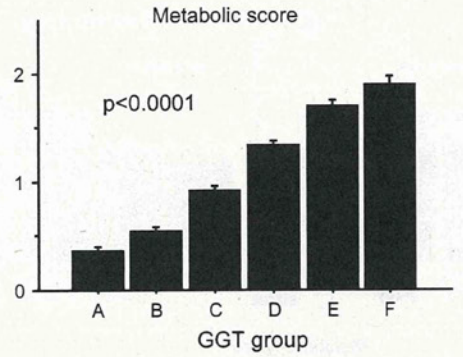

Fig. 2 Correlation between serum GGT level and metabolic syndrome. *Bar graph* shows metabolic score according to sextiles of serum GGT level

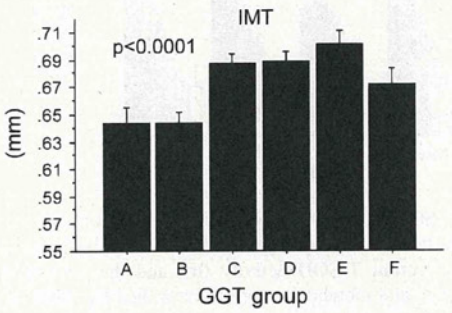

Fig. 3 Correlation between serum GGT level and atherosclerosis. *Bar graph* shows intima-media thickness (*IMT*) according to sextiles of serum GGT level

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

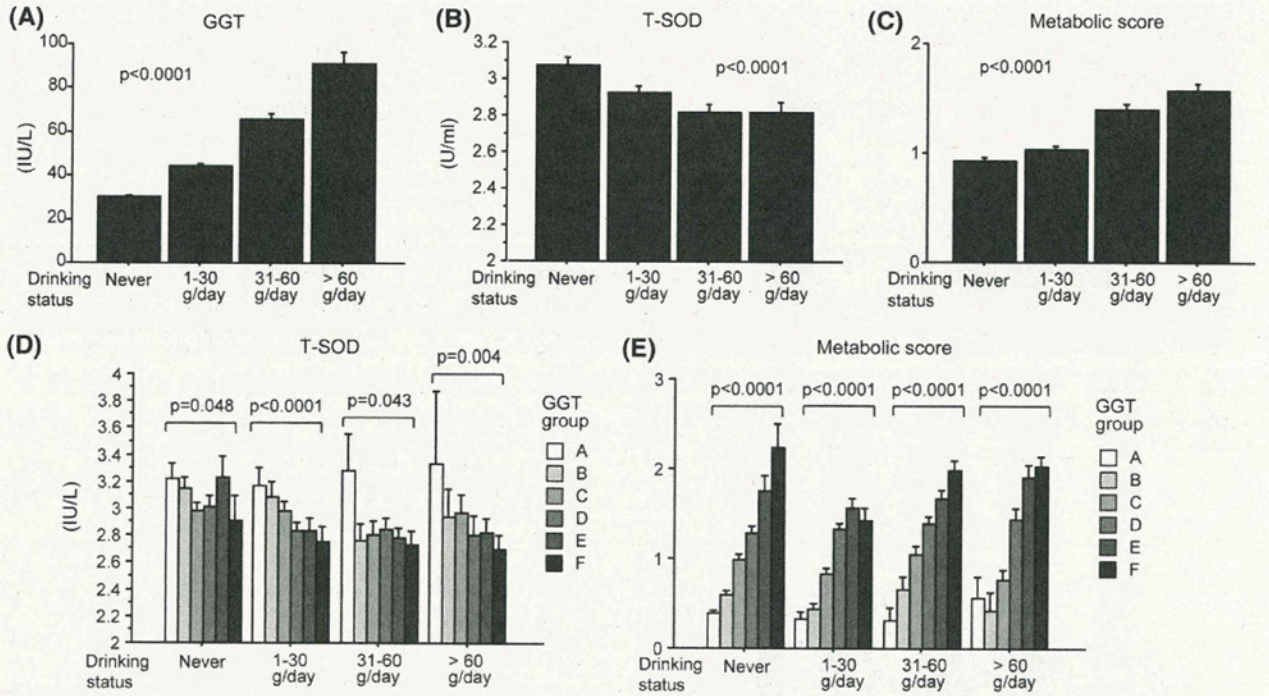

Fig. 4 Influence of drinking status on the relationship of serum GGT level to serum T-SOD activity and metabolic syndrome. Influence of drinking status on serum GGT level, serum T-SOD activity, and metabolic score is shown. *Bar graph* shows serum GGT level (**a**), serum T-SOD activity (**b**), and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

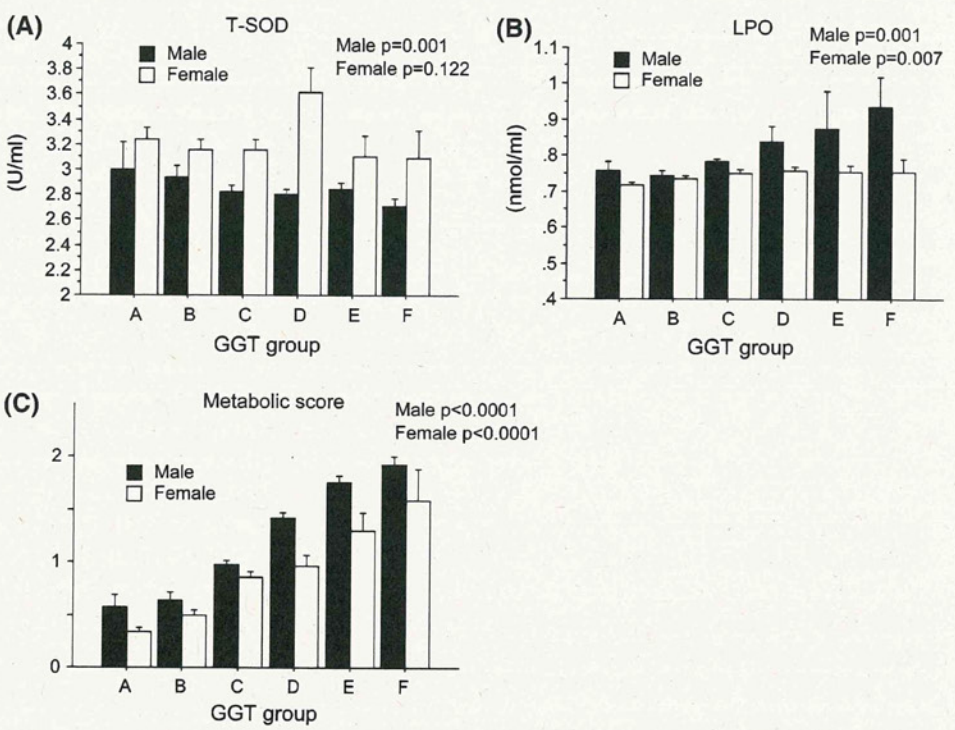

Fig. 5 Gender separated analysis of serum GGT level as the marker of oxidative stress and metabolic syndrome. *Bar graph* shows (**a**) serum T-SOD level, (**b**) serum lipid peroxide level, and (**c**) metabolic score according to sextiles of serum GGT level

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.

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Conflict of interest None.

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Research Article

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

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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.

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.

Keywords: Mitochondrial isoenzyme of creatine kinase; Hepatocellular carcinoma; Alpha-fetoprotein; Des-gamma-carboxy prothrombin.
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Abbreviations: HCC, hepatocellular carcinoma; AFP, alpha-fetoprotein; DCP, des-gamma-carboxy prothrombin; CK, creatine kinase; MtCK, mitochondrial isoenzyme of creatine kinase; HBV, hepatitis B virus; HCV, hepatitis C virus; CK-MB, MB fraction of creatine kinase; CK-M, MM fraction of creatine kinase; RFA, radiofrequency ablation; APRI, aspartate aminotransferase-to-platelet ratio index; BCLC, Barcelona Clinic Liver Cancer.

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′-TAATGCTTGGTGTGGATGAC-3′) and *18s* rRNA primers (5′-GTAACCCGTTGAACCCCATT-3′ and 5′-CCATCCAATCGGTAGTAGCG-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

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.

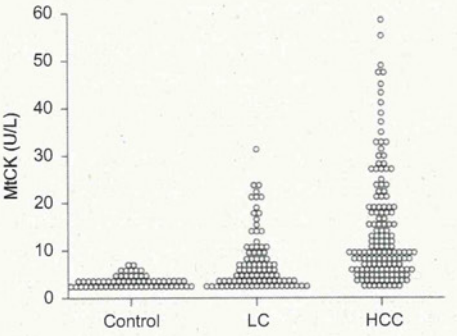

Fig. 1. Scatter plots showing serum MtCK activity in control subjects (Control), cirrhotic patients without HCC (Cirrhosis), and cirrhotic patients with HCC (HCC). Mean serum MtCK activity in cirrhotic patients with HCC (14.3 U/L) was significantly higher than that in patients without HCC (7.3 U/L; p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

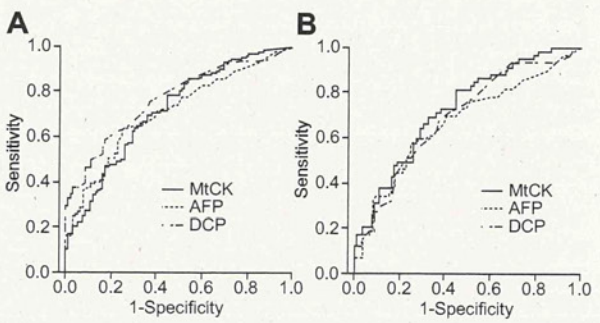

Fig. 2. ROC curves comparing MtCK, AFP, and DCP in patients with cirrhosis without HCC and with HCC. The ROC statistical analyses were performed to compare the diagnostic accuracy of MtCK, AFP, and DCP for (A) HCC in cirrhotic patients and for (B) HCC of a single lesion and smaller than 2 cm.

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

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)

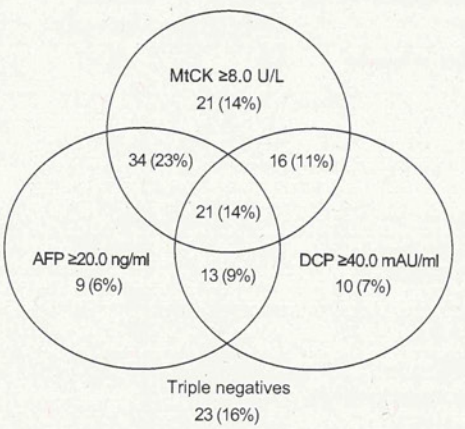

Fig. 3. Number of HCC patients with positive or negative MtCK, AFP, and DCP. The cut-off for serum MtCK activity for the prediction of HCC was defined as 8.0 U/L. The cut-offs for AFP and DCP for the prediction of HCC were 20 ng/ml and 40 mAu/ml, respectively.

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

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.

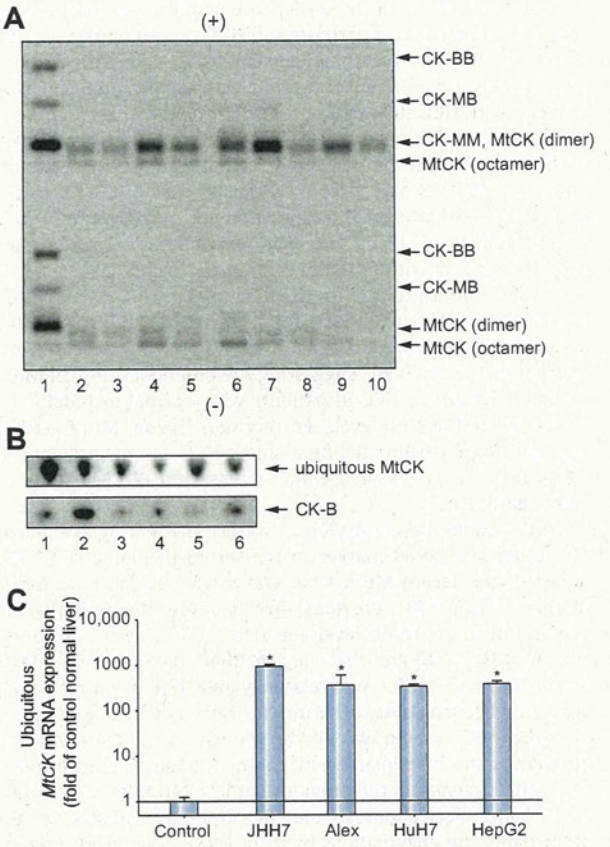

Fig. 4. CK and MtCK isoenzymes in the sera of HCC patients and in HCC cell lines. (A) MtCK and other CK isoenzymes in the sera of HCC patients. The sera of HCC patients with high MtCK activities (>30 U/L; lanes 2–8) and low MtCK activities (<8 U/L; lanes 9–10) were analyzed using electrophoresis with 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.

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, 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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