterisk (*) indicates a significant difference from the control normal liver.

ischemic-reperfusion injury [25] or tumor formation [15]. The enzyme described as "Macro CK" [26,27] in previous reports has in fact been identified as ubiquitous MtCK, and a correlation between serum ubiquitous MtCK level and the pathological condition of nephrotoxicity in HIV patients receiving tenofovir has been reported [28]. Although the role of CK expression in the pathological liver has not been fully elucidated, CK expression in the liver of transgenic mice reportedly provokes tolerance against tumor necrosis factor-α-induced apoptosis [29], protection against hypoxia or endotoxin perfusion [30–32], and inhibition of pro-apoptotic mechanisms [33], suggesting a beneficial role of CK expression in the liver.

In the current study, serum activity of MtCK was significantly higher in patients with cirrhosis and HCC caused by HBV or HCV virus than in subjects with no liver diseases. Among the patients with cirrhosis, serum MtCK activity was significantly higher in patients with HCC than in those without HCC. We also observed that serum MtCK activity decreased significantly after treatment with RFA, although the number of patients analyzed was small. Thus, our findings may raise a possibility that MtCK, measured by the novel immune-inhibition method, may be useful as a serum marker of HCC. The ROC curve comparing cirrhotic patients with or without HCC showed that MtCK was superior to AFP but inferior to DCP for the diagnosis of HCC. Serum MtCK activity above this cut-off was found in 52.9% and 63.2% of HCC patients with AFP levels below 20 ng/ml and DCP levels below 40 mAu/ml, respectively, suggesting the potential utility of MtCK for the diagnosis of HCC in patients with normal or mildly elevated AFP and/or DCP levels. Furthermore, serum MtCK activity was also useful for predicting a single HCC $\leqslant 2$ cm in diameter, suggesting the potential usefulness of serum MtCK activity to detect early HCC.

As described earlier, MtCK once attracted attention as a potential tumor-associated marker in the serum including HCC [15], however, the serum MtCK level was not judged to be a useful marker of HCC [18]. Previous investigations reported that an increase in serum MtCK level was detectable only in cases with advanced HCC [16] and that the sensitivity of serum MtCK level for the diagnosis of HCC was relatively low [18]. In contrast, a relatively higher sensitivity of serum MtCK activity for the detection of a single HCC $\leqslant 2$ cm was observed in the current study. These differences can be explained by the methodology used to measure MtCK activity. In the previous studies, MtCK level was measured using electrophoresis and densitometry [16,18]. On the other hand, the enzymatic activity of MtCK was directly determined following the immuno-inhibition in the current study. The presently reported method may be superior to previous methods for quantifying MtCK activity. Furthermore, MtCK is known to exist in the serum as a dimer and an octamer [14]. After electrophoresis, dimeric MtCK is found close to the electrophoretic position of CK-MM, while the octameric MtCK is electrophoresed cathodic to CK-MM [34]. This close migration of the dimeric MtCK to the position of CK-MM in the zymogram could cause overlapping of the dimeric MtCK with the CK-MM band. In fact, the dimeric MtCK was missed in the evaluation of MtCK activity in a previous study [16] and the current study. In contrast, our current method is free from this problem, being capable of measuring both dimeric and octameric MtCK [19]. Collectively, the utility of MtCK as a serum marker for HCC has been clarified as a result of this improved methodology.

Another advantage of this novel method is its applicability for an automatic analyzer. Using this method, serum MtCK activity of a large number of serum samples can be quickly measured, reducing the turnaround time of routine laboratory tests and ultimately increasing its value when used in the clinical setting.

When considering serum MtCK activity as a potential marker for HCC, its limitation is that the correlation between serum MtCK activity and the stage of HCC was not observed in contrast to the previous reports [16,18]. Because CK including MtCK is not naturally secreted from the cells, it is speculated that the active release of MtCK from the tissue with the higher expression of MtCK may be necessary for its serum activity to be increased. Although a higher mRNA expression of ubiquitous MtCK in four

HCC cell lines than in the normal liver tissue was determined in the current study, the releasing mechanism of MtCK into the blood stream in HCC remains to be clarified. If this releasing mechanism might not be correlated with the stage of HCC, it may explain the failed correlation between serum MtCK activity and the stage of HCC. This potential releasing mechanism may include mitochondrial dysfunction as the commitment step in hepatocyte cell death [35]. Because continuous hepatocyte cell death is a main feature of liver cirrhosis [36], mitochondrial dysfunction may be linked to the abundant appearance of MtCK in the blood of cirrhotic patients. It should be further elucidated whether this mitochondrial dysfunction may be involved in the release of MtCK also in the HCC tissue.

As another limitation of this study, it should be noted that the analyzed HCC patients were predominantly those with recurrence, because they were enrolled consecutively. Thus, the performance of MtCK to predict HCC at the first occurrence in cirrhotic patients, especially a less than 2 cm HCC detected at an ultrasound screening, should be further evaluated.

Although the correlation between serum MtCK activity and the stage of HCC was not observed, its increase in patients with early HCC should be noted. Unlike AFP or DCP, the performance of MtCK for the prediction of early HCC was not reduced compared to that of all HCCs. On the other hand, the increase of serum MtCK activity has not been observed in early stage of gastric cancer or colorectal cancer (data not shown). It is possible that the increase of serum MtCK activity in its early stage may be a specific phenomenon of HCC. In conclusion, serum MtCK activity merits consideration as a novel marker for HCC to be further tested as for its diagnostic and prognostic power.

Conflict of interest

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

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CME

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Radiofrequency Ablation for Hepatocellular Carcinoma: 10-Year Outcome and Prognostic Factors

LIVER

Shuichiro Shiina, MD, PhD[1], Ryosuke Tateishi, MD, PhD[1], Toru Arano, MD[1], Koji Uchino, MD[1], Kenichiro Enooku, MD, PhD[1], Hayato Nakagawa, MD, PhD[1], Yoshinari Asaoka, MD, PhD[1], Takahisa Sato, MD, PhD[1], Ryota Masuzaki, MD, PhD[1], Yuji Kondo, MD, PhD, Tadashi Goto, MD, PhD[1], Haruhiko Yoshida, MD, PhD[1], Masao Omata, MD, PhD[1] and Kazuhiko Koike, MD, PhD[1]

OBJECTIVES: Radiofrequency ablation (RFA) is widely performed for hepatocellular carcinoma (HCC). However, there has been no report on 10-year outcome of RFA. The objective of this study was to report a 10-year consecutive case series at a tertiary referral center.

METHODS: We performed 2,982 RFA treatments on 1,170 primary HCC patients and analyzed a collected database.

RESULTS: Final computed tomography images showed complete tumor ablation in 2,964 (99.4%) of 2,982 treatments performed for the 1,170 primary HCC patients. With a median follow-up of 38.2 months, 5- and 10-year survival rates were 60.2% (95% confidence interval (CI): 56.7–63.9%) and 27.3% (95% CI: 21.5–34.7%), respectively. Multivariate analysis demonstrated that age, antibody to hepatitis C virus (anti-HCV), Child-Pugh class, tumor size, tumor number, serum des-γ-carboxy-prothrombin (DCP) level, and serum lectin-reactive α-fetoprotein level (AFP-L3) were significantly related to survival. Five- and 10-year local tumor progression rates were both 3.2% (95% CI: 2.1–4.3%). Serum DCP level alone was significantly related to local tumor progression. Five- and 10-year distant recurrence rates were 74.8% (95% CI: 71.8–77.8%) and 80.8% (95% CI: 77.4–84.3%), respectively. Anti-HCV, Child-Pugh class, platelet count, tumor size, tumor number, serum AFP level, and serum DCP level were significantly related to distant recurrence. There were 67 complications (2.2%) and 1 death (0.03%).

CONCLUSIONS: RFA could be locally curative for HCC, resulting in survival for as long as 10 years, and was a safe procedure. RFA might be a first-line treatment for selected patients with early-stage HCC.

INTRODUCTION

Hepatocellular carcinoma (HCC) is the fifth most common malignant neoplasm in the world (1). Only 20% of HCC patients are candidates for resection (2). Furthermore, recurrence is frequent even after apparently curative resection. Liver transplantation is restricted by organ donor shortage. Thus, various nonsurgical therapies have been introduced (3–5). Among these, image-guided percutaneous ablation is considered best for early-stage HCC.

Ethanol injection was formerly the standard procedure among the various percutaneous ablation techniques. Randomized controlled trials, however, have demonstrated that radiofrequency ablation (RFA) has a more reliable local antitumor effect, leading to a lower local tumor progression risk and higher survival rates (6–9). RFA has largely replaced ethanol injection (10).

Several reports on 5-year outcome of RFA exist (11–17); however, no study has covered 10-year outcome. We report on a 10-year consecutive case series at a tertiary referral center. We analyzed antitumor effect, patient survival, local tumor progression, and distant recurrence rates, variables relevant to these outcomes, and complications. To our knowledge, this study documents the largest number of RFA treatments performed at a single institution.

METHODS

RFA indications

RFA was the treatment of choice in HCC patients satisfying the following criteria: (i) ineligible for surgical resection/liver transplantation or patient refusal for surgery; (ii) no extrahepatic metastasis/vascular invasion; and (iii) no other malignancies that
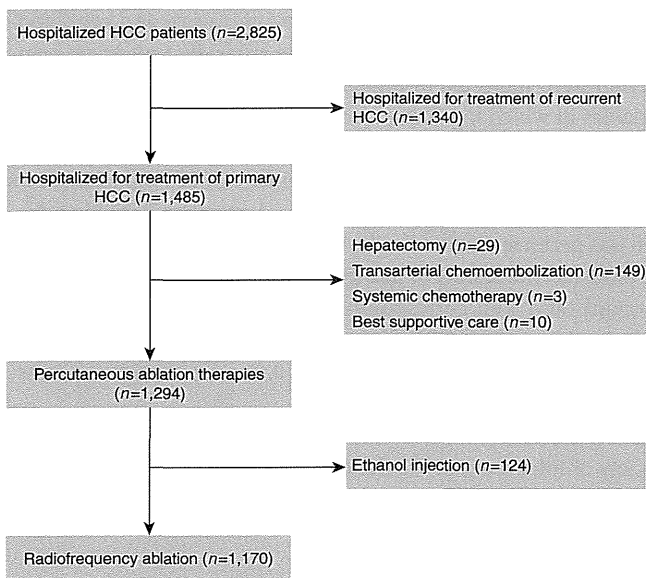
Figure 1. Flow of patients in this study. HCC, hepatocellular carcinoma.

may determine the patient's prognosis. Exclusion criteria were as follows: (i) tumor not visualized by ultrasonography/not accessible percutaneously; (ii) total bilirubin level ≥3.0 mg/dl; (iii) platelet count < 50×10⁹/l or prothrombin activity < 50%; (iv) refractory ascites; (v) enterobiliary reflux; and (vi) adhesion between the tumor and the gastrointestinal tract. In general, we performed RFA on Child-Pugh class A or B patients, a single tumor ≤5 cm in diameter, or three or fewer tumors ≤3 cm in diameter. In cases beyond these conditions, we performed RFA on patients who were likely to benefit from this procedure for possible cure or prolongation of life. No patients were excluded solely on account of tumor location (18). Informed consent was obtained from each patient. This study was conducted according to the ethical guidelines of the 1975 Declaration of Helsinki and approved by the institutional review board (Registration ID: P98C05-11Y).

Patients

In this cohort study, we analyzed a prospectively collected computerized database. Between February 1999 and December 2009, 2,825 HCC patients were admitted once or more to the Department of Gastroenterology, the University of Tokyo (**Figure 1**). At initial hospitalization, 1,485 had primary HCC and the remaining 1,340 had recurrent HCC. In the recurrent HCC patients, primary HCC had previously been treated by therapies other than RFA.

Of the 1,485 primary HCC patients, 1,294 (87.1%) underwent percutaneous ablation as the initial treatment, including RFA. The remaining 191 patients underwent other therapies: hepatic resection, 29 patients with good liver function and who consented to an operation; transarterial chemoembolization, 149 with multinodular or large tumors that could not be treated by ablation therapies; systemic chemotherapy, three with extrahepatic metastasis; and only supportive care, 10 with decompensated cirrhosis or poor general condition.

Of the 1,294 patients treated by percutaneous ablation, 1,170 underwent RFA and the other 124 underwent ethanol injection. The choice of therapy was made as follows: between April 1999 and January 2001, 232 patients with three or fewer tumors, each ≤3 cm in diameter, and Child-Pugh class A or B liver function were entered into a randomized controlled trial to compare RFA with ethanol injection (6). Patients outside these inclusion criteria were mostly treated by RFA. After this trial, RFA was generally the treatment of choice, and ethanol injection was administered only to those considered unsuitable for RFA; ethanol injection was administered to those with either enterobiliary reflux or adhesion of the tumor to the gastrointestinal tract.

HCC was diagnosed based on typical imaging findings; that is, *early-phase enhancement* and *late-phase contrast washout* on dynamic computed tomography (CT) (19). HCC diagnosis was also confirmed by biopsy in 1,078 (92.1%) of the 1,170 patients with RFA-treated primary HCC. A total of 998 (85.3%) were diagnosed as having liver cirrhosis.

In general, transarterial chemoembolization was combined with RFA in patients with either ≥4 tumors or those with even one tumor > 3.0 cm in diameter, although indication criteria of this combination had changed over time. The combination of transarterial chemoembolization with RFA was performed in 324 primary HCC patients.

Treatment methods

RFA was performed on an inpatient basis. Preoperative planning including evaluation of all imaging studies, and careful ultrasound examination was performed to identify the tumors and determine the access routes.

The procedure was performed according to an institutional protocol and in the presence of three physicians. One physician inserted the electrode under ultrasound guidance while another assisted the procedure; at least one had 8-year or longer experience of percutaneous ablation therapies. The remaining physician was responsible for the ultrasound machine and data recording. Video recording was performed occasionally to improve and standardize the procedure.

The precise techniques of RFA are described elsewhere (6). Briefly, all RFA procedures were performed percutaneously under ultrasound guidance (Power Vision 8000, Aplio XV or Aplio XG; Toshiba, Tokyo, Japan). We used artificial pleural effusion (20) or artificial ascites (21) for tumors, which were in the hepatic dome or adjacent to the gastrointestinal tract. After administration of sedatives and local anesthesia, a 17-gauge cooled-tip electrode (Cool-Tip; RF Ablation System, Covidien, Boulder, Colombia, CO) was inserted. Radiofrequency energy was delivered for 6–12 min for each application. For large tumors, the electrode was repeatedly inserted into different sites, such that the entire tumor could be enveloped by assumed necrotic volumes. Following the procedure, the patient remained in bed until the next morning.

A CT scan with a 5-mm section thickness was performed 1–3 days after RFA to evaluate technique effectiveness (22). Complete ablation was defined as hypoattenuation of the entire tumor. We intended to ablate not only the tumor but also some of the liver

parenchyma surrounding it. When we suspected that unablated tumor portions remained, the procedure was repeated. We did not predefine the procedure number in a treatment: treatment was generally continued until CT imaging demonstrated necrosis of the entire tumor.

Follow-up
To detect recurrence at an early stage, serum α-fetoprotein (AFP), lectin-reactive AFP (AFP-L3), and des-γ-carboxy-prothrombin (DCP) levels were measured monthly, and CT and ultrasonography were performed every 4 months. Local tumor progression was defined as the appearance of viable cancer tissue touching the initially treated tumor (22) and distant recurrence as the emergence of one or several tumor(s) separate from the primary site. Chest CT or bone scintigraphy was performed if extrahepatic recurrence was suspected. RFA was used for recurrence if the patient still met the indication criteria. If multiple recurrences were not treatable with RFA, transarterial chemoembolization was generally performed.

Statistical analysis
This is a report of a consecutive case series: all RFA treatments performed on primary HCC patients at the Department of Gastroenterology, University of Tokyo between February 1999 and December 2009 were included and none was excluded. Data are presented as mean±s.d. for quantitative variables, and as absolute frequencies for qualitative variables.

A "procedure" was defined as a single intervention episode comprising one or more ablation performed on one or more tumors and a "treatment" as the completed effort to ablate one or more tumors. A treatment comprised one or more procedures (22).

"Technique effectiveness" rate was defined as the percentage of successfully eradicated macroscopic tumors, as evidenced by CT scan 1–3 days after the last procedure (22).

Overall survival was calculated in the 1,170 primary HCC patients. Survival curves were generated by the Kaplan–Meier method. In addition to overall survival, some subgroup analyses were performed with clinical characteristics including tumor size, tumor number, and liver function. Recurrence was evaluated in 1,138 of the 1,170 primary HCC patients; the remaining 32 patients were excluded from the recurrence analysis because some small tumors had been left untreated by RFA on account of detection failure by ultrasonography. Recurrence rates were calculated by the Gaynor's method (23). All time estimates were made from the date of the first RFA. The follow-up was finalized at either death or the last visit to the outpatient clinic before 31 December 2009. Transplanted patients were censored from this study at the date of transplantation.

The prognostic relevance of 19 baseline variables (**Table 1**), the combination of transcatheter arterial chemoembolization (TACE) with RFA, HCC recurrence, and the number of RFA sessions to survival was analyzed by univariate and multivariate Cox proportional hazards regression models. The prognostic relevance of 19 baseline variables (**Table 1**), the combination of TACE with RFA, and the number of RFA sessions to local tumor progression and

distant recurrence was also analyzed by univariate and multivariate models. All variables with a P value <0.05 by univariate comparison were subjected to multivariate analysis. Some continuous variables in which log-linearity could not be assumed were transformed into categorical variables. In multivariate analysis, we evaluated two models that contained either Child-Pugh class or its components to avoid multicollinearity. A stepwise variable selection was performed with Akaike Information Criteria in multivariate analysis. The results of multivariate analyses were presented as a hazard ratio with corresponding 95% confidence interval (CI), with P values from the Wald test. All significance tests were two-tailed, and differences with a P value <0.05 were considered statistically significant.

Complications were defined according to the guidelines of the Society of Interventional Radiology (24).

RESULTS
Antitumor effect
We performed a total of 2,982 RFA treatments for the 1,170 primary HCC patients, comprising 4,514 procedures. Thus, procedure number per treatment was 1.52±0.78. Many patients undergoing RFA for treatment of primary HCC received iterative RFA treatments for recurrence. A total of 485 patients underwent RFA treatment once, 247 twice, 177 thrice, 94 four times, 70 five times, 35 six times, 23 seven times, 14 eight times, 7 nine times, 7 ten times, 6 eleven times, 2 twelve times, 2 thirteen times, and 1 fourteen times.

Technique effectiveness rate was 99.4% (2,964/2,982 treatments). It was similar between the initial RFA treatments and the other RFA treatments for recurrence (P=0.98). Complete ablation of the tumor was achieved in 1,163 (99.4%) of the 1,170 initial treatments and in 1,801 (99.4%) of the 1,812 other RFA treatments. However, technique effectiveness rate significantly differed with tumor size (P=0.023). No apparent viable portions remained in the treated tumor in 1,642 (99.6%) of 1,648 treatments for tumors ≤2.0 cm in diameter, in 923 (99.2%) of 930 treatments for tumors 2.1–3.0 cm, in 356 (98.9%) of 360 treatments for tumors 3.1–5.0 cm, and in 43 (97.7%) of 44 treatments for tumors >5.0 cm. Final CT imaging demonstrated residual cancer tissue in the remaining 18 treatments. We decided against performing additional procedures, because liver failure rather than HCC seemed to determine the prognosis in 10 treatments, and because additional RFA would have caused complications on account of poor visualization or inaccessibility in the other eight treatments.

Survival
The 19 baseline clinical characteristics of the 1,170 patients who underwent RFA for treatment of primary HCC are shown in **Table 1**. A total of 269 patients (23.0%) were >75 years old. In all, 422 patients had tumors ≤2.0 cm in diameter, 467 had tumors 2.1–3.0 cm, 246 had tumors 3.1–5.0 cm, and 35 had tumors >5.0 cm; 685 patients had 1 tumor, 395 had 2 or 3 tumors, and 90 had ≥4 tumors.

As of December 2009 (with a median follow-up of 38.2 months), 692 patients (59.1%) remained alive, 39 (3.3%) were lost to

Table 1. Baseline characteristics of the 1,170 patients undergoing radiofrequency ablation for primary hepatocellular carcinoma

Variable	
Age (years)	68.3±8.6
Males, n (%)	751 (64.1)
Viral infection	
HBs-Ag-positive, n (%)	127 (10.9)
Anti-HCV-positive, n (%)	870 (74.4)
Both positive, n (%)	13 (1.1)
Both negative, n (%)	159 (13.6)
Alcohol consumption >80 g/d	170 (14.5)
Ascites, n (%)	117 (10.0)
Encephalopathy, n (%)	24 (2.1)
Albumin (g/dl)	3.65±0.47
Total bilirubin (mg/dl)	0.95±0.49
Prothrombin time (%)	79.6±14.1
Platelet count ($\times 10^4$/mm^3)	11.9±5.6
AST (IU/l)	61.5±35.9
ALT (IU/l)	57.3±40.8
Child-Pugh class, n (%)	
A	868 (74.2)
B	291 (24.9)
C	11 (0.9)
Tumor size (cm)	2.54±1.04
Tumor number	1.8±1.2
Serum AFP (ng/dl), n (%)	
≤100	928 (793)
101–400	146 (12.5)
>400	96 (8.2)
Serum DCP (mAU/ml), n (%)[a]	
≤100	964 (83.1)
101–400	126 (10.9)
>400	70 (6.0)
Serum AFP-L3 (%), n (%)	
≤15	1,015 (86.8)
15.1–40	74 (6.3)
>40	81 (6.9)

AFP, α-fetoprotein; AFP-L3, lectin-reactive α-fetoprotein; ALT, alanine aminotransferase; AST, asparatate aminotransferase; DCP, des-γ-carboxy-prothrombin; HCV, hepatitis C virus.
Data are expressed as mean±s.d.
[a]Serum DCP level could not be measured in 10 patients because they were being administered warfarin.
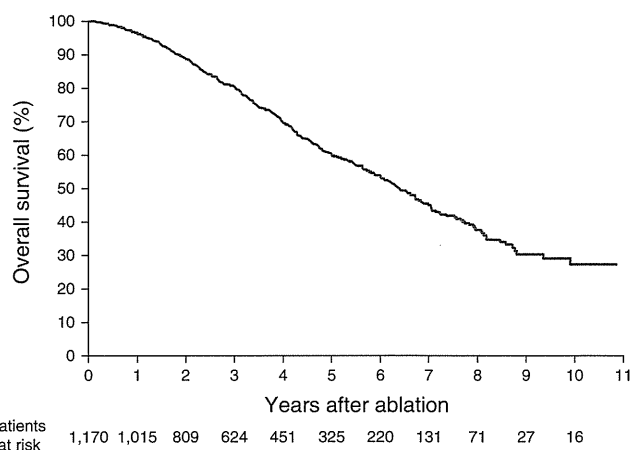

Figure 2. Overall survival in 1,170 primary hepatocellular carcinoma patients who underwent radiofrequency ablation.

follow-up, and 439 (37.5%) had died. Of the 1,170 patients, two were transplanted. The number of 5-, 7-, and 10-year survivors was 325, 131, and 16, respectively. The cause of death was HCC in 245 patients (55.8%), liver failure in 89 (20.3%), upper gastrointestinal bleeding in 11 (2.5%), complications related to the procedure in 3 (0.7%), liver-unrelated diseases in 81 (18.5%), and undetermined in 10 (2.3%).

The 1-, 3-, 5-, 7-, and 10-year survival rates of all 1,170 primary HCC patients were 96.6% (95% CI: 95.5–97.7%), 80.5% (95% CI: 78.0–83.1%), 60.2% (95% CI: 56.7–63.9%), 45.1% (95% CI: 40.9–49.6%), and 27.3% (95% CI: 21.5–34.7%), respectively (**Figure 2**; **Table 2**). Survival rates differed significantly with tumor size ($P<0.0001$), tumor number ($P=0.0003$), and Child-Pugh class ($P<0.0001$). In the Child-Pugh class A or B patients with a single tumor ≤5 cm in diameter, or three or fewer tumors ≤3 cm in diameter, the 5-year survival rate was 63.8% (95% CI: 59.9–67.9%), while in those outside these criteria, it was 46.4% (95% CI: 39.4–54.8%).

Univariate analysis showed 19 of the 22 variables relevant to survival. In multivariate analysis that contained Child-Pugh class but not its components, a model that contained age, antibody to hepatitis C virus (anti-HCV), Child-Pugh class, tumor size, tumor number, serum DCP level, and serum AFP-L3 level was selected (**Table 3**). The other model that contained the components of Child-Pugh class is shown in **Supplementary Table** online.

Recurrence

Recurrence developed in 741 patients. Local tumor progression alone was found in 25, local tumor progression with distant recurrence was found in 9, and distant recurrence alone was found in the other 707 patients. Of these 707 patients, 13 had the first recurrence in extrahepatic sites: 7 had lymph node metastasis, 3 had peritoneal seeding, 1 had lung metastasis, 1 had bone metastasis, and the remainder had both peritoneal seeding and lung metastasis. No recurrence developed in the remaining 397 patients.

Of the 741 patients, the first recurrence was treated by iterative RFA in 659 (88.9%), transarterial chemoembolization in 69 (9.3%), systemic chemotherapy in 4 (0.5%), surgical resection in 3 (0.4%), radiation therapy in 2 (0.3%), and supportive care in 4 (0.5%).

Table 2. Survival of patients undergoing radiofrequency ablation, based on tumor number, tumor size, and Child-Pugh class

Grading	n	Survival (%)					Median (years)	P value
		1-Year	3-Year	5-Year	7-Year	10-Year		
Overall survival	1,170	96.6	80.5	60.2	45.1	27.3	6.4	—
Tumor number								
Solitary	685	97.2	82.6	64.6	50.5	32.0	7.0	0.0003
2–3	395	95.7	77.9	54.4	39.4	19.9	5.6	
≥4	90	96.5	76.4	53.6	30.1	17.6	5.3	
Tumor size								
≤3 cm	889	97.2	83.8	65.1	47.3	30.7	6.7	<0.0001
>3 cm	281	94.8	71.0	46.5	38.0	18.6	4.6	
Child-Pugh class								
A	868	98.0	86.0	65.9	50.2	30.1	7.0	<0.0001
B	291	93.2	66.4	46.5	32.4	20.6	4.6	
C	11	81.8	58.4	23.4	23.4	—	3.1	
Combination of tumor number, tumor size, and Child-Pugh class								
Solitary, ≤3 cm	534	97.6	84.7	68.0	51.4	34.3	7.1	—
Solitary, ≤3 cm, Child-Pugh A	401	98.7	90.1	74.0	57.4	41.3	8.2	—
1–3 Tumors, ≤3 cm	822	97.1	83.7	65.2	48.8	32.5	6.9	—
Solitary, ≤5 cm, or 1–3 tumors, ≤3 cm	947	97.2	82.8	63.8	48.8	30.6	6.9	—
Child-Pugh A/B								
Satisfied the indication criteria of surgical resection proposed in the BCLC protocol[a]	237	98.6	90.5	75.9	61.1	38.1	8.7	—

BCLC, Barcelona Clinic Liver Cancer; HCC, hepatocellular carcinoma.
[a]Child-Pugh class A with a normal level of bilirubin, no significant portal hypertension, and a single HCC.

The 1-, 3-, 5-, 7-, and 10-year rates of local tumor progression with or without distant recurrence were 1.4% (95% CI: 0.7–2.1%), 3.2% (95% CI: 2.1–4.3%), 3.2% (95% CI: 2.1–4.3%), 3.2% (95% CI: 2.1–4.3%), and 3.2% (95% CI: 2.1–4.3%), respectively (**Figure 3**). Univariate analysis demonstrated that prothrombin time and serum AFP, DCP, and AFP-L3 levels were correlated to local tumor progression, whereas multivariate analysis showed that serum DCP level alone was significantly correlated. Tumor size was not correlated to local tumor progression.

The 1-, 3-, 5-, 7-, and 10-year rates of distant recurrence without local tumor progression were 25.6% (95% CI: 23.0–28.2%), 63.3% (95% CI: 60.2–66.4%), 74.8% (95% CI: 71.8–77.8%), 78.1% (95% CI: 75.1–81.2%), and 80.8% (95% CI: 77.4–84.3%), respectively. Univariate analysis demonstrated 14 variables relevant to distant recurrence, whereas multivariate analysis showed that anti-HCV, Child-Pugh class, platelet count, tumor size, tumor number, serum AFP level, and serum DCP level were significantly related to distant recurrence (**Table 3**).

Complications
A total of 67 complications were encountered (**Table 4**). The incidence rates of complications per treatment and per procedure were 2.2% (67/2,982) and 1.5% (67/4,514), respectively. One patient

died of hepatic failure on account of massive hepatic infarction 7 days after the last RFA procedure. He had undergone 12 RFA treatments in 8 years. The treatment mortality rate was 0.03%.

DISCUSSION
This study describes our 10-year clinical experience with RFA at a high-volume center. We performed the 2,982 RFA treatments on a total of the 1,170 primary HCC patients, showing that RFA has a high antitumor effect. Tumors were judged to have been completely ablated by final CT imaging in 99.4% of the treatments. Complete response was achieved not only in the first RFA but also in iterative RFA for recurrence. Although complete response rate differed with tumor size, there was not a sharp drop-off in effectiveness. The complete response rate may be higher in this study than others probably because we generally repeated the procedure until CT imaging demonstrated complete tumor necrosis, whereas many other studies limited the procedure number of RFA to 2–3 (11,13,15). Complete ablation of tumors has been reported to be related to improved survival (25). There were the 18 treatments in which we did not perform additional RFA for residual cancer tissue. In those treatments, usefulness of RFA had been unclear at the initial session because of liver dysfunction or tumor burden.

Table 3. Multivariate analysis of variables relevant to survival, local tumor progression, and distant recurrence

Variable	Multivariate analysis Hazard ratio (95% CI)	P value
Survival		
Age (per year)	1.03 (1.02–1.04)	<0.0001
Anti-HCV-positive	1.34 (1.03–1.76)	0.03
Child-Pugh class		
A	1	
B or C	2.08 (1.69–2.56)	<0.0001
Tumor size (cm)		
≤2.0	1	
2.1–3.0	1.40 (1.10–1.80)	0.007
3.1–5.0	1.80 (1.37–2.38)	<0.0001
>5.0	1.50 (0.90–2.49)	0.12
Tumor number		
Solitary	1	
2–3	1.28 (1.04–1.59)	0.02
≥4	1.58 (1.13–2.21)	0.008
Serum DCP (mAU/ml)		
≤100	1	
101–400	1.22 (0.88–1.69)	0.24
>400	1.66 (1.14–2.42)	0.008
Serum AFP-L3 (%)		
≤15	1	
>15	1.45 (1.11–1.91)	0.008
Local tumor progression		
Serum DCP (mAU/ml)		
≤100	1	
101–400	2.51 (1.02–6.20)	0.05
>400	6.52 (2.63–16.1)	<0.0001
Distant recurrence		
Anti-HCV-positive	1.44 (1.19–1.75)	0.0002
Child-Pugh class		
A	1	
B or C	1.23 (1.03–1.45)	0.02
Platelet count (/l)		
>10^{11}	1	
≤10^{11}	1.36 (1.12–1.64)	0.002
Tumor size (cm)		
≤2.0	1	
2.1–3.0	1.30 (1.10–1.55)	0.003
3.1–5.0	1.29 (1.05–1.60)	0.02
>5.0	1.25 (0.75–2.08)	0.4

Table 3. Continued

Variable	Multivariate analysis Hazard ratio (95% CI)	P value
Tumor number		
Solitary	1	
2–3	1.36 (1.16–1.59)	0.0002
≥4	2.02 (1.53–2.66)	<0.0001
Serum AFP (ng/dl)		
≤100	1	
101–400	1.15 (0.92–1.44)	0.22
>400	1.36 (1.03–1.81)	0.03
Serum DCP (mAU/ml)		
≤100	1	
101–400	1.19 (0.92–1.54)	0.19
>400	1.72 (1.22–2.42)	0.002

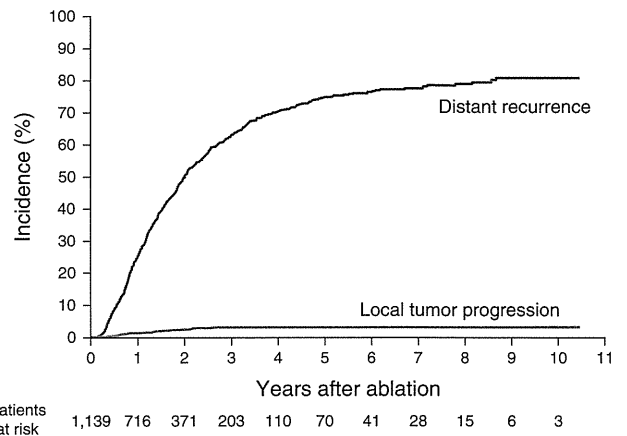AFP, α-fetoprotein; CI, confidence interval; DCP, des-γ-carboxy-prothrombin; HCV, hepatitis C virus.

Figure 3. Local tumor progression and distant recurrence in patients who underwent radiofrequency ablation.

This study shows that RFA could achieve long-term survival for as long as 10 years. Sixteen patients treated by RFA survived for >10 years. The variables relevant to survival were similar to those found in previous studies on ethanol injection (26,27), RFA, hepatic resection (28), and transarterial chemoembolization (29). Both liver function and tumor-related factors were associated with survival. In addition, age and anti-HCV were relevant to survival in this study. Age was among the prognostic factors, probably because 23.0% of the patients were >75 years old, which resulted in a higher percentage (18.5%) of liver-unrelated deaths in this study compared with others. Anti-HCV was among the prognostic factors, probably because anti-HCV-positive patients developed distant recurrence more frequently.

HCC frequently recurred after RFA; most recurrences were, however, not local tumor progression but distant recurrence. Frequent recurrence is not specific to RFA. After hepatic resection, the

Table 4. Complications in 2,982 treatments of radiofrequency ablation for hepatocellular carcinoma

Complication	No. of complications
Neoplastic seeding	24
Liver abscess	6
Hemoperitoneum	12
Hemothorax	5
Symptomatic pleural effusion	1
Massive hepatic infarction	6
Gastrointestinal perforation or penetration	5
Hemobilia	2
Skin burn	1
Pneumothorax	3
Gallbladder injury	1
Cerebral infarction	1

tumor recurrence rate exceeds 70% at 5 years (30,31). In this study, periodic follow-up detected most recurrences at limited stage. RFA was performed again for first recurrence in almost 90% of cases, although multimodal treatments were used in a long-term follow-up. On the other hand, repeat resection rate for first recurrence has been reported to range from 10.4 to 30.6% (31,32). Because RFA is less invasive than hepatic resection, iterative RFA can be performed for recurrence more easily.

Local tumor progression was found less frequently in this study than in other studies, having been reported to be around 10% at 3 years following RFA (13,14). Furthermore, different from the findings in previous reports (33,34), tumor size was not related to local tumor progression in this study. These differences are probably because we repeated RFA until we considered we had ablated not only the tumor itself but also some of the liver tissue surrounding it. Furthermore, to avoid local tumor progression, we were more cautious in the treatment of larger tumors when deciding whether sufficient ablation had been performed. Only serum DCP level was significantly related to local tumor progression in this study. Elevated serum DCP level may be related to the malignant potential of HCC such as the development of portal venous invasion (35).

The frequency of distant recurrence in this study was similar to that reported in other studies (13). Among the variables significantly related to distant recurrence, tumor size, tumor number, serum AFP level, and serum DCP level were probably related to micrometastasis, which had not been detected by imaging modalities before the treatment, while anti-HCV, Child-Pugh class, and platelet count were related to metachronous multicentric carcinogenesis, which developed based on underlying chronic liver disease.

From the viewpoint of survival and distant recurrence, patients with 2.1–5.0 cm tumors had significantly worse outcomes than those with ≤2.0 cm tumors while those with tumors >5.0 cm did not have worse rates than those with tumors ≤2 cm. This is probably

because the number of patients with tumors >5.0 cm ($n = 35$) were not large enough for the difference to be statistically significant. Another possibility is selection bias. It is possible that patient with tumors >5.0 cm who underwent RFA had more favorable conditions for survival and distant recurrence except tumor size than those with 2.1–5.0 cm tumors.

In this study, 324 of the 1,170 patients were treated with combination of TACE and RFA at the initial treatment. Thus, we evaluated the combination as a possible variable that influences survival or recurrence. Univariate analysis demonstrated that the combined therapy was significantly correlated to overall survival, whereas multivariate analysis did not show the relationship. TACE was generally combined with RFA in patients with either ≥4 tumors or those with even one tumor >3.0 cm in diameter. This is why the correlation was significant in univariate analysis, while it was not in multivariable model in which the effect of other risk factors, such as tumor number and tumor size were adjusted. The combination of TACE and RFA was not significantly related to either local tumor progression or distant recurrence.

RFA was a safe procedure. Although many patients treated by RFA in this study were at high risk for surgical treatment because of advanced cirrhosis or other comorbidities, complications occurred in only 2.2% of the treatments. Other investigators have also reported low complication rates of 0–6.1% (11,13–16). For hepatic resection, morbidity rates of 38–47% have been reported even in recent studies (36–38).

To date, percutaneous ethanol injection has been considered the standard in ablation (5). However, randomized controlled trials have demonstrated the superiority of RFA (6–9), with RFA now largely replacing ethanol injection. We have also shifted from ethanol injection to RFA (10). At our department, RFA is currently the first option and ethanol injection is performed only on patients on whom RFA cannot be performed safely because of either enterobiliary reflux, adhesion between the tumor and the gastrointestinal tract, or other reasons.

Surgical resection has been considered the treatment of choice for HCC. Our first option for resectable HCC was also surgery. However, most patients who came to our department visited us because they did not want surgical resection. Thus, many patients in this study underwent RFA not because of unresectable tumor but because of refusal of surgery. Those who preferred surgery would have directly gone to the surgical department that has extensive experience in hepatic resection (38).

It is not easy to compare outcomes between RFA and surgical resection; the indications are different between the two treatments. Furthermore, indications for each treatment are different from institution to institution. Thus, a case adjudged to be treatable by RFA or surgical resection at an institution may not be given the same treatment at another. The best known indication criteria for surgical resection may be those proposed in the Barcelona Clinic Liver Cancer (BCLC) protocol (5), which states that surgical resection should be restricted to patients with performance status 0, Child-Pugh class A, single HCC, normal portal pressure, and normal serum bilirubin level. In patients satisfying those criteria, the 5-year survival rate is expected to be >70% (30). In this study, 237

(20.3%) of 1,170 patients satisfied those criteria and were thus considered good candidates for surgical resection; their 5-year survival rate was 75.9%, which appears satisfactory when compared with outcomes following surgical resection. Furthermore, in all 1,170 primary HCC patients treated by RFA, 5- and 10-year survival rates were 60.2% and 27.3%, respectively. In patients treated by surgical resection, 5- and 10-year survival rates were 34.4–70.0% and 10.5–52.0%, respectively (32,39–45). Although this is an observational study with no control, survivals following RFA appear comparable to those reported following surgical resection.

Two recent randomized controlled trials showed no significant difference in survival between RFA and surgical resection (46,47). Several nonrandomized controlled trials reported that RFA had similar overall survival rates to resection (48–50), while others found resection to be associated with higher survival rates (51–53). Further studies are necessary to resolve comparison of RFA with resection.

We have made strenuous efforts to standardize the RFA procedure. Although many physicians have participated in RFA at our institution, the procedure was invariably performed according to the institutional protocol and in the presence of experienced physicians. Video recording was also used to monitor the procedure. Additionally, preoperative planning and postoperative evaluation of technique effectiveness were also carried out by at least three physicians. We also believe that not only proficient practice of RFA but also detailed preoperative planning, cautious postoperative evaluation of therapeutic effect, and careful follow-up are vital to achieve satisfactory outcomes.

Source population in this study may represent selection bias, as we performed RFA on most patients who were hospitalized at our department; however, many patients with unfavorable tumor conditions for RFA might not have been referred to us. Therefore, caution is required when extrapolating our findings to the general population of HCC patients.

A second limitation is that study population cannot be clearly defined. This study was based on daily clinical practice over a 10-year period. Indication criteria of RFA have changed over time, mainly because another percutaneous ablation, that is, ethanol injection has also been performed. Furthermore, various treatments besides percutaneous ablation were available for HCC, such as surgical resection and transarterial chemoembolization, with frequently overlapping indications.

One further limitation is the fact that this was a single-center study; these results might not be reproducible consistently in other settings. To extrapolate the findings in this study to patients at other institutions, careful consideration should be given to differences in the indications, methods, expertise, performance of available ultrasound and CT equipment, and others. Treatment outcome may be influenced by the physicians' expertise and the institution's volume of care. We started ethanol injection in 1985 and microwave ablation in 1995, that is, before the introduction of RFA. Recently, we have performed over 900 RFA treatments per year, which may represent a far greater number of treatments than those in most other institutions. We would not recommend any change in daily clinical practice solely on the strength of our study findings.

In conclusion, our 10-year clinical experience shows that RFA could be locally curative, resulting in survival for as long as 10 years, and was a safe procedure. RFA might be a first-line treatment for selected patients with early-stage HCC.

Study Highlights

WHAT IS CURRENT KNOWLEDGE

✓ Radiofrequency ablation (RFA) has been widely performed for hepatocellular carcinoma (HCC).

✓ RFA has a more reliable local antitumor effect and higher survival than ethanol injection.

✓ There has been no report on 10-year outcome of RFA.

WHAT IS NEW HERE

✓ Five- and 10-year survival rates in 1,170 patients with primary hepatocellular carcinoma (HCC) were 60.2 and 27.3%, respectively.

✓ Age, antibody to hepatitis C virus, Child-Pugh class, tumor size, tumor number, serum des-γ-carboxy-prothrombin level, and serum lectin-reactive α-fetoprotein level were significantly related to survival.

✓ Five- and 10-year local tumor progression rates were both 3.2%. Five- and 10-year distant recurrence rates were 74.8 and 80.8%, respectively.

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