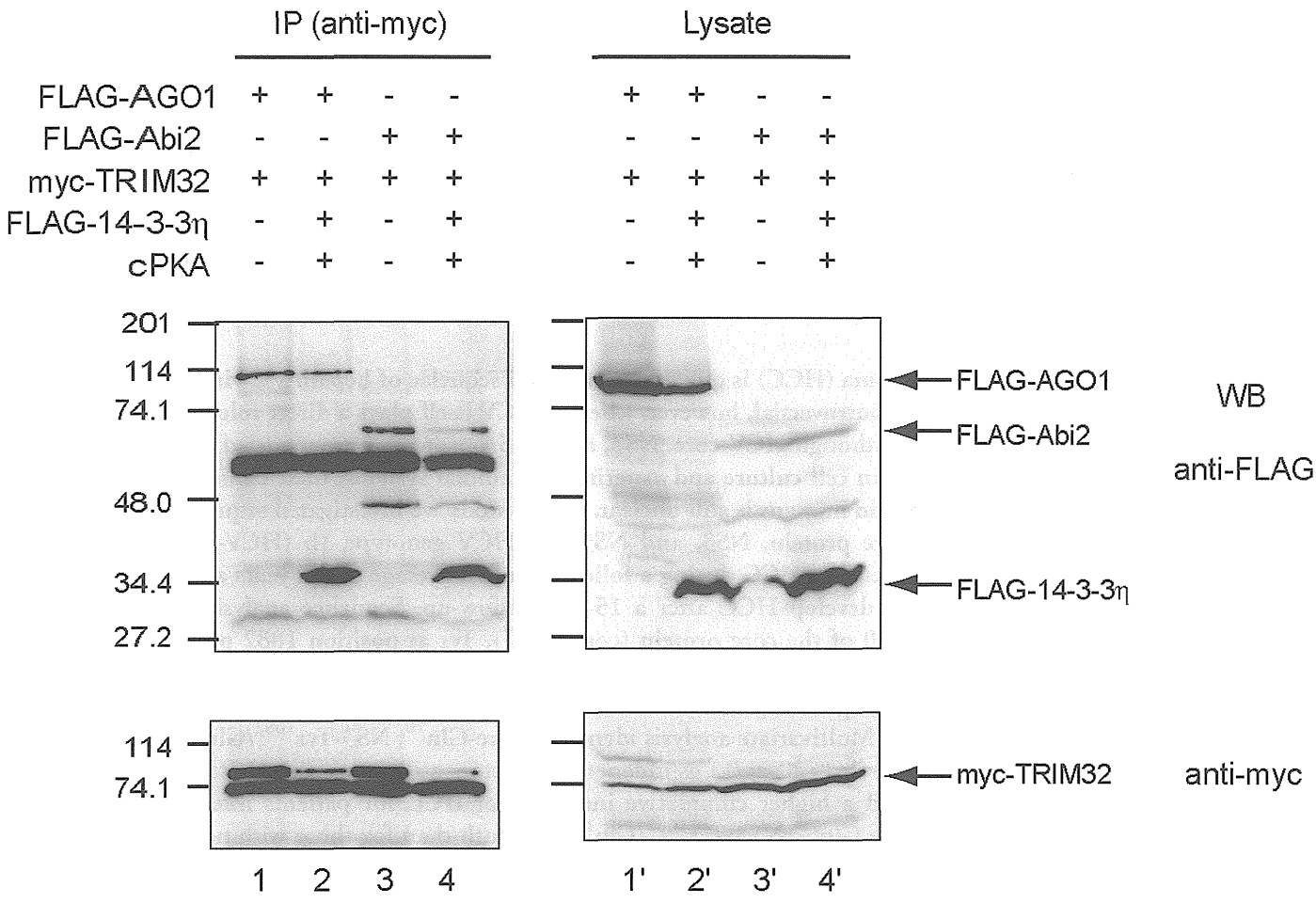

Fig. S5. 14-3-3 binding does not significantly disrupt the interaction of TRIM32 with AGO1 or Abi2. HEK293 cells transiently transfected with the indicated plasmids (5 μg each) were lysed with 1% Triton X-100 and then subjected to immunoprecipitation (IP) with anti-myc after normalizing levels of expressed proteins in each IP experiment. The amounts of myc- and FLAG-tagged proteins in the immunoprecipitates (left panels) and in lysates (right panels) were analyzed by western blotting (WB) with the indicated antibodies. Molecular size markers, in kDa, are indicated to the left.

Table S1. List of 14-3-3-associated proteins consistently identified in at least two LC-MS/MS analyses

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Polymorphisms of the Core, NS3, and NS5a Proteins of Hepatitis C Virus Genotype 1b Associate With Development of Hepatocellular Carcinoma

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Hepatocellular carcinoma (HCC) is one of the common sequelae of hepatitis C virus (HCV) infection. It remains controversial, however, whether HCV itself plays a direct role in the development of HCC. Although HCV core, NS3, and NS5A proteins were reported to display tumorigenic activities in cell culture and experimental animal systems, their clinical impact on HCC development in humans is still unclear. In this study we investigated sequence polymorphisms in the core protein, NS3, and NS5A of HCV genotype 1b (HCV-1b) in 49 patients who later developed HCC during a follow-up of an average of 6.5 years and in 100 patients who did not develop HCC after a 15-year follow-up. Sequence analysis revealed that Gln at position 70 of the core protein (core-Gln^{70}), Tyr at position 1082 plus Gln at 1112 of NS3 (NS3-Tyr^{1082}/Gln^{1112}), and six or more mutations in the interferon/ribavirin resistance-determining region of NS5A (NS5A-IRRDR≥6) were significantly associated with development of HCC. Multivariate analysis identified core-Gln^{70}, NS3-Tyr^{1082}/Gln^{1112}, and α-fetoprotein (AFP) levels (>20 ng/L) as independent factors associated with HCC. Kaplan-Meier analysis revealed a higher cumulative incidence of HCC for patients infected with HCV isolates with core-Gln^{70}, NS3-Tyr^{1082}/Gln^{1112} or both than for those with non-(Gln^{70} plus NS3-Tyr^{1082}/Gln^{1112}). In most cases, neither the residues at position 70 of the core protein nor positions 1082 and 1112 of the NS3 protein changed during the observation period. *Conclusion:* HCV isolates with core-Gln^{70} and/or NS3-Tyr^{1082}/Gln^{1112} are more closely associated with HCC development compared to those with non-(Gln^{70} plus NS3-Tyr^{1082}/Gln^{1112}). (HEPATOLOGY 2013;58:555-563)

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Hepatitis C virus (HCV) is a major etiologic agent of chronic hepatitis worldwide, with the estimated number of infected individuals being more than 180 million. Approximately 15% to 20% of chronically infected individuals undergo liver cirrhosis in a decade or so after infection, with hepatocellular carcinoma (HCC) arising from cirrhosis at an estimated rate of 1% to 4% per year.[1-3] Several host factors such as male gender, older age, elevated α-fetoprotein (AFP) level, advanced liver fibrosis as well as nonresponsiveness to interferon (IFN) therapy have been reported as important predictors of HCC development.[4,5] Recently, a host genetic factor, i.e., the *DEPDC5* locus polymorphism, was reported to be associated with progression to HCC in HCV-infected individuals.[6] On the other hand, it remains controversial as to whether HCV itself plays a direct role in the development of HCC. Experimental data suggest that HCV contributes to HCC by modulating pathways that promote malignant transformation of hepatocytes. HCV core, NS3, and NS5A proteins were shown to be involved in a

Abbreviations: HCC, hepatocellular carcinoma; HCV, hepatitis C virus; IFN, interferon; IRRDR, interferon/ribavirin resistance-determining region; ISDR, interferon sensitivity-determining region.

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Received September 3, 2012; accepted December 9, 2012.

Supported in part by Health and Labour Sciences Research Grants from the Ministry of Health, Labour and Welfare, Japan, and a SATREPS Grant from Japan Science and Technology Agency (JST) and Japan International Cooperation Agency (JICA). This study was also carried out as part of Japan Initiative for Global Research Network on Infectious Diseases (J-GRID), Ministry of Education, Culture, Sports, Science and Technology, Japan, and the Global Center of Excellence (G-COE) Program at Kobe University Graduate School of Medicine.

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number of potentially oncogenic pathways in cell culture and experimental animal systems.[7] HCV core protein rendered cultured cells more resistant to apoptosis[8,9] and promoted *ras* oncogene-mediated transformation.[10,11] Moreover, transgenic mice expressing the HCV core protein in the liver developed HCC.[12] However, the clinical impact of HCV proteins on HCC development in humans and whether all HCV isolates are equally associated with HCC is yet to be determined. In a clinical setting, HCV core protein mutations at positions 70 (Gln^{70}) and/or 91 (Met^{91}) were closely associated with HCC development.[13-16] Gln^{70} and/or Met^{91} were also linked to resistance to PEG-IFN/ribavirin (RBV) treatment.[17-20] In addition, we and other investigators reported that an N-terminal part of the NS3 protein has the capacity to transform NIH3T3 and rat fibroblast cells[21,22] and to render NIH3T3 cells more resistant to DNA damage-induced apoptosis, which is thought to be a prerequisite for malignant transformation of the cell.[23] Also, the NS5A protein is a pleiotropic protein with key roles in both viral RNA replication and modulation of the host cell functions.[24] In particular, the links between NS5A and the IFN responses have been widely discussed. It was proposed initially that sequence variations within a region in NS5A spanning from amino acids (aa) 2209 to 2248, called the IFN sensitivity-determining region (ISDR), were correlated with IFN responsiveness.[25] Subsequently, in the era of PEG-IFN/RBV combination therapy, we identified a new region near the C-terminus of NS5A spanning from aa 2334 to 2379, which we referred to as the IFN/RBV resistance-determining region (IRRDR).[26,27] The degree of sequence variations within the IRRDR was significantly associated with the clinical outcome of PEG-IFN/RBV therapy. In the context of HCC, several retrospective studies suggested that IFN-based therapy might reduce the risk of HCC development.[4,28-30]

In an attempt to clarify whether viral factors, in particular those within the core, NS3, and NS5A proteins, are involved in HCC development, we carried out a comparative analysis of the aa sequences obtained from HCV patients who developed HCC and those who did not. In addition, we studied the sequence evolution of these genes in the interval between chronic hepatitis C and HCC development over a period of 15 years.

Patients and Methods

Ethics Statement. The study protocol, which conforms to the provisions of the 1975 Declaration of Helsinki, was approved beforehand by the Ethic Committees in Akashi City Hospital and Kobe University Graduate School of Medicine, and written informed consent was obtained from each patient enrolled in this study.

Patients. A total of 49 HCV-infected patients who developed HCC (HCC group) were retrospectively examined. They were followed up (from 1988 to 2003) with an average period until HCC development being 6.5 ± 2.9 years. Paired serum samples at the time of chronic hepatitis C (pre-HCC sample) and HCC development (post-HCC sample) were collected. As a control group, 100 HCV-infected patients who were followed up over a period of 15 years (from 1988 to 2003) without HCC development were retrospectively examined. Serum samples of the control group were available at the time of first visit to the clinic. All patients enrolled in this study were chronically infected with HCV genotype 1b (HCV-1b). HCV subtype was determined as reported previously.[31] Serum HCV RNA titers were quantitated by reverse-transcription polymerase chain reaction (RT-PCR0 with an internal RNA standard derived from the 5′ noncoding region of HCV (Amplicor HCV Monitor test, v. 2.0, Roche Diagnostics, Tokyo, Japan). All patients underwent liver biopsy and were diagnosed as chronic hepatitis. All HCC and 68% (68/100) of non-HCC patients received IFN-monotherapy, either natural IFN alpha (Sumiferon, Dainipponsumitomo Pharmaceutical, Osaka, Japan) at a dose of 6 million units (MU) or recombinant IFN alpha 2b (Intron A; Schering-Plough, Osaka, Japan) at a dose of 10 MU, 3 times a week for 6 months. All HCC patients were nonresponders (NR), who had detectable viremia during the entire course of IFN treatment. On the other hand, 18 (26%) of the 68 non-HCC patients treated with IFN achieved HCV RNA negativity at the end of treatment followed by rebound viremia within 6 months after the treatment and, therefore, they were referred to as relapsers. The other 50 IFN-treated, non-HCC patients were NR. The remaining 32 non-HCC patients did not receive IFN. All patients were

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View this article online at wileyonlinelibrary.com.
DOI 10.1002/hep.26205
Potential conflict of interest: Nothing to report.

seen every 2 months and tested for liver function markers during the follow-up period.

Sequence Analysis of HCV Core, NS3, and NS5A Proteins. HCV RNA was extracted from 140 μL of serum using a commercially available kit (QIAmp viral RNA kit; Qiagen, Tokyo, Japan). The core, NS3, and NS5A regions of the HCV genome were amplified as described elsewhere.[26,32-34] The sequences of the amplified fragments were determined by direct sequencing. The aa sequences were deduced and aligned using GENETYX Win software version 7.0 (GENETYX, Tokyo, Japan). The numbering of aa was according to the polyprotein of the prototype of HCV-1b; HCV-J.[35]

Statistical Analysis. Statistical differences in the baseline parameters of HCC and control groups were determined by Student's t test for numerical variables and Fisher's exact probability or chi-square tests for categorical variables. Likewise, statistical differences in viral mutations between HCC and control groups were determined by Fisher's exact probability test. Kaplan-Meier analysis was performed to estimate the cumulative incidence of HCC. The data obtained were evaluated by the log-rank test. Univariate and multivariate logistic analyses were performed to identify variables that independently associated with HCC development. Variables with $P < 0.1$ in univariate analysis were included in a backward stepwise multivariate logistic regression analysis. The odds ratios and 95% confidence intervals (95% CI) were calculated. All statistical analyses were performed using SPSS v. 16 software (Chicago, IL). Unless otherwise stated, $P < 0.05$ was considered statistically significant.

Nucleotide Sequence Accession Numbers. The sequence data reported in this article have been deposited in the DDBJ/EMBL/GenBank nucleotide sequence databases with the accession numbers AB719460 through AB719842.

Results

Demographic Characteristics of HCC and Control Groups. The clinical characteristics of HCC and control groups are shown in Table 1. The HCC group had significantly higher titers of ALT, AST, and AFP, and higher fibrosis staging score than that of the control group. There was no significant difference in viremia titers between the two groups.

Correlation Between Core Protein Sequence Polymorphism and HCC Development. HCV core protein sequences were obtained from all (49/49) and 94% (94/100) of pre-HCC and control patients' sera, respectively. Comparative sequence analysis revealed that 22 (45%) of 49 HCV isolates in the pre-HCC sera (pre-HCC isolates) and 59 (63%) of 94 HCV isolates from the control group (control isolates) had wild-core (Arg^{70}/Leu^{91}) (Table 2). The difference between HCC and control groups was hovering at a statistically significant level ($P = 0.05$). When the sequence pattern at position 70 alone was examined, a stronger association with HCC was observed. We found that 21 (43%) of 49 pre-HCC isolates had Gln^{70} while only 13 (14%) of 94 control isolates did ($P = 0.0002$). On the other hand, there was no significant correlation between sequence pattern at position 91 and HCC. Thus, a single mutation at position 70 (Gln^{70}) was the only polymorphic factor within core protein that was significantly associated with HCC development. It should be noted that there was no significant correlation between Gln^{70} and the degree of fibrosis progression (data not shown).

Table 1. Demographic Characteristics of HCC and Control Groups

Factor	HCC	Control	P Value
Age	57.3 ± 7.0*	56.4 ± 8.3	0.54
Sex (male/female)	31/18	54/46	0.29
ALT (IU/L)	159.4 ± 79.8	129.7 ± 51.5	0.007
AST (IU/L)	113.0 ± 62.2	91.6 ± 44.1	0.017
AFP (ng/L)	29.1 ± 33.7	18.4 ± 4.4	0.002
Platelets (x $10^4/mm^3$)	16.2 ± 2.8	16.2 ± 2.4	0.88
Inflammation grading score	8.7 ± 0.9	8.4 ± 1.2	0.05
Fibrosis staging score	2.4 ± 0.5	2.2 ± 0.5	0.02
HCV-RNA (KIU/mL)	593.4 ± 112.3	618.1 ± 95.9	0.17

*Mean ± SD. HCC, hepatocellular carcinoma; ALT, alanine aminotransferase; AST, aspartate transaminase; AFP; α-fetoprotein.

Correlation Between NS3 Protein Sequence Polymorphism and HCC Development. Sequences of NS3 serine protease domain (aa 1027 to 1146) were obtained from 94% (46/49) and 93% (93/100) of pre-HCC and control isolates, respectively. We found that 29 (63%) of 46 pre-HCC isolates had Tyr and Gln at positions 1082 and 1112, respectively (Tyr^{1082}/Gln^{1112}), while 39 (42%) of 93 control isolates did (Table 2). The difference in the proportion between pre-HCC and control isolates was statistically significant ($P = 0.029$). On the other hand, there was no significant correlation between Tyr^{1082}/Gln^{1112} and the degree of fibrosis progression (data not shown).

Correlation Between NS5A Protein Sequence Polymorphism and HCC Development. NS5A protein sequences were obtained from 92% (45/49) and 74% (74/100) of pre-HCC and control isolates, respectively. Twenty-four (53%) of 45 pre-HCC isolates had IRRDR of 6 or more mutations (IRRDR≥6)

Table 2. Correlation Between HCC and Sequence Polymorphic Factors of Core, NS3 and NS5A

HCV Protein	Factor	No. of Subjects / No. of Total* HCC	Control	*P* Value
Core	Wild-core (Arg^{70}/Leu^{91})	22/49 (45%)	59/94 (63%)	0.05
	Non-wild-core	27/49 (55%)	35/94 (37%)	
	Gln^{70}	21/49 (43%)	13/94 (14%)	0.0002
	Non-Gln^{70}	28/49 (57%)	81/94 (86%)	
	Leu^{91}	37/49 (76%)	70/94 (74%)	1.0
	Non- Leu^{91}	12/49 (24%)	24/94 (26%)	
NS3	Tyr^{1082} / Gln^{1112}	29/46 (63%)	39/93 (42%)	0.029
	Non-(Tyr^{1082} / Gln^{1112})	17/46 (37%)	54/93 (58%)	
NS5A	IRRDR≥6	24/45 (53%)	15/74 (20%)	0.0003
	IRRDR≤5	21/45 (47%)	59/74 (80%)	
	ISDR≥3	11/45 (24%)	8/74 (11%)	0.07
	ISDR≤2	34/45 (76%)	66/74 (89%)	
	Asn^{2218}	11/45 (24%)	3/74 (4%)	0.002
	Non-Asn^{2218}	34/45 (76%)	71/74 (96%)	

*Number of subjects with a given factor / total number of HCC or control. HCC, hepatocellular carcinoma; Arg^{70}, arginine at position 70 of the core protein; Leu^{91}, leucine at position 91 of the core protein; Gln^{70}, glutamine at position 70 of the core protein; Tyr^{1082}, tyrosine at position 1082 of NS3; Gln^{1112}, glutamine at position 1112 of NS3; IRRDR, interferon/ribavirin resistance-determining region; ISDR, interferon sensitivity-determining region; Asn^{2218}, asparagine at position 2218 of NS5A-ISDR.

while only 15 (20%) of 74 control isolates did (Table 2; $P = 0.0003$). We also found that pre-HCC isolates tended to have a higher degree of sequence heterogeneity in ISDR than control isolates, although not statistically significant due probably to the small number of cases examined; 11 (24%) of 45 pre-HCC isolates and 8 (11%) of 74 of control isolates had ISDR with three or more mutations ($P = 0.07$). Moreover, Asn at position 2218 (Asn^{2218}) within the ISDR was found in 24% (11/45) of pre-HCC isolates and only in 4% (3/74) of the control isolates ($P = 0.002$), suggesting that Asn^{2218} is significantly associated with development of HCC.

Cumulative HCC Incidence on the Basis of Core-Gln^{70}, NS3-Tyr^{1082}/Gln^{1112}, NS5A-IRRDR≥6, and NS5A-Asn^{2218}. Follow-up study revealed that the cumulative HCC incidence in patients infected with HCV-1b isolates with core protein of Gln^{70} and those of non-Gln^{70}, respectively, was 29% and 5% at the end of 5 years, 56% and 23% at the end of 10 years, and 63% and 26% at the end of 15 years (Fig. 1A), with the differences between the two groups being statistically significant ($P < 0.0001$; Log-rank test). Likewise, the cumulative HCC incidence in patients infected with HCV-1b isolates with NS3 of Tyr^{1082}/Gln^{1112} and those of non-(Tyr^{1082}/Gln^{1112}), respectively, was 15% and 7% at the end of 5 years, 37% and 24% at the end of 10 years, and 45% and 24% at the end of 15 years ($P = 0.02$) (Fig. 1B). Also, the cumulative HCC incidence in patients infected with HCV-1b isolates of IRRDR≥6 and those of IRRDR≤5, respectively, was 18% and 10% at the end of 5 years, 59% and 22% at the end of 10 years, and 63% and 27% at the end of 15 years ($P = 0.0002$) (Fig. 1C). Similarly, the cumulative HCC incidence in patients infected with HCV-1b isolates of Asn^{2218} and those of non-Asn^{2218}, respectively, was 31% and 9% at the end of 5 years, 77% and 28% at the end of 10 years, and 77% and 33% at the end of 15 years ($P = 0.0003$) (Fig. 1D).

Identification of Independent Factors Correlated With HCC Development by Univariate and Multivariate Logistic Regression Analyses. In order to identify significant independent factors associated with HCC development, all available data of baseline patients' parameters and core, NS3, and NS5A polymorphic factors were first analyzed by univariate logistic analysis. This analysis yielded eight factors that were significantly associated with HCC development: core-Gln^{70}, NS3-(Tyr^{1082}/Gln^{1112}), NS5A-IRRDR≥6, NS5A-Asn^{2218}, increased levels of ALT (>165 IU/L), AST (>65 IU/L), and AFP (>20 ng/L), and fibrosis staging score (≥3). Subsequently, those eight factors were entered in multivariate logistic regression analysis. This analysis identified two viral factors, core-Gln^{70} and NS3-(Tyr^{1082}/Gln^{1112}), and a host factor, AFP levels (>20 ng/L), as independent factors associated with HCC development (Table 3).

The vast majority of pre-HCC isolates (85%; 39/46) had core-Gln^{70} and/or NS3-Tyr^{1082}/Gln^{1112} and only 15% (7/46) had non-(Gln^{70} plus NS3-Tyr^{1082}/Gln^{1112}). By contrast, about a half of control isolates (52%; 46/89) had non-(Gln^{70} plus NS3-Tyr^{1082}/Gln^{1112}) (Fig. 2A). The difference in the proportion between HCC and control groups was statistically significant ($P < 0.0001$). Furthermore, the cumulative HCC incidence after 15-year follow-up was highest (63%) among patients with core-Gln^{70} plus NS3-(Tyr^{1082}/Gln^{1112}), whereas it was lowest (11%) among patients with non-(Gln^{70} plus NS3-Tyr^{1082}/Gln^{1112}) (Fig. 2B), with the difference being statistically significant ($P < 0.0001$; Log-rank test).

Evolution of the Sequences of the Core, NS3, and NS5A Proteins During the Follow-up Period From Chronic Hepatitis to HCC Development. Finally, we investigated sequence evolution of the core protein, NS3 and NS5A (IRRDR and ISDR) during the follow-up period from chronic hepatitis to HCC development by comparing the sequences between pre-HCC and

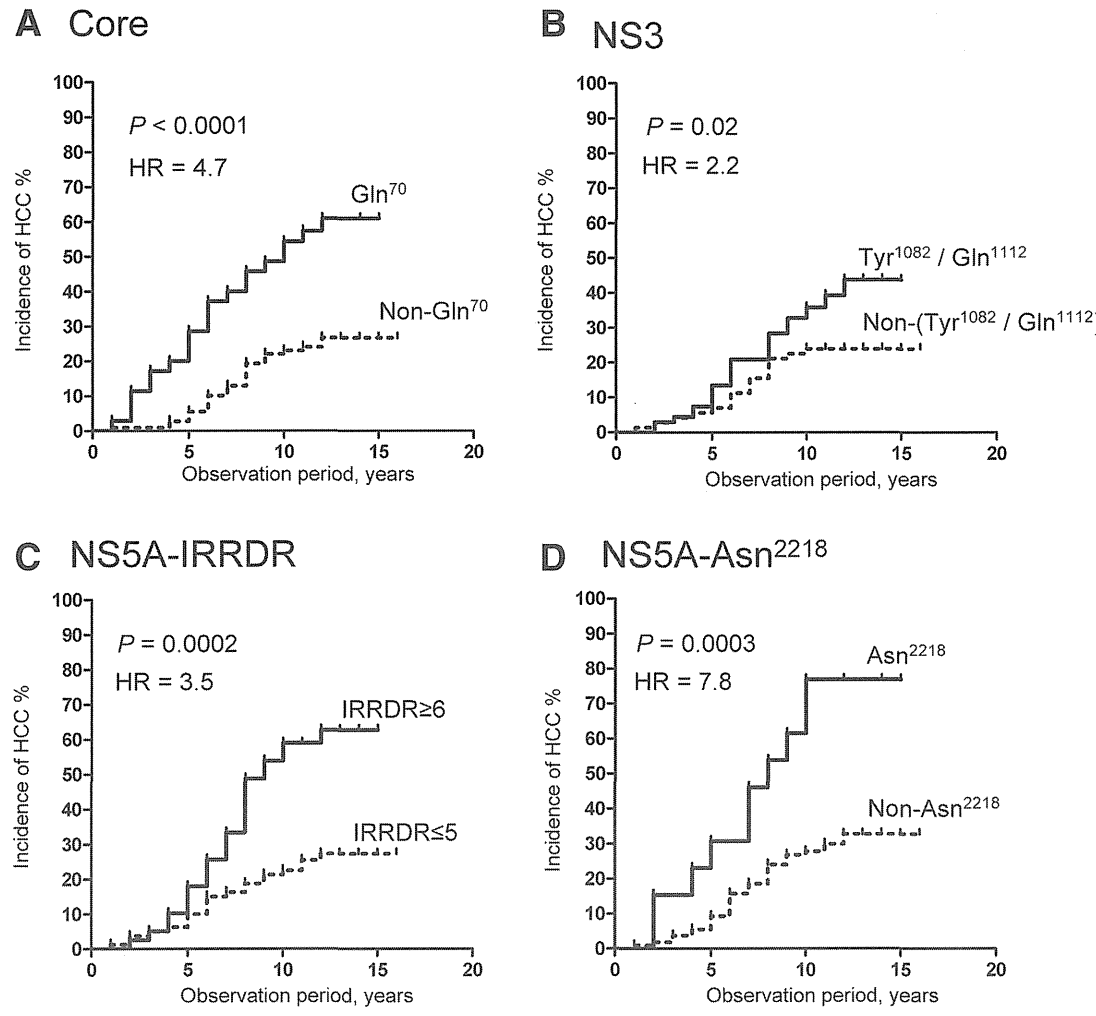

Fig. 1. Cumulative HCC incidence on the basis of HCV-1b sequence patterns. (A) Position 70 of the core protein. The numbers of core-Gln^{70} and non-Gln^{70} analyzed were 34 and 109, respectively. (B) Positions 1082 and 1112 of NS3. The numbers of NS3-(Tyr^{1082}/Gln^{1112}) and non-(Tyr^{1082}/Gln^{1112}) analyzed were 68 and 71, respectively. (C) NS5A-IRRDR. The numbers of NS5A-IRRDR≥6 and IRRDR≤5 analyzed were 39 and 80, respectively. (D) NS5A-Asn^{2218}. The numbers of NS5A-Asn^{2218} and non-Asn^{2218} analyzed were 14 and 105, respectively.

post-HCC isolates. The residue at position 70 of the core protein was conserved in 91% (41/45) of sequence pairs analyzed. The substitutions observed at this position were from Arg^{70} and His^{70} each to Gln^{70} in two cases and from Gln^{70} to Arg^{70} in the other two cases. The residues at positions 1082 and 1112 of NS3 were conserved in 95% (41/43) and 100% (43/43), respectively, of the sequence pairs analyzed.

Table 3. Univariate and Multivariate Regression Analyses to Identify Independent Factors Associated With HCC

	Univariate		Multivariate	
Variable	Odds Ratio (95% CI)	*P* Value	Odds Ratio (95% CI)	*P* Value
Core-Gln^{70}	0.23 (0.10 - 0.52)	0.0004	6.8 (2.1 - 23.0)	0.001
NS3-Tyr^{1082} / Gln^{1112}	2.4 (1.1 - 4.9)	0.029	3.4 (1.1 - 10.0)	0.03
NS5A-IRRDR≥6	4.5 (2.0 - 10.0)	0.0003		
NS5A-Asn^{2218}	7.7 (2.0 - 29.0)	0.002		
AFP (>20 ng/L)	12 (5.1 - 30.0)	0.0001	19.5 (4.7 - 80.0)	0.0001
ALT (>165 IU/L)	4.0 (1.8 - 8.6)	0.0006		
AST (>65 IU/L)	3.9 (1.5 - 10.0)	0.003		
Fibrosis staging score (≥3)	2.4 (1.1 - 4.9)	0.02		

Gln^{70}, glutamine at position 70 of the core protein; Tyr^{1082}, tyrosine at position 1082 of NS3; Gln^{1112}, glutamine at position 1112 of NS3; IRRDR, interferon/ribavirin resistance-determining region; Asn^{2218}, asparagine at position 2218 of NS5A-ISDR, ALT, alanine aminotransferase; AST, aspartate transaminase; AFP; α-fetoprotein; IFN; interferon.

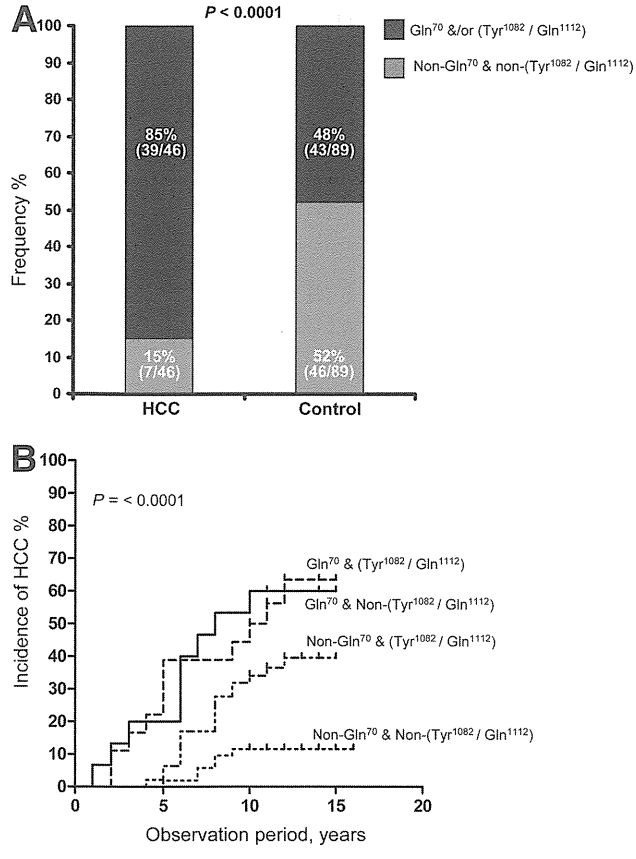

Fig. 2. (A) Proportions of HCV-1b isolates of the HCC high-risk group (core-Gln^{70} and/or NS3-[Tyr^{1082}/Gln^{1112}]) and the low-risk group (non-Gln^{70} and non-[Tyr^{1082}/Gln^{1112}]) among HCC and control groups. (B) Cumulative HCC incidence on the basis of different combined sequence patterns of position 70 of the core protein and positions 1082 and 1112 of NS3. Core-Gln^{70} and NS3-(Tyr^{1082}/Gln^{1112}), n = 18; core-Gln^{70} and non-(Tyr^{1082}/Gln^{1112}), n = 16; non-Gln^{70} and NS3-(Tyr^{1082}/Gln^{1112}), n = 48; non-Gln^{70}/non-(Tyr^{1082}/Gln^{1112}), n = 53.

IRRDR and ISDR showed a high degree of sequence evolution. IRRDR sequences were different between pre-HCC and post-HCC isolates in 66% (25/38) of cases analyzed (Fig. 3). IRRDR sequences tended to be more polymorphic at the time of HCC occurrence. Frequency of HCV isolates with IRRDR≥6 was significantly higher in post-HCC isolates than in pre-HCC isolates; IRRDR≥6 was found in 47% (18/38) of post-HCC isolates compared to 24% (9/38) of pre-HCC isolates ($P = 0.03$). On the other hand, ISDR≥3 was found in 21% (8/38) of post-HCC isolates compared to 11% (4/38) of pre-HCC isolates, with the difference between the two groups being not statistically significant ($P = 0.3$).

Discussion

HCC is one of the common long-term complications of HCV infection. However, whether HCV itself plays a direct role in the development of HCC and whether all HCV isolates are equally associated with HCC development remain to be determined. HCV core, NS3, and NS5A proteins have been reported to affect a wide variety of potentially oncogenic pathways in cell culture and experimental animal systems.[7] In the present study, we demonstrated that HCV isolates with core-Gln^{70}, NS3-Tyr^{1082}/Gln^{1112} or NS5A-IRRDR≥6 were closely associated with HCC development. In addition, a follow-up study revealed that sequence patterns at position 70 of the core protein and positions 1082 and 1112 of NS3 did not significantly alter during the progression from chronic hepatitis to HCC while NS5A-IRRDR showed a significantly higher degree of sequence heterogeneity in post-HCC than in pre-HCC isolates.

Correlation between polymorphisms at positions 70 and 91 of HCV-1b core protein and IFN-based treatment outcome was extensively studied, especially in a Japanese population.[17-20] Interestingly, the same mutations were also associated with progression to HCC in the Japanese population with HCV-1b infection.[13] Results obtained in the present study confirmed and emphasized the significant association between the mutation at position 70 (core-Gln^{70}), but not at position 91, and HCC development (Tables 2, 3; Fig. 1A). Despite the clinical evidence that strongly supports the correlation between core-Gln^{70} and HCC development, the molecular mechanism underlying this correlation is still obscure. Delhem et al.[36] found that tumor-derived HCV core proteins, but not nontumor-derived ones, interact with and activate double-stranded RNA-dependent protein kinase (protein kinase R or PKR), which might modulate viral persistence and carcinogenesis. Gln^{70} was found in two of the three tumor-derived sequences, whereas Arg^{70} was found in two of the three nontumor-derived ones.

As for the NS3 protein of HCV, the possible link between an N-terminal portion of NS3 encoding viral serine protease (aa 1027 to 1146) and hepatocarcinogenesis was reported.[21,22] However, information about the relationship between NS3 sequence diversity and HCC development is still limited. We previously reported a significant correlation between predicted secondary structure of an N-terminal portion of NS3 and HCC development.[34] In the present study, we demonstrated that HCV patients infected with HCV isolates with NS3-(Tyr^{1082}/Gln^{1112}) were at a higher risk to develop HCC than those infected with HCV isolates with non-Tyr^{1082}/Gln^{1112} (Tables 2, 3; Fig. 2B). Computer-assisted secondary structure analysis of NS3 revealed that Tyr^{1082} was associated with the

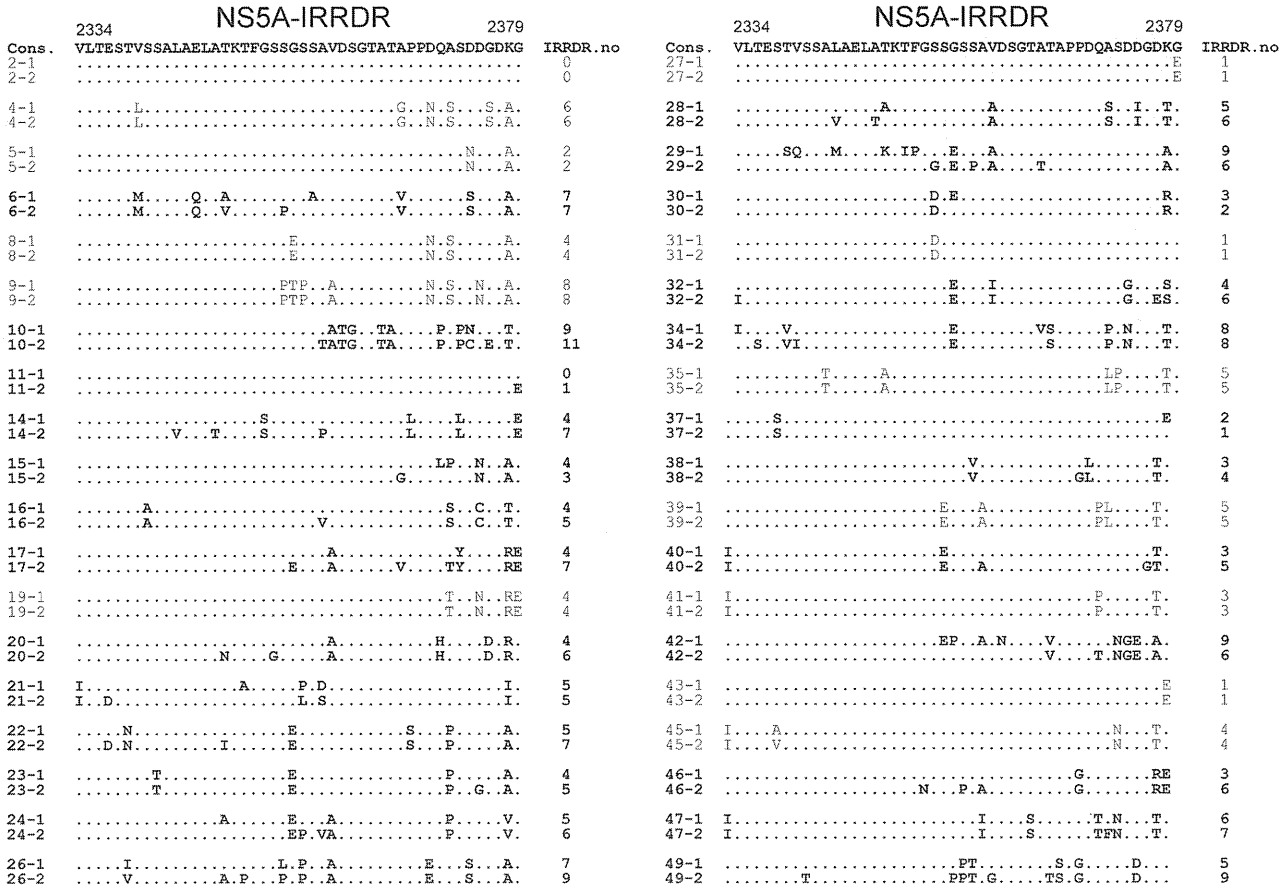

Fig. 3. Pairwise comparison of IRRDR sequences of HCV-1b during the follow-up period between chronic hepatitis and HCC development. Sequence pairs that differ between pre-HCC (numbered with -1) and post-HCC isolates (numbered with -2) are shown. The consensus sequence (Cons.) is shown at the top. The numbers along the sequence indicate the aa positions. Dots indicate residues identical to those of the Cons. sequence. The numbers of IRRDR mutations are shown on the right.

presence of a turn structure at around position 1083 while Phe[1082] was associated with the absence of the turn structure.[34] Notably, the catalytic triad of NS3 serine protease consists of His[1083], Asp[1107], and Ser[1165].[37] Since positions 1082 and 1112 are in close vicinity of the catalytic triad, sequences diversity at these positions might influence the serine protease activity and also pathogenicity of HCV. Large-scale, multicenter clinical studies as well as more detailed experimental studies at the molecular and cellular levels are needed to clarify the importance of sequence diversity at positions 1082 and 1112 of NS3 in HCV-mediated hepatocarcinogenesis.

HCV heterogeneity in NS5A-ISDR and NS5A-IRRDR are correlated with IFN-responsiveness.[17,18,25,26] As IFN-based therapy reduces the risk of HCC development,[4,28-30] we were interested to investigate whether there is a correlation between sequence heterogeneity in NS5A and development of HCC. Our present results revealed that a high degree of sequence heterogeneity in IRRDR (IRRDR≥6) was closely associated with HCC development (Table 2). We previously reported that IRRDR≥6 was significantly associated with good responses to PEG-IFN/RBV combination therapy.[26,27] These results collectively suggest that oncogenic properties and PEG-IFN/RBV responsiveness are independent viral characteristics and that PEG-IFN/RBV therapy helps eliminate oncogenic HCV isolates, thus reducing the risk of HCC development.

Position 2218 of NS5A, located within ISDR, appears to tolerate a wide range of aa substitutions as observed in different HCV-1b isolates.[25,38,39] Interestingly, Asn at position 2218 (Asn[2218]) was detected significantly more frequently in pre-HCC isolates than in the control isolates. Further studies are needed to determine the possible importance of this residue in hepatocarcinogenesis.

Another focus of attention is how the sequences of the core protein, NS3, and NS5A-IRRDR evolve during the interval between chronic hepatitis and HCC development. One of the significant advantages of the

present study was that we could conduct a longitudinal investigation by analyzing the target sequences of pre- and post-HCC isolates. We found that core-Gln^{70} and NS3-(Tyr^{1082}/Gln^{1112}) were well conserved in each paired sample. This indicates that core-Gln^{70} and NS3-(Tyr^{1082}/Gln^{1112}) were already present before the development of HCC. Non-Gln^{70} of the core protein and non-Tyr^{1082} and non-Gln^{1112} of NS3 were also well conserved in each paired sample. These results imply the possibility that these sequence patterns were not a result of HCC but, rather, they were a possible causative factor for the development of HCC. We hypothesize, therefore, that HCV isolates with core-Gln^{70} and/or NS3-(Tyr^{1082}/Gln^{1112}) are highly oncogenic, whereas those with non-(Gln^{70} plus NS3-Tyr^{1082}/Gln^{1112}) are less oncogenic. It is not clear yet as to whether these oncogenic mutations were present from the very beginning of HCV infection or if they emerged at a certain timepoint (before the initiation of follow-up) during the long-term persistence through an adaptive viral evolution in the host. More comprehensive follow-up study is needed to address this issue. In any case, the core-Gln^{70} and NS3-(Tyr^{1082}/Gln^{1112}) would be considered an index for prediction of HCC development. On the other hand, IRRDR in NS5A is more tolerant for sequence evolution. IRRDR in post-HCC isolates showed a significantly higher degree of sequence heterogeneity compared with that in pre-HCC isolates. This observation suggests that IRRDR is under strong selective pressure during the course of HCV infection and that the high degree of IRRDR heterogeneity (IRRDR$\geq$6) in HCV isolates from patients with HCC may not be a causative factor for development of HCC.

In conclusion, the present results suggest the possibility that patients infected with HCV isolates with core-Gln^{70} and/or NS3-(Tyr^{1082}/Gln^{1112}) are at a higher risk to develop HCC compared to those with non-(Gln^{70} plus NS3-Tyr^{1082}/Gln^{1112}).

References

1. Lauer GM, Walker BD. Hepatitis C virus infection. N Engl J Med 2001;345:41-52.
2. Niederau C, Lange S, Heintges T, Erhardt A, Buschkamp M, Hurter D, et al. Prognosis of chronic hepatitis C: results of a large, prospective cohort study. HEPATOLOGY 1998;28:1687-1695.
3. Ikeda K, Saitoh S, Suzuki Y, Kobayashi M, Tsubota A, Koida I, et al. Disease progression and hepatocellular carcinogenesis in patients with chronic viral hepatitis: a prospective observation of 2215 patients. J Hepatol 1998;28:930-938.
4. Yoshida H, Shiratori Y, Moriyama M, Arakawa Y, Ide T, Sata M, et al. Interferon therapy reduces the risk for hepatocellular carcinoma: national surveillance program of cirrhotic and noncirrhotic patients with chronic hepatitis C in Japan. IHIT Study Group. Inhibition of hepatocarcinogenesis by interferon therapy. Ann Intern Med 1999;131: 174-181.
5. Lok AS, Seeff LB, Morgan TR, di Bisceglie AM, Sterling RK, Curto TM, et al. Incidence of hepatocellular carcinoma and associated risk factors in hepatitis C-related advanced liver disease. Gastroenterology 2009;136:138-148.
6. Miki D, Ochi H, Hayes CN, Abe H, Yoshima T, Aikata H, et al. Variation in the DEPDC5 locus is associated with progression to hepatocellular carcinoma in chronic hepatitis C virus carriers. Nat Genet 2011; 43:797-800.
7. Banerjee A, Ray RB, Ray R. Oncogenic potential of hepatitis C virus proteins. Viruses 2010;2:2108-2133.
8. Marusawa H, Hijikata M, Chiba T, Shimotohno K. Hepatitis C virus core protein inhibits Fas- and tumor necrosis factor alpha-mediated apoptosis via NF-κB activation. J Virol 1999;73:4713-4720.
9. Ray RB, Meyer K, Ray R. Suppression of apoptotic cell death by hepatitis C virus core protein. Virology 1996;226:176-182.
10. Chang J, Yang SH, Cho YG, Hwang SB, Hahn YS, Sung YC. Hepatitis C virus core from two different genotypes has an oncogenic potential but is not sufficient for transforming primary rat embryo fibroblasts in cooperation with the H-*ras* oncogene. J Virol 1998;72: 3060-3065.
11. Ray RB, Lagging LM, Meyer K, Ray R. Hepatitis C virus core protein cooperates with ras and transforms primary rat embryo fibroblasts to tumorigenic phenotype. J Virol 1996;70:4438-4443.
12. Moriya K, Fujie H, Shintani Y, Yotsuyanagi H, Tsutsumi T, Ishibashi K, et al. The core protein of hepatitis C virus induces hepatocellular carcinoma in transgenic mice. Nat Med 1998;4:1065-1067.
13. Akuta N, Suzuki F, Kawamura Y, Yatsuji H, Sezaki H, Suzuki Y, et al. Amino acid substitutions in the hepatitis C virus core region are the important predictor of hepatocarcinogenesis. HEPATOLOGY 2007;46: 1357-1364.
14. Akuta N, Suzuki F, Hirakawa M, Kawamura Y, Sezaki H, Suzuki Y, et al. Amino acid substitutions in hepatitis C virus core region predict hepatocarcinogenesis following eradication of HCV RNA by antiviral therapy. J Med Virol 2011;83:1016-1022.
15. Akuta N, Suzuki F, Kawamura Y, Yatsuji H, Sezaki H, Suzuki Y, et al. Substitution of amino acid 70 in the hepatitis C virus core region of genotype 1b is an important predictor of elevated alpha-fetoprotein in patients without hepatocellular carcinoma. J Med Virol 2008;80: 1354-1362.
16. Kobayashi M, Akuta N, Suzuki F, Hosaka T, Sezaki H, Kobayashi M, et al. Influence of amino-acid polymorphism in the core protein on progression of liver disease in patients infected with hepatitis C virus genotype 1b. J Med Virol 2010;82:41-48.
17. El-Shamy A, Shoji I, Saito T, Watanabe H, Ide YH, Deng L, et al. Sequence heterogeneity of NS5A and core proteins of hepatitis C virus and virological responses to pegylated-interferon/ribavirin combination therapy. Microbiol Immunol 2011;55:418-426.
18. El-Shamy A, Kim SR, Ide YH, Sasase N, Imoto S, Deng L, et al. Polymorphisms of hepatitis C virus non-structural protein 5A and core protein and clinical outcome of pegylated-interferon/ribavirin combination therapy. Intervirology 2012;55:1-11.
19. Akuta N, Suzuki F, Sezaki H, Suzuki Y, Hosaka T, Someya T, et al. Association of amino acid substitution pattern in core protein of hepatitis C virus genotype 1b high viral load and non-virological response to interferon-ribavirin combination therapy. Intervirology 2005;48: 372-380.
20. Akuta N, Suzuki F, Kawamura Y, Yatsuji H, Sezaki H, Suzuki Y, et al. Predictive factors of early and sustained responses to peginterferon plus ribavirin combination therapy in Japanese patients infected with hepatitis C virus genotype 1b: amino acid substitutions in the core region and low-density lipoprotein cholesterol levels. J Hepatol 2007;46: 403-410.

21. Sakamuro D, Furukawa T, Takegami T. Hepatitis C virus nonstructural protein NS3 transforms NIH 3T3 cells. J Virol 1995;69:3893-3896.
22. Zemel R, Gerechet S, Greif H, Bachmatove L, Birk Y, Golan-Goldhirsh A, et al. Cell transformation induced by hepatitis C virus NS3 serine protease. J Viral Hepat 2001;8:96-102.
23. Fujita T, Ishido S, Muramatsu S, Itoh M, Hotta H. Suppression of actinomycin D-induced apoptosis by the NS3 protein of hepatitis C virus. Biochem Biophys Res Commun 1996;229:825-831.
24. Macdonald A, Harris M. Hepatitis C virus NS5A: tales of a promiscuous protein. J Gen Virol 2004;85:2485-2502.
25. Enomoto N, Sakuma I, Asahina Y, Kurosaki M, Murakami T, Yamamoto C, et al. Mutations in the nonstructural protein 5A gene and response to interferon in patients with chronic hepatitis C virus 1b infection. N Engl J Med 1996;334:77-81.
26. El-Shamy A, Nagano-Fujii M, Sasase N, Imoto S, Kim SR, Hotta H. Sequence variation in hepatitis C virus nonstructural protein 5A predicts clinical outcome of pegylated interferon/ribavirin combination therapy. HEPATOLOGY 2008;48:38-47.
27. Kim SR, El-Shamy A, Imoto S, Kim KI, Ide YH, Deng L, et al. Prediction of response to pegylated interferon/ribavirin combination therapy for chronic hepatitis C genotype 1b and high viral load. J Gastroenterol 2012;47:1143-1151.
28. Ikeda K, Saitoh S, Arase Y, Chayama K, Suzuki Y, Kobayashi M, et al. Effect of interferon therapy on hepatocellular carcinogenesis in patients with chronic hepatitis type C: A long-term observation study of 1,643 patients using statistical bias correction with proportional hazard analysis. HEPATOLOGY 1999;29:1124-1130.
29. Okanoue T, Itoh Y, Minami M, Sakamoto S, Yasui K, Sakamoto M, et al. Interferon therapy lowers the rate of progression to hepatocellular carcinoma in chronic hepatitis C but not significantly in an advanced stage: a retrospective study in 1148 patients. Viral Hepatitis Therapy Study Group. J Hepatol 1999;30:653-659.
30. Benvegnu L, Chemello L, Noventa F, Fattovich G, Pontisso P, Alberti A. Retrospective analysis of the effect of interferon therapy on the clinical outcome of patients with viral cirrhosis. Cancer 1998;83:901-909.
31. Okamoto H, Sugiyama Y, Okada S, Kurai K, Akahane Y, Sugai Y, et al. Typing hepatitis C virus by polymerase chain reaction with type-specific primers: application to clinical surveys and tracing infectious sources. J Gen Virol 1992;73:673-679.
32. El-Shamy A, Sasayama M, Nagano-Fujii M, Sasase N, Imoto S, Kim SR, et al. Prediction of efficient virological response to pegylated interferon/ribavirin combination therapy by NS5A sequences of hepatitis C virus and anti-NS5A antibodies in pre-treatment sera. Microbiol Immunol 2007;51:471-482.
33. Ogata S, Nagano-Fujii M, Ku Y, Yoon S, Hotta H. Comparative sequence analysis of the core protein and its frameshift product, the F protein, of hepatitis C virus subtype 1b strains obtained from patients with and without hepatocellular carcinoma. J Clin Microbiol 2002;40:3625-3630.
34. Ogata S, Florese RH, Nagano-Fujii M, Hidajat R, Deng L, Ku Y, et al. Identification of hepatitis C virus (HCV) subtype 1b strains that are highly, or only weakly, associated with hepatocellular carcinoma on the basis of the secondary structure of an amino-terminal portion of the HCV NS3 protein. J Clin Microbiol 2003;41:2835-2841.
35. Kato N, Hijikata M, Ootsuyama Y, Nakagawa M, Ohkoshi S, Sugimura T, et al. Molecular cloning of the human hepatitis C virus genome from Japanese patients with non-A, non-B hepatitis. Proc Natl Acad Sci U S A 1990;87:9524-9528.
36. Delhem N, Sabile A, Gajardo R, Podevin P, Abadie A, Blaton MA, et al. Activation of the interferon-inducible protein kinase PKR by hepatocellular carcinoma derived-hepatitis C virus core protein. Oncogene 2001;20:5836-5845.
37. Love RA, Parge HE, Wickersham JA, Hostomsky Z, Habuka N, Moomaw EW, et al. The crystal structure of hepatitis C virus NS3 proteinase reveals a trypsin-like fold and a structural zinc binding site. Cell 1996;87:331-342.
38. Saiz JC, Lopez-Labrador FX, Ampurdanes S, Dopazo J, Forns X, Sanchez-Tapias JM, et al. The prognostic relevance of the nonstructural 5A gene interferon sensitivity determining region is different in infections with genotype 1b and 3a isolates of hepatitis C virus. J Infect Dis 1998;177:839-847.
39. Sarrazin C, Berg T, Lee JH, Teuber G, Dietrich CF, Roth WK, et al. Improved correlation between multiple mutations within the NS5A region and virological response in European patients chronically infected with hepatitis C virus type 1b undergoing combination therapy. J Hepatol 1999;30:1004-1013.

IJC
International Journal of Cancer

High ubiquitous mitochondrial creatine kinase expression in hepatocellular carcinoma denotes a poor prognosis with highly malignant potential

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We previously reported the increased serum mitochondrial creatine kinase (MtCK) activity in patients with hepatocellular carcinoma (HCC), mostly due to the increase in ubiquitous MtCK (uMtCK), and high uMtCK mRNA expression in HCC cell lines. We explored the mechanism(s) and the relevance of high uMtCK expression in HCC. In hepatitis C virus core gene transgenic mice, known to lose mitochondrial integrity in liver and subsequently develop HCC, uMtCK mRNA and protein levels were increased in HCC tissues but not in non-tumorous liver tissues. Transient overexpression of ankyrin repeat and suppressor of cytokine signaling box protein 9 (ASB9) reduced uMtCK protein levels in HCC cells, suggesting that increased uMtCK levels in HCC cells may be caused by increased gene expression and decreased protein degradation due to reduced ASB9 expression. The reduction of uMtCK expression by siRNA led to increased cell death, and reduced proliferation, migration and invasion in HCC cell lines. Then, consecutive 105 HCC patients, who underwent radiofrequency ablation with curative intent, were enrolled to analyze their prognosis. The patients with serum MtCK activity >19.4 U/L prior to the treatment had significantly shorter survival time than those with serum MtCK activity ≤19.4 U/L, where higher serum MtCK activity was retained as an independent risk for HCC-related death on multivariate analysis. In conclusion, high uMtCK expression in HCC may be caused by hepatocarcinogenesis *per se* but not by loss of mitochondrial integrity, of which ASB9 could be a negative regulator, and associated with highly malignant potential to suggest a poor prognosis.

Key words: ubiquitous mitochondrial creatine kinase, ankyrin repeat and suppressor of cytokine signaling box protein 9, hepatocellular carcinoma, prognostic factor

Abbreviations: AFP: alpha-fetoprotein; ALT: alanine aminotransferase; ASB: ankyrin repeat and suppressor of cytokine signaling box protein; AST: aspartate aminotransferase; DCP: des-gamma-carboxy prothrombin; GGT: gamma-glutamyltransferase; HCC: hepatocellular carcinoma; HCV: hepatitis C virus; RFA: radiofrequency ablation; ROC: receiver operating characteristic; SOCS: suppressor of cytokine signaling; uMtCK: ubiquitous mitochondrial creatine kinase

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DOI: 10.1002/ijc.28547
History: Received 2 July 2013; Accepted 1 Oct 2013; Online 15 Oct 2013

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Primary liver cancer, 95% of which is hepatocellular carcinoma (HCC), is ranked third in men and fifth in women as a cause of death from malignant neoplasms in Japan.[1] Furthermore, the worldwide incidence of HCC has increased over several decades, and HCC has recently received considerable attention as a common cause of mortality.[2] HCC often arises in background of liver cirrhosis, which is formed as a result of chronic viral infections, alcoholic injury and some other disorders in the liver.[3,4] Of note, HCC has recently been linked to non-alcoholic fatty liver disease, and this association may contribute to the rising incidence of HCC witnessed in many industrialized countries. It is also problematic that HCC may complicate non-cirrhotic, non-alcoholic fatty liver disease with mild or absent fibrosis, greatly expanding the population potentially at higher risk.[5] Because HCC has a poor prognosis due to its aggressive nature, surgical resection and radiofrequency ablation (RFA) are effective only in early stage of HCC.[4,6] Recurrence occurs almost in 70% of patients with HCC of the first occurrence within 5 years.[7] Regarding the treatment of HCC in United

What's new?

The identification of biomolecules associated with hepatocellular carcinoma (HCC) could greatly improve screening for early disease detection. Ubiquitous mitochondrial creatine kinase (uMtCK) could be a promising marker in this context, though its relevance in HCC is unclear, as it may be associated with mitochondrial stability rather than carcinogenesis. Here, in transgenic mice susceptible to the loss of liver mitochondrial integrity, uMtCK was found to be elevated in HCC tissue but not in non-tumorous liver tissue. Increased uMtCK was further linked to reduced expression of ASB9 and elevated risk for HCC-related death.

States veterans, approximately 40% of patients were reportedly diagnosed during hospitalization. Most patients were not seen by a surgeon or oncologist for treatment evaluation and only 34% received treatment.[8] Although there was no effective chemotherapy for advanced HCC for a long time, a novel anti-cancer therapy such as anti-angiogenesis pathway therapy has just recently been developed to prolong survival in patients with the advanced disease.[9,10] However, its effect is rather limited, just extending median survival from 7.9 months to 10.7 months in patients with advanced HCC.[10] Thus, the effective way for early detection of HCC is urgently needed. To this end, the recommended screening strategy for patients with cirrhosis includes the determination of serum alpha-fetoprotein (AFP) levels and an abdominal ultrasound every 6 months to detect HCC at an earlier stage. AFP, however, is a marker characterized by poor sensitivity and specificity.[11] Although other potential markers such as des-gamma-carboxy prothrombin (DCP) and squamous cell carcinoma antigen-immunoglobulin M complex have been proposed to use for diagnosis of HCC, none of them is optimal; however, when used together, their sensitivity in detecting HCC is increased.[11–14] For cholangiocarcinoma, which is a relatively rare type of primary liver cancer that originates in the bile duct epithelium, carbohydrate antigen 19-9, carcinogenic embryonic antigen and cancer antigen 125 have shown sufficient sensitivity and specificity to detect and monitor it. In particular, the combination of these markers seems to increase their efficiency in diagnosing of cholangiocarcinoma.[15]

In this context, we have recently reported that serum mitochondrial creatine kinase (MtCK) activity is increased in patients with HCC, even in those with early stage, suggesting that MtCK may be useful to detect early stage of HCC.[16] Among two tissue-specific isozymes of MtCK, that is, ubiquitous MtCK (uMtCK) and sarcomeric MtCK, we have found that the increase in serum MtCK activity in HCC patients was mostly due to that in serum uMtCK activity but not in serum sarcomeric MtCK activity.[16] Then, we have further observed the higher expression of uMtCK mRNA in HCC cell lines than in normal human liver tissues.[16] Of note, the increased uMtCK expression occurred not only upon malignant changes in the liver, but also in several other malignant tumors such as gastric cancer, breast cancer and lung cancer, where the high expression of uMtCK suggests a poor prognosis.[17–19] In contrast, uMtCK was down-regulated in oral squamous cell carcinoma,[20] and sarcomeric MtCK was also down-regulated during sarcoma development in leg muscle in mice.[21] Therefore, we aimed to elucidate the mechanism(s) and the significance of high uMtCK expression in HCC in this study.

We first examined whether loss of mitochondrial integrity might be involved in high uMtCK expression in HCC, using hepatitis C virus (HCV) core gene transgenic mice. HCV core protein has been first demonstrated to play a pivotal role in HCC development within these transgenic mice, which are known to lose mitochondrial integrity and subsequently develop HCC without apparent inflammation and fibrosis in the liver.[22,23] As a regulatory factor for uMtCK expression, we have focused on the ankyrin repeat and suppressor of cytokine signaling (SOCS) box protein (ASB) family, which reportedly plays an important role in biological processes and regulations of cell proliferation and differentiation. The ASBs have two functional domains: a SOCS box and a variable number of N-terminal ankyrin repeats. Although SOCS domain uses the SH2 domain to recruit substrates, the ankyrin repeat regions serve as a specific protein–protein interaction domain to recruit target substrates.[24] One of ASB family protein, ASB9, was found to interact with brain type of creatine kinase, leading to its degradation.[25] Recently, uMtCK was found to be another ASB9 target.[26] Ankyrin repeat domains of ASB9 associates with the substrate binding site of uMtCK and induce its ubiquitination. Thus, we analyzed the potential association between uMtCK and ASB9 in HCC cell lines, HepG2, PLC/PRF/5, HuH7, in which the expression of uMtCK mRNA was shown to be increased compared with normal liver tissues.[16] To clarify the significance of high uMtCK expression in HCC, we used the siRNA approach to silence uMtCK expression and study its effects on HCC cell lines. Finally, we analyzed the clinical significance of high uMtCK expression in HCC patients who were treated with RFA.

Material and Methods

Materials

Human normal liver RNA was purchased from Cell Applications (San Diego, CA), and human whole liver cell pellets from DV Biologics (Costa Mesa, CA). Specific antibodies against uMtCK and ASB9 were obtained from Abcam (Cambridge, UK), an antibody against caspase 3 from Cell Signaling Technology (3G2; Boston, MA), and an antibody against beta-actin from Sigma–Aldrich (MO).

Cells and cell culture

HCC cell lines, HepG2 and PLC/PRF/5 were obtained from RIKEN BioResource Center (Tsukuba, Ibaraki, Japan) and HuH7 from Health Science Research Resources Bank, Japan Health Science Foundation. HepG2 and PLC/PRF/5 were maintained in RPMI-1640 containing 10% of fetal bovine serum, and HuH7, in Dulbecco's Modified Eagle Medium containing 10% of fetal bovine serum.

Transgenic mice

HCV core gene transgenic mice were produced as previously described.[22] Nontransgenic littermates of the transgenic mice were used as controls. All mice were fed a standard pelleted diet and water *ad libitum* under normal laboratory conditions of 12 hr-light/dark cycles, and received humane care. The experimental protocol was approved by Animal Research Committee of the University of Tokyo.

Quantitative real-time PCR

Total RNA of HCC cell lines (HepG2, PLC/PRF/5 and HuH7), human normal liver and livers from non-transgenic and HCV core gene transgenic mice were extracted using TRIZOL reagent (Invitrogen, CA). One microgram of purified total RNA was transcribed using a SuperScript™ First-Strand Synthesis System for RT-PCR (Invitrogen). Quantitative real-time PCR was performed with a LightCycler FastStart DNA Master SYBR Green I kit (Roche Molecular Diagnostics, CA) or TaqMan Universal Master Mix. The primer pairs used were as follows: human ASB9: 5′-CCTGGCATCAGGCTTCTTTC-3′ and 5′-ACCCCTGGCTGATGAGGTTC-3′[27]; human beta-actin: 5′-GGGTCAGAAGGATTCCTATG-3′ and 5′-CCTTAATGTC ACGCACGATTT-3′.[26] Mouse uMtCK primers and probe were obtained from Applied Biosystems, TaqMan Gene Expression Assays (Mm00438221_m1). The samples were incubated for 10 min at 95°C, followed by 40 cycles at 95°C for 10 sec, 60°C for 10 sec and 72°C for 10 sec. The target gene mRNA expression level was relatively quantified to beta-actin using $2^{-\Delta\Delta Ct}$ method (Applied Biosystems, User Bulletin No 2).

ASB9 transfection

Cells, transiently expressing human ASB9 protein, were constructed using mammalian cell expression vector p3FLAG CMV-10 containing the corresponding cDNA which derived from human normal liver RNA. The primers used for cloning were 5′-GCGGATCCGTCATGGATGGCAAACAAGGG-3′ and 5′-GAGCGGCCGCTTAAGATGTAGGAGAAACTGTT T-3′ which were designed based on human ASB9 reference sequence (NM_001031739.2). The ASB9 cDNA was created by PCR and verified by DNA sequencing.

Immunoblot analysis

Cell and tissue extracts were prepared using M-PER Mammalian Protein Extraction Reagent (Thermo Fisher Scientific, IL) plus Halt™ Protease Inhibitor Cocktail (Thermo Fisher Scientific). Immunoblot analysis was performed as previously described,[28] using NuPAGE SDS-PAGE Gel (Invitrogen) and iBlot Dry Blotting System (Invitrogen) with specific antibodies against uMtCK (dilution 1:1,000), ASB9 (dilution 1:500), caspase 3 (dilution 1:1,000) and beta-actin (dilution 1:2,000). Immunoreactive proteins were visualized using a chemiluminescence kit (GE Healthcare, Buckinghamshire, UK), and recorded using a LAS-4000 image analyzer (Fuji Film, Tokyo, Japan). The intensities of immunodetected bands were quantified with NIH Image J software.

uMtCK siRNA transfection

Cells were transfected with the human uMtCK-specific 23/27mer RNA duplex or a universal negative control duplex at 20 nM, respectively, according to the vender instructions (Integrated DNA Technologies, IA). The human uMtCK-specific RNA duplex used was 5′-UGAAGCACACCACGGAUCU-3′ and 3′-ACUUCGUGUGGUGCCUAGA-5′,[29] negative control RNA duplex, 5′-CGUUAAUCGCGUAUAAUACGCGUAT-3′ and 3′-CAGCAAUUAGCGCAUAUUAUGCGCAUA-5′ (Integrated DNA Technologies). The transfection was performed using Lipofectamine Plus™ (Invitrogen) as described.[29]

Cell membrane integrity and proliferation assays

Cell membrane integrity was determined using the In Vitro Toxicology Assay Kit, Lactic Dehydrogenase based (Sigma–Aldrich). HCC cell lines were inoculated in six-well plates at 2.5×10^5 cells/well and cultured for 24 hr before uMtCK siRNA or universal negative control transfection. Dead cells were assessed at 48 hr after transfection.

Cell proliferation in HCC cell lines was measured at 48 hr after transfection with uMtCK siRNA or universal negative control by determination of BrdU incorporation using the Cell Proliferation ELISA, BrdU colorimetric assay (Roche Applied Science, Upper Bavaria, Germany). In the above two assays, absorbance was measured by plate reader (SPECTRA Thermo, TECAN, Männedorf, Switzerland).

Cell migration and invasion assays

Cell migration and invasion assays were performed according to the vender's instruction (BD, NJ). Cells transfected with uMtCK siRNA or universal negative control were cultured for 24 hr, then 2×10^4 cells were plated into the upper chamber of 24-well plates with 8 μm of pore size in serum-starved condition to examine cell migration and polycarbonate transwell filter chamber coated with Matrigel (BD BioCoat Matrigel Invasion Chamber) to check cell invasion. In both assays, 750 μL medium supplemented with 10% serum was added into the lower chambers. Cells were incubated at 37°C for 22 hr, and the inside chambers were removed with cotton swabs and cells that had transferred to the lower membrane surface were fixed and stained with Diff-Quik stain. Cell counts (four random 100× fields per well) are expressed as the mean number of cells per field of view.

Patients and measurement of MtCK activity

Consecutive 147 HCC patients with cirrhosis caused by hepatitis B virus or HCV, who were admitted into the Department of Gastroenterology, the University of Tokyo Hospital, Tokyo, Japan, between January and April 2010, were previously enrolled to analyze serum MtCK activity.[16] Diagnosis of cirrhosis was based on the presence of clinical and laboratory features indicating portal hypertension, and diagnosis of HCC was made by dynamic CT or MRI.[30,31] Prior to the treatment of HCC, serum MtCK activity was measured[16] with an immuno-inhibition method using the two types of anti-MtCK monoclonal antibodies.[32] Among these patients, 105 patients, who had been successfully treated by RFA without residual HCC after the treatment, were enrolled in the current prognosis analysis. The detailed procedure of RFA has been meticulously described elsewhere.[33] Overall survival of these 105 patients was analyzed from the time of measurement of serum MtCK activity to death related to HCC, excluding the death not associated with HCC expansion or liver insufficiency, such as cardiovascular events or other organ malignancy, or to March 2013.

This study was performed 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. A written informed consent was obtained for the use of the samples in this study.

Statistical analysis

The results of *in vitro* experiments are expressed as the means and standard error of the mean. Student's *t* test (two tailed) was used for comparison unless indicated otherwise. The results were considered significant when *p*-values were 0.05. In the analysis of risk factors for HCC-related death, we tested the following variables obtained at the time of entry on the univariate and multivariate Cox proportional hazard regression analysis: age, sex, hepatitis B infection, serum MtCK activity, serum albumin concentration, aspartate aminotransferase (AST) levels, alanine aminotransferase (ALT) levels, gamma-glutamyltransferase (GGT) levels, total bilirubin concentration, AFP concentration, DCP concentration, platelet count, prothrombin activity and liver stiffness values. Survival and recurrence curves were created using Kaplan–Meier method and compared *via* log-rank test. Data processing and analysis were performed using S-PLUS 2000 (MathSoft, Seattle, WA) and SAS Software version 9.1 (SAS Institute, Cary, NC).

Results

Loss of mitochondrial integrity may not contribute to high expression of uMtCK in HCC

Mutations of mitochondrial DNA have been reported to be involved in hepatocarcinogenesis in humans.[34,35] Furthermore, in a mouse model for hepatocarcinogenesis, oxidative stress was shown to lead to loss of mitochondrial integrity in the liver and ultimately hepatocarcinogenesis.[23] Thus, we wondered whether loss of mitochondrial integrity in the liver might be associated with increased expression of uMtCK in HCC. To examine this idea, we used a transgenic mouse model of HCC in HCV infection (transgenic line S-N/863), with which the direct association between HCV and HCC was first described.[22] In these HCV core gene transgenic mice, loss of mitochondrial integrity has been reported to be observed as early as 2 months of age and increased in an age-dependent manner,[23] and ultimately HCC develops at 19 months of age without apparent inflammation or fibrosis in the liver.[22]

We examined uMtCK mRNA levels in the liver of these HCV core protein transgenic mice at 6 months and 19 months of age. These mice at 6 months of age reportedly develop hepatic steatosis[22] as well as loss of mitochondrial integrity.[23] In these mice at 19 months of age, tumor tissues of HCC and non-tumorous tissues of the liver were analyzed. Non-transgenic mice at 6 months of age were used as control. uMtCK mRNA levels were increased in tumor tissues of HCC in HCV core gene transgenic mice at 19 months of age by 7.7-fold compared to the liver tissues of control mice ($p = 0.02$; Fig. 1*a*). In these HCV core transgenic mice at 19 months of age, uMtCK protein expression was detected in HCC tissues but not in non-tumorous tissues by immunoblot analysis (Fig. 1*b*). These results suggest that hepatocarcinogenesis *per se* but not loss of mitochondrial integrity may contribute to the increase in uMtCK levels in HCC.

Transient expression of ASB9 negatively regulates uMtCK protein levels in HCC cells

It has been reported that ASB protein family is importantly involved in ubiquitination-mediated proteolysis pathway and each member of this large protein family has a different target to be degraded. In ASB protein family, we paid attention to ASB9, which reportedly plays a crucial role in the regulation of the brain type of creatine kinase and uMtCK. HCC cell lines, HepG2, PLC/PRF/5 and HuH7, were selected for *in vitro* experiments, because they had been reported to express high levels of uMtCK mRNA compared to human normal liver tissue.[16] To study whether ASB9 could regulate uMtCK protein levels in these HCC cells, we first measured ASB9 mRNA expression in those cells. Figure 2*a* demonstrates the low ASB9 mRNA expression in HCC cell lines, contrasting with high uMtCK mRNA expression levels in those cells.[16] In line with our mRNA expression data, ASB9 protein levels were almost undetectable in HepG2, PLC/PRF/5 and HuH7 cells comparing to normal whole liver cell pellets (Fig. 2*b*). Further, we investigated the effect of transient overexpression of ASB9 on uMtCK protein levels in HepG2, PLC/PRF/5 and HuH7 cells. Cells were transiently transfected with mammalian cell expression vector p3FLAG-CMV10 containing human ASB9 DNA and harvested at 36 hr after transfection to analyze protein levels. Down-regulation of uMtCK protein levels by transient

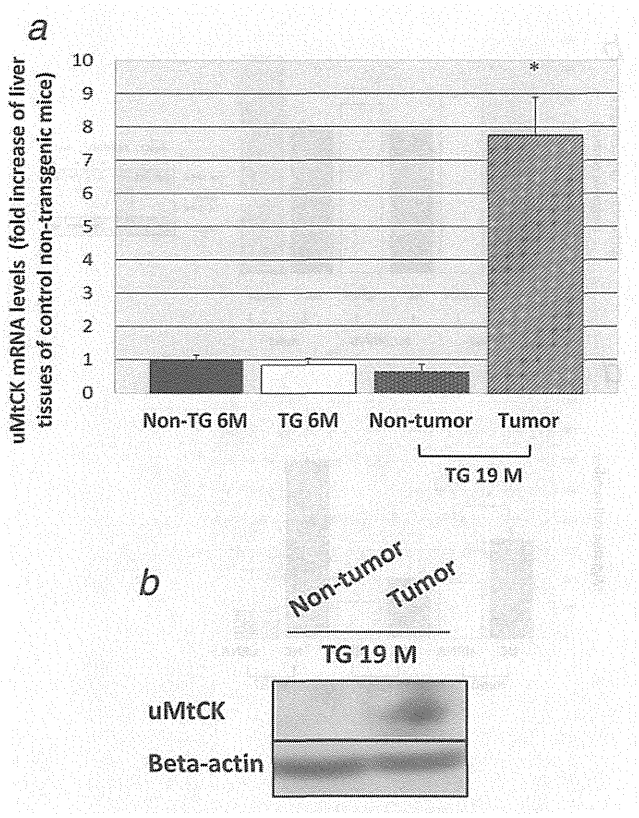

Figure 1. uMtCK mRNA and protein levels in liver tissues of the control non-transgenic, HCV core gene transgenic mice. (*a*) uMtCK mRNA levels were examined by real-time PCR in liver tissues of the control non-transgenic mice (Non-TG) at 6 months of age ($n = 4$), and HCV core gene transgenic mice (TG) at 6 ($n = 4$) and 19 months of age ($n = 4$). For HCV core gene transgenic mice at 19 months of age, HCC tissues and non-tumorous tissues were separately evaluated. Results represent a fold increase level of liver tissues of control non-transgenic mice. An asterisk indicates a significant difference ($p = 0.02$) from liver tissues of non-transgenic mice. (*b*) uMtCK protein levels were analyzed by immunoblotting in HCC tissues and non-tumorous tissues in the livers of HCV core gene transgenic mice at 19 months of age.

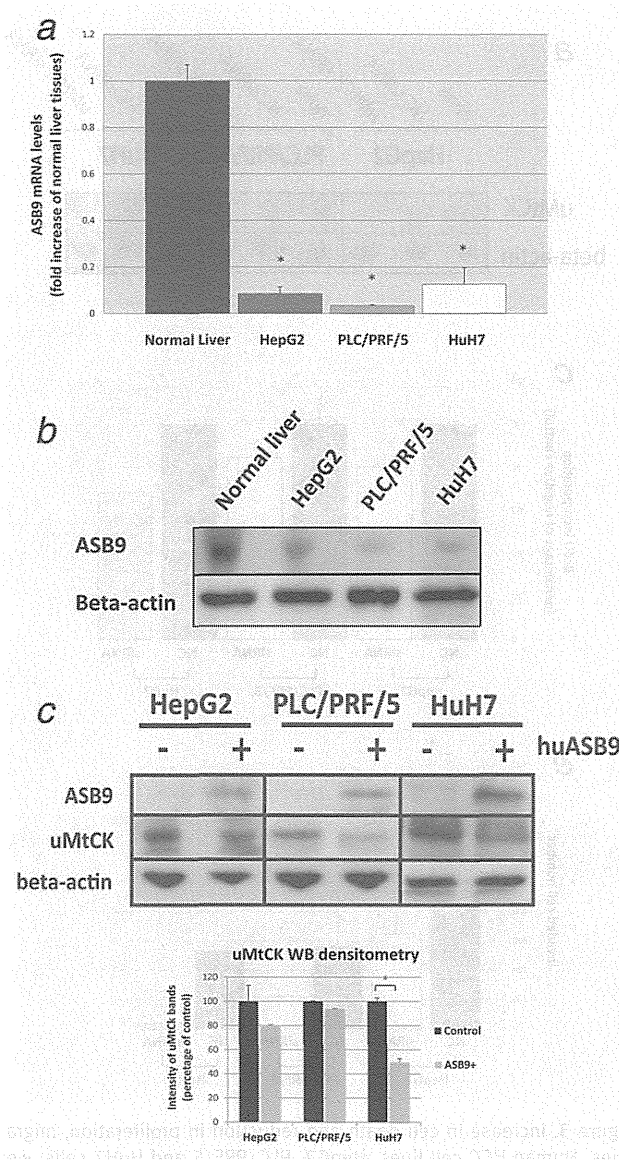

Figure 2. ASB9 expression and the effect of ASB9 transfection on uMtCK protein levels in HCC cells. ASB9 mRNA (*a*) and protein (*b*) levels in HepG2, PLC/PRF/5 and HuH7 cells were examined by real-time PCR and immunoblot analysis, respectively. As a positive control for ASB9 mRNA and protein expressions, human normal liver RNA and human whole liver cell pellets were used. An asterisk indicates a significant difference from normal liver tissue; $p = 0.006$ for HepG2, $p = 0.005$ for PLC/PRF/5 and $p = 0.01$ for HuH7. Increased expression of ASB9 by transfection caused reduced protein levels of uMtCK in HepG2, PLC/PRF/5 and HuH7 cells (*c*). An asterisk indicates a significant difference ($p = 0.007$) from control without ASB9 transfection.

overexpression of ASB9 was observed significantly in HuH7 cells ($p = 0.007$), and a trend of decreased uMtCK protein levels was found in HepG2 and PLC/PRF/5 cells, although not statistically significant (Fig. 2*c*). These results suggest a functional interaction of ASB9 with uMtCK may lead to degradation of uMtCK protein in HCC cell lines, as previously described.[26]

Reduction in uMtCK expression led to increased cell death, and reduced proliferation, migration and invasion of HCC cells

To inhibit high uMtCK expression in HepG2, PLC/PRF/5 and HuH7 cells,[16] isoform-specific siRNA was chosen as described[29] and successfully silenced target protein expression; the results from immunoblot analysis of untransfected and transfected cell lysates with universal negative control and uMtCK siRNA are shown in Figure 3*a*. As expected, in all HCC cell lines transfected with uMtCK siRNA, the expression levels of uMtCK were clearly reduced at 36 hr after transfection (Fig. 3*a*).

Then, the effects of a reduction in uMtCK expression on cell membrane integrity and proliferation were determined in HepG2, PLC/PRF/5 and HuH7 cells. In the first step, we have checked cell membrane integrity by measuring lactate

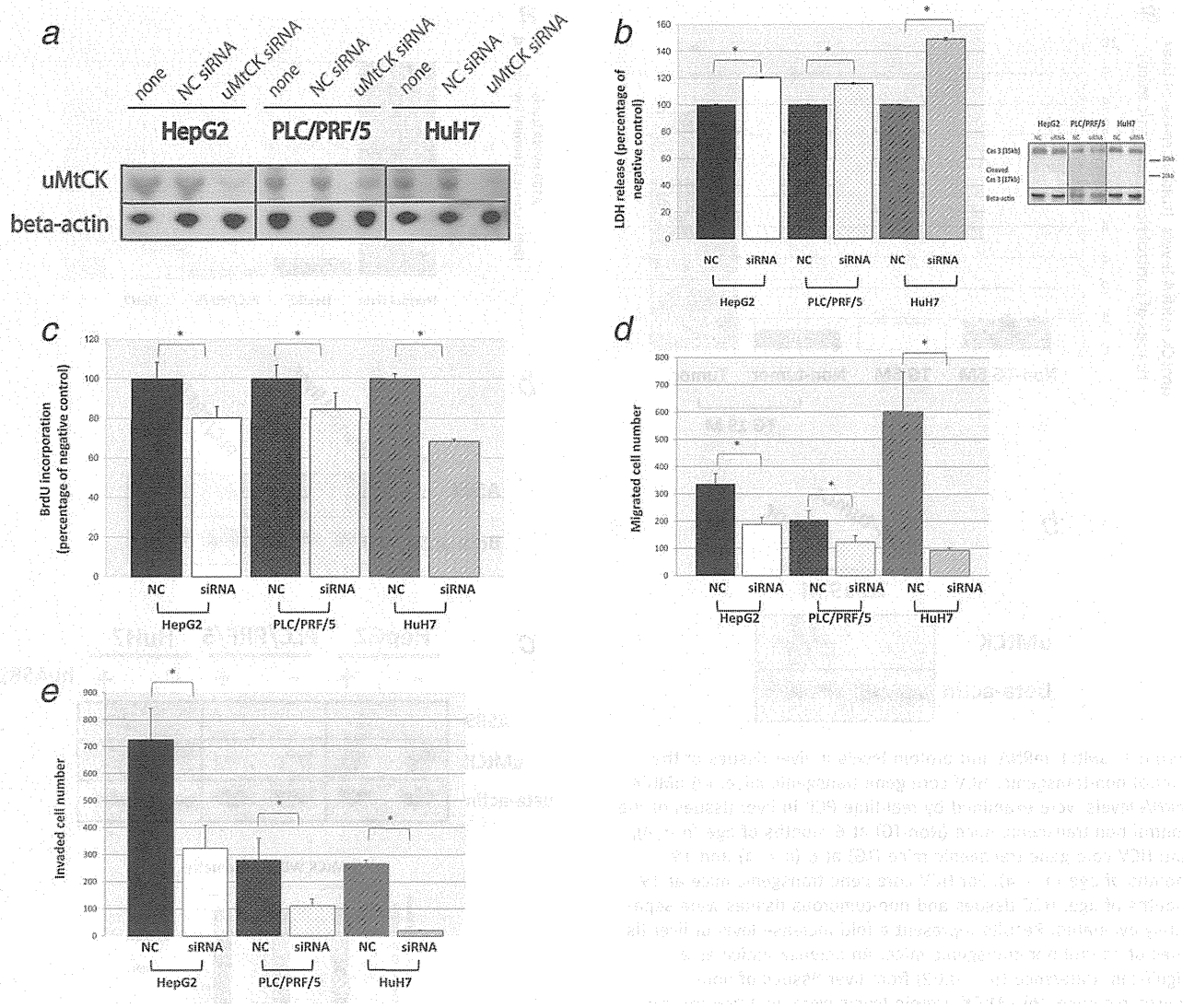

Figure 3. Increase in cell death and reduction in proliferation, migration and invasion by reduced uMtCK expression with siRNA in HCC cell lines. Human HCC cell lines, HepG2, PLC/PRF/5 and HuH7 cells, were transfected with 20 nM uMtCK siRNA or universal negative control, and uMtCK levels were examined by immunoblot analysis. None, no transfection; NC, negative control (*a*). Cell death (*b*), proliferation (*c*), migration (*d*) and invasion (*e*) were assessed in these HCC cell lines treated with or without uMtCK siRNA. An asterisk indicates a significant difference; $p < 0.001$ for cell death and proliferation, $p < 0.02$ for cell migration and invasion from NC.

dehydrogenase released into the culture medium in universal negative control- and uMtCK siRNA-transfected cells (Fig. 3*b*). In all three cells, transfection with uMtCK siRNA led to an increase in the rate of cell lysis by 20.3% in HepG2, by 15.9% in PLC/PRF/5 and by 49.2% in HuH7, compared to respective control cells transfected with universal negative control ($p < 0.001$). However, caspase 3 activity was not altered in uMtCK siRNA-transfected cells compared to universal negative control-transfected cells (Fig. 3*b*), suggesting that lactate dehydrogenase release may be explained by some non-specific cell lysis but not by programmed cell death.

Next, to examine a potential association of the reduction in uMtCK expression with cell proliferation rate, BrdU incorporation assay was performed (Fig. 3*c*). A reduction in cell proliferation was detected in all three HCC cell lines by 19.8% in HepG2, by 15.5% in PLC/PRF/5 and by 31.7% in HuH7, compared to respective control cells transfected with universal negative control ($p < 0.001$). These results suggest that high expression of uMtCK may play a role in sustaining active proliferation of HCC cells.

The ability of a cancer cell to undergo migration and invasion allows the cell to change position within the tissues. To spread within the tissues, tumor cells use migration and invasion mechanisms. Thus, we investigated the effects of uMtCK inhibition on HCC cell migration and invasion by conducting assays for Matrigel-coated chamber migration and invasion. As shown in Figure 3*d*, silencing of uMtCK decreased migration rate by 44.1% in HepG2, by 40.0% in

Table 1. Baseline characteristics

Parameter	*N* = 105
Age (year)[1]	70.7 ± 6.7 (49–84)
Male[2]	63 (60.0)
Hepatitis B/C	8 / 97
MtCK (U/L)[3]	9.71 (5.99–19.44)
Albumin (g/dL)[3]	3.4 (3.1–3.9)
AST (U/L)[3]	55 (35–76)
ALT (U/L)[3]	45 (26–60)
GGT (U/L)[3]	37 (28–62)
Total bilirubin (mg/dL)[3]	0.9 (0.7–1.3)
AFP (ng/dL)[3]	18 (8–66)
DCP (mAU/mL)[3]	26 (17–58)
Platelet ($\times 10^4/\mu L$)[3]	9.3 (6.3–11.7)
Prothrombin time (sec)[3]	12.1 (11.5–13.1)
Liver stiffness (kPa)[3]	26.3 (18.8–42.2)

[1]Data were expressed as mean ± SD (range).
[2]Data were expressed as number (%).
[3]Data were expressed as median (first to third quartile).

PLC/PRF/5 and by 84.1% in HuH7 cells in comparison with the universal negative control-transfected cells ($p < 0.02$). Furthermore, the results from Matrigel invasion assay indicate that the reduction of uMtCK expression by siRNA transfection inhibited the invasion of HepG2, PLC/PRF/5 and HuH7 cells by 51.7, 62.6 and 92.4%, compared to the universal negative control-transfected cells ($p < 0.02$) (Fig. 3*e*). Collectively, high expression of uMtCK may contribute to active migration and invasion of HCC cells.

HCC patients with higher serum MtCK activity had a poorer prognosis after RFA

Because above *in vitro* results using HCC cell lines suggest that HCC cells with higher expression of uMtCK may have more malignant potential, we next examined a potential association between serum MtCK activity and prognosis in patients with HCC. As described earlier, among two tissue-specific isozymes of MtCK, that is, uMtCK and sarcomeric MtCK, the increase in serum MtCK activity in HCC patients was mostly due to that in serum uMtCK activity but not in serum sarcomeric MtCK activity.[16] To this end, a prognosis of HCC patients, who had been previously enrolled to examine their serum MtCK activity and successfully treated by RFA without residual HCC after the treatment, was analyzed. Characteristics of these 105 HCC patients are shown in Table 1. During the mean follow-up period of 848 days, HCC-related death was observed in 17 patients. First, to evaluate the potential ability of MtCK values to predict survivals or death, a receiver operating characteristic (ROC) curve was generated. The ROC curve showed that a MtCK cutoff of 19.4 U/L had a sensitivity of 76.9% and a specificity of 83.8% for discriminating survivors and deceased patients (Fig. 4*a*). Then, Figure 4*b* shows the actuarial survival curves of these patients subdivided according to their serum MtCK activity prior to RFA for HCC, that is, ≤19.4 U/L and >19.4 U/L; overall survival was shorter in patients with serum MtCK activity >19.4 U/L than in those with ≤19.4 U/L ($p = 0.0002$; log-rank test; Fig. 4*b*). Then, risk factors for HCC-related death were analyzed. On the univariate analysis, high serum MtCK activity (>19.4 U/L) was a significant risk factor for HCC-related death (Table 2). Other significant risk factors for HCC-related death included serum albumin concentration, serum AST levels, serum total bilirubin concentration, platelet count and prothrombin time (Table 2). Then, multivariate Cox proportional hazard regression analysis revealed that serum MtCK activity >19.4 U/L was an independent risk for HCC-related death, with a hazard ratio of 2.32 (95% confidence interval: 1.03–5.25; $p = 0.042$; Table 2). Serum albumin concentration and serum AST levels were also independently associated with HCC-related death (Table 2). Regarding recurrence, HCC in patients with serum MtCK activity >19.4 U/L recurred earlier than HCC in those with serum MtCK activity ≤19.4 U/L, as depicted in Figure 4*c* ($p = 0.004$; log-rank test); median (interquartile range) time to recurrence was 189 (107–292) days in patients with serum MtCK activity >19.4 U/L, whereas 278 (160–445) days in those with serum MtCK activity ≤19.4 U/L. Collectively, these findings suggest that HCC patients with higher serum MtCK activity may have shorter survival time possibly due to more malignant potential.

Discussion

Little is known about whether there might be an association between the status of mitochondria and uMtCK expression. Kwon *et al.* have reported that ASB9 negatively regulated uMtCK expression with the inhibition of mitochondrial function,[26] suggesting that low uMtCK expression could be associated with loss of mitochondrial integrity. There could be several possibilities regarding the status of mitochondria and uMtCK expression in the liver or HCC; one is that loss of mitochondrial integrity might be associated with reduced uMtCK expression as previously reported.[26] As another possibility, uMtCK expression might be increased as a compensatory mechanism with loss of mitochondrial integrity. In fact, this is exactly the case with sarcomeric MtCK in mitochondrial myopathies.[36] It is also possible that there might be no association in general between loss of mitochondrial integrity and uMtCK expression. In this context, we wondered whether loss of mitochondrial integrity in the liver might be involved in the mechanism of increased uMtCK expression in HCC. To examine this, HCV core gene transgenic mice were used, because these mice develop HCC with loss of mitochondrial integrity in the liver in the absence of inflammation and fibrosis.[22,23] As a result, uMtCK expression was essentially not altered in non-tumorous liver tissues with loss of mitochondrial integrity but clearly enhanced in HCC tissues, suggesting that hepatocarcinogenesis *per se* but not

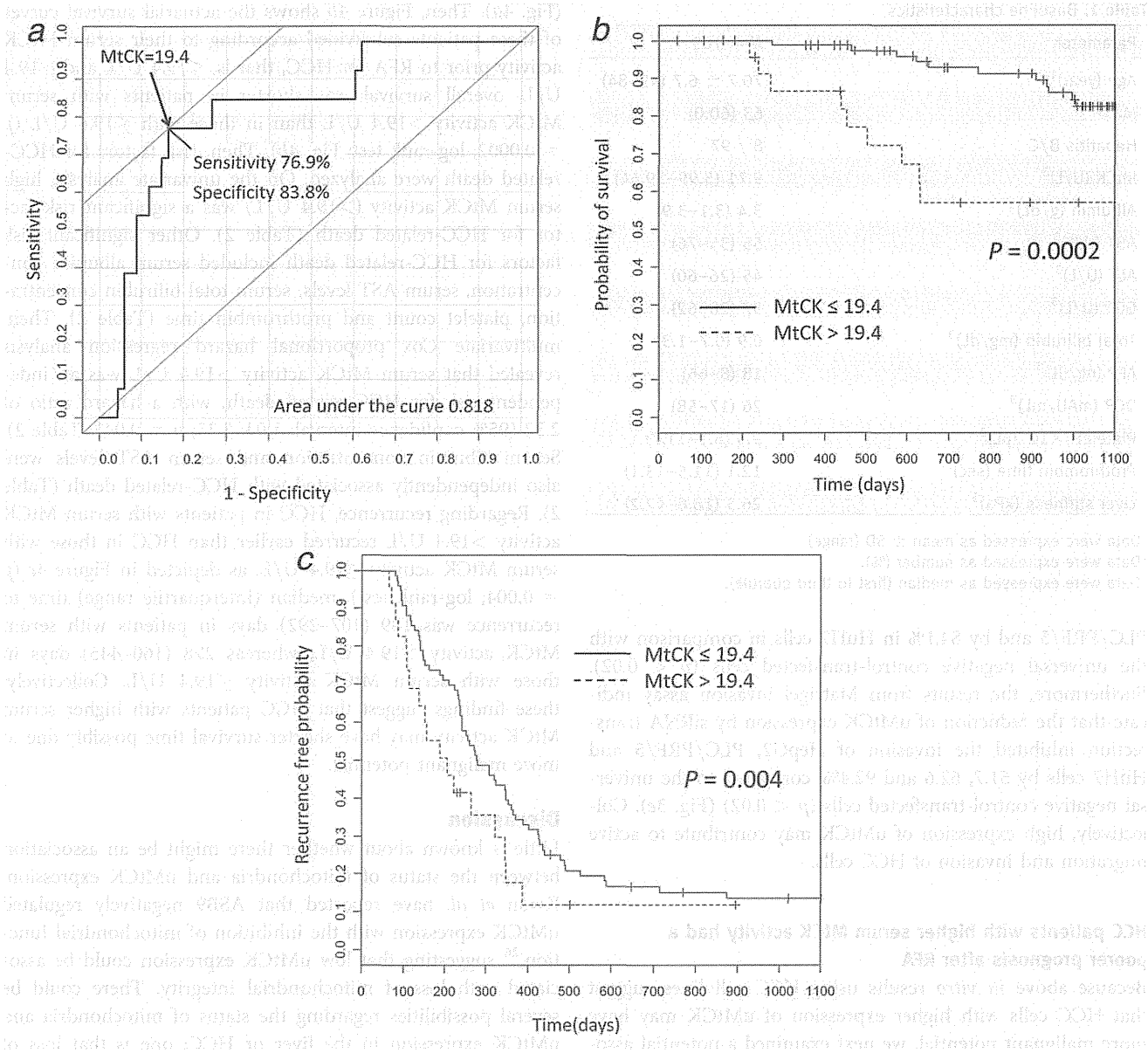

Figure 4. (*a*) ROC curve showing the overall accuracy of serum MtCK activity for discriminating between survivors and deceased patients. The arrow identifies the best cutoff value (*i.e.*, 19.4 U/L) of serum MtCK activity. Kaplan–Meier survival (*b*) and recurrence (*c*) curve of the studied patients subdivided according to their serum MtCK activity prior to RFA for HCC. Solid line, ≤19.4 U/L; dashed line, >19.4 U/L.

loss of mitochondrial integrity may contribute to increased uMtCK expression in HCC.

Regarding the regulatory mechanism(s) of increased uMtCK expression in HCC, we have found that ASB9 interacted with uMtCK to reduce its protein levels in HCC cells, similarly to HEK293 cells as previously described.[26] In normal liver, uMtCK levels are generally at a very low level, while sarcomeric MtCK as a muscle-specific isoform is not expressed at all,[37] whereas ASB9 mRNA expression is reportedly abundant.[26] Thus, ASB9 may play a physiological role to keep uMtCK protein levels low in the liver. Regarding HCC, ASB9 mRNA expression in HCC cells were much lower than that in normal liver tissue in the current study. This finding raises the possibility that low expression of ASB9 may explain, at least in part, high protein levels of uMtCK in HCC. Collectively, we may suggest that the two possible mechanisms of increased uMtCK protein levels in HCC cells should be increased gene expression and decreased protein degradation due to reduced ASB9 expression. It has been reported that colorectal cancer with low ASB9 expression may have a higher malignant potential and a poorer prognosis than that with high ASB9 expression,[27] suggesting a negative association of ASB9 with uMtCK protein levels also in colorectal cancer cells. Nonetheless, a potential role of ASB9 in the regulation of uMtCK expression in HCC *in vivo* should be further elucidated.

Table 2. Risk factors for HCC-related death evaluated by univariate/multivariate Cox proportional hazard regression

	Univariate		Multivariate	
Parameter	HR (95% CI)	*p* value	HR (95% CI)	*p* value
Age (year)	1.02 (0.95–1.10)	0.60		
Female	1.45 (0.56–3.77)	0.44		
Hepatitis B	1.37 (0.18–10.3)	0.76		
MtCK >19.4 (U/L)	5.03 (1.93–13.1)	<0.001	2.32 (1.03–5.25)	0.042
Albumin	0.15 (0.05–0.44)	<0.001	0.26 (0.09–0.71)	0.009
AST	1.02 (1.01–1.03)	<0.001	1.01 (1.00–1.02)	0.028
ALT	1.01 (0.99–1.02)	0.13		
GGT	1.00 (0.98–1.01)	0.45		
Total bilirubin	3.23 (1.98–5.29)	<0.001	1.72 (0.97–3.04)	0.064
AFP >100 (ng/dL)	2.28 (0.84–6.18)	0.11		
DCP >80 (mAU/mL)	2.74 (0.99–7.45)	0.59		
Platelet	0.83 (0.71–0.97)	0.017	0.89 (0.76–1.04)	0.14
Prothrombin time	1.32 (1.11–1.57)	0.002	0.91 (0.70–1.17)	0.45
Liver stiffness	1.02 (0.98–1.04)	0.25		

Reduction of uMtCK expression in HCC cells led to the inhibition in their proliferation, migration and invasion. The similar effects of inhibition of uMtCK expression were reported in Hela cells[29] and breast cancer cells.[17] This finding may be in agreement with the notion that the creatine kinase system is generally essential for the control of cellular energetics in tissues or cells with high and fluctuating energy requirements.[37] Indeed, overexpression has been reported for different creatine kinase isoforms in different types of cancer and has provided a more general growth advantage to solid tumors.[37,38] Overexpression of uMtCK in different Hodgkin-derived cell lines has been described as a marker for poor prognosis.[39] Increased uMtCK levels in cancer cells might be a part of metabolic adaptation of those cells to perform high growth rate under oxygen and glucose restriction as typical for many cancers; it could help to sustain energy turnover, but would be also protective against stress situations such as hypoxia and possibly protect cells from death.[40] Nonetheless, these *in vitro* findings raise the possibility that high expression of uMtCK in HCC may be associated with its active growth and metastasis.

Then, we performed a follow-up study of the HCC patients, with whom we showed the increased serum MtCK activity.[16] Among the entire HCC patients in the previous study, we enrolled the patients who underwent RFA with curative intent to examine the potential association between serum MtCK activity and prognosis in this study. In the previous report, serum MtCK activity was also enhanced in the patients with liver cirrhosis compared to healthy control, although less prominent than in those with HCC and liver cirrhosis,[16] suggesting that background liver status of HCC may also affect serum MtCK activity. In this context, because RFA with curative intent was performed on patients without advanced liver damages such as high serum total bilirubin concentration, low platelet counts or massive ascites,[33] the potential association between serum MtCK activity and prognosis of HCC patients could be assessed with less bias from background liver status. Furthermore, of note, HCC patients treated with RFA had no extended tumor lesions, that is, three or fewer lesions, each 3.0 cm in diameter.[33] As a result, the HCC patients with higher serum MtCK activity had a significantly poorer prognosis than those with lower serum MtCK activity on a survival analysis, and higher serum MtCK activity was retained as a significant risk for HCC-related death on multivariate analysis. Thus, in line with the current *in vitro* findings, it is suggested that HCC with increased uMtCK expression may have highly malignant potential.

In conclusion, high uMtCK expression in HCC may be caused by hepatocarcinogenesis *per se* but not by loss of mitochondrial integrity, and associated with highly malignant potential, where ASB9 could be one of the regulators of uMtCK expression. In the clinical setting, higher serum MtCK activity was associated with a poorer prognosis of HCC, suggesting that HCC with high serum MtCK activity should be thoroughly treated when considered to be curative.

References

1. Umemura T, Ichijo T, Yoshizawa K, et al. Epidemiology of hepatocellular carcinoma in Japan. *J Gastroenterol* 2009;44 Suppl 19:102–7.
2. Ferlay J, Shin HR, Bray F, et al. Estimates of worldwide burden of cancer in 2008: GLOBOCAN 2008. *Int J Cancer* 2010;127:2893–917.
3. Bosch FX, Ribes J, Cleries R, et al. Epidemiology of hepatocellular carcinoma. *Clin Liver Dis* 2005; 9:191–211.

4. El-Serag HB, Rudolph KL. Hepatocellular carcinoma: epidemiology and molecular carcinogenesis. *Gastroenterology* 2007;132:2557–76.
5. Baffy G, Brunt EM, Caldwell SH. Hepatocellular carcinoma in non-alcoholic fatty liver disease: an emerging menace. *J Hepatol* 2012;56:1384–91.
6. El-Serag HB, Mason AC. Rising incidence of hepatocellular carcinoma in the United States. *N Engl J Med* 1999;340:745–50.
7. Nakakura EK, Choti MA. Management of hepatocellular carcinoma. *Oncology (Williston Park)* 2000;14:1085–98; discussion 98–102.
8. Davila JA, Kramer JR, Duan Z, et al. Referral and receipt of treatment for hepatocellular carcinoma in United States veterans: effect of patient and nonpatient factors. *Hepatology* 2013;57:1858–68.
9. El-Serag HB, Marrero JA, Rudolph L, et al. Diagnosis and treatment of hepatocellular carcinoma. *Gastroenterology* 2008;134:1752–63.
10. Llovet JM, Ricci S, Mazzaferro V, et al. Sorafenib in advanced hepatocellular carcinoma. *N Engl J Med* 2008;359:378–90.
11. Bertino G, Ardiri A, Malaguarnera M, et al. Hepatocellualar carcinoma serum markers. *Semin Oncol* 2012;39:410–33.
12. Bertino G, Ardiri AM, Boemi PM, et al. A study about mechanisms of des-gamma-carboxy prothrombin's production in hepatocellular carcinoma. *Panminerva Med* 2008;50:221–6.
13. Bertino G, Neri S, Bruno CM, et al. Diagnostic and prognostic value of alpha-fetoprotein, des-gamma-carboxy prothrombin and squamous cell carcinoma antigen immunoglobulin M complexes in hepatocellular carcinoma. *Minerva Med* 2011; 102:363–71.
14. Bertino G, Ardiri AM, Calvagno GS, et al. Prognostic and diagnostic value of des-gamma-carboxy prothrombin in liver cancer. *Drug News Perspect* 2010;23:498–508.
15. Malaguarnera G, Paladina I, Giordano M, et al. Serum markers of intrahepatic cholangiocarcinoma. *Dis Markers* 2013;34:219–28.
16. Soroida Y, Ohkawa R, Nakagawa H, et al. Increased activity of serum mitochondrial isoenzyme of creatine kinase in hepatocellular carcinoma patients predominantly with recurrence. *J Hepatol* 2012;57:330–6.
17. Qian XL, Li YQ, Gu F, et al. Overexpression of ubiquitous mitochondrial creatine kinase (uMtCK) accelerates tumor growth by inhibiting apoptosis of breast cancer cells and is associated with a poor prognosis in breast cancer patients. *Biochem Biophys Res Commun* 2012;427:60–6.
18. Kanemitsu F, Kawanishi I, Mizushima J, et al. Mitochondrial creatine kinase as a tumor-associated marker. *Clin Chim Acta* 1984;138:175–83.
19. Pratt R, Vallis LM, Lim CW, et al. Mitochondrial creatine kinase in cancer patients. *Pathology* 1987;19:162–5.
20. Onda T, Uzawa K, Endo Y, et al. Ubiquitous mitochondrial creatine kinase downregulated in oral squamous cell carcinoma. *Br J Cancer* 2006; 94:698–709.
21. Patra S, Bera S, SinhaRoy S, et al. Progressive decrease of phosphocreatine, creatine and creatine kinase in skeletal muscle upon transformation to sarcoma. *FEBS J* 2008;275:3236–47.
22. Moriya K, Fujie H, Shintani Y, et al. The core protein of hepatitis C virus induces hepatocellular carcinoma in transgenic mice. *Nat Med* 1998;4: 1065–7.
23. Moriya K, Nakagawa K, Santa T, et al. Oxidative stress in the absence of inflammation in a mouse model for hepatitis C virus-associated hepatocarcinogenesis. *Cancer Res* 2001;61:4365–70.
24. Kile BT, Schulman BA, Alexander WS, et al. The SOCS box: a tale of destruction and degradation. *Trends Biochem Sci* 2002;27:235–41.
25. Debrincat MA, Zhang JG, Willson TA, et al. Ankyrin repeat and suppressors of cytokine signaling box protein asb-9 targets creatine kinase B for degradation. *J Biol Chem* 2007;282: 4728–37.
26. Kwon S, Kim D, Rhee JW, et al. ASB9 interacts with ubiquitous mitochondrial creatine kinase and inhibits mitochondrial function. *BMC Biol* 2010;8:23.
27. Tokuoka M, Miyoshi N, Hitora T, et al. Clinical significance of ASB9 in human colorectal cancer. *Int J Oncol* 2010;37:1105–11.
28. Ikeda H, Nagashima K, Yanase M, et al. Involvement of Rho/Rho kinase pathway in regulation of apoptosis in rat hepatic stellate cells. *Am J Physiol Gastrointest Liver Physiol* 2003;285:G880–6.
29. Lenz H, Schmidt M, Welge V, et al. Inhibition of cytosolic and mitochondrial creatine kinase by siRNA in HaCaT- and HeLaS3-cells affects cell viability and mitochondrial morphology. *Mol Cell Biochem* 2007;306:153–62.
30. Makuuchi M, Kokudo N, Arii S, et al. Development of evidence-based clinical guidelines for the diagnosis and treatment of hepatocellular carcinoma in Japan. *Hepatol Res* 2008;38:37–51.
31. Torzilli G, Minagawa M, Takayama T, et al. Accurate preoperative evaluation of liver mass lesions without fine-needle biopsy. *Hepatology* 1999;30:889–93.
32. Hoshino T, Sakai Y, Yamashita K, et al. Development and performance of an enzyme immunoassay to detect creatine kinase isoenzyme MB activity using anti-mitochondrial creatine kinase monoclonal antibodies. *Scand J Clin Lab Invest* 2009;69:687–95.
33. Omata M, Tateishi R, Yoshida H, et al. Treatment of hepatocellular carcinoma by percutaneous tumor ablation methods: ethanol injection therapy and radiofrequency ablation. *Gastroenterology* 2004;127:S159–66.
34. Nishikawa M, Nishiguchi S, Shiomi S, et al. Somatic mutation of mitochondrial DNA in cancerous and noncancerous liver tissue in individuals with hepatocellular carcinoma. *Cancer Res* 2001;61:1843–5.
35. Tamori A, Nishiguchi S, Nishikawa M, et al. Correlation between clinical characteristics and mitochondrial D-loop DNA mutations in hepatocellular carcinoma. *J Gastroenterol* 2004;39: 1063–8.
36. Stadhouders AM, Jap PH, Winkler HP, et al. Mitochondrial creatine kinase: a major constituent of pathological inclusions seen in mitochondrial myopathies. *Proc Natl Acad Sci USA* 1994; 91:5089–93.
37. Schlattner U, Tokarska-Schlattner M, Wallimann T. Mitochondrial creatine kinase in human health and disease. *Biochim Biophys Acta* 2006;1762: 164–80.
38. Wyss M, Kaddurah-Daouk R. Creatine and creatinine metabolism. *Physiol Rev* 2000;80: 1107–213.
39. Kornacker M, Schlattner U, Wallimann T, et al. Hodgkin disease-derived cell lines expressing ubiquitous mitochondrial creatine kinase show growth inhibition by cyclocreatine treatment independent of apoptosis. *Int J Cancer* 2001;94: 513–9.
40. Dang CV, Semenza GL. Oncogenic alterations of metabolism. *Trends Biochem Sci* 1999;24:68–72.