Research Article

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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.

Keywords: Mitochondrial isoenzyme of creatine kinase; Hepatocellular carcinoma; Alpha-fetoprotein; Des-gamma-carboxy prothrombin.
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* Corresponding author. Address: Department of Clinical Laboratory Medicine, Graduate School of Medicine, The University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-8655, Japan. Tel.: +81 3 3815 5411; fax: +81 3 5689 0495.
E-mail address: ikeda-1im@h.u-tokyo.ac.jp (H. Ikeda).
† The authors contributed equally to this work.
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.

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.

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 (×10^4/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.

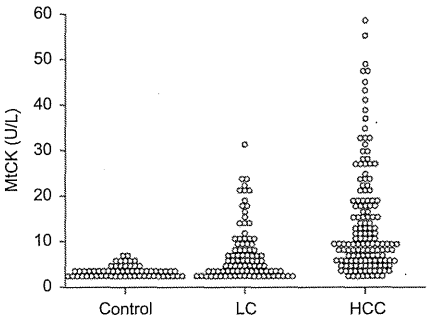

Fig. 1. Scatter plots showing serum MtCK activity in control subjects (Control), cirrhotic patients without HCC (Cirrhosis), and cirrhotic patients with HCC (HCC). Mean serum MtCK activity in cirrhotic patients with HCC (14.3 U/L) was significantly higher than that in patients without HCC (7.3 U/L; p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

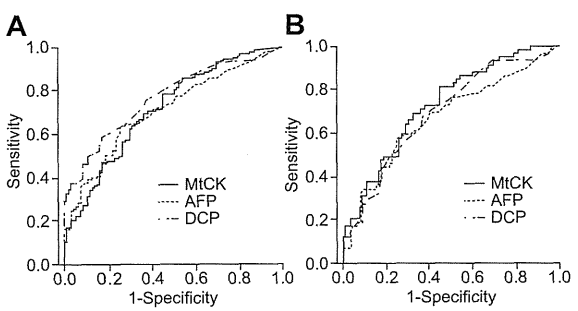

Fig. 2. ROC curves comparing MtCK, AFP, and DCP in patients with cirrhosis without HCC and with HCC. The ROC statistical analyses were performed to compare the diagnostic accuracy of MtCK, AFP, and DCP for (A) HCC in cirrhotic patients and for (B) HCC of a single lesion and smaller than 2 cm.

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)

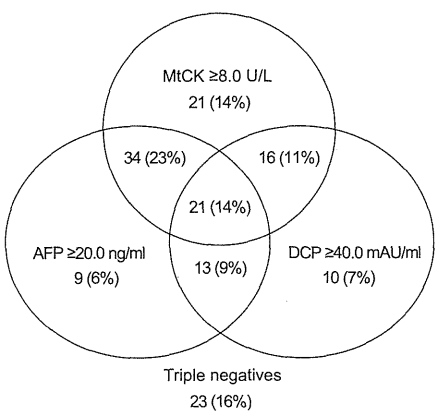

Fig. 3. Number of HCC patients with positive or negative MtCK, AFP, and DCP. The cut-off for serum MtCK activity for the prediction of HCC was defined as 8.0 U/L. The cut-offs for AFP and DCP for the prediction of HCC were 20 ng/ml and 40 mAu/ml, respectively.

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.

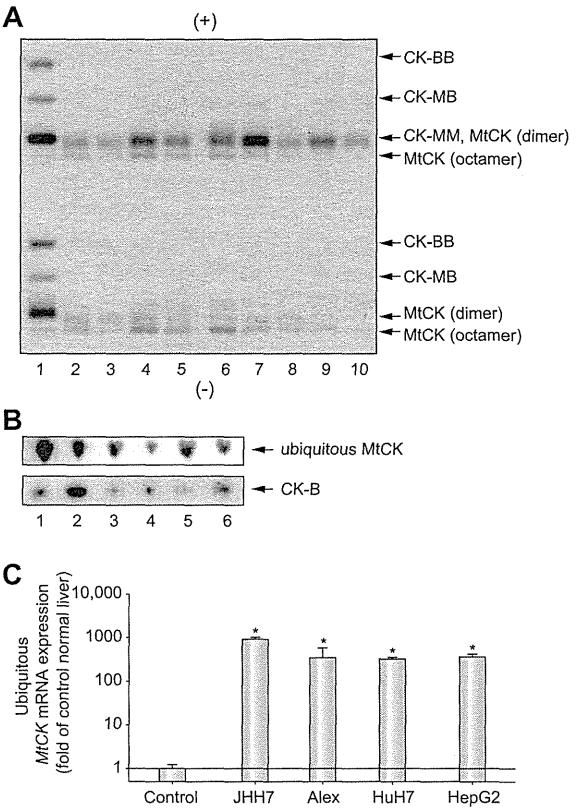

Fig. 4. CK and MtCK isoenzymes in the sera of HCC patients and in HCC cell lines. (A) MtCK and other CK isoenzymes in the sera of HCC patients. The sera of HCC patients with high MtCK activities (>30 U/L; lanes 2–8) and low MtCK activities (<8 U/L; lanes 9–10) were analyzed using electrophoresis with 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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A miRNA machinery component DDX20 controls NF-κB via microRNA-140 function

Akemi Takata, Motoyuki Otsuka *, Takeshi Yoshikawa, Takahiro Kishikawa, Yotaro Kudo, Tadashi Goto, Haruhiko Yoshida, Kazuhiko Koike

Department of Gastroenterology, Graduate School of Medicine, The University of Tokyo, Tokyo 113-8655, Japan

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ABSTRACT

Hepatocellular carcinoma is the third leading cause of cancer mortality worldwide, but the molecular mechanisms in tumorigenesis remain largely unknown. Previously, a DEAD-box protein DDX20, a component of microRNA-containing ribonucleoprotein complexes, was identified as a liver tumor suppressor candidate in an oncogenomics-based *in vivo* RNAi screen. However, the molecular mechanisms were unknown. Here, we show that deficiency of DDX20 results in the enhancement of NF-κB activity, a crucial intracellular signaling pathway closely linked with hepatocarcinogenesis. While DDX20 normally suppresses NF-κB activity by regulating NF-κB-suppressing miRNA-140 function, this suppressive effect was lost in DDX20-deficient cells. The impairment of miRNA function due to DDX20 deficiency appears to be miRNA species-specific at the point of loading miRNAs into the RNA-induced silencing complex. These results indicate that DDX20 deficiency enhances NF-κB activity by impairing the NF-κB-suppressive action of microRNAs, and suggest that dysregulation of the microRNA machinery components may also be involved in pathogenesis in various human diseases.

1. Introduction

The incidence of hepatocellular carcinoma, the third most common cause of cancer-related mortality worldwide [1], is increasing in Western countries [2]. While numerous studies have investigated molecular abnormalities in hepatocarcinogenesis, the development of this disease cannot be attributed to any single oncogenic event. Thus, drugs targeting various molecular pathways must be evaluated in combination with, or in comparison with, the current therapeutic options [3]. Although recent findings on the effectiveness of sorafenib, a multi-kinase inhibitor, are promising, the survival benefit is only less than 3 months [4]. As no effective therapy currently exists, a better understanding of the exact mechanisms involved in hepatocarcinogenesis remains the fundamental foundation for developing new candidate drugs.

DDX20 (also known as Gemin3 or DP103) was originally isolated as a DEAD-box protein that associated with the Epstein–Barr virus nuclear proteins EBNA2 and EBNA3C [5]. This protein has also been isolated independently as an interactant of survival motor neuron protein (SMN) in the gems, and in cytoplasmic spliceosomal small nuclear ribonucleoprotein complexes (snRNPs) [6]. These results suggest that DDX20 is involved in both transcriptional regulation and RNA processing. More evidence has indicated that DDX20 acts as a transcriptional regulator [7–9]. Concurrently, DDX20 was identified as a major component of microRNA (miRNA)-containing ribonucleoprotein complexes (miRNPs) that also contain eIF2C2 (Argonaute 2; Ago2) [10,11], and which perform translational control in the miRNA pathway. In addition, attempts to create DDX20 knockout mice have resulted in embryonic lethality, suggesting that this protein has essential biologic roles [12].

Recently, an oncogenomics-based *in vivo* RNAi screen identified 13 new tumor-suppressor genes in murine liver cancers [13], one of which was DDX20. Because DDX20 has not been clearly linked to liver cancer previously, the molecular mechanisms by which the dysregulation of this gene causes hepatocellular carcinoma are unknown. To address these, we examined the deregulated intracellular signaling pathway caused by DDX20 deficiency and identified a previously unknown intracellular signaling pathway.

2. Methods

2.1. Cell culture

PLC/PRF/5, Huh7, and 293T cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS). Hep3B cells were cultured in DMEM supplemented with 10% nonessential amino acids and 10% FBS.

* Corresponding author. Address: Department of Gastroenterology, Graduate School of Medicine, The University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-8655, Japan. Fax: +81 3 3814 0021.
E-mail address: otsukamo-tky@umin.ac.jp (M. Otsuka).

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2.2. Plasmids

FLAG-tagged human DDX20-expressing plasmids were kindly provided by Dr. C. Glass and Dr. G. Dreyfuss [8,10]. FLAG-tagged human DDX20-expression plasmids used for generation of DDX20-overexpressing lentiviruses were constructed by inserting the PCR-amplified DDX20 cDNA at the NotI site of the pCDH vector (System Biosciences). Plasmids expressing microRNA precursors (miRNA-22 and miRNA-140 precursors) were purchased from System Biosciences (Mountain View, CA). Reporter plasmids to analyze miRNA function were constructed by inserting annealed synthetic primers containing two tandem sequences, complementary to each miRNA, into the 3′-UTR of the firefly luciferase gene, driven by the CMV promoter (pGL3-basic; Promega, Madison, WI), at the FseI site. Primers used for PCR amplification of DDX20 were: forward, 5′-GCG GCC GCG CCG CCA TGG ACT ACA AGG ACG ACG ACG ACA AGG ACT ACA AGG ACG ACG ACG ACA AGA TGG CGG CGG CAT TTG AAG C-3′ and reverse, 5′-GCG GCC GCT CAC TGG TTA CTA TGC ATC AT TTC-3′. The sequences of the primers used for reporter plasmid construction were as follows: miR-22, 5′-ACA GTT CTT CAA CTG GCA GCT TAA TTA CAG TTC TTC AAC TGG CAG CTT CTC GAG CCG G-3′; miRNA-140–3p, 5′-CCG TGG TTC TAC CCT GTG GTA AAT TCC GTG GTT CTA CCC TGT GGT ACT CGA GCC GG-3′; miRNA-140-5p, 5′-CTA CCA TAG GGT AAA ACC ACT GAA TTC TAC CAT AGG GTA AAA CCA CTG CTC GAG CCG G-3′.

2.3. Lentiviral production and transduction

Cells were transduced with DDX20 (Gemin3)-shRNA and control-shRNA lentiviral particles (Santa Cruz Biotechnology) and then selected on puromycin. To produce FLAG-tagged DDX20 expressing-lentiviruses, 293T cells were transfected with pPACKH1 Packaging Plasmid Mix (System Biosciences) and pCDH-FLAG-tagged DDX20 expressing-lentivector constructs. After 2 days, the supernatants were collected and the viruses were concentrated using PEG-it Virus Precipitation Solution (System Biosciences).

2.4. Transfection and luciferase assay

Transfection was performed using Fugene6 (Promega). Luciferase activities were measured by use of a Dual Luciferase Reporter Assay System (Promega) as described previously [14].

2.5. RNA isolation and reverse transcription

Total RNA was isolated using Trizol Reagent (Invitrogen, Carlsbad, CA). cDNA was synthesized from RNA using the SuperScript III First-Strand Synthesis System (Invitrogen).

2.6. Antibodies

The following antibodies were used: mouse anti-Gemin3 (Ddx20) (sc-57007), rabbit anti-TRADD (sc-7868), rabbit anti-RIP (sc-7881), mouse anti-IKKα (sc-7183), mouse anti-NF-κB p65 (sc-8008), and mouse anti-NF-κB p50 (sc-7188), all purchased from Santa Cruz Biotechnology (Santa Cruz, CA); mouse anti-β-actin (A5316), purchased from Sigma (St. Louis, MO); mouse anti-TRAF2 (#558890) and mouse anti-IKKγ (#559675), purchased from BD Pharmingen (San Diego, CA); rabbit anti-TAK1 (#4505) and rabbit anti-IKKβ (#2370), purchased from Cell Signaling Technology (Danvers, MA); mouse anti-IκBα (#610690), purchased from BD Transduction Laboratories (Lexington, KY); mouse anti-Ago2 (#015-22031) and mouse anti-DYKDDDDY (FLAG)-tag (#018-22381), purchased from Wako (Osaka, Japan).

2.7. Western blotting

Western blotting was performed as described previously [15].

2.8. Reporter plasmids for signal transduction

The following reporter plasmids were used to examine how DDX20 modulated intracellular signaling: pNF-κB-luc, pGAS (IFNγ-activated sequences)-luc, pSRE-luc, pAP-1-luc, and p53-luc were purchased from Stratagene (La Jolla, CA). IL-8-luc, and p3TP-luc (to determine TGFβ pathway activity), were described previously [16]. To construct a reporter plasmid containing mutated NF-κB binding sites, the NF-κB binding motifs, GGGAATTTCC, in pNF-κB-luc were mutated to ATCAATTTCA, as previously reported [17]. Synthetic oligonucleotides with four mutant binding sites (forward, 5′-CTA GCA TCA ATT TCA ATC AAT TTC AAT CAA TTT CAA TCA ATT TCA A-3′; reverse, 5′-GAT CTT GAA ATT GAT TGA AAT TGA TTG AAA TTG ATT GAA ATT GAT G-3′) were annealed and cloned into the NheI and BglII sites of pNF-κB-luc to replace the original NF-κB binding motifs. As positive controls, the following were used: transfection with pFC-MEKK, a MEKK-expressing plasmid, for NF-κB-luc, SRE-luc, and AP-1-luc; transfection with p53-expressing plasmid for p53-luc; incubation for 6 h with 5 ng/mL IFNγ (ProSpec-Tany TechnoGene, Rehovot, Israel) for GAS-luc, and with 5 ng/mL TGFβ (Peprotech, Rocky Hill, NJ) for p3TP-luc.

2.9. EMSA

Nuclear extracts were prepared as described previously [18]. Five micrograms of nuclear extract were incubated with a double-stranded biotin-labeled DNA probe containing NF-κB binding sites (5′-AGT TGA GGG GAC TTT CCC AGG C-3′) plus 1 μg of poly (dI-dC) in a binding buffer (50 mM Tris [pH 7.5], 250 mM NaCl, 2.5 mM DTT, 2.5 mM EDTA, 5 mM $MgCl_2$, and 20% glycerol) at 15 °C for 30 min. DNA–protein complexes were separated on a 6% non-denaturing polyacrylamide gel in 0.5x TBE, and then transferred to nylon membrane (Hybond-N^+; GE Healthcare Life Sciences). Oligonucleotides were visualized using the LightShift Chemiluminescent EMSA Kit (Thermo Scientific, Rockford, IL). DNA–protein complex specificity was tested by adding a 100-fold excess of unlabeled (cold) NF-κB probe. To confirm equal loading of nuclear-extracts, the amounts of TFIID, a nuclear protein, were examined by Western blotting using an anti-TFIID antibody (sc-273; Santa Cruz).

2.10. miRNA isolation and quantitation

To measure the amounts of different microRNAs in cells, a Mir-X miRNA qRT-PCR SYBR Kit (Clontech, Mountain View, CA) was used. The levels of U6 snRNA were used for the normalization of cellular miRNA levels. To purify miRNAs from Ago2-related RISCs and DDX20-associated miRNP complexes, microRNAs fractions were isolated using the Human Ago2 MicroRNA Isolation Kit (Wako, Osaka, Japan), which uses antibodies raised to Ago2 and DDX20 to precipitate miRNAs from Ago2-related RISCs and DDX20-associated miRNPs, respectively. The primers used in the quantitative PCR analysis for miRNAs were miRNA-140-5p, CAG TGG TTT TAC CCT ATG GTA G; miRNA-140-3p, TAC CAC AGG GTA GAA CCA CGG; miRNA-22, AAG CTG CCA GTT GAA GAA CTG T.

2.11. Quantitative PCR

Quantitative PCR was performed using the TaqMan Gene Expression system and SYBR Green (Applied Biosystems, Foster City, CA). All target gene expression values were normalized to the expression values for the housekeeping gene, GAPDH, and

relative expression levels were calculated by the $\Delta\Delta C_T$ method: $\Delta\Delta C_T = \Delta C_{Tsample} - \Delta C_{Tgapdh}$ The primers used included (5′–3′): IL-6 forward, CAC AGA CAG CCA CTC ACC TC; IL-6 reverse, TTT TCT GCC AGT GCC TCT TT; IL-8 forward, ATG ACT TCC AAG CTG GCC GTG GCT; IL-8 reverse, TCT CAG CCC TCT TCA AAA ACT TCT C; GAPDH forward, ATC AAC GAC CCC TTC ATT GAC C, and GAPDH reverse, CCA GTA GAC TCC ACG ACA TAC TCA GC.

2.12. Immunoprecipitation

For immunoprecipitation, 293T cells were transfected with FLAG-tagged DDX20-expressing plasmids. FLAG-tagged DDX20 protein was precipitated by incubation with anti-FLAG M2 agarose (Sigma) for 8 h. Cell extracts were prepared as described previously [16].

2.13. Statistical analysis

Statistically significant differences were determined using Student's *t*-test, when variances were equal. When variances were unequal, Welch's *t*-test was instead used.

3. Results

3.1. DDX20 modulates the NF-κB activity

Because it was reported that DDX20 regulates transcriptions [7–9,19], we first examined the effects of altered DDX20 levels on intracellular signaling pathways by a reporter assay (Fig. 1A). While SRE was repressed moderately by DDX20 overexpression consistent with a previous report (Fig. 1A) [8], NF-κB activity was also decreased significantly in our study (Fig. 1A). Thus, we next examined NF-κB activity in stable DDX20-knockdown PLC/PRF/5 cells (Fig. 1B). Whereas DDX20-knockdown cells showed slightly higher NF-κB activity than control cells (Fig. 1C), the response was significantly enhanced by TNFα, which induces NF-κB activity and is involved in the pathogenesis of hepatitis, leading to HCC [20–23] (Fig. 1C). DDX20-knockdown cells consistently showed significantly higher promoter activity for the interleukin (IL)-8 gene, a gene known to be induced by NF-κB [24] (Fig. 1D). To exclude the possibility of cell-specific effects, we established DDX20-knockdown Huh7 cells and observed a similar trend in these cell lines (Supplementary Fig. S1a, b, and c). To further confirm these findings in an overexpression model, we established FLAG-tagged DDX20-overexpressing stable cell lines (Supplementary Fig. S2a). Restoration of NF-κB activity and mRNA levels of IL-6 and IL-8 resulted from DDX20 overexpression, consistent with the results from DDX20-knockdown cells (Supplementary Fig. S2b and c). These results also suggest that DDX20 normally functions to suppress NF-κB activity and the resulting downstream effects of this pathway.

3.2. DDX20 does not modulate or interact with molecules in the NF-κB canonical pathway

To determine how DDX20 deficiency enhances NF-κB activity, we examined the DNA-binding activity of NF-κB, which was increased in DDX20-knockdown cells (Fig. 2A). Although we

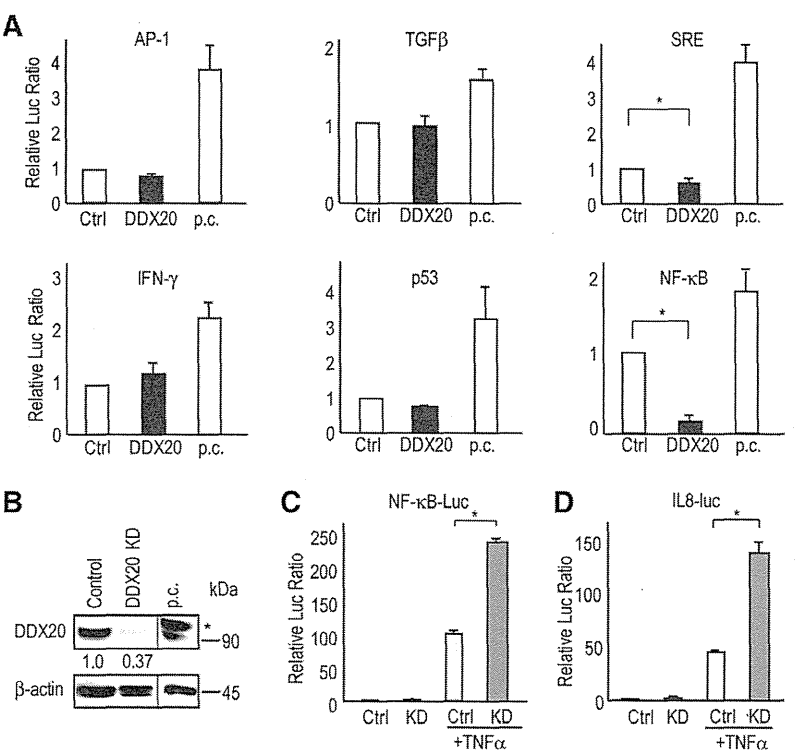

Fig. 1. Modulation of the NF-κB pathway by DDX20. (A) The effects of DDX20 on intracellular signaling pathways as assessed by a reporter assay. Huh7 cells were transiently transfected with a luciferase reporter plasmid and a DDX20-expressing (DDX20) or control plasmid (Ctrl). Luciferase values from control cells were set to 1. The data shown represent the means ± s.d. from at least four independent experiments. p.c.; positive control. (B) Establishment of stable DDX20-knockdown PLC/PRF/5 cells. *FLAG-tagged human DDX20 as a positive control (p.c.). (C, D) Reporter assay data showing that DDX20 deficiency enhances the TNFα-induced activity of NF-κB. Reporter plasmids for NF-κB (C) and its target gene, IL-8 (D), were transiently transfected into control (Ctrl) or DDX20-knockdown (KD) cells. The cells were treated with TNFα (5 ng/mL) or vehicle for 6 h before the reporter assay was performed. *$p < 0.05$. The data shown represent the means ± s.d. from three independent experimental trials. Similar results were obtained in DDX20-knockdown Huh7 cells.

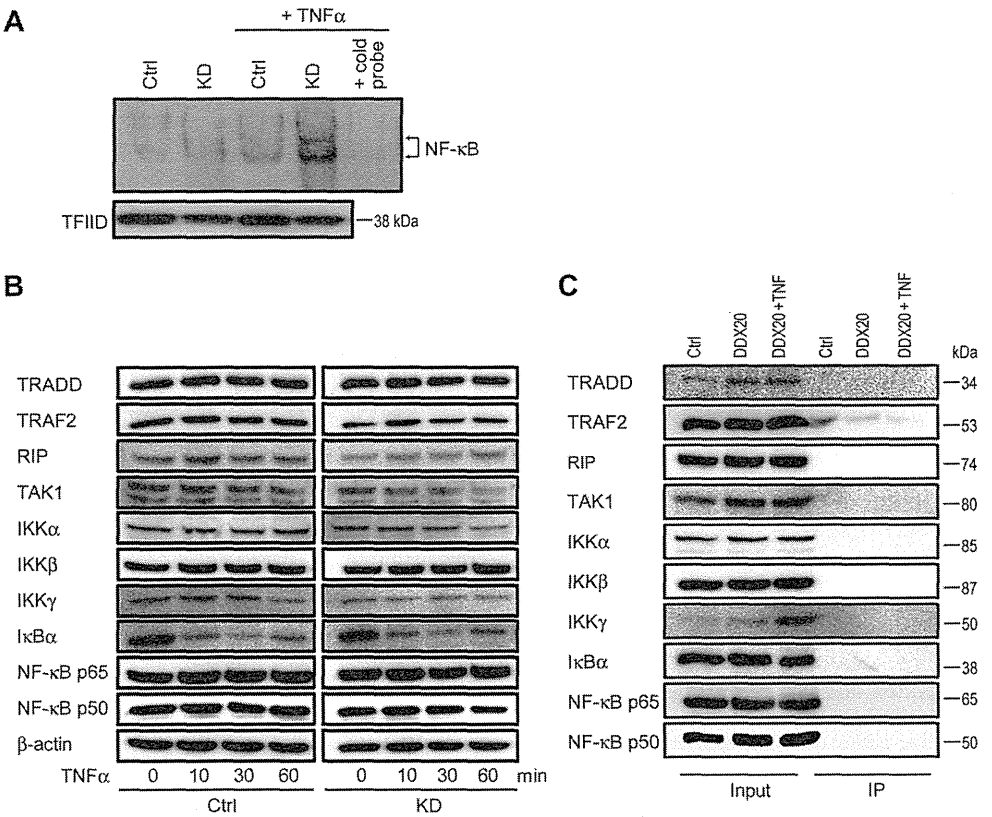

Fig. 2. NF-κB DNA binding is increased in DDX20 knockdown cells after TNFα stimulation. (A) Nuclear extracts from unstimulated and TNFα-stimulated control (Ctrl) and DDX20-knockdown (KD) PLC/PRF/5 cells were analyzed by electrophoretic mobility-shift assay. The specificity of the DNA–protein complex was tested by adding unlabeled NF-κB probe (cold probe) to the TNFα-stimulated KD nuclear extracts. TFIID amounts were examined to confirm equal nuclear extract loading. A representative result is shown from four independent experiments. Similar results were obtained using Huh7 cells. (B) Control (Ctrl) and DDX20-knockdown (KD) PLC/PRF/5 cells were stimulated with 5 ng/mL TNFα for the times indicated. Western blotting was performed using antibodies against the indicated proteins. A representative result is shown from two independent experiments. Similar results were obtained using Huh7 cells. (C) 293T cells (treated with or without 5 ng/mL TNFα for 6 h before the assay) were transfected with a control vector (Ctrl) or a FLAG-tagged DDX20-expressing (DDX20) plasmid. FLAG-tagged DDX20 was immunoprecipitated using anti-FLAG agarose. Co-precipitated proteins were blotted using antibodies against the indicated proteins. Five percent of the total cell lysates were loaded as an input control. Representative results from two independent experiments are shown.

observed IκB-α protein degradation after TNFα stimulation, as expected, the degree of degradation was similar in the control and DDX20-knockdown cells (Fig. 2B) and the levels of the other proteins examined were also similar (Fig. 2B). In addition, we were unable to detect any interactions between DDX20 and the NF-κB pathway-related molecules examined (Fig. 2C). These results suggest that it is unlikely that DDX20 modulates NF-κB activity either by interacting directly with these molecules or by altering their expression levels.

3.3. DDX20 deficiency enhances NF-κB activity by impairing miRNA-140-3p function

Because DDX20 interacts with Ago2 (Fig. 3A), which plays a central role in miRNA function [10,11], we hypothesized that DDX20 deficiency leads to impaired miRNA function and to subsequent activation of NF-κB. Because we previously identified by a comprehensive liver-expressing miRNA library screen that miRNA-22 and miRNA-140 are the critical suppressors of NF-κB activities [25], we hypothesized that DDX20 deficiency enhances NF-κB activity by impairing the NF-κB-suppressing miRNA function.

In DDX20-knockdown cells, the effects of miRNA-140-3p were reduced significantly (Fig. 3B). This impairment of miRNA function had miRNA species-specificity because the degree of functional impairment in DDX20-knockdown cells was much greater for miRNA-140-3p than for miRNA-140-5p and miRNA-22 (Fig. 3B).

While miRNA-22 and miRNA-140 suppressed NF-κB activity in the control cells as we previously reported [25] (Fig. 3C), the suppressive effects of miRNA-140 on NF-κB activity were reduced significantly in DDX20-knockdown cells (Fig. 3C). The reversal of the suppressive effect of miRNA-140 appeared to depend on miRNA-140-3p as miRNA-140-5p showed less functional impairment than did miRNA-140-3p when the corresponding reporter constructs were used as described above (Fig. 3B). The effect was NF-κB specific because no effects were observed for reporters with mutations in the NF-κB binding sites (Fig. 3D).

3.4. DDX20 preferentially interacts with miRNA-140-3p

To elucidate the mechanisms by which DDX20 preferentially impairs certain miRNA functions, we compared the levels of mature miRNAs (miR-22, 140-3p, and 140-5p) in control cells and in DDX20-knockdown cells and found them to be comparable (Fig. 4A). This suggests that DDX20 was not involved in miRNA maturation. We next considered the possibility that the preferential impairment of miRNA function in DDX20-knockdown cells was caused by preferential loading of miRNAs into the RNA-induced silencing complex (RISC). To test this, we immunoprecipitated

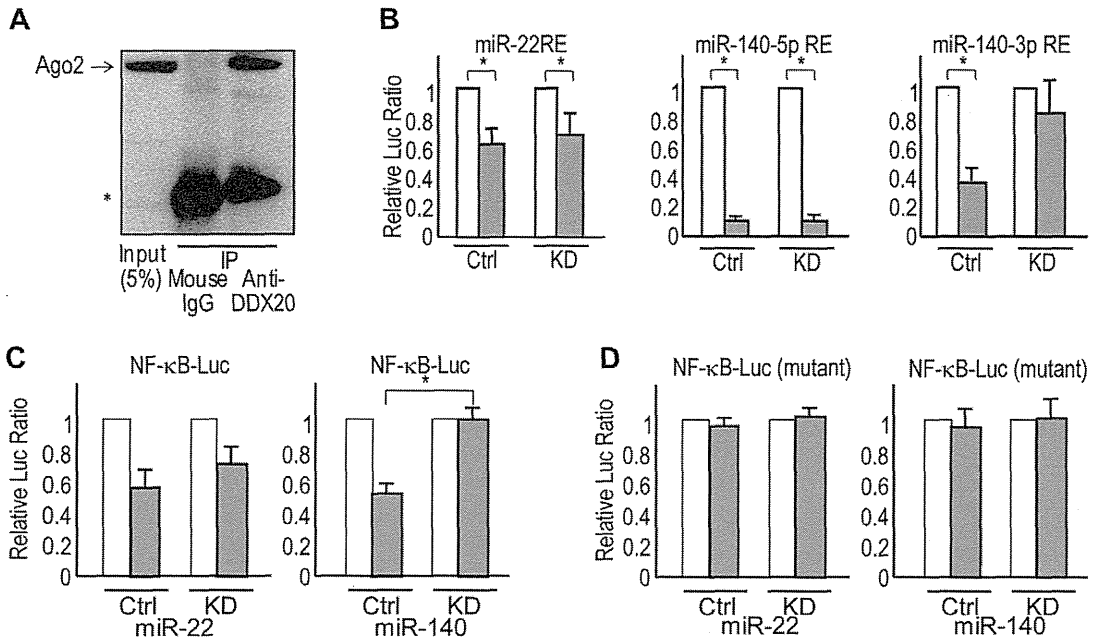

Fig. 3. DDX20 deficiency enhances NF-κB activity by impairing miRNA function. (A) DDX20 binds Ago2. 293T cells were transfected with a FLAG-tagged DDX20-expressing plasmid, and immunoprecipitated using monoclonal antibodies against FLAG-tag or non-immune mouse IgG. Precipitates were blotted with a human anti-Ago2 antibody. Five per cent of each cell lysate was used as an "input" to show the endogenous Ago2 protein. *Bands derived from immunoglobulin light-chain. (B) Overexpression of the miRNA precursors suppresses the activities of the corresponding promoter-reporter constructs in control cells (Ctrl), whereas DDX20 knockdown (DDX KD) reverses the suppressive effects of miRNA-140-3p in PLC/PRF/5 cells. The white and gray bars indicate results with the empty vector and with miRNA precursor overexpression, respectively. $^*p < 0.05$. (C) Expression of the miRNA-140 precursor suppresses NF-κB reporter activity in control cells (Ctrl), but this effect was attenuated in DDX20-knockdown PLC/PRF/5 cells (KD) as determined by NF-κB-Luc. The cells were treated with 5 ng/mL TNFα for 6 h. White and gray bars indicate empty vector and miRNA precursor overexpression, respectively. $^*p < 0.05$. (D) The suppressive effects by miR-22 and miR-140 were NF-κB-specific. Mutant NF-κB reporter plasmids were transfected with an empty vector (white bar) or the corresponding miRNA precursor-expression plasmids (black bar), into control (Ctrl) and DDX20-knockdown (KD) PLC/PRF/5 cells. Data were normalized and represent the mean ± s.d. of three independent experiments. Similar results were obtained using DDX20-knockdown Huh7 cells.

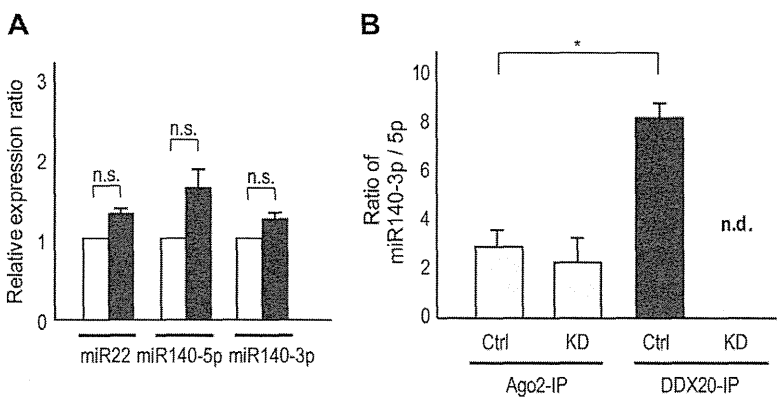

Fig. 4. DDX20 preferentially interacts with miRNA-140-3p. (A) Relative expression of indicated mature miRNAs in the total cellular RNA were measured and normalized to the level of U6 snRNA. The relative ratios of the miRNAs in control cells (white bars) and DDX20-knockdown cells (black bars) were calculated. The data represent the means ± s.d. of six independent experiments. n.s.; non significant. (B) The ratios of miRNA-140-3p to miRNA-140-5p in Ago2- and DDX20-associated RISCs as determined from anti-Ago2 or anti-DDX20 immunoprecipitates (IP) in PLC/PRF/5 control (Ctrl) and DDX20-knockdown (KD) cells. The ratios shown are the means ± s.d. of six independent experiments. Because DDX20-knockdown cells are deficient in DDX20, the anti-DDX20 miRNA ratio was not determined in these cells (n.d.).

Ago2-related RISCs and measured the amount of different miRNAs in the complexes. Because there are no standard small RNAs that can be used as a "house-keeping gene" to adjust for variation in sample loading, we compared miRNA140-5p:miRNA140-3p ratios (since both miRNAs are derived from a single precursor). This ratio in Ago2-related RISCs was approximately two in both control and DDX20-knockdown cells, suggesting that DDX20 was not involved in the preferential loading of specific miRNAs into Ago2-related RISCs (Fig. 4B). However, the ratio in the DDX20-associated miRNPs precipitated from control cells was approximately one to eight (Fig. 4B), indicating that DDX20 preferentially interacts with certain miRNAs, such as miRNA-140-3p, in DDX20-associated miRNPs. The results suggest that functional impairment of certain miRNAs in DDX20-knockdown cells might be caused by the preferential loading of certain miRNAs into DDX20-associated miRNPs. These results further suggest that DDX20 deficiency impairs the function of certain subsets of miRNAs and that this impairment could enhance NF-κB activity by deregulating the NF-κB-suppressive actions of miRNAs, especially miRNA-140-3p, in DDX20-deficient cells.

4. Discussion

Here we report that DDX20 deficiency may enhance NF-κB activities through impairing the NF-κB-suppressing miRNA-140 functions. It was reported that DDX20 is a possible liver tumor suppressor in mice [13]. NF-κB activation is a common feature of human HCCs, particularly those linked to hepatitis [26,27]. In fact, experiments using patient samples suggest that NF-κB activation in the liver leads to the development of HCC [28]. Taken together, these results suggest that the enhancement of NF-κB activity that occurs when DDX20 is deficient may play a role on hepatocarcinogenesis.

Our results indicate that, although DDX20 is one of the major components of Ago2-related RISCs, which play central roles in global miRNA functions [10], impairment of miRNA function due to DDX20 deficiency appears to be miRNA species-specific at the point of loading miRNAs into RISCs. While the precise mechanisms how DDX20 preferentially loads specific miRNAs into RISCs remain to be elucidated, because not all miRNPs have the same components, variation in RISC complexes may determine the properties or specificities of individual miRNAs [10].

Because DDX20 is a multifunctional protein, the miRNA functional impairment reported in the present study may not be the only consequence of DDX20 deficiency. In addition, because novel miRNAs are continually being discovered, other currently unknown miRNAs may also be involved in the biologic role of DDX20. Nonetheless, this study shows that DDX20 is involved in the function of specific miRNAs and the resulting control of NF-κB activity. These results suggest that it is important to investigate not only aberrant miRNA expression levels [29–32] but also deregulation of miRNP components with the subsequent impairment of miRNA function as a path of pathogenesis in human diseases.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2012.03.034.

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BRIEF ARTICLE

Assessment of disease progression in patients with transfusion-associated chronic hepatitis C using transient elastography

Ryota Masuzaki, Ryosuke Tateishi, Haruhiko Yoshida, Toru Arano, Koji Uchino, Kenichiro Enooku, Eriko Goto, Hayato Nakagawa, Yoshinari Asaoka, Yuji Kondo, Tadashi Goto, Hitoshi Ikeda, Shuichiro Shiina, Masao Omata, Kazuhiko Koike

Ryota Masuzaki, Ryosuke Tateishi, Haruhiko Yoshida, Toru Arano, Koji Uchino, Kenichiro Enooku, Eriko Goto, Hayato Nakagawa, Yoshinari Asaoka, Yuji Kondo, Tadashi Goto, Shuichiro Shiina, Kazuhiko Koike, Department of Gastroenterology, Graduate School of Medicine, The University of Tokyo, 1138655 Tokyo, Japan
Hitoshi Ikeda, Department of Clinical Laboratory, The University of Tokyo Hospital, 1138655 Tokyo, Japan
Masao Omata, Yamanashi Prefectural Hospital Organization, 4008506 Yamanashi, Japan
Author contributions: Masuzaki R, Tateishi R and Yoshida H designed research; Masuzaki R, Arano T, Uchino K, Enooku K, Goto E, Nakagawa H, Asaoka Y, Kondo Y and Goto T collected data; Tateishi R and Yoshida H analyzed data; Masuzaki R and Yoshida H wrote the paper; Tateishi R, Ikeda H and Shiina S reviewed the paper; Omata M and Koike K supervised the entire project.
Correspondence to: Dr. Ryota Masuzaki, Department of Gastroenterology, Graduate School of Medicine, The University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, 113-8655 Tokyo, Japan. ryota-m@umin.net
Telephone: +81-3-38155411 Fax: +81-3-38140021
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Abstract

AIM: To evaluate the relationship between liver stiffness and duration of infection in blood transfusion-associated hepatitis C virus (HCV) patients with or without hepatocellular carcinoma (HCC).

METHODS: Between December 2006 and June 2008, a total of 524 transfusion-associated HCV-RNA positive patients with or without HCC were enrolled. Liver stiffness was obtained noninvasively by using Fibroscan (Echosens, Paris, France). The date of blood transfusion was obtained by interview. Duration of infection was derived from the interval between the date of blood transfusion and the date of liver stiffness measurement (LSM). Patients were stratified into four groups based on the duration of infection (17-29 years; 30-39 years; 40-49 years; and 50-70 years). The difference in liver stiffness between patients with and without HCC was assessed in each group. Multiple linear regression analysis was used to determine the factors associated with liver stiffness.

RESULTS: A total of 524 patients underwent LSM. Eight patients were excluded because of unsuccessful measurements. Thus 516 patients were included in the current analysis (225 with HCC and 291 without). The patients were 244 men and 272 women, with a mean age of 67.8 ± 9.5 years. The median liver stiffness was 14.3 kPa (25.8 in HCC group and 7.6 in non-HCC group). The patients who developed HCC in short duration of infection were male dominant, having lower platelet count, with a history of heavier alcohol consumption, showing higher liver stiffness, and receiving blood transfusion at an old age. Liver stiffness was positively correlated with duration of infection in patients without HCC (r = 0.132, P = 0.024) but not in patients with HCC (r = -0.103, P = 0.123). Liver stiffness was significantly higher in patients with HCC than in those without in each duration group (P < 0.0001). The factors significantly associated with high liver stiffness in multiple regression were age at blood transfusion (P < 0.0001), duration of infection (P = 0.0015), and heavy alcohol consumption (P = 0.043)

CONCLUSION: Although liver stiffness gradually increases over time, HCC develops in patients with high stiffness value regardless of the duration of infection.

Key words: Transfusion-associated hepatitis C; Transient elastography; Hepatocellular carcinoma; Liver stiffness; Ultrasonography; Liver fibrosis

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INTRODUCTION

Hepatitis C virus (HCV) is a leading cause of chronic liver diseases, presenting serious public health problems worldwide[1,2]. HCV infection generally shows an asymptomatic onset and slow fibrosis progression, with cirrhosis developing after 20-50 years[3-7]. Progression of disease is known to depend on patients' characteristics at the onset of infection[8-12]. Infection at old age, male gender and heavy alcohol consumption are reported to be independently associated with rapid disease progression.

The onset of HCV infection can be reliably estimated in transfusion-associated chronic hepatitis C patients, in contrast to repeating injecting-drug users. In Japan, about 40% of chronic hepatitis C patients and HCV-related HCC patients have a history of blood transfusion typically in 1950s and 1960s[13], when blood supply depended on paid blood donors. Not a few of the blood donors were also injecting-drug users, mainly methamphetamine, among whom HCV spread first after the end of World War II. Viral spread started to decline in Japan after commercial blood banks were entirely abolished in 1969[14].

Chronic hepatitis C with cirrhosis is a strong risk factor for hepatocellular carcinoma (HCC)[15,16]. It has been shown that the risk of HCC increases with the degree of liver fibrosis[17]. Until recently, however, the degree of liver fibrosis could be reliably assessed only with liver biopsy, an invasive procedure with the possibility of serious complications[18,19].

Liver stiffness, which can be noninvasively measured with transient elastography, has been recently reported to be well correlated with histologically assessed liver fibrosis stage[20]. We previously reported that liver stiffness is strongly associated with the risk of HCC[21,22]. The calculated stratum-specific likelihood ratio indicated that the post-test odds for the presence of HCC increase five-fold when liver stiffness is higher than 25 kPa and decrease to one-fifth when lower than 10 kPa. Furthermore, we have confirmed in a prospective study that liver stiffness is a significant risk factor for HCC development, together with male gender, clinical cirrhosis and serum albumin level. However, in those studies we did not fully consider the duration of HCV infection and the age at the onset of infection, which are indicated in several studies to be associated with disease progression.

The aim of this study is to evaluate the association between liver stiffness and the duration of infection in blood transfusion-associated hepatitis C patients with and without HCC, focusing on the risk of HCC development.

MATERIALS AND METHODS

Patients

This study conformed to the ethical guideline of the 1975 Helsinki Declaration and the ethical guidelines for epidemiologic research designed by Japanese Ministry of Education, Culture, Sports, Science and Technology and Ministry of Health, Labor and Welfare. The study design was approved by the ethics committee of the authors' institution. Between December 2006 and June 2008, a total of 1562 patients positive for HCV RNA visited the liver clinic of authors' institution. Among these patients, those with a history of receiving blood transfusion were consecutively enrolled (229 with HCC and 295 without). We excluded from the present study those patients with concomitant hepatitis B virus surface antigen positivity, patients with uncontrollable ascites, patients on interferon therapy, and patients who received multiple blood transfusions.

Transient elastography

Liver stiffness was obtained noninvasively by using Fibroscan (Echosens, Paris, France), a newly developed medical device based on elastometry. Liver stiffness measurement (LSM) was considered valid only when at least eight acquisitions were successful with a success rate of at least 60% and the ratio of inter-quartile range (IQR) to the median value was larger than 30%.

Diagnosis of hepatocellular carcinoma

In patients with HCC, the cancer was diagnosed by dynamic computed tomography (CT), where intrahepatic nodules with hyperattenuation in the arterial phase with washout in the late phase were considered as definite HCC[23,24]. Histopathological diagnosis, using ultrasound-guided biopsy samples, was also performed when required. In patients without HCC, the cancer was ruled out by ultrasonography. No HCC was detected in the subsequent six-month follow-up period among these patients.

Laboratory tests

We determined the following parameters on the day of LSM: serum albumin and total bilirubin concentrations, serum aspartate aminotransferase (AST) and alanine aminotransferase (ALT) levels, prothrombin activity and platelet count. Serogrouping of HCV was assessed by enzyme-linked immunosorbent assay (ELISA) (Immucheck F-HCV Gr Kokusai; Sysmex, Kobe, Japan)[25]. Based on the prevalence of each HCV genotype in Japan, serogroup 1 was assumed to represent genotype 1b and serogroup 2 to represent genotype 2a or 2b.

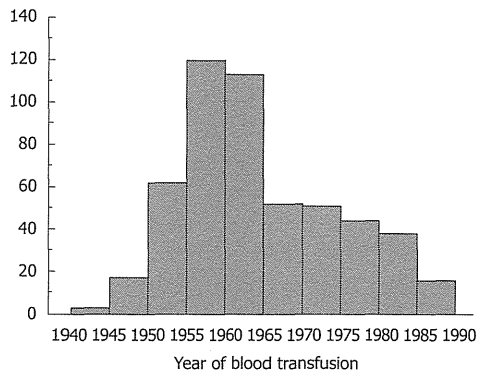

Figure 1 Frequency distribution of the year of receiving blood transfusion among the subjects. There is a peak around the year 1960.

Table 1 Characteristics of patients according to presence of hepatocellular carcinoma *n* (%)

Characteristics	HCC	Non-HCC	*P* value
Overall patients	*n* = 225	*n* = 291	
Gender (M/F)	126/99	118/173	0.0005
Age (yr)[1]	71.2 (66.1-75.7)	68.1 (58.7-72.4)	< 0.0001
Platelet count (10^9/L)[1]	95 (74-133)	161 (111-200)	< 0.0001
ALT (IU/L)[1]	48 (34-68)	42 (25-69)	0.006
Alcohol consumption > 50 g/d	51 (22.7)	28 (9.6)	< 0.0001
Liver stiffness (kPa)[1]	25.8 (17.3-37.4)	7.6 (5.6-13.9)	< 0.0001
IQR (kPa)[1]	4.0 (2.5-6.0)	1.6 (1.2-2.6)	< 0.0001
Duration (17-29 yr)	*n* = 34	*n* = 64	
Gender (M/F)	25/9	38/26	0.0028
Age (yr)[1]	73.1 (65.7-77.1)	59.7 (47.2-69.2)	0.033
Platelet count (10^9/L)[1]	95 (76-154)	180 (116-229)	< 0.0001
ALT (IU/L)[1]	51 (34-89)	42 (22-77)	0.2071
Alcohol consumption > 50 g/d	12 (35.3)	9 (14.1)	0.023
Liver stiffness (kPa)[1]	26.1 (16.8-53.3)	5.9 (4.9-12.1)	< 0.0001
Duration (30-39 yr)	*n* = 40	*n* = 59	
Gender (M/F)	16/24	23/36	0.9191
Age (yr)[1]	72.0 (65.4-76.7)	62.3 (55.7-68.6)	< 0.0001
Platelet count (10^9/L)[1]	93 (68-120)	151 (97-215)	< 0.0001
ALT (IU/L)[1]	42 (33-65)	48 (27-80)	0.7591
Alcohol consumption > 50 g/d	6 (15)	7 (11.9)	0.7641
Liver stiffness (kPa)[1]	28.7 (20.1-37.8)	7.4 (5.7-13.8)	< 0.0001
Duration (40-49 yr)	*n* = 101	*n* = 127	
Gender (M/F)	58/43	51/76	0.0113
Age (yr)[1]	69.2 (65.8-73.6)	69.9 (65.7-72.7)	0.8107
Platelet count (10^9/L)[1]	97 (67-136)	163 (112-195)	< 0.0001
ALT (IU/L)[1]	48 (34-69)	38 (23-64)	0.0080
Alcohol consumption > 50 g/d	25 (24.8)	8 (6.3)	0.0001
Liver stiffness (kPa)[1]	25.1 (17.5-37.4)	8.2 (5.8-14.1)	< 0.0001
Duration (50-70 yr)	*n* = 50	*n* = 41	
Gender (M/F)	27/23	18/23	0.4016
Age (yr)[1]	74.4 (70.0-78.1)	73.7 (66.3-79.2)	0.5658
Platelet count (10^9/L)[1]	97 (81-141)	147 (117-189)	0.0001
ALT (IU/L)[1]	52 (36-69)	46 (32-63)	0.1700
Alcohol consumption > 50 g/d	8 (16)	4 (9.8)	0.5363
Liver stiffness (kPa)[1]	16.0 (8.0-36.3)	7.9 (6.5-15.8)	< 0.0001

[1]Data are expressed as median (25th-75th percentiles). ALT: Alanine aminotransferase; IQR: Inter-quartile range; HCC: Hepatocellular carcinoma; M: Male; F: Female.

Duration of infection and liver stiffness progression

The date of blood transfusion was obtained by interview. Duration of infection was derived from the interval between the date of blood transfusion and the date of LSM. The rate of liver stiffness progression was calculated as follows: present liver stiffness-minimal stiffness value in the cohort (kPa)/interval after blood transfusion (years).

Statistical analysis

Data were expressed as median and 25th-75th percentiles in parenthesis unless otherwise indicated. The categorical variables were compared by χ^2 tests, whereas continuous variables were compared with unpaired Student's *t* test (parametric) or Mann-Whitney *U* test (non-parametric). A *P* value < 0.05 on two-tailed test was considered significant.

The correlation between liver stiffness and the interval from blood transfusion was assessed by Spearman's rank correlation. The duration of infection was arbitrarily stratified into 4 groups, 17-29 years; 30-39 years; 40-49 years; and 50-70 years. The difference in liver stiffness according to the presence of HCC was assessed in each group. Multiple linear regression analysis was used to determine the factors associated with liver stiffness. Processing and analysis were performed by using the S-plus Version 7 (TIBCO Software Inc., Palo Alto, CA, United States).

RESULTS

Patients' profile

A total of 524 patients underwent LSM. Eight patients were excluded because of unsuccessful measurements, mostly due to obesity (four patients had IQR/median > 30% and four had a success rate lower than 60%). Thus 516 patients were included in the current analysis (225 with HCC and 291 without). Their characteristics at the time of LSM are summarized in Table 1. The patients were 244 men and 272 women, with a mean age of 67.8 ± 9.5 years. The median liver stiffness was 14.3 kPa (25.8 in HCC group and 7.6 in non-HCC group). Figure 1 shows the frequency distribution of the year of receiving blood transfusion among the subjects. A peak is noted around the year 1960.

Characteristics of patients according to the duration of infection

Characteristics of patients were compared between patients with and without HCC in each duration of infection group (Table 1). The patients who developed HCC in short duration of infection were male dominant, having low platelet count, with a history of heavier alcohol consumption, showing higher liver stiffness, and receiving blood transfusion at an older age.

Correlation between liver stiffness and duration of infection

The correlation between liver stiffness and duration of

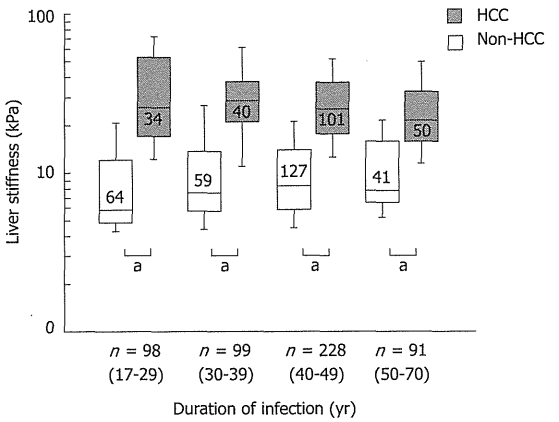

Figure 2 Duration of infection and liver stiffness. Liver stiffness was higher in patients with HCC than in patients without in each infection duration group ($^aP < 0.0001$ by Mann-Whitney *U* test).

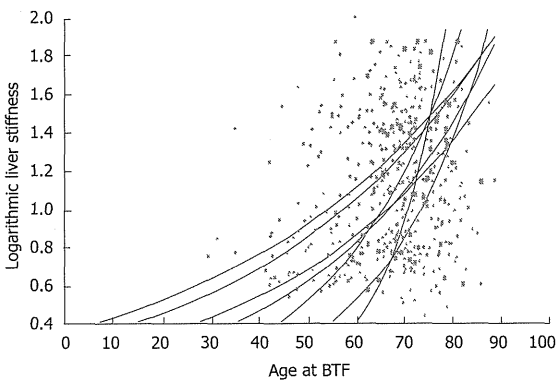

Figure 3 Age at blood transfusion and liver stiffness. Stiffness at present (each dot) and stiffness at BTF (assumed to normal value) were connected approximate logarithmic curve. Stiffness progressions become rapid in older age at BTF.

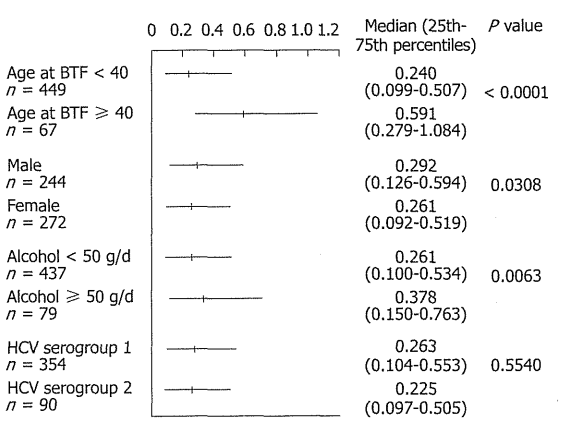

Figure 4 Liver stiffness progression rate. The progression rate is significantly higher in patients who were older than 40 at the time of blood transfusion, whose alcohol consumption is more than 50 g/d, and who are male. There is no significant difference according to hepatitis C virus (HCV) serotypes. Horizontal bar represents median value and 25th-75th percentiles.

infection was significant in patients without HCC (r = 0.132, Z = 2.256, P = 0.024) but not in patients with HCC (r = -0.103, Z = -1.54, P = 0.123). When the duration of infection was stratified into 4 groups, 17-29 years; 30-39 years; 40-49 years; and 50-70 years, liver stiffness was higher in patients with HCC than in patients without in each group ($P < 0.0001$, Figure 2).

Multiple regression analysis

The relationship between present liver stiffness and patients' characteristics, i.e., the age at blood transfusion, duration of infection, gender, and alcohol consumption (alcohol > 50 g/d) was analyzed with multiple linear regression analysis. The results showed that the age at blood transfusion was positively correlated with liver stiffness, with a coefficient of +0.336 per year for kPa, $P < 0.0001$, independently of the duration of infection (coefficient +0.272 per year for kPa, P = 0.0015). This suggests that fibrosis progression is more rapid when infection is acquired at older ages. Alcohol consumption was also significantly correlated with a positive coefficient (coefficient +4.183 for kPa, P = 0.043).

Stiffness progression and the age at blood transfusion

The progression of liver fibrosis, as represented by the increase in liver stiffness, must have been rapid in patients who have high liver stiffness in spite of short duration of HCV infection. We assumed that the liver stiffness was normal, that is, 2.9 kPa, when patients received blood transfusion. In Figure 3, the slopes represent the estimated increase rates of liver stiffness. In accordance with the results of multiple regression, the estimated increase rate was higher when patients received blood transfusion at older ages.

The progression rate of liver stiffness was assessed in subgroups according to three parameters (Figure 4). The progression rate was significantly higher in patients who were older than 40 at the time of blood transfusion ($P <$ 0.0001), which is in accordance with the results of multiple regression. Heavy alcohol consumption (more than 50 g ethanol/d, P = 0.0308) and male gender (P = 0.0063) also showed significant difference by Mann-Whitney *U* test. There was no significant difference among HCV genotypes.

DISCUSSION

The natural history of chronic hepatitis C concerning liver fibrosis progression has been vigorously studied using liver biopsy specimens. The extent of liver fibrosis is usually evaluated as categorical stages. For example, METAVIR Score uses five stages, F0-F4, for fibrosis evaluation[26]. The fibrosis progression in hepatitis C patients, calculated by using paired liver biopsy, was reported to be 0.1-0.133 Unit per year[12,13]. Liver stiffness measured by transient elastography is now widely accepted as a surrogate marker of liver fibrosis[27]. Liver stiffness is expressed as a continuous variable in kPa unit. The cut-

off for cirrhosis is reportedly 13-17 kPa, and the upper limit of measurement is currently 75 kPa. Thus LSM has a wider dynamic range than histological staging, and the rate of fibrosis progression may be more accurately analyzed with LSM.

In the present study, the increase rate of liver stiffness was positively correlated with the age at blood transfusion, as shown by the steeper slopes of approximation curves when patients received BTF at older ages. The cause of this phenomenon is not clear but age-related changes in immunity may be involved. If this is the case, the increase rate is likely to become higher in the same patient with age. Indeed, each approximation curve in the figure apparently becomes steeper with age, suggesting age-related acceleration. This is to be confirmed in future longitudinal studies.

LSM is useful not only as a surrogate of liver biopsy but also as a risk indicator of HCC development. Indeed, in the present study, liver stiffness is high in patients with HCC regardless of duration of infection. The patients who developed HCC with short duration of infection received blood transfusion at an older age and were older at the time of LSM, male dominant, and showed higher liver stiffness than patients without HCC with similar duration of infection. The difference between patients with and without HCC became smaller with longer duration of infection, as the average liver stiffness in patients with HCC became lower and that in patients without HCC became higher. We speculated that patients with high liver stiffness who received blood transfusion in the early period have already died of HCC or liver failure and were eliminated from the study population. Another possibility is that HCC may develop in patients with relatively low liver stiffness when infection has lasted a long time.

In the present study, the median increase in liver stiffness was calculated as 0.275 kPa per year. Using 13.01 kPa as a cut-off for cirrhosis[28], it will take around 40 years on average to develop cirrhosis, which is consistent with previous reports based on liver biopsy[29]. Admittedly, the present study is basically cross-sectional, and prospective longitudinal LSM will be obviously superior in understanding the natural course of liver fibrosis progression. However, the estimated average increase rate of liver stiffness indicates that such studies will require repeated LSM at an interval of at least five years.

Age at viral infection, alcohol consumption, and male gender were reported to be associated with accelerated fibrosis progression[8-11]. In the present study, we performed subgroup analysis and indeed found that blood transfusion at an age older than 40, male gender, and alcohol consumption more than 50 g ethanol/d were significantly associated with rapid increase in liver stiffness. There is consensus that heavy alcohol consumption is associated with fibrosis progression[30]. Alcohol, which by itself can cause liver disease and fibrosis, may affect liver stiffness and worsen fibrosis in hepatitis C[31]. We did not find a difference in liver stiffness increase rate between HCV genotypes 1 (mostly 1b) and 2 (2a/2b), although we could not evaluate genotypes 1a, 3 or 4.

This study has some limitations. First, since this is a cross-sectional study performed after LSM became available, patients with more rapid disease progression may have died and been excluded from the study. Second, because transfusion-associated HCV infection has been virtually eliminated in Japan since 1992, we could not include patients with shorter duration of infection. Lastly, we did not perform paired LSM but assumed that liver stiffness was normal at the time of infection. Longitudinal observation is now on-going but will take several years to obtain results.

In conclusion, although liver stiffness gradually increases over time from the onset of infection in general, HCC develops in patients with high liver stiffness regardless of the duration of infection. Patients who acquired HCV infection at older ages showed higher increase rate of liver stiffness and probably more rapid disease progression.

COMMENTS

Background

Liver stiffness, which can be noninvasively measured with transient elastography, has been recently reported to be well correlated with histologically assessed liver fibrosis stage.

Research frontiers

This study evaluated the association between liver stiffness and the duration of infection in blood transfusion-associated hepatitis C patients with and without hepatocellular carcinoma (HCC), focusing on the risk of HCC development.

Innovations and breakthroughs

Liver stiffness is expressed as a continuous variable in kPa unit. The cut-off for cirrhosis is reportedly 13-17 kPa, and the upper limit of measurement is currently 75 kPa. Thus liver stiffness measurement (LSM) has a wider dynamic range than histological staging, and the rate of fibrosis progression may be more accurately analyzed with LSM.

Applications

Although liver stiffness gradually increases over time from the onset of infection in general, HCC develops in patients with high liver stiffness regardless of the duration of infection. Patients who acquired hepatitis C virus (HCV) infection at older ages showed higher increase rate of liver stiffness and probably more rapid disease progression.

Terminology

Transient elastography (Fibro-Scan®; EchoSens, Paris, France) is a rapid, reliable and well-tolerated imaging technique for the assessment of liver fibrosis by measuring liver stiffness.

Peer review

This is an interesting and timely study on liver stiffness in patients with transfusion associatated HCV. The authors show that HCC develops in patients with high liver stiffness regardless of the duration of infection. Patients who acquired HCV infection at older ages showed higher increase rate of liver stiffness. Co-exposure to alcohol is critical. The methodology is sound and the paper is well and clearly written.

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Loss of 5-hydroxymethylcytosine is accompanied with malignant cellular transformation

Yotaro Kudo,[1] Keisuke Tateishi,[1,3] Keisuke Yamamoto,[1] Shinzo Yamamoto,[1] Yoshinari Asaoka,[1] Hideaki Ijichi,[1] Genta Nagae,[2] Haruhiko Yoshida,[1] Hiroyuki Aburatani[2] and Kazuhiko Koike[1]

[1]Department of Gastroenterology, Graduate School of Medicine, and [2]Genome Science Division, Research Center for Advanced Science and Technology, University of Tokyo, Tokyo, Japan

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Dysregulated DNA methylation followed by abnormal gene expression is an epigenetic hallmark in cancer. DNA methylation is catalyzed by DNA methyltransferases, and the aberrant expression or mutations of DNA methyltransferase genes are found in human neoplasm. The enzymes for demethylating 5-methylcytosine were recently identified, and the biological significance of DNA demethylation is a current focus of scientific attention in various research fields. Ten–eleven translocation (TET) proteins have an enzymatic activity for the conversion from 5-methylcytosine to 5-hydroxymethylcytosine (5-hmC), which is an intermediate of DNA demethylation. The loss-of-function mutations of *TET2* gene were reported in myeloid malignancies, suggesting that impaired TET-mediated DNA demethylation could play a crucial role in tumorigenesis. It is still unknown, however, whether DNA demethylation is involved in biological properties in solid cancers. Here, we show the loss of 5-hmC in a broad spectrum of solid tumors: for example, a significant reduction of 5-hmC was found in 72.7% of colorectal cancers (CRCs) and 75% of gastric cancers compared to background tissues. *TET1* expression was decreased in half of CRCs, and a large part of them was followed by the loss of 5-hmC. These findings suggest that the amount of 5-hmC in tumors is often reduced via various mechanisms, including the downregulation of *TET1*. Consistently, in the *in vitro* experiments, the downregulation of *TET1* was clearly induced by oncogene-dependent cellular transformation, and loss of 5-hmC was seen in the transformed cells. These results suggest the critical roles of aberrant DNA demethylation for oncogenic processes in solid tissues. (*Cancer Sci* 2012; 103: 670–676)

Patterns of DNA methylation, histone modification and chromatin structure are profoundly altered in human cancers.[1–5] In particular, aberrant promoter hypermethylation leading to inappropriate transcriptional silencing of genes, especially tumor suppressor genes, is often found in various types of human neoplasm, including colorectal and gastric cancers.[6–9] DNA methylation is catalyzed by DNA methyltransferases (DNMTs), and it is reported that the increased level of DNMT1 is correlated with the histological grade or poor prognosis of human cancers.[10–12] In addition, a recent report demonstrated somatic mutations in the *DNMT3A* gene from acute myeloid leukemia patients.[13]

Global loss of methylated DNA in paternal genome after fertilization suggests active DNA demethylation pathway in mammalian cells, although the molecular mechanism has been unknown for a long time. The recent discovery of ten–eleven translocation (TET) proteins those are capable of converting from 5-methylcytosine to 5-hydroxymethylcytosine (5-hmC) gave a breakthrough to the epigenetic research field.[14–19] Following studies showed that the activation-induced cytidine deaminase family convert cytosine to uracil and 5-hmC to 5-hydroxymethyluracil,[20,21] and that TET1 mediates further oxidation of 5-hmC to 5-formylcytosine and 5-carboxylcytosine.[17,18] These reports indicate that the active DNA demethylation may be established through multiple steps generating various forms of intermediates.[22]

5-hydroxymethylcytosine (5-hmC), a proposed intermediate of DNA demethylation, is abundant in embryonic stem (ES) cells and adult neural cells.[15,23–25] Accordingly, 5-hmC and TET proteins have been vigorously discussed from the aspect of cellular differentiation and pluripotency of ES cells.[15,23] Meanwhile, the biological significance of 5-hmC and TETs remains elusive in human cancers. It was recently reported that myeloid cancers have the mutations of *TET2* gene compromising the catalytic activity and show the lower levels of 5-hmC.[26–29] In contrast, in human solid cancers, biological significance of TETs and 5-hmC remains elusive. A recent study revealed that 5-hmC levels were decreased in solid tumors compared to normal tissues by immunohistochemistry;[30] however, there was no analysis comparing the 5-hmC levels among matched-pair samples.

Here, we semiquantitatively demonstrate the reduced level of 5-hmC in human cancers by examining paired matched tissues. In addition, we found that the *TET1* mRNA is suppressed under oncogene-induced cellular transformation, resulting in loss of 5-hmC.

Materials and Methods

Immunostaining. All procedures involving animals were approved by the institutional committee for animal research at the University of Tokyo and complied with the Guide for the Care and Use of Laboratory Animals. A frozen acetone-fixed tumor and normal tissue arrays were purchased from BioChain (Hayward, CA, USA). Slides were treated with 2 M hydrochloric acid followed by blocking with 10% goat serum in PBS for 1 h at room temperature and incubated with primary anti-5-hmC polyclonal antibody (1:10 000; Active Motif, Carlsbad, CA, USA) in 1% goat serum and 0.1% Triton X-100 in PBS at 4°C overnight, and were further labeled with secondary antibodies conjugated with Alexa 488 dyes (Invitrogen, Tokyo, Japan). Cell nuclei were counterstained with Hoechst 33342 dye (Dojindo, Kumamoto, Japan). All florescent images were taken using an Olympus AX80 microscope (Olympus, Tokyo, Japan).

Clinical human tissue samples. A total of 22 colorectal and 12 gastric adenocarcinoma samples were obtained from Motojima General Hospital (Gumma, Japan). All patients gave

[3]To whom correspondence should be addressed.
E-mail: ktate-tky@umin.ac.jp

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