Research Article

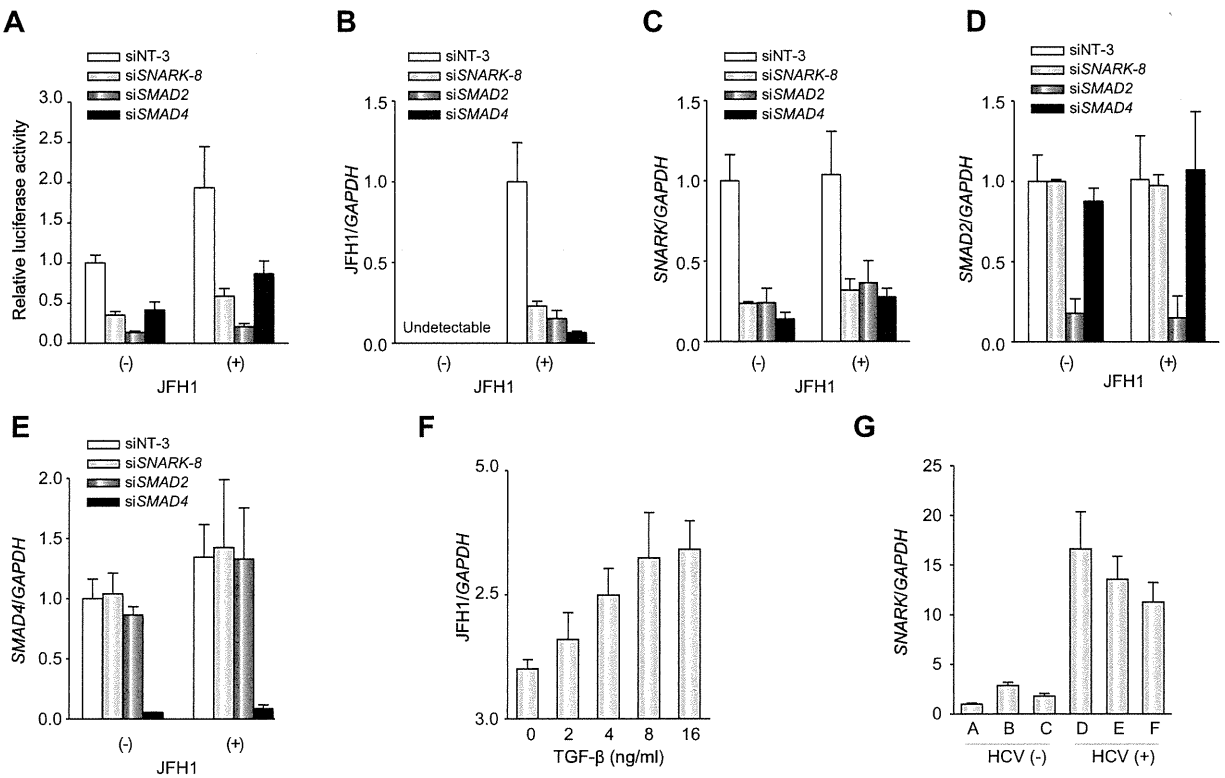

Fig. 4. Reciprocal regulation between SNARK and HCV in TGF-β signaling. (A) 48 hours after transfection of siRNAs, HuH7.5.1 cells were infected with JFH1, which was followed by transfection of PAI/L reporter 24 hours later and lysis 72 hours later for dual luciferase assay (A) and quantification of RNA levels for JFH1 (B), *SNARK* (C), *SMAD2* (D), and *SMAD4* (E) by real-time PCR with normalization to *GAPDH*. JFH1 RNA was not detected in the uninfected HuH7.5.1 cells (Undetectable, B). (F) 24 hours after the infection with JFH1, HuH7.5.1 cells were treated with TGF-β for 48 hours at indicated doses (0–16 ng/ml). Then cells were lysed and the viral RNA levels were measured by real-time PCR and normalized to *GAPDH*. (G) mRNA levels of *SNARK* in liver biopsies from patients were measured by real-time PCR and normalized to *GAPDH*. Patients A, B, and C were HCV-negative, and D, E, and F were infected with HCV. Details of patient characteristics are provided in Supplementary Table 2.

Reciprocal enhancement of TGF-β signaling and HCV infection via SNARK

Our data agree with the reported observation of TGF-β signaling elevated by HCV [14,25] and resultant proviral effects in a replicon cell line [15]. We therefore examined the effects of HCV replication on TGF-β signaling and immediate involvement of SNARK in the JFH1-HuH7.5.1 system. JFH1 replication (Fig. 4B) upregulated PAI/L luciferase activity, which was abolished by siRNA-mediated knockdown of SNARK (Fig. 4A and C), as was observed with the knockdown of either SMAD2 or SMAD4 (Fig. 4D and E). The data clearly demonstrate that SNARK expression plays a role in TGF-β signaling in JFH1 replication. Intriguingly, knockdown of either SMAD2 or SMAD4 led to the reduced expression of SNARK (Fig. 4C), implying converse regulation of SNARK by SMAD pathway. Next, the effects of enhanced TGF-β signaling on HCV replication were tested in this *bona fide* HCV infection system. JFH1-infected cells were treated with increasing quantities of TGF-β, and we observed that TGF-β enhanced viral replication in a dose-dependent fashion (Fig. 4F) with no cytotoxicity at the concentrations indicated (data not shown).

Lastly, SNARK expression was examined in human liver tissue to investigate its pathophysiological dynamics. The levels of *SNARK* mRNA were prominently elevated in HCV-infected patients in comparison to HCV-negative controls (Fig. 4G). These findings strongly suggest that HCV-mediated induction of SNARK facilitates both proviral and profibrogenic signaling of TGF-β, leading to reciprocal amplification of HCV and profibrogenic signals. Collectively, these factors could interact to accelerate hepatic fibrosis progression in HCV infection.

Discussion

SNARK is an AMPK-related kinase identified through our previous genome-wide RNAi screen as a host cellular cofactor for HCV replication [6]. Our present studies reveal an intersection of TGF-β-SMAD signaling, LKB1-AMPK-related kinase signaling, and viral replication, in which there is reciprocal stimulation. This convergence could well explain the relationship between HCV replication and its pathogenic effects observed *in vitro* and *in vivo*, providing strong support for the concept that the TGF-β signaling pathway is one of the key cellular pathways targeted by HCV to promote replication and may be therefore a future therapeutic target.

Growing numbers of host cellular cofactors for HCV replication have been discovered so far to support the viral lifecycle through interaction with viral proteins and alteration of host signaling pathways. Here we demonstrated that SNARK contributes to HCV replication through a reportedly proviral cytokine, TGF-β [15,26]. Simultaneously, the induction of SNARK by prolonged HCV replication in cell culture and patients demonstrates its reciprocal regulation by HCV. SNARK was transcriptionally upregulated in an NF-κB-dependent manner in a breast cancer cell line

[27] and was identified in a microarray analysis as the only kinase substantially induced in endothelial cells by tumor necrosis factor (TNF)-α, a well-known NF-κB activator [28]. Moreover, we and others reported that HCV infection activates NF-κB phosphorylation [29] and that NS5A stimulates NF-κB-dependent luciferase activity [30], respectively. Thus, SNARK expression may be transcriptionally induced by sustained HCV infection through virally-triggered NF-κB activation even though its basal expression may be quite low as illustrated by Western blot in HuH7.5.1 cells. Despite these low basal expression levels, the activation by infection of SNARK appears to enable further viral replication.

TGF-β is a pivotal cytokine and the central driver of hepatic fibrogenesis during HCV infection [31]. An increasing variety of proteins mediating TGF-β signaling have been described [32] and phosphorylation of SMAD2 and SMAD3 by non-TGF-βRI kinases such as Mps1 [33] and Rho/ROCK [34] also promoted TGF-β signaling. Therefore, SNARK associated with SMAD2 can be responsible for phosphorylation of SMAD2, enhancing SMAD-signaling. On the other hand, partial dependence of SNARK expression on SMADs observed in our knockdown experiments (Fig. 4C) may better explain reciprocal regulation between SNARK and TGF-β pathway. Possible modes of participation of SNARK in other phosphorylation-dependent processes in TGF-β signaling and TGF-β signaling-regulated SNARK expression, together with those in mediators in cross-talking pathways leading to epithelial-mesenchymal transition through cell dedifferentiation including Notch1, whose expression was mildly induced by SNARK overexpression in the presence of TGF-β in HuH7.5.1 cells (data not shown), remain an open subject for further investigation. On the other hand, SNARK alone was also capable of moderately inducing PAI-1 promoter-driven luciferase activity, underscoring its potential transcription-modulatory functions suggested by the nuclear localization of SNARK in HuH7.5.1 (Supplementary Fig. 1A and B), PLC/PRF/5, and HeLa cells [35], similarly to the HCV-activated transcription factor Elk1 [36], responsible for epidermal growth factor (EGF)-driven enhancement of PAI-1 expression [37].

Knockdown experiments in HuH7.5.1 cells demonstrate the importance of LKB1 in the TGF-β signaling pathway and in its promotion by SNARK, consistent with the decreased SMAD2 and TGF-β pathway activities in *Stk11*$^{-/-}$ mice, a model of Peutz-Jeghers syndrome [38]. It is tempting to conjecture critical roles of SNARK in HCV-induced liver disease in consideration of SNARK as a LKB1 signaling molecule directly activated by LKB1 in the TGF-β pathway. Currently, a wide variety of inhibitors of mediators in TGF-β/PAI-1 expression control are under clinical evaluation [39]. Thus SNARK, whose expression and activity are closely linked to TNF-α and TGF-β, activators of hepatic stellate cells [40], appears to be an attractive target for antifibrotic development.

Metformin has been widely used for the treatment of type 2 diabetes for 50 years [41], primarily decreasing hepatic glucose production [42] via AMPK activation [43]. Simultaneously, metformin was also revealed to exert multifaceted actions through AMPK-independent mechanisms targeting several kinases [44]. Indeed, the phosphotransferase activity of SNARK was inhibited by metformin in a human hepatocellular carcinoma cell line [20]. The phosphotransferase-dependent phosphorylation level of overexpressed SNARK was diminished by metformin in our immunoprecipitation assay as well, again indicating metformin-mediated inhibitory effects on SNARK phosphorylation partly via interference with its autophosphorylation. Hence it is rational that we observed an antiviral action of metformin against JFH1, and indeed a trial in Spain showed that the addition of metformin to standard anti-HCV treatment improved SVR [45], which suggests a possible productive application of metformin and SNARK inhibitors to the anti-HCV armamentarium. Furthermore, SNARK phosphotransferase activity-driven stimulation of TGF-β signaling in HuH7.5.1 cells allowed us to confirm the suppressive effects of metformin on TGF-β signaling accentuation by SNARK overexpression, implying SNARK as a target in HCV pathogenesis. Further studies to elucidate the mechanisms and consequences of inhibition of SNARK and metformin itself are warranted. Besides, since another pathogenic effect of HCV is insulin resistance, and type 2 diabetes is a risk factor for HCC [46], novel SNARK-mediated antiviral and antifibrotic properties of the antidiabetic metformin could offer an important and multifaceted agent for long term HCV disease management.

On the heels of development of anti-HCV agents targeting viral proteins, proviral host cellular cofactors have been discovered [3] and subsequent HTAs are emerging, best typified by cyclophilin (CyP) inhibitors [47], overcoming the drug resistance against virally-targeted inhibitors. In this study, we provide an example of a potential target for host-directed antiviral and anti-pathogenic therapies, which target a key host cellular cofactor involved not only in viral replication but also in viral pathogenesis. In fact, HCV modulates and depends on lipid metabolism enhancing lipogenesis for the establishment of efficient viral infection [48], and cholesterol-lowering statins were not only antiviral [49], but also effective in reducing steatosis and retarding fibrosis in viral and non-alcoholic fatty liver disease (NAFLD) patients [50], nicely exemplifying the notion above.

Taken together, our data overall suggest that SNARK is a novel host cellular factor for HCV replication and an additional mediator of TGF-β-SMAD signaling. Involvement of its activity as a kinase in proviral and pathogenic pathways positions SNARK as a potentially critical and druggable target for new therapies against hepatitis C.

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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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Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jhep.2013. 06.025.

References

[1] Schaefer EA, Chung RT. Anti-hepatitis C virus drugs in development. Gastroenterology 2012;142:e1341.

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[2] Chung RT. A watershed moment in the treatment of hepatitis C. N Engl J Med 2012;366:273–275.
[3] Salloum S, Tai AW. Treating hepatitis C infection by targeting the host. Transl Res 2012;159:421–429.
[4] Flisiak R, Jaroszewicz J, Flisiak I, Lapinski T. Update on alisporivir in treatment of viral hepatitis C. Expert Opin Investig Drugs 2012;21: 375–382.
[5] Adinolfi LE, Restivo L, Zampino R, Lonardo A, Loria P. Metabolic alterations and chronic hepatitis C: treatment strategies. Expert Opin Pharmacother 2011;12:2215–2234.
[6] Tai AW, Benita Y, Peng LF, Kim SS, Sakamoto N, Xavier RJ, et al. A functional genomic screen identifies cellular cofactors of hepatitis C virus replication. Cell Host Microbe 2009;5:298–307.
[7] Lizcano JM, Goransson O, Toth R, Deak M, Morrice NA, Boudeau J, et al. LKB1 is a master kinase that activates 13 kinases of the AMPK subfamily, including MARK/PAR-1. EMBO J 2004;23:833–843.
[8] Li Q, Brass AL, Ng A, Hu Z, Xavier RJ, Liang TJ, et al. A genome-wide genetic screen for host factors required for hepatitis C virus propagation. Proc Natl Acad Sci U S A 2009;106:16410–16415.
[9] Ng TI, Mo H, Pilot-Matias T, He Y, Koev G, Krishnan P, et al. Identification of host genes involved in hepatitis C virus replication by small interfering RNA technology. Hepatology 2007;45:1413–1421.
[10] Tsuchihara K, Ogura T, Fujioka R, Fujii S, Kuga W, Saito M, et al. Susceptibility of Snark-deficient mice to azoxymethane-induced colorectal tumorigenesis and the formation of aberrant crypt foci. Cancer Sci 2008;99:677–682.
[11] Koh HJ, Toyoda T, Fujii N, Jung MM, Rathod A, Middelbeek RJ, et al. Sucrose nonfermenting AMPK-related kinase (SNARK) mediates contraction-stimulated glucose transport in mouse skeletal muscle. Proc Natl Acad Sci U S A 2010;107:15541–15546.
[12] Mankouri J, Tedbury PR, Gretton S, Hughes ME, Griffin SD, Dallas ML, et al. Enhanced hepatitis C virus genome replication and lipid accumulation mediated by inhibition of AMP-activated protein kinase. Proc Natl Acad Sci U S A 2010;107:11549–11554.
[13] Derynck R, Akhurst RJ, Balmain A. TGF-beta signaling in tumor suppression and cancer progression. Nat Genet 2001;29:117–129.
[14] Blackard JT, Komurian-Pradel F, Perret M, Sodoyer M, Smeaton L, St Clair JB, et al. Intrahepatic cytokine expression is downregulated during HCV/HIV co-infection. J Med Virol 2006;78:202–207.
[15] Lin W, Weinberg EM, Tai AW, Peng LF, Brockman MA, Kim KA, et al. HIV increases HCV replication in a TGF-beta1-dependent manner. Gastroenterology 2008;134:803–811.
[16] Wilson LE, Torbenson M, Astemborski J, Faruki H, Spoler C, Rai R, et al. Progression of liver fibrosis among injection drug users with chronic hepatitis C. Hepatology 2006;43:788–795.
[17] Barrios-Rodiles M, Brown KR, Ozdamar B, Bose R, Liu Z, Donovan RS, et al. High-throughput mapping of a dynamic signaling network in mammalian cells. Science 2005;307:1621–1625.
[18] Zhang L, Jilg N, Shao RX, Lin W, Fusco DN, Zhao H, et al. IL28B inhibits hepatitis C virus replication through the JAK-STAT pathway. J Hepatol 2010;55:289–298.
[19] Shao RX, Zhang L, Peng LF, Sun E, Chung WJ, Jang JY, et al. Suppressor of cytokine signaling 3 suppresses hepatitis C virus replication in an mTOR-dependent manner. J Virol 2010;84:6060–6069.
[20] Lefebvre DL, Rosen CF. Regulation of SNARK activity in response to cellular stresses. Biochim Biophys Acta 2005;1724:71–85.
[21] Watashi K, Hijikata M, Hosaka M, Yamaji M, Shimotohno K. Cyclosporin A suppresses replication of hepatitis C virus genome in cultured hepatocytes. Hepatology 2003;38:1282–1288.
[22] Gronborg M, Kristiansen TZ, Stensballe A, Andersen JS, Ohara O, Mann M, et al. A mass spectrometry-based proteomic approach for identification of serine/threonine-phosphorylated proteins by enrichment with phospho-specific antibodies: identification of a novel protein, Frigg, as a protein kinase A substrate. Mol Cell Proteomics 2002;1:517–527.
[23] Lefebvre DL, Bai Y, Shahmolky N, Sharma M, Poon R, Drucker DJ, et al. Identification and characterization of a novel sucrose-non-fermenting protein kinase/AMP-activated protein kinase-related protein kinase, SNARK. Biochem J 2001;355:297–305.
[24] Abe M, Harpel JG, Metz CN, Nunes I, Loskutoff DJ, Rifkin DB. An assay for transforming growth factor-beta using cells transfected with a plasminogen activator inhibitor-1 promoter-luciferase construct. Anal Biochem 1994;216:276–284.
[25] Woodhouse SD, Narayan R, Latham S, Lee S, Antrobus R, Gangadharan B, et al. Transcriptome sequencing, microarray, and proteomic analyses reveal cellular and metabolic impact of hepatitis C virus infection in vitro. Hepatology 2010;52:443–453.
[26] Presser LD, Haskett A, Waris G. Hepatitis C virus-induced furin and thrombospondin-1 activate TGF-beta1: role of TGF-beta1 in HCV replication. Virology 2011;412:284–296.
[27] Legembre P, Schickel R, Barnhart BC, Peter ME. Identification of SNF1/AMP kinase-related kinase as an NF-kappaB-regulated anti-apoptotic kinase involved in CD95-induced motility and invasiveness. J Biol Chem 2004;279:46742–46747.
[28] Yamamoto H, Takashima S, Shintani Y, Yamazaki S, Seguchi O, Nakano A, et al. Identification of a novel substrate for TNFalpha-induced kinase NUAK2. Biochem Biophys Res Commun 2008;365:541–547.
[29] Lin W, Tsai WL, Shao RX, Wu G, Peng LF, Barlow LL, et al. Hepatitis C virus regulates transforming growth factor beta1 production through the generation of reactive oxygen species in a nuclear factor kappaB-dependent manner. Gastroenterology 2010;138:2509–2518, 2518 e2501.
[30] Gong G, Waris G, Tanveer R, Siddiqui A. Human hepatitis C virus NS5A protein alters intracellular calcium levels, induces oxidative stress, and activates STAT-3 and NF-kappa B. Proc Natl Acad Sci U S A 2001;98:9599–9604.
[31] Dabrowska MM, Panasiuk A, Flisiak R. Signal transduction pathways in liver and the influence of hepatitis C virus infection on their activities. World J Gastroenterol 2009;15:2184–2189.
[32] Schmierer B, Hill CS. TGFbeta-SMAD signal transduction: molecular specificity and functional flexibility. Nat Rev Mol Cell Biol 2007;8:970–982.
[33] Zhu S, Wang W, Clarke DC, Liu X. Activation of Mps1 promotes transforming growth factor-beta-independent Smad signaling. J Biol Chem 2007;282:18327–18338.
[34] Samarakoon R, Higgins CE, Higgins SP, Higgins PJ. Differential requirement for MEK/ERK and SMAD signaling in PAI-1 and CTGF expression in response to microtubule disruption. Cell Signal 2009;21:986–995.
[35] Kuga W, Tsuchihara K, Ogura T, Kanehara S, Saito M, Suzuki A, et al. Nuclear localization of SNARK; its impact on gene expression. Biochem Biophys Res Commun 2008;377:1062–1066.
[36] Fukuda K, Tsuchihara K, Hijikata M, Nishiguchi S, Kuroki T, Shimotohno K. Hepatitis C virus core protein enhances the activation of the transcription factor, Elk1, in response to mitogenic stimuli. Hepatology 2001;33:159–165.
[37] Wyrzykowska P, Stalinska K, Wawro M, Kochan J, Kasza A. Epidermal growth factor regulates PAI-1 expression via activation of the transcription factor Elk-1. Biochim Biophys Acta 2010;1799:616–621.
[38] Katajisto P, Vaahtomeri K, Ekman N, Ventela E, Ristimaki A, Bardeesy N, et al. LKB1 signaling in mesenchymal cells required for suppression of gastrointestinal polyposis. Nat Genet 2008;40:455–459.
[39] Samarakoon R, Higgins PJ. Integration of non-SMAD and SMAD signaling in TGF-beta1-induced plasminogen activator inhibitor type-1 gene expression in vascular smooth muscle cells. Thromb Haemost 2008;100:976–983.
[40] Ming-Ju H, Yih-Shou H, Tzy-Yen C, Hui-Ling C. Hepatitis C virus E2 protein induce reactive oxygen species (ROS)-related fibrogenesis in the HSC-T6 hepatic stellate cell line. J Cell Biochem 2010;112:233–243.
[41] Wong AK, Howie J, Petrie JR, Lang CC. AMP-activated protein kinase pathway: a potential therapeutic target in cardiometabolic disease. Clin Sci (Lond) 2009;116:607–620.
[42] Kirpichnikov D, McFarlane SI, Sowers JR. Metformin: an update. Ann Intern Med 2002;137:25–33.
[43] Shaw RJ, Lamia KA, Vasquez D, Koo SH, Bardeesy N, Depinho RA, et al. The kinase LKB1 mediates glucose homeostasis in liver and therapeutic effects of metformin. Science 2005;310:1642–1646.
[44] Labuzek K, Liber S, Gabryel B, Okopien B. Metformin has adenosine-monophosphate activated protein kinase (AMPK)-independent effects on LPS-stimulated rat primary microglial cultures. Pharmacol Rep 2010;62:827–848.
[45] Romero-Gomez M, Diago M, Andrade RJ, Calleja JL, Salmeron J, Fernandez-Rodriguez CM, et al. Treatment of insulin resistance with metformin in naive genotype 1 chronic hepatitis C patients receiving peginterferon alfa-2a plus ribavirin. Hepatology 2009;50:1702–1708.
[46] Donadon V, Balbi M, Zanette G. Hyperinsulinemia and risk for hepatocellular carcinoma in patients with chronic liver diseases and Type 2 diabetes mellitus. Expert Rev Gastroenterol Hepatol 2009;3:465–467.
[47] Gallay PA. Cyclophilin inhibitors: a novel class of promising host-targeting anti-HCV agents. Immunol Res 2012;52:200–210.
[48] Syed GH, Amako Y, Siddiqui A. Hepatitis C virus hijacks host lipid metabolism. Trends Endocrinol Metab 2010;21:33–40.
[49] Bader T, Fazili J, Madhoun M, Aston C, Hughes D, Rizvi S, et al. Fluvastatin inhibits hepatitis C replication in humans. Am J Gastroenterol 2008;103:1383–1389.
[50] Ekstedt M, Franzen LE, Mathiesen UL, Holmqvist M, Bodemar G, Kechagias S. Statins in non-alcoholic fatty liver disease and chronically elevated liver enzymes: a histopathological follow-up study. J Hepatol 2007;47:135–141.

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ORIGINAL ARTICLE—LIVER, PANCREAS, AND BILIARY TRACT

IL28B minor allele is associated with a younger age of onset of hepatocellular carcinoma in patients with chronic hepatitis C virus infection

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Abstract
Background IL28B polymorphisms were shown to be associated with a response to peg-interferon-based treatment in chronic hepatitis C (CHC) and spontaneous clearance. However, little is known about how this polymorphism affects the course of CHC, including the development of hepatocellular carcinoma (HCC). We evaluated the influence of IL28B polymorphisms on hepatocarcinogenesis in CHC patients.
Methods We genotyped the rs8099917 single-nucleotide polymorphism in 351 hepatitis C-associated HCC patients without history of IFN-based treatment, and correlated the age at onset of HCC in patients with each genotype.
Results Frequencies of TT, TG, and GG genotypes were 74.3 % (261/351), 24.8 % (87/351), and 0.9 % (3/351), respectively. The mean ages at onset of HCC for TT, TG, and GG genotypes were 69.9, 67.5 and 66.8, respectively. In multivariate analysis, IL28B minor allele (TG and GG genotypes) was an independent risk factor for younger age at onset of HCC ($P = 0.02$) in males ($P < 0.001$) with higher body mass index (BMI; $P = 0.009$). The IL28B minor allele was also associated with a lower probability of having aspartate aminotransferase-to-platelet ratio index (APRI) >1.5 (minor vs. major, 46.7 vs. 58.6 %; $P = 0.01$), lower AST (69.1 vs. 77.7 IU/L, $P = 0.02$), lower ALT (67.8 vs. 80.9 IU/L, $P = 0.002$), higher platelet count (12.8 vs. 11.2 $\times$ 10^4/μL, $P = 0.002$), and higher prothrombin time (79.3 vs. 75.4 %, $P = 0.002$).
Conclusions The IL28B minor allele was associated with lower inflammatory activity and less progressed fibrosis of the liver; however, it constituted a risk factor for younger-age onset of HCC in CHC patients.

Keywords rs8099917 · Hepatocarcinogenesis · Interferon-λ · Risk allele · Fibrosis

Abbreviations

AFP	α-Fetoprotein
APRI	Aminotransferase platelet ratio index
CHC	Chronic hepatitis C
GWAS	Genome-wide association study
HCC	Hepatocellular carcinoma
HCV	Hepatitis C virus
IL28B	Interleukin 28B
PCR	Polymerase chain reaction
peg-IFN	peg-Interferon
RIG- I	Retinoic acid-inducible gene-I
SNP	Single-nucleotide polymorphism
SVR	Sustained viral response
TLR3	Toll-like receptor 3

Introduction

Hepatitis C virus (HCV) infection is one of the major causes of chronic hepatitis, liver cirrhosis, and hepatocellular carcinoma (HCC) [1]. Currently, patients with chronic

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hepatitis C (CHC) are treated with a combination of peg-interferon (peg-IFN) and ribavirin [2, 3]. Recently, HCV nonstructural 3/4A serine protease inhibitors combined with PEG-IFN and RBV were reported to achieve higher sustained viral response (SVR) rates in genotype 1 patients compared to conventional PEG-IFN/RBV. These triple therapies are considered to be the next standard of care for patients with CHC virus infection [4, 5].

Genetic variations near the interleukin 28B (IL28B) gene, encoding the type III IFN-λ3, were shown to be strongly associated with the response to peg-IFN and ribavirin treatment in patients with CHC [6–8] and also spontaneous clearance of HCV [9]. Host immune cells produce IFN and other cytokines in response to viral infection. In response to HCV, cellular sensors detect the double-stranded RNA via the retinoic acid-inducible gene-I (RIG-I) and toll like receptor 3 (TLR3) and activate a pathway to produce antiviral cytokines, including alpha and beta IFNs that trigger an antiviral response to eradicate the virus [10, 11].

Genetic polymorphisms of genes involved in innate immunities are likely to influence the strength and nature of this defense system [12]. Besides its antiviral properties, IFN-λ exhibits antitumor activity; in fact, several experimental studies in cell lines and in animal models demonstrated that the activation of type III IFN induces apoptosis [13] and antitumor activities [14–16]. Thus, this genetic factor is thought to influence the natural course of HCV infection, including the development of HCC. However, little is known about the influence of IL28B polymorphisms on hepatocarcinogenesis in patients with CHC.

In the present study, we examined the association between the rs8099917 single-nucleotide polymorphism (SNP) at the IL28B locus with the age at onset of HCC and other clinical findings in patients with CHC who had no history of receiving IFN-based treatment.

Materials and methods

Patients

The patients analyzed in the present study were derived from an HCV study cohort of the University of Tokyo Hospital. In this cohort, we enrolled the patients who visited the liver clinic at our institute between August 1997 and April 2009, and agreed to provide blood samples for human genome studies along with written informed consent according with the Declaration of Helsinki. All patients underwent laboratory blood tests at the time of enrollment in our cohort. The result of the blood tests were recorded with the information on alcohol consumption and BMI of each patient. The patients who were positive for hepatitis B surface antigen and had a history of biliary disease were excluded. All subjects in our cohort were Japanese, and this research project was approved by the ethics committees of the University of Tokyo (No. 400).

From this cohort, we examined the patients who had developed new-onset HCC and received initial therapy in our institute by January 31, 2010, and with available sample for genotyping. We excluded the patients with a history of receiving IFN-based treatment. Finally, 351 patients were enrolled for this study, and the association between the age at onset of HCC and the IL28B genotype was analyzed. Patient follow-up and Diagnosis of HCC was performed as previously described [17, 18].

IL28B genotyping

Human genomic DNA was extracted from the whole blood of each patient. Genotyping for the IL28B rs8099917 T/G polymorphism was performed by polymerase chain reaction (PCR) using the TaqMan predesigned SNP Genotyping Assay (Applied Biosystems, Foster City, CA) as recommended by the manufacturer. Allele-specific primers were labeled with fluorescent dye (FAM or HEX) and used in the PCR reaction. Aliquots of the PCR products were genotyped using an allele-specific probe of the SNP on a real-time PCR thermocycler (MX3000P, Stratagene, La Jolla, CA). Samples were subjected to 50 cycles of denaturation for 15 s at 92 °C, annealing of primers for 30 s at 60 °C, and elongation for 30 s at 60 °C.

Study endpoint

We analyzed the relationship between the age at onset of HCC (the primary endpoint of this study) and host factors, including the IL28B genotypes, sex, BMI, alcoholic consumption, and HCV genotype. We also examined the relationship between IL28B genotypes and the clinical findings at the time of enrollment in our cohort (the secondary endpoint), such as the biochemical markers and presence of liver fibrosis. Liver biopsies were only available in a small number of patients (48); liver fibrosis was assessed using the aspartate aminotransferase platelet ratio index (APRI), and an APRI of >1.5 was classified as bridging fibrosis or cirrhosis (F stage 3–4) [19].

Statistical analysis

Continuous variables were presented as the mean ± standard deviation (SD) while categorical variables were expressed as frequencies (%). Categorical data were analyzed using the Chi square test, and stepwise logistic regression analyses were used to adjust the influence of IL28B genotype by other covariates such as sex, BMI (<25

or not), and alcoholic consumption (<50 g/day or not). For continuous data, the univariate associations were evaluated using the Student's t test or nonparametric Wilcoxon rank-sum test as appropriate. Since the age at onset of HCC (the primary endpoint of this study) satisfied the assumption of normal distribution (Kolmogorov–Smirnov test, $P > 0.05$), we used stepwise regression analysis to adjust the influence of IL28B genotype by sex, BMI (<25 or not), and alcoholic consumption (<50 g/day or not). All statistical analyses were two-sided, and the threshold of the reported P values for significance was accepted as <0.05. All statistical analyses were performed using R 2.13.1 software (http://www.r-project.org).

Results

Patient characteristics

Patient characteristics are shown in Table 1. Frequencies of the rs8099917 TT, TG, and GG genotype were 74.3 % (261/351), 24.8 % (87/351), and 0.9 % (3/351), respectively. The SNP genotype distribution was in Hardy–Weinberg equilibrium (P value was not significant). We defined the IL28 major genotype as homozygous for the major sequence (TT) and the IL28B minor genotype as homozygous (GG) or heterozygous (TG) for the minor sequence. The mean age at onset of the HCC patients was 69.3 years, and approximately 60 % were male. The mean age at the time of enrollment was 67.2 years and the follow-up period was 27.9 months in average.

Table 1 Clinical characteristics and genotype distributions in the study cohort ($n = 351$)

Parameter	Values
Mean age at onset of HCC, in years	69.26 ± 8.07
Mean age at the time of enrollment, in years	67.16 ± 8.32
Male sex	200 (57.0 %)
BMI >25	70 (20.0 %)
Alcohol consumption (>50 g/day)	75 (21.4 %)
IL28B genotype	
TT	261 (74.3 %)
TG	87 (24.8 %)
GG	3 (0.9 %)
T allele frequency	0.87
HCV genotype	
Genotype 1	240 (68.4 %)
Genotype 2	91 (25.9 %)
Not tested	20 (5.7 %)

Continuous variables were represented as the mean ± standard deviation (SD) and categorical variables were as number and frequencies (%)

Primary endpoint

Table 2 shows the age at onset of patients with HCC and the associations among IL28B genotypes, sex, BMI, alcohol consumption, and HCV genotype. The mean age at onset in patients with HCC for the IL28B major and minor genotypes were 69.88 ± 7.97 and 67.48 ± 8.17, respectively, and significantly higher in patients with the IL28B major genotype than in those with the minor genotype ($P = 0.02$). In multivariate analysis, the age at onset of HCC was significantly younger in patients with the IL28B minor genotype ($P = 0.02$, Fig. 1), independently of male sex ($P < 0.001$) and higher BMI ($P = 0.009$). The characters of HCC, such as sizes (2.56 vs. 2.40 cm, $P = 0.41$) or the numbers (1.94 vs. 2.23, $P = 0.54$) at diagnosis were not significantly different between IL28B major and minor genotypes. We also analyzed the interval between blood transfusion and the onset of HCC in 161 patients who have histories of blood transfusion which had been the major cause of HCV infection in Japan [20]. The mean interval between blood transfusion and the onset of HCC for the IL28B major and minor genotypes were 39.09 ± 9.99 and 38.86 ± 9.27 years, respectively ($P = 0.9$; data not shown).

Secondary endpoint

Table 3 shows the clinical findings and associations between the IL28B genotypes at the time of enrollment in our cohort. The IL28B major genotype was significantly associated with a higher probability of having an APRI >1.5 (58.62 vs. 46.67 %, $P = 0.01$; Fig. 2), a lower platelet count (11.15 vs. 12.80 × 10^4/μL, $P = 0.002$), a higher AST level (77.69 vs. 69.12 IU/L, $P = 0.02$), a higher ALT level (80.92 vs. 67.79 IU/L, $P = 0.002$), and a lower prothrombin time (75.40 vs. 79.27 %, $P = 0.002$) compared to the IL28B minor genotype after adjustment for sex, BMI, alcoholic consumption, and the age at enrollment of our cohort. A lower γ-GTP level was significantly associated with the IL28B major genotype in univariate analysis, and alcoholic consumption, sex, and age were stronger factors associated with the γ-GTP level. Thus, after adjustment for these factors, the IL28B genotype was not extracted as a significant factor associated with the γ-GTP level. Histological assessments of liver fibrosis were performed in 248 patients at the time of initial therapy. The prevalence of histologically proved liver cirrhosis (F4) was 65.6 % (118/180) in patients with major genotype and 51.5 % (35/68) in those with minor genotype. The prevalence of liver cirrhosis was significantly higher in patients with major genotype after adjustment for sex, BMI, alcoholic consumption, and the age at the time of initial therapy for HCC ($P = 0.045$, data not shown).

Table 2 Factors associated with the age at onset of HCC

Variable	Mean	Standard deviation (SD)	*P* value	
			Univariate	Multivariate[a]
IL28B genotype			0.02	0.02
Major (TT)	69.88	7.97		
Minor (TG/GG)	67.48	8.17		
Sex			<0.001	<0.001
Male	67.94	8.48		
Female	71.02	7.16		
BMI			0.01	0.009
>25	66.87	9.11		
≤25	69.86	7.70		
Alcohol consumption			0.11	–
>50 (g/day)	67.78	9.37		
≤50 (g/day)	69.67	7.65		
HCV genotype			0.29	–
Genotype 1	69.65	7.59		
Genotype 2	68.22	8.79		

[a] Stepwise regression analysis for the age at onset of HCC (the dependent variable) using IL28B genotype, sex, BMI, alcohol consumption, and HCV genotype as independent variables

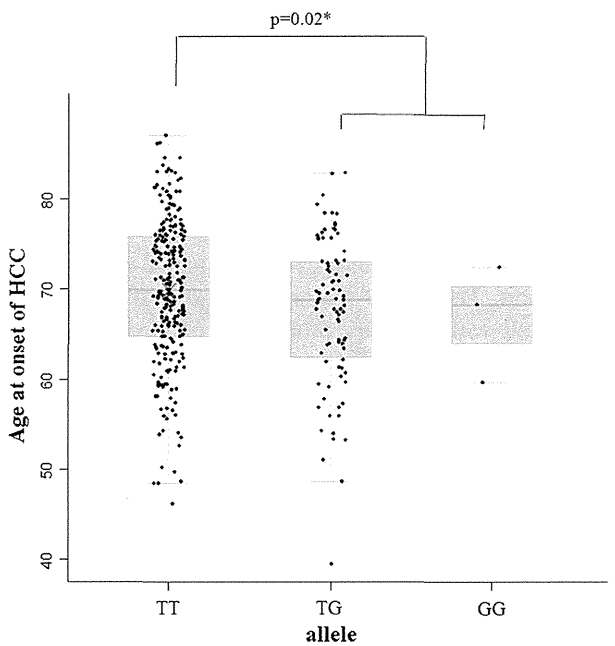

Fig. 1 *Box and whisker and dot plot* distributions of the age at onset of HCC in each genotype. The mean age at onset of HCC for the IL28B major and minor genotypes were 69.88 ± 7.97 and 67.48 ± 8.17, respectively, and was significantly higher in patients with the IL28B major genotype than in those with the minor genotype ($P = 0.02$). *P values after adjustment for sex, BMI, and alcoholic consumption

Discussion

In the present study, we evaluated the association between the IL28B polymorphism and the age at onset of HCC in patients with CHC. The IL28B minor genotype was significantly associated with younger age at onset of HCC with well known risk factors for the development of HCC such as male gender and higher BMI [21] without prior IFN-based treatment. Our previous study analyzing a susceptibility locus for HCV-induced HCC using a genome-wide association study (GWAS) could not detect the significant association between IL28B genotypes and the development of HCC in a cross-sectional distribution analysis between patients with and without HCC in more than 3,000 samples [22]. Also, IL28B alleles were not identified as a susceptibility locus for HCV-induced HCC in another GWAS study [23]. The cross-sectional distribution analyses may have underestimated the susceptibility to HCC because it could not take into consideration the future development of HCC and the duration after the past onset of HCC. Moreover, although GWAS would provide an effective and unbiased approach for revealing risk alleles for genetically complex non-Mendelian disorders, the risk of multiple comparisons made in a GWAS have resulted in reports of false positive results (Type 1 errors), and if the correction is overly conservative or the power is inadequate, false negative results (Type 2 errors) [24–26]. The relation between IL28B polymorphism and the susceptibility to HCC is still controversial. A previous study from Japan reported that the rs8099917 TT genotype was associated with a lower incidence of HCC even in non-responders to IFN based treatment [27] that was in agreement with the present study. Another study from Italy evaluating the association between genome frequency and the presence of cirrhosis due to hepatitis C, hepatitis B, alcohol use, and other factors also showed a higher prevalence of the IL28B minor allele in patients with HCC

Table 3 Associations between the IL28B genotype and clinical findings at the time of enrollment in our cohort

Variable	Mean/proportion (standard deviation; SD)		*P* values	
	Major (TT)	Minor (TG/GG)	*P* value	Adjusted *P* value[¶]
APRI >1.5[a]	58.62 % (52.38–64.66)	46.67 % (36.07–57.69)	0.07	0.01[‖]
Platelet count ($\times 10^4/\mu L$)	11.15 (5.00)	12.80 (5.43)	0.01	0.002**
AST (IU/L)	77.69 (45.14)	69.12 (38.16)	0.12	0.02**
ALT (IU/L)	80.92 (60.45)	67.79 (41.78)	0.17	0.002**
T.B (mg/dL)	0.90 (0.40)	0.83 (0.39)	0.02	–
Alb (g/dL)	3.69 (0.46)	3.71 (0.46)	0.9	–
ALP (IU/L)[b]	236.4 (81.75)	216.4 (58.96)	0.08	0.11**
γGTP (IU/L)[c]	76.83 (65.34)	87.23 (42.92)	0.005	–
PT (%)[d]	75.40 (13.36)	79.27 (13.13)	0.02	0.002**

[¶] Adjusted for sex, BMI, alcoholic consumption, and the age at enrollment (independent variables). The dependent variables of each *P* values are the items in the leftmost fields of corresponding rows (the proportion of having APRI >1.5, platelet count, AST, ALT and so on)

[‖] *P* value by stepwise logistic regression analysis

** *P* value by stepwise regression analysis

[a] Odds ratio (95 % CI) for major allele was 1.88 (1.13–3.11), and 95 % confidence interval (CI) of each proportion is parenthesized for this outcome

[b] Missing in 115 patients

[c] Missing in 112 patients

[d] Missing in 4 patients

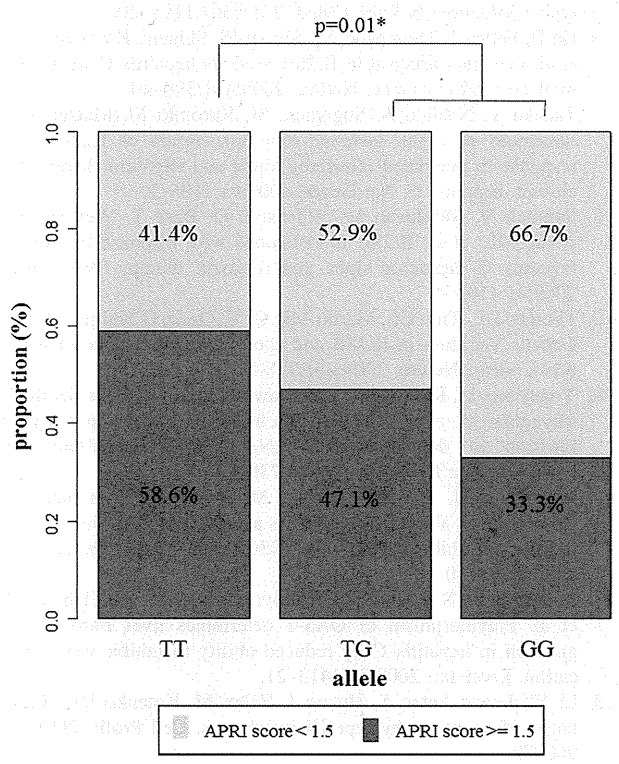

Fig. 2 *Bar plot* the proportion of having an AST-to-platelet ratio (APRI) score >1.5 in each allele. **P* values after adjustment for sex, BMI, alcoholic consumption, and the age at enrollment

compared to those without HCC [28]. However, other studies showed no relation between IL28B polymorphism and the susceptibility to HCC [29–32]. Some studies have reported the HCV genotype 1 as a risk factor associated with HCC in patients who had CHC [33–35]; however, we could not find a significant association between the HCV genotype and hepatocarcinogenesis in the present study. Our data showed no relationship between the duration of HCV infection in the patients with a history of blood transfusion. The mean age of blood transfusion was not significantly different between patients with major and minor genotypes (28.99 in major genotype vs. 27.60 in minor genotype, $P = 0.18$). Moreover, older age at HCV infection was reported to be associated with more rapid disease progression [36]. Thus, the difference in the duration of HCV infection may have little effect on the result of the present study. The IL28B genotype may have a critical role in the onset of HCC. Moreover, only about 45 % of all patients in the present study have the history of blood transfusion; hence, further analysis with larger samples may be indicated.

Previous studies evaluating patients with chronic HCV infection showed severer histological inflammatory activity and fibrosis, as well as higher ALT levels and APRI scores in patients homozygous for the IL28B major alleles [29, 32, 37, 38]. Similarly, in the present study, the IL28B

major genotype was significantly associated with a higher probability of having an APRI >1.5 and a higher ALT level; and the prevalence of histologically proved liver cirrhosis (F4) was significantly higher in patients with major genotype at the age at the time of initial therapy for HCC. Given the association between the IL28B major allele and the severe inflammatory activity or progressed fibrosis, the IL28B allele is thought to be associated with the susceptibility to HCC via a mechanism that is independent of controlling an activity of HCV infection.

Recent experimental studies have suggested that IFN-λ has an antitumor activity. In esophageal cancer cell lines expressing IFN-λ receptor complexes, IFN-λ1 suppressed growth via the induction of the G1 phase arrest or apoptosis [39]. An antitumor activity of IFN-λ was also shown in the B16 melanoma, BNL hepatoma, Colon 26, and neuroendocrine BON1 tumor cells [40–43]. One probable explanation for the paradoxical result of the present study is that the more aggressive inflammatory activity of patients with IL28B major genotype may reflect a stronger immune response to the virus, which may also have anti-tumor effects. However, the innate immune responses and antitumor activity via IFN-λ, as well as the mechanism underlying the association of the IL28B genotype, have not been elucidated. Further studies are needed to determine the functional role of the IL28B gene in relation to the course of chronic HCV infection, including hepatocarcinogenesis.

Because of the retrospective design, this study is limited by the absence of some important clinical details such as information about the histological findings of fibrosis and inflammation. Although the APRI is a useful index for the prediction of fibrosis, the limitation of this score has been reported in previous studies [44, 45]. Prospectively designed studies are needed to confirm our findings. However, observing chronic HCV-infected patients without antiviral treatment would be nearly impossible in the future. In this regard, the present study may have important implications.

In conclusion, the IL28B minor genotype was associated with a younger age of onset of HCC in patients with CHC, and this association was completely independent of the response to IFN-based treatment. Hepatocarcinogenesis appeared to be suppressed in patients who had CHC with the IL28B major genotype, despite higher inflammatory activity and progressed fibrosis of liver. The current findings may provide a clinically important information in the follow-up or HCC screening of cirrhotic patients.

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Conflict of interest None of the authors have any conflicts of interest.

References

1. Barrera JM, Bruguera M, Ercilla MG, Gil C, Celis R, Gil MP, et al. Persistent hepatitis C viremia after acute self-limiting posttransfusion hepatitis C. Hepatology. 1995;21:639–44.
2. Hadziyannis SJ, Sette H Jr, Morgan TR, Balan V, Diago M, Marcellin P, et al. Peginterferon-alpha2a and ribavirin combination therapy in chronic hepatitis C: a randomized study of treatment duration and ribavirin dose. Ann Intern Med. 2004;140:346–55.
3. Manns MP, McHutchison JG, Gordon SC, Rustgi VK, Shiffman M, Reindollar R, et al. Peginterferon alfa-2b plus ribavirin compared with interferon alfa-2b plus ribavirin for initial treatment of chronic hepatitis C: a randomised trial. Lancet. 2001;358:958–65.
4. McHutchison JG, Everson GT, Gordon SC, Jacobson IM, Sulkowski M, Kauffman R, et al. Telaprevir with peginterferon and ribavirin for chronic HCV genotype 1 infection. N Engl J Med. 2009;360:1827–38.
5. Poordad F, McCone J Jr, Bacon BR, Bruno S, Manns MP, Sulkowski MS, et al. Boceprevir for untreated chronic HCV genotype 1 infection. N Engl J Med. 2011;364:1195–206.
6. Ge D, Fellay J, Thompson AJ, Simon JS, Shianna KV, Urban TJ, et al. Genetic variation in IL28B predicts hepatitis C treatment-induced viral clearance. Nature. 2009;461:399–401.
7. Tanaka Y, Nishida N, Sugiyama M, Kurosaki M, Matsuura K, Sakamoto N, et al. Genome-wide association of IL28B with response to pegylated interferon-alpha and ribavirin therapy for chronic hepatitis C. Nat Genet. 2009;41:1105–9.
8. Suppiah V, Moldovan M, Ahlenstiel G, Berg T, Weltman M, Abate ML, et al. IL28B is associated with response to chronic hepatitis C interferon-alpha and ribavirin therapy. Nat Genet. 2009;41:1100–4.
9. Thomas DL, Thio CL, Martin MP, Qi Y, Ge D, O'Huigin C, et al. Genetic variation in IL28B and spontaneous clearance of hepatitis C virus. Nature. 2009;461:798–801.
10. Yoneyama M, Kikuchi M, Natsukawa T, Shinobu N, Imaizumi T, Miyagishi M, et al. The RNA helicase RIG-I has an essential function in double-stranded RNA-induced innate antiviral responses. Nat Immunol. 2004;5:730–7.
11. Moriyama M, Kato N, Otsuka M, Shao RX, Taniguchi H, Kawabe T, et al. Interferon-beta is activated by hepatitis C virus NS5B and inhibited by NS4A, NS4B, and NS5A. Hepatol Int. 2007;1:302–10.
12. Li CZ, Kato N, Chang JH, Muroyama R, Shao RX, Dharel N, et al. Polymorphism of OAS-1 determines liver fibrosis progression in hepatitis C by reduced ability to inhibit viral replication. Liver Int. 2009;29:1413–21.
13. Li W, Lewis-Antes A, Huang J, Balan M, Kotenko SV. Regulation of apoptosis by type III interferons. Cell Prolif. 2008;41:960–79.
14. Numasaki M, Tagawa M, Iwata F, Suzuki T, Nakamura A, Okada M, et al. IL-28 elicits antitumor responses against murine fibrosarcoma. J Immunol. 2007;178:5086–98.
15. Li M, Liu X, Zhou Y, Su SB. Interferon-lambdas: the modulators of antivirus, antitumor, and immune responses. J Leukoc Biol. 2009;86:23–32.

16. Maher SG, Sheikh F, Scarzello AJ, Romero-Weaver AL, Baker DP, Donnelly RP, et al. IFNalpha and IFNlambda differ in their antiproliferative effects and duration of JAK/STAT signaling activity. Cancer Biol Ther. 2008;7:1109–15.
17. Tateishi R, Shiina S, Teratani T, Obi S, Sato S, Koike Y, et al. Percutaneous radiofrequency ablation for hepatocellular carcinoma. An analysis of 1000 cases. Cancer. 2005;2005(103): 1201–9.
18. Masuzaki R, Tateishi R, Yoshida H, Goto E, Sato T, Ohki T, et al. Prospective risk assessment for hepatocellular carcinoma development in patients with chronic hepatitis C by transient elastography. Hepatology. 2009;49:1954–61.
19. Wai CT, Greenson JK, Fontana RJ, Kalbfleisch JD, Marrero JA, Conjeevaram HS, et al. A simple noninvasive index can predict both significant fibrosis and cirrhosis in patients with chronic hepatitis C. Hepatology. 2003;38:518–26.
20. Kiyosawa K, Umemura T, Ichijo T, Matsumoto A, Yoshizawa K, Gad A, et al. Hepatocellular carcinoma: recent trends in Japan. Gastroenterology. 2004;127:S17–26.
21. El-Serag HB, Rudolph KL. Hepatocellular carcinoma: epidemiology and molecular carcinogenesis. Gastroenterology. 2007;132: 2557–76.
22. Kumar V, Kato N, Urabe Y, Takahashi A, Muroyama R, Hosono N, et al. Genome-wide association study identifies a susceptibility locus for HCV-induced hepatocellular carcinoma. Nat Genet. 2011;43:455–8.
23. 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.
24. McCarthy MI, Abecasis GR, Cardon LR, Goldstein DB, Little J, Ioannidis JP, et al. Genome-wide association studies for complex traits: consensus, uncertainty and challenges. Nat Rev Genet. 2008;9:356–69.
25. Cantor RM, Lange K, Sinsheimer JS. Prioritizing GWAS results: a review of statistical methods and recommendations for their application. Am J Hum Genet. 2010;86:6–22.
26. Johnson RC, Nelson GW, Troyer JL, Lautenberger JA, Kessing BD, Winkler CA, et al. Accounting for multiple comparisons in a genome-wide association study (GWAS). BMC Genomics. 2010; 11:724.
27. Asahina Y, Tanaka K, Suzuki Y, Tamaki N, Hoshioka T, Kato T, et al. Association between IL28B gene variation and development of hepatocellular carcinoma after interferon therapy in patients with chronic hepatitis C. J Hepatol. 2011;54:S37.
28. Fabris C, Falleti E, Cussigh A, Bitetto D, Fontanini E, Bignulin S, et al. IL-28B rs12979860 C/T allele distribution in patients with liver cirrhosis: role in the course of chronic viral hepatitis and the development of HCC. J Hepatol. 2011;54:716–22.
29. Bochud PY, Bibert S, Kutalik Z, Patin E, Guergnon J, Nalpas B, et al. IL28B alleles associated with poor hepatitis C virus (HCV) clearance protect against inflammation and fibrosis in patients infected with non-1 HCV genotypes. Hepatology. 2012;55:384–94.
30. Joshita S, Umemura T, Katsuyama Y, Ichikawa Y, Kimura T, Morita S, et al. Association of IL28B gene polymorphism with development of hepatocellular carcinoma in Japanese patients with chronic hepatitis C virus infection. Hum Immunol. 2012;73:298–300.
31. Miura M, Maekawa S, Kadokura M, Sueki R, Komase K, Shindo H, et al. Analysis of viral amino acids sequences and the IL28B SNP influencing the development of hepatocellular carcinoma in chronic hepatitis C. Hepatol Int. 2012;6:386–96.
32. Agundez JA, Garcia-Martin E, Maestro ML, Cuenca F, Martinez C, Ortega L, et al. Relation of IL28B gene polymorphism with biochemical and histological features in hepatitis C virus-induced liver disease. PLoS ONE. 2012;7:e37998.
33. Bruno S, Crosignani A, Maisonneuve P, Rossi S, Silini E, Mondelli MU. Hepatitis C virus genotype 1b as a major risk factor associated with hepatocellular carcinoma in patients with cirrhosis: a seventeen-year prospective cohort study. Hepatology. 2007;46:1350–6.
34. Bruno S, Silini E, Crosignani A, Borzio F, Leandro G, Bono F, et al. Hepatitis C virus genotypes and risk of hepatocellular carcinoma in cirrhosis: a prospective study. Hepatology. 1997;25: 754–8.
35. Silini E, Bottelli R, Asti M, Bruno S, Candusso ME, Brambilla S, et al. Hepatitis C virus genotypes and risk of hepatocellular carcinoma in cirrhosis: a case-control study. Gastroenterology. 1996;111:199–205.
36. Freeman AJ, Dore GJ, Law MG, Thorpe M, Von Overbeck J, Lloyd AR, et al. Estimating progression to cirrhosis in chronic hepatitis C virus infection. Hepatology. 2001;34:809–16.
37. Moghaddam A, Melum E, Reinton N, Ring-Larsen H, Verbaan H, Bjoro K, et al. IL28B genetic variation and treatment response in patients with hepatitis C virus genotype 3 infection. Hepatology. 2011;53:746–54.
38. Abe H, Ochi H, Maekawa T, Hayes CN, Tsuge M, Miki D, et al. Common variation of IL28 affects gamma-GTP levels and inflammation of the liver in chronically infected hepatitis C virus patients. J Hepatol. 2010;53:439–43.
39. Li Q, Kawamura K, Ma G, Iwata F, Numasaki M, Suzuki N, et al. Interferon-lambda induces G1 phase arrest or apoptosis in oesophageal carcinoma cells and produces anti-tumour effects in combination with anti-cancer agents. Eur J Cancer. 2010;46: 180–90.
40. Lasfar A, Lewis-Antes A, Smirnov SV, Anantha S, Abushahba W, Tian B, et al. Characterization of the mouse IFN-lambda ligand-receptor system: IFN-lambdas exhibit antitumor activity against B16 melanoma. Cancer Res. 2006;66:4468–77.
41. Abushahba W, Balan M, Castaneda I, Yuan Y, Reuhl K, Raveche E, et al. Antitumor activity of type I and type III interferons in BNL hepatoma model. Cancer Immunol Immunother. 2010;59: 1059–71.
42. Sato A, Ohtsuki M, Hata M, Kobayashi E, Murakami T. Antitumor activity of IFN-lambda in murine tumor models. J Immunol. 2006;176:7686–94.
43. Zitzmann K, Brand S, Baehs S, Goke B, Meinecke J, Spottl G, et al. Novel interferon-lambdas induce antiproliferative effects in neuroendocrine tumor cells. Biochem Biophys Res Commun. 2006;344:1334–41.
44. Khan DA, Fatima Tuz Z, Khan FA, Mubarak A. Evaluation of diagnostic accuracy of APRI for prediction of fibrosis in hepatitis C patients. J Ayub Med Coll Abbottabad. 2008;20:122–6.
45. Sebastiani G, Vario A, Guido M, Noventa F, Plebani M, Pistis R, et al. Stepwise combination algorithms of non-invasive markers to diagnose significant fibrosis in chronic hepatitis C. J Hepatol. 2006;44:686–93.

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Increased serum mitochondrial creatine kinase activity as a risk for hepatocarcinogenesis in chronic hepatitis C patients

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Serum mitochondrial creatine kinase (MtCK) activity was reportedly increased in cirrhotic patients although less prominent than that in hepatocellular carcinoma (HCC) patients. To elucidate the clinical significance of serum MtCK activity in chronic liver disease, 171 chronic hepatitis C patients were enrolled. Serum MtCK activity in study subjects was correlated with serum albumin, platelet counts, liver stiffness values and serum aspartate and alanine aminotransferase. In mouse fibrotic liver induced by bile duct ligation, ubiquitous MtCK mRNA and protein expressions were significantly enhanced and its immunoreactivity was increased, predominantly in hepatocytes. During the mean follow-up period of 2.7 years, HCC developed in 21 patients, in whom serum MtCK activity was significantly higher than that in patients without HCC development. Multivariate Cox regression analysis revealed that higher serum MtCK activity was a risk for HCC development. A cutoff value of MtCK for the prediction of HCC development was determined as 9.0 U/L on receiver operating characteristics analysis, where area under receiver operating characteristics curve was 0.754, with a sensitivity of 61.9%, a specificity of 92.8% and a high negative predictive value of 94.2%. Cumulative incidence of HCC was significantly higher in patients with serum MtCK activity of >9.0 U/L compared to those with serum MtCK activity of ≤9.0 U/L even in patients with elevated liver stiffness value, >15 kPa. In conclusion, serum MtCK activity may be increased correlatively with the stage of liver fibrosis and hepatocellular damage. Increased serum MtCK activity is an independent risk for hepatocarcinogenesis in chronic hepatitis C patients.

Hepatocellular carcinoma (HCC) is one of the common malignancies worldwide,[1] and the number of patients suffering from HCC is currently increasing in many countries.[2,3] As HCC has a specific feature that it usually develops in the setting of chronic liver injury,[2] especially liver cirrhosis,[4] cancer surveillance, when performed intensively in patients with chronic liver injury, could lead to HCC detection in its early stage, where biomarkers for HCC may play an important role. Although novel therapies have been developed to prolong survival in patients with advanced HCC, their effects are rather limited,[5] suggesting that the effective way for early detection of HCC is urgently needed. To this end, many attempts have been made to explore a novel biomarker for HCC,[6,7] among which we have recently found that serum mitochondrial creatine kinase (MtCK) activity was increased in patients with HCC. Among two tissue-specific isozymes of MtCK, that is, ubiquitous MtCK (uMtCK) and sarcomeric MtCK (sMtCK), we have found that the increase in serum MtCK activity in HCC patients was mostly owing to uMtCK, not sMtCK.[8] We have further found high expression of uMtCK mRNA in human HCC cell lines compared to normal human liver tissue.[8] Recently, we have reported that high uMtCK expression in HCC denotes a poor prognosis with highly malignant potential.[9] It is worth noting 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.[10–13]

In our previous report, we have observed that serum MtCK activity was also increased in patients with liver cirrhosis compared to healthy control although less prominent than in HCC patients.[8] In fact, an elevated serum MtCK

Key words: ubiquitous mitochondrial creatine kinase, hepatocellular carcinoma, liver fibrosis

Abbreviations: AFP: alpha-fetoprotein; ALT: alanine aminotransferase; AST: aspartate aminotransferase; CT: computed tomography; DCP: des-gamma-carboxy prothrombin; GGT: γ-glutamyltransferase; HCC: hepatocellular carcinoma; HCV: hepatitis C virus; MtCK: mitochondrial isoenzyme of creatine kinase; sMtCK: sarcomeric mitochondrial creatine kinase; uMtCK: ubiquitous mitochondrial creatine kinase

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What's new?
Chronic liver injury such as viral hepatitis increases the risk to develop hepatocellular carcinoma (HCC). Here, the authors show that serum mitochondrial creatine kinase activity, a potential new biomarker for progressive liver damage, was increased in patients with chronic hepatitis C virus infection and correlated with the stage of liver fibrosis and hepatocellular damage. Similar results were reproduced in mice after liver damage via bile duct ligation. Notably, high serum mitochondrial creatine kinase activity was an independent risk factor for hepatocarcinogenesis in viral hepatitis patients, underscoring the promise of this new marker in the prediction and possibly pathogenesis of HCC.

activity was previously reported in patients with liver cirrhosis,[14] where MtCK was described as "Macro CK type 2."[14,15] However, the clinical significance of increased serum MtCK activity in cirrhotic patients has not been clarified yet. In our study, we wondered whether serum MtCK activity might be increased in patients with not only liver cirrhosis but also chronic liver disease, in general, with less fibrosis, and if so, what would be the clinical significance of increased serum MtCK activity in patients with chronic liver disease. To address these questions, we sought to analyze serum MtCK activity in patients with chronic hepatitis C without the presence and the history of HCC.

Material and Methods

Subjects

One-hundred seventy-one patients with chronic hepatitis C, who visited the Department of Gastroenterology, The University of Tokyo Hospital, Tokyo, Japan, between January 2010 and April 2011, were enrolled. Chronic hepatitis C was defined as serum anti-hepatitis C virus antibody positivity and a detectable HCV RNA level, having persistent liver damage for more than 6 months, where other causes of liver disease such as hepatitis B and alcohol abuse had been excluded. Patients with HCC at the time of enrollment or with past history of HCC were excluded from this analysis, where HCC was ruled out by ultrasonography, dynamic computed tomography (CT) and/or magnetic resonance imaging. To assess a potential relationship between serum MtCK activity and liver fibrosis, all the enrolled patients undertook liver stiffness measurement.

Our 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. In our study, informed consent was obtained for the use of the samples.

Measurement of MtCK activity

MtCK activity was measured with an immune-inhibition method using two types of anti-MtCK monoclonal antibodies, that is, an anti-uMtCK monoclonal antibody and an anti-sMtCK monoclonal antibody in addition to an anticreatine kinase-M antibody[16] as described previously.[8] JCA-BM8040 (JEOL, Tokyo, Japan) was used as an automatic analyzer. The regression line of this assay was linear up to at least 1,800 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.

Measurements of other parameters

Ordinary serum chemistry parameters, albumin, aspartate aminotransferase (AST), alanine aminotransferase (ALT), γ-glutamyl transpeptidase (GGT) and total bilirubin, were analyzed using JCA-BM8040 (JEOL, Tokyo, Japan). Complete blood count examination was performed using XE-5000 (Sysmex, Kobe, Japan). Prothrombin time was measured using ACL TOP (Mitsubishi Chemical Medience, Tokyo, Japan). Alpha-fetoprotein (AFP) and des-gamma-carboxy prothrombin (DCP) were analyzed by a two-site immunoenzymetric assay using ST AIA-PACK AFP (TOSOH, Tokyo, Japan) and Lumipulse Presto PIVKAII (EIDIA, Tokyo, Japan), in automatic analyzers, AIA 2000 (TOSOH) and Lumipulse® PrestoII (FUJIREBIO, Tokyo, Japan), respectively. Liver stiffness was measured using transient elastography (FibroScan 502; EchoSens, Paris, France) as described previously.[17]

Animals and induction of liver fibrosis

Liver fibrosis was induced in C57BL/6N mice (CLEA Japan, Japan) by bile duct ligation at 4 weeks after the operation as described previously.[18]

All animals received humane care and the experimental protocol was approved by Animal Research Committee of the University of Tokyo.

Quantitative real-time polymerase chain reaction

Total RNA of mouse livers was extracted using TRIZOL reagent (Invitrogen, Carlsbad, CA). One microgram of purified total RNA was transcribed using a Transcriptor First Strand cDNA Synthesis Kit (Roche Diagnostics, Mannheim, Germany). Quantitative real-time polymerase chain reaction (PCR) was performed with a TaqMan Universal Master Mix No AmpErase UNG (Applied Biosystems, Foster City, CA). 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 15 sec and 60°C for 60 sec. The target gene mRNA expression level was relatively quantified to 18S

ribosomal RNA using $2^{-\Delta\Delta Ct}$ method (Applied Biosystems,, Foster City, CA, User Bulletin No. 2).

Immunoblot analysis

Liver tissue extracts were prepared by using M-PER® Mammalian Protein Extraction Reagent (Thermo Fisher Scientific, Rockford, IL) plus Halt™ Protease Inhibitor Cocktail (Thermo Fisher Scientific, Rockford, IL). Immunoblot analysis was performed with specific antibodies against uMtCK (dilution, 1:1,000; Abcam, Cambridge, United Kingdom) and beta-actin (dilution, 1:2,000; Sigma-Aldrich, St. Louis, MO) as described-previously.[9] Immunoreactive proteins were visualized using a chemiluminescence kit (GE Healthcare, Buckinghamshire, United Kingdom), and recorded using a LAS-4000 image analyzer (Fuji Film, Tokyo, Japan). The intensities of immunodetected bands were quantified with NIH Image J software.

Immunohistochemical analysis

Excised liver specimens were fixed immediately in 10% formalin and embedded in paraffin. Serial 4-μm-thick liver tissue sections were deparaffinized, and incubated in citrate buffer at 95°C for 40 min for antigen retrieval, and then incubated overnight at 4°C with anti-uMtCK antibody (Proteintech, Chicago, IL). Biotinylated secondary antibodies (Pharmingen, San Diego, CA) were added and incubated for 20 min at room temperature. Streptavidin–horseradish peroxidase (Pharmingen, San Diego, CA) was added and after 30 min the sections were developed with 3,3′-diaminobenzidine substrate and counterstained hematoxylin.

Patient follow-up and diagnosis of HCC

Patients were followed up at the outpatient clinic with blood tests including tumor markers every 1–3 months, and ultrasonography every 4–6 months. Contrast-enhanced CT was performed when serum tumor markers showed an abnormal rise and/or tumor(s) was detected as possible HCC on ultrasonography. The diagnosis of HCC was based on the typical findings on CT, that is, hyperattenuation in the arterial phase and hypoattenuation in the equilibrium phase.[19,20]

The end points consisted of the interval between the first measurement of serum MtCK activity and the detection of HCC development, death without HCC development or the last examination until May 30, 2013, whichever came first. Death without HCC development was treated as censored data.

Statistical analysis

Categorical data were compared by χ^2-test or Fisher's exact test. Distributions of continuous variables were analyzed with Student's *t*-test for two groups. All tests of significance were two-tailed, and $p < 0.05$ was considered statistically significant. The potential associations between the MtCK and the following factors were assessed using Spearman's rank correlation coefficient: age, serum albumin, AST, ALT, GGT, total bilirubin, AFP, DCP, platelet count, prothrombin time and liver stiffness measured by Fibroscan. Cumulative incidence of hepatocarcinogenesis was calculated by the Kaplan–Meier method, and differences among groups were assessed using the log-rank test. The following factors were assessed as candidate risk factors for hepatocarcinogenesis by time-fixed Cox proportional hazard regression: age, sex, hepatitis virus, serum albumin, AST, ALT, GGT, total bilirubin, AFP, DCP, platelet count, prothrombin time, liver stiffness and MtCK. We used univariate and multivariate time-fixed Cox proportional hazard models and stepwise variable selection based on Akaike Information Criteria. Data processing and analysis were performed using SPSS software version 17.0 or 19.0 (SPSS, Chicago, IL).

Table 1. Characteristics of the enrolled chronic hepatitis C patients

Parameter	*N* = 171
Age (year)[1]	68 (60–75.5)
Female[2]	75 (43.9)
MtCK (U/L)[1]	4.50 (3.20–7.19)
Albumin (g/dL)[1]	4.0 (3.7–4.3)
AST (U/L)[1]	40 (29–63)
ALT (U/L)[1]	35 (23–55.5)
GGT (U/L)[1]	28 (20–49.5)
Total bilirubin (mg/dL)[1]	0.8 (0.6–1.2)
AFP (ng/dL)[1]	5.0 (3.0–10.1)
DCP (mAU/mL)[1]	16 (12–22.5)
Platelet ($\times 10^4/\mu L$)[1]	12.1 (8.8–17.5)
Prothrombin time (sec)[1]	11.7 (11.2–12.5)
LSV measured by Fibroscan (kPa)[1]	10.5 (5.7–17.0)

[1]Data were expressed as mean (1st–3rd. quartile).
[2]Data were expressed as number (%).

Results

Increased serum MtCK activity in patients with chronic hepatitis C

Clinical and laboratory variables of the enrolled patients are listed in Table 1. The mean level of serum albumin and total bilirubin and the mean platelet count in the enrolled patients were 4.0 g/dL, 0.8 mg/dL and 12.1 × $10^4/\mu L$, suggesting that the patients would have developed various stages of liver fibrosis, not exclusively liver cirrhosis. In agreement with this fact, the mean liver stiffness value in the enrolled patients was 10.5 kPa, suggesting the fibrosis stage of F3.[17] In these patients, serum MtCK activity was higher than the previously reported values in healthy subjects ($p < 0.001$): the mean serum MtCK activity was 4.5 U/L in patients with chronic hepatitis C, whereas 3.4 U/L in healthy subjects as described previously.[8]

Relationships between serum MtCK activity and various parameters

Relationships between serum MtCK activity and various clinical parameters are summarized in Table 2. Serum MtCK activity was significantly correlated with serum albumin levels, platelet counts and liver stiffness values ($p < 0.001$, 0.026

Table 2. Relation between serum MtCK activity and various parameters

Parameter	Spearman's ρ	*p*-Value
Age (year)	0.1829	0.016
Albumin (g/dL)	−0.4041	<0.001
AST (U/L)	0.2419	0.0014
ALT (U/L)	0.1556	0.042
GGT (U/L)	0.0427	0.58
Total bilirubin (mg/dL)	−0.0044	0.96
AFP (ng/dL)	0.2207	0.0037
DCP (mAU/mL)	0.0667	0.39
Platelet ($\times 10^4/\mu L$)	−0.1703	0.026
Prothrombin time (sec)	0.1482	0.086
LSV measured by Fibroscan (kPa)	0.2843	<0.001

and <0.001), suggesting that the increase in serum MtCK activity may be associated with the stage of liver fibrosis. On the other hand, the significant correlations between serum MtCK activity and serum levels of AST ($p = 0.0014$) and ALT ($p = 0.042$) were observed, which may suggest that serum MtCK activity is increased in association with hepatocellular damage. Furthermore, serum MtCK activity was significantly correlated with serum AFP levels ($p = 0.0037$).

Increased uMtCK mRNA and protein expressions and immunoreactivity for uMtCK in fibrotic livers in mice

As described earlier, among two tissue-specific isozymes of MtCK, that is, uMtCK and sMtCK, we have found that the increase in serum MtCK activity in HCC patients was mostly owing to that in serum uMtCK activity but not in serum sMtCK activity.[8] As the current evidence suggests that serum MtCK activity may be increased in association with the stage of liver fibrosis, we wondered whether uMtCK expression might be enhanced in fibrotic livers. To test this hypothesis, we first measured uMtCK mRNA levels in the livers of mice treated with bile duct ligation for 4 weeks. As shown in Figure 1*a*, uMtCK mRNA levels in the livers were significantly enhanced in bile duct-ligated mice at 4 weeks after the operation compared to sham-operated mice ($p = 0.02$; Fig. 1*a*). An increased immunoreactivity for uMtCK was detected in bile duct-ligated mouse livers, predominantly in hepatocytes at the periductular area, as compared to sham-operated livers, where immunoreactivity was very low or absent (Fig. 1*b*). This increased immunoreactivity was confirmed to be owing to uMtCK protein expression by immunoblot analysis (Fig. 1*c*). These results suggest that uMtCK expression may be increased in fibrotic livers predominantly in hepatocytes, possibly leading to enhanced serum MtCK activity.

Increased serum MtCK activity as an independent risk for hepatocarcinogenesis

The enrolled patients were then followed up to detect HCC occurrence. During the mean follow-up period of 2.7 years (1st–3rd quartile: 2.4–3.1 years), HCC developed in 21 patients. To carefully exclude MtCK production by HCC, HCC was ruled out at the enrollment by ultrasonography, dynamic CT and/or magnetic resonance imaging. The cumulative incidence rates of HCC at 1, 2 and 3 years estimated by the Kaplan–Meier method were 3.5, 8.8 and 12.3%, respectively, as shown in Figure 2*a*. In these patients who developed HCC, serum MtCK activity was significantly higher than that in patients who did not develop HCC ($p < 0.001$) as shown in Figure 2*b*; serum MtCK activity was 10.6 U/L (interquartile range, 4.4–20.7) in patients who developed HCC and 4.3 U/L (interquartile range, 3.1–6.6) in patients who did not develop HCC. Then, significant risk factors for HCC occurrence by univariate Cox regression analysis were as follows (Table 3): older age ($p = 0.018$), lower albumin ($p < 0.001$), higher AST ($p = 0.017$), higher AFP ($p < 0.001$), lower platelet count ($p = 0.0025$), longer prothrombin time ($p = 0.0013$), elevated liver stiffness value ($p < 0.001$) and higher serum MtCK activity ($p < 0.001$). Multivariate analysis using stepwise variable selection based on Akaike Information Criteria identified higher serum MtCK activity (HR: 1.09/year, $p < 0.001$), higher AFP (HR: 1.01/year, $p = 0.002$) and longer prothrombin time (HR: 1.48/year, $p = 0.002$) as the significant risk factors.

As our multivariate analysis identified serum MtCK activity as an independent factor associated with a risk for HCC development, we determined a cutoff value of serum MtCK activity for the prediction of HCC development by receiver operating characteristics (ROC) analysis. From this analysis, serum MtCK activity of 9.0 U/L was identified as a cutoff value (Fig. 3*a*), and with this cutoff value, area under receiver operating characteristics curve for serum MtCK activity was 0.754 (95% confidence interval [CI]: 0.613–0.894), with a sensitivity of 61.9%, a specificity of 92.8%, a positive predictive value of 56.5% and a negative predictive value of 94.2%. As this negative predictive value was high, the patients with serum MtCK activity of ≤9.0 U/L are suggested to be at a lower risk for HCC development. In fact, as shown in Figure 3*b*, patients with serum MtCK activity of >9.0 U/L were at a significantly higher risk for HCC development compared to those with serum MtCK activity of ≤9.0 U/L ($p < 0.001$). As serum MtCK activity seemed to be correlated with liver fibrosis as observed above, a relationship between serum MtCK activity and HCC development was analyzed in stratified patients by liver stiffness values. As shown in Figures 3*c* and 3*d*, in both patient groups with liver stiffness values of >15 and ≤15 kPa, serum MtCK activity of >9.0 U/L was a significantly higher risk for HCC development compared to those with serum MtCK activity of ≤9.0 U/L ($p < 0.001$). Notably, the cumulative incidence of HCC at 1,100 days of follow-up period in patients with serum MtCK activity of >9.0 U/L was comparable, approximately 0.5, irrespective of their liver stiffness values, that is ≤15 or >15 kPa. Collectively, the higher serum MtCK activity may be an independent risk for HCC development in chronic hepatitis C patients.

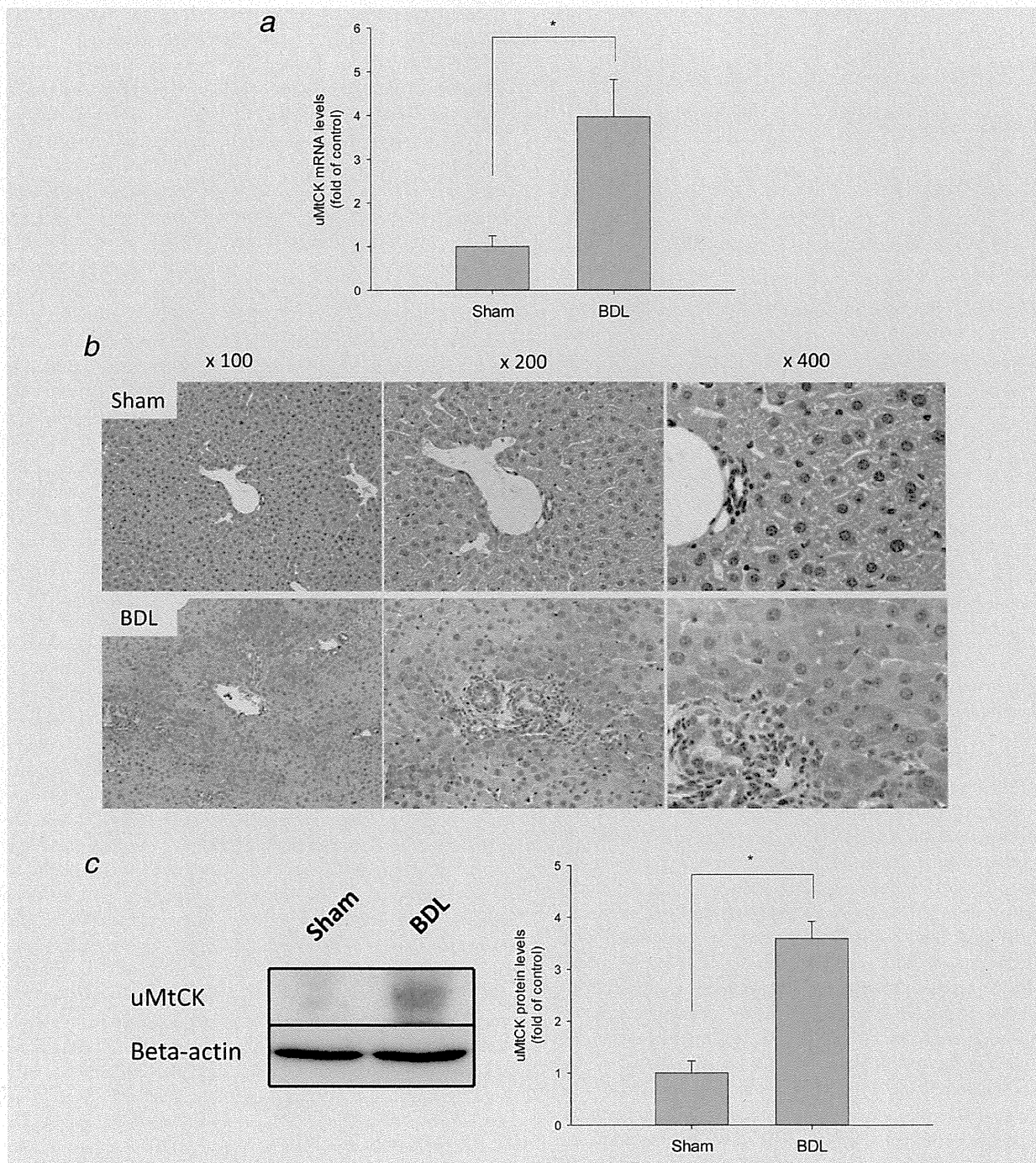

Figure 1. uMtCK mRNA and protein expressions in fibrotic livers induced by bile duct ligation in mice. (*a*) uMtCK mRNA expressions were evaluated by quantitative real-time PCR in the livers of bile duct-ligated and sham-operated mice at 4 weeks after the operation. Results represent a fold of control mice (means ± SEM, $n = 4$). uMtCK mRNA expressions were significantly enhanced in fibrotic livers induced by bile duct ligation in mice ($p = 0.02$) compared to control livers; an asterisk indicates a significant difference. (*b*) uMtCK protein expressions were evaluated immunohistochemically in fibrotic livers induced by bile duct ligation in mice in comparison with control livers. Increased immunoreactivity for uMtCK was observed predominantly in hepatocytes in fibrotic livers compared to control livers. (*c*) uMtCK protein expressions, evaluated by immmunoblot analysis, were significantly enhanced in fibrotic livers induced by bile duct ligation in mice ($p = 0.03$) compared to control livers; an asterisk indicates a significant difference.

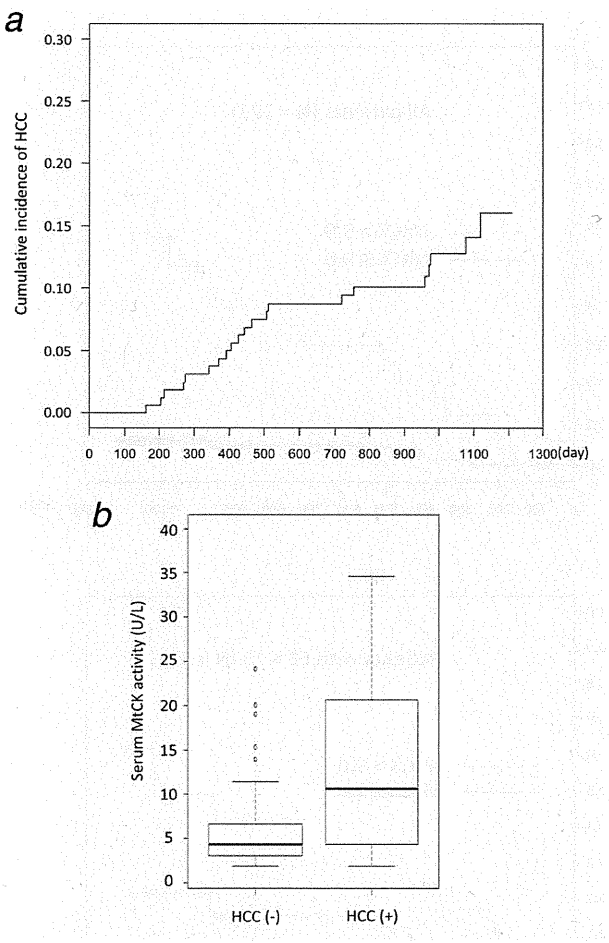

Figure 2. Serum MtCK activity and HCC development in chronic hepatitis C patients. (*a*) Cumulative incidence of HCC in chronic hepatitis C patients. During the mean follow-up period of 2.7 years, HCC developed in 21 patients. The cumulative incidence rates of HCC at 1, 2 and 3 years estimated by the Kaplan–Meier method were 3.5, 8.8 and 12.3%, respectively. (*b*) Serum MtCK activity in chronic hepatitis C patients with or without HCC development. The mean serum MtCK activity in patients with HCC development was 10.6 U/L and significantly higher than that in patients without HCC development, 4.3 U/L ($p < 0.001$).

Table 3. Risk factors for HCC evaluated by univariate and multivariate analyses

	Univariate		Multivariate	
Parameter	HR (95% CI)	*p*-Value	HR (95% CI)	*p*-Value
Age (year)	1.06 (1.01–1.12)	0.018	1.04 (0.98–1.09)	0.28
Female	0.74 (0.31–1.78)	0.50		
MtCK (U/L)	1.08 (1.05–1.11)	<0.001	1.09 (1.04–1.13)	<0.001
Albumin (g/dL)	0.15 (0.07–0.36)	<0.001		
AST (U/L)	1.01 (1.00–1.02)	0.017		
ALT (U/L)	1.002 (0.998–1.010)	0.66		
GGT (U/L)	1.001 (0.997–1.006)	0.54		
Total bilirubin (mg/dL)	2.36 (0.99–5.61)	0.053		
AFP (ng/dL)	1.02 (0.98–1.02)	<0.001	1.01 (1.004–1.02)	0.002
DCP (mAU/mL)	1.02 (0.98–1.04)	0.020		
Platelet ($\times 10^4/\mu L$)	0.87 (0.80–0.95)	0.0025		
Prothrombin time (sec)	1.53 (1.18–1.98)	0.0013	1.48 (1.28–1.91)	0.002
LSV (kPa)	1.06 (1.04–1.08)	<0.001		

Discussion

In our study, we aimed to explore the clinical significance of serum MtCK activity in chronic hepatitis C patients without HCC. As a result, we have found that serum MtCK activity may be increased correlatively with the stage of liver fibrosis and hepatocellular damage, and that the increased serum MtCK activity is an independent risk for hepatocarcinogenesis, which could be the important information for physicians.

As MtCK is not naturally secreted from the cells, the active production of MtCK in a certain tissue or organ and its active release into the blood stream are assumed to be necessary for the increase in serum MtCK activity. Indeed, the increased uMtCK mRNA expression and the increased immunoreactivity for uMtCK were observed predominantly in hepatocytes of fibrotic livers in mice induced by bile duct ligation in our study, suggesting that the active production of uMtCK in fibrotic livers. Furthermore, the strong correlations between serum MtCK activity and serum levels of AST and ALT may suggest that serum MtCK activity is increased in association with hepatocellular damage, leading to the active release of MtCK from hepatocytes into the blood stream.

It is well known that HCV-related cirrhosis is associated with an extremely high risk of HCC development, with a reported annual incidence ranging between 3 and 8%,[4,21,22] indicating that advanced liver fibrosis is one of the strongest risk factors for HCC development in chronic hepatitis C patients. As our current results suggest that serum MtCK activity may be increased in association with the stage of liver fibrosis, the increased serum MtCK activity as a risk factor for hepatocarcinogenesis in chronic hepatitis C patients could be explained, at least in part, by the association between serum MtCK activity and liver fibrosis. In our study, higher serum MtCK activity but not elevated liver stiffness value was determined as a risk for HCC development on multivariate analysis. This finding may be explained by that liver stiffness value, being strongly correlated with serum MtCK

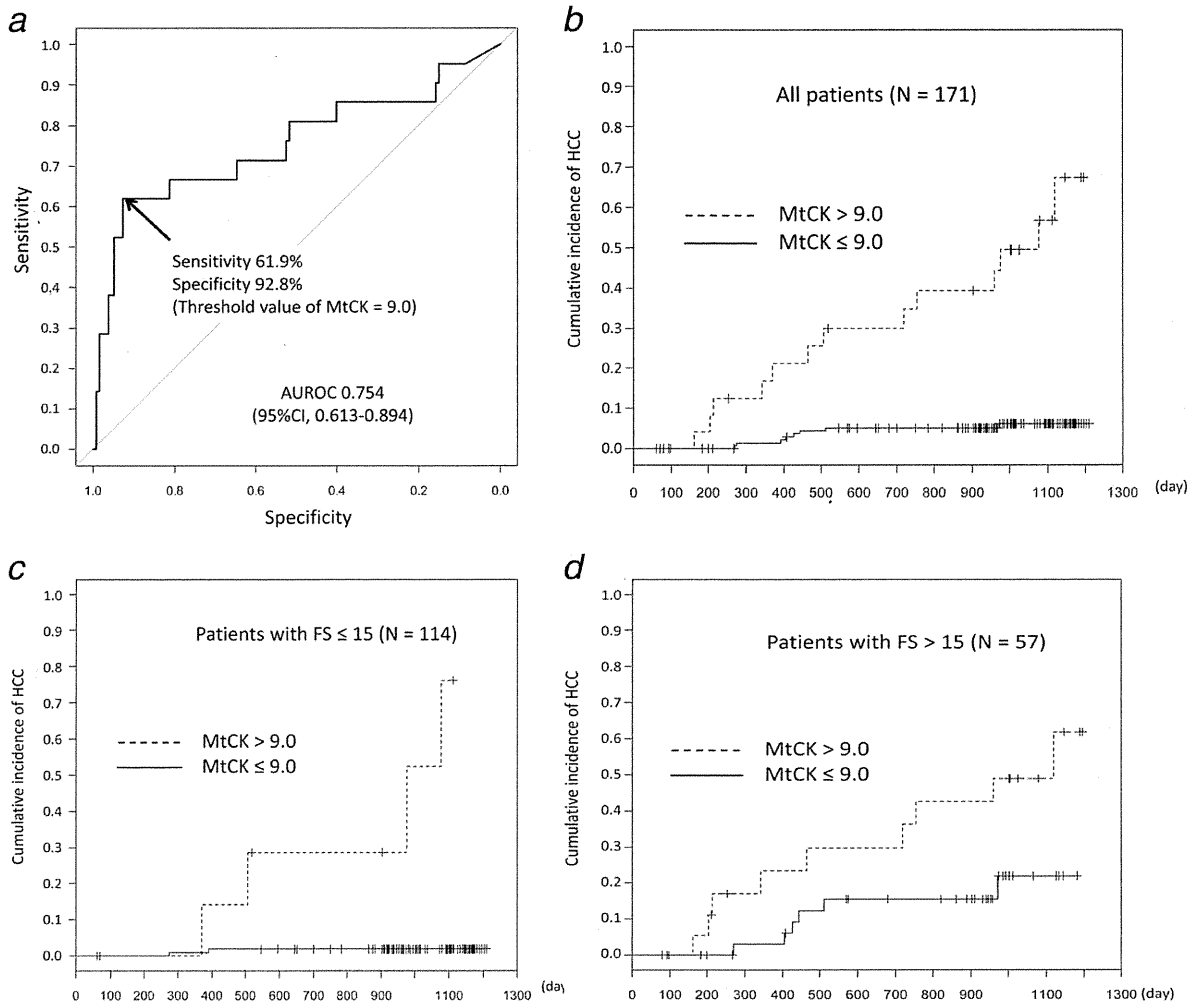

Figure 3. ROC curve showing the overall accuracy of serum MtCK activity for the prediction of HCC development and cumulative incidence of HCC subdivided according to serum MtCK activity in chronic hepatitis C patients. (*a*) ROC curve showing the overall accuracy of serum MtCK activity for the prediction of HCC development in chronic hepatitis C patients. The arrow identifies the best cutoff value (*i.e.*, 9.0 U/L) of serum MtCK activity. Then, cumulative incidence rates of HCC were estimated by the Kaplan–Meier method in all patients (*b*), in patients with liver stiffness value (LSV) of ≤15 kPa (*c*), and in patients with LSV of >15 kPa (*d*) subdivided according to their serum MtCK activity of 9.0 U/L. Serum MtCK activity of >9.0 U/L was a significantly higher risk for HCC development compared to those with serum MtCK of <9.0 U/L ($p < 0.001$) in all patient groups. Solid line, MtCK ≤ 9.0 U/L; dashed line, MtCK > 9.0 U/L.

activity as a predicting factor for liver fibrosis, was not retained as an independent risk for HCC development as a confounding factor. When evaluating this result, we should also bear in mind that another factor other than liver fibrosis may be responsible for the strong association between serum MtCK activity and HCC development. In this context, of interest is the evidence that the higher serum ALT levels were associated with the higher rate of HCC development[23] and HCC recurrence after the surgical treatment[24] in HCV-related cirrhosis, suggesting that the active hepatocellular damage may also be a risk for HCC development. Thus, the association between serum MtCK activity and hepatocellular damage, in addition to liver fibrosis, may explain the reason why serum MtCK activity was retained as an independent risk for hepatocarcinogenesis on multivariate analysis.

In our study, a significant association between serum MtCK activity and serum AFP levels was observed. As it is well known, serum AFP levels have been widely used as a serological marker for HCC[25] although the combination with other serological markers and imaging techniques is recommended to increase diagnostic accuracy.[26] However, elevated serum AFP levels are often observed in patients with chronic hepatitis C without HCC.[27–29] Although the mechanism(s) underlying this finding has not been fully understood yet, it was reported that serum AFP levels were independently associated with liver fibrosis and serum AST levels.[28,30] Thus, it

may be reasonable to assume that serum MtCK activity would behave similarly to serum AFP levels, both of which may be associated with liver fibrosis and hepatocellular damage. Indeed, in our study, both serum MtCK activity and serum AFP levels were retained as a risk for hepatocarcinogenesis, which may be in line with the evidence that the higher serum AFP levels were a risk for HCC development in cirrhotic patients.[31,32] Serum MtCK activity as a risk for HCC development should be further evaluated in comparison with serum AFP levels in a larger cohort with a variety of etiology.

As healthy liver tissue is known to be one of the few tissues that, in general, does not express detectable amounts of uMtCK,[33] uMtCK expression in the liver is assumed to be a sign of pathological development associated with, for example, ischemic–reperfusion injury[34] or tumor formation.[35] In agreement with this notion, in our study, serum MtCK activity was increased in association particularly with liver fibrosis and hepatocellular damage. Although a role of MtCK expression in pathological liver tissues remains to be elucidated, the evidence from CK gene transgenic mice, which showed that CK expression in the liver led to inhibition of apoptosis[36,37] and protection against hypoxia or endotoxin perfusion,[38–40] may suggest a protective role of MtCK expression in injured liver tissues. Indeed, MtCK has been assumed to be important for the energetics of oxidative tissues to control cellular energy homeostasis by building up a large pool of rapidly diffusing phosphocreatine for temporal and spatial buffering of ATP levels.[33] Hence, it is speculated that the increased MtCK activity may support active proliferation of the injured liver tissues to regenerate, which may ultimately lead to hepatocarcinogenesis as a result of enhanced proliferative activity as suggested previously.[32]

One of the limitations of our study is that serum MtCK activity was analyzed in a relatively small number of patients with chronic hepatitis C. In addition, the enrolled patients were at an older age (mean age, 68 years), which may be in line with the trend that the prevalence of older patients with chronic hepatitis C has been increasing in Japan.[41] In our study, as our cohort had a relatively narrow age distribution, age might not be retained as a risk for hepatocarcinogenesis. Nonetheless, serum MtCK activity as a risk for hepatocarcinogenesis should be further validated in a larger number of patients with other etiology, such as chronic hepatitis B or nonalcoholic steatohepatitis.

References

1. 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.
2. Llovet JM, Burroughs A, Bruix J. Hepatocellular carcinoma. *Lancet* 2003;362: 1907–17.
3. El-Serag HB. Epidemiology of hepatocellular carcinoma in USA. *Hepatol Res* 2007;37:S88–94.
4. Yoshida H, Shiratori Y, Moriyama 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;131:174–81.
5. Bertino G, Di Carlo I, Ardiri A, et al. Systemic therapies in hepatocellular carcinoma: present and future. *Future Oncol* 2013;9:1533–48.
6. Biondi A, Malaguarnera G, Vacante M, et al. Elevated serum levels of Chromogranin A in hepatocellular carcinoma. *BMC Surg* 2012;12:S7.
7. Bertino G, Ardiri A, Malaguarnera M, et al. Hepatocellualar carcinoma serum markers. *Semin Oncol* 2012;39:410–33.
8. 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;57:330–36.
9. Uranbileg B, Enooku K, Soroida Y, et al. High ubiquitous mitochondrial creatine kinase expression in hepatocellular carcinoma denotes a poor prognosis with highly malignant potential. *Int J Cancer* 2013 Oct 15. doi: 10.1002/ijc.28547. [Epub ahead of print]
10. Kanemitsu F, Kawanishi I, Mizushima J, et al. Mitochondrial creatine kinase as a tumor-associated marker. *Clin Chim Acta* 1984;138:175–83.
11. Pratt R, Vallis LM, Lim CW, et al. Mitochondrial creatine kinase in cancer patients. *Pathology* 1987;19:162–65.
12. 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;427:60–66.
13. 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.
14. Stein W, Bohner J, Renn W, et al. Macro creatine kinase type 2: results of a prospective study in hospitalized patients. *Clin Chem* 1985;31:1959–64.
15. Lee KN, Csako G, Bernhardt P, et al. Relevance of macro creatine kinase type 1 and type 2 isoenzymes to laboratory and clinical data. *Clin Chem* 1994;40:1278–83.
16. 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.
17. Castera L, Vergniol J, Foucher J, et al. Prospective comparison of transient elastography, Fibrotest, APRI, and liver biopsy for the assessment of fibrosis in chronic hepatitis C. *Gastroenterology* 2005;128:343–50.
18. Kageyama Y, Ikeda H, Watanabe N, et al. Antagonism of sphingosine 1-phosphate receptor 2 causes a selective reduction of portal vein pressure in bile duct-ligated rodents. *Hepatology* 2012;56:1427–38.
19. Torzilli G, Minagawa M, Takayama T, et al. Accurate preoperative evaluation of liver mass lesions without fine-needle biopsy. *Hepatology* 1999;30:889–93.
20. 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.
21. Tsukuma H, Hiyama T, Tanaka S, et al. Risk factors for hepatocellular carcinoma among patients with chronic liver disease. *N Engl J Med* 1993; 328:1797–801.
22. Ikeda K, Saitoh S, Koida I, et al. A multivariate analysis of risk factors for hepatocellular carcinogenesis: a prospective observation of 795 patients with viral and alcoholic cirrhosis. *Hepatology* 1993;18:47–53.
23. Tarao K, Rino Y, Ohkawa S, et al. Association between high serum alanine aminotransferase levels and more rapid development and higher rate of incidence of hepatocellular carcinoma in patients with hepatitis C virus-associated cirrhosis. *Cancer* 1999;86:589–95.
24. Tarao K, Takemiya S, Tamai S, et al. Relationship between the recurrence of hepatocellular carcinoma (HCC) and serum alanine aminotransferase levels in hepatectomized patients with hepatitis C virus-associated cirrhosis and HCC. *Cancer* 1997; 79:688–94.
25. El-Serag HB, Marrero JA, Rudolph L, et al. Diagnosis and treatment of hepatocellular carcinoma. *Gastroenterology* 2008;134:1752–63.
26. 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.
27. Bayati N, Silverman AL, Gordon SC. Serum alpha-fetoprotein levels and liver histology in

patients with chronic hepatitis C. *Am J Gastroenterol* 1998;93:2452–56.
28. Goldstein NS, Blue DE, Hankin R, et al. Serum alpha-fetoprotein levels in patients with chronic hepatitis C. Relationships with serum alanine aminotransferase values, histologic activity index, and hepatocyte MIB-1 scores. *Am J Clin Pathol* 1999;111:811–16.
29. Chu CW, Hwang SJ, Luo JC, et al. Clinical, virologic, and pathologic significance of elevated serum alpha-fetoprotein levels in patients with chronic hepatitis C. *J Clin Gastroenterol* 2001;32:240–44.
30. Hu KQ, Kyulo NL, Lim N, et al. Clinical significance of elevated alpha-fetoprotein (AFP) in patients with chronic hepatitis C, but not hepatocellular carcinoma. *Am J Gastroenterol* 2004;99:860–65.
31. Oka H, Tamori A, Kuroki T, et al. Prospective study of alpha-fetoprotein in cirrhotic patients monitored for development of hepatocellular carcinoma. *Hepatology* 1994;19:61–66.
32. Sangiovanni A, Colombo E, Radaelli F, et al. Hepatocyte proliferation and risk of hepatocellular carcinoma in cirrhotic patients. *Am J Gastroenterol* 2001;96:1575–80.
33. Schlattner U, Tokarska-Schlattner M, Wallimann T. Mitochondrial creatine kinase in human health and disease. *Biochim Biophys Acta* 2006;1762:164–80.
34. Vaubourdolle M, Chazouilleres O, Poupon R, et al. Creatine kinase-BB: a marker of liver sinusoidal damage in ischemia-reperfusion. *Hepatology* 1993;17:423–28.
35. Kanemitsu F, Kawanishi I, Mizushima J. A new creatine kinase found in mitochondrial extracts from malignant liver tissue. *Clin Chim Acta* 1983;128:233–40.
36. Dolder M, Walzel B, Speer O, et al. Inhibition of the mitochondrial permeability transition by creatine kinase substrates. Requirement for microcompartmentation. *J Biol Chem* 2003;278:17760–66.
37. Hatano E, Tanaka A, Kanazawa A, et al. Inhibition of tumor necrosis factor-induced apoptosis in transgenic mouse liver expressing creatine kinase. *Liver Int* 2004;24:384–93.
38. Miller K, Halow J, Koretsky AP. Phosphocreatine protects transgenic mouse liver expressing creatine kinase from hypoxia and ischemia. *Am J Physiol* 1993;265:C1544–51.
39. Hatano E, Tanaka A, Iwata S, et al. Induction of endotoxin tolerance in transgenic mouse liver expressing creatine kinase. *Hepatology* 1996;24:663–69.
40. Miller K, Sharer K, Suhan J, et al. Expression of functional mitochondrial creatine kinase in liver of transgenic mice. *Am J Physiol* 1997;272:C1193–202.
41. Tanaka Y, Hanada K, Mizokami M, et al. A comparison of the molecular clock of hepatitis C virus in the United States and Japan predicts that hepatocellular carcinoma incidence in the United States will increase over the next two decades. *Proc Natl Acad Sci USA* 2002;99:15584–89.

Changes in Risk of Immediate Adverse Reactions to Iodinated Contrast Media by Repeated Administrations in Patients with Hepatocellular Carcinoma

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Abstract

Background: To elucidate whether repeated exposures to iodinated contrast media increase the risk of adverse reaction.

Materials and Methods: We retrospectively reviewed 1,861 patients with hepatocellular carcinoma who visited authors' institution, a tertiary referral center, between 2004 and 2008. We analyzed cumulative probability of adverse reactions and risk factors. We categorized all symptoms into hypersensitivity reactions, physiologic reactions, and other reactions, according to the American College of Radiology guidelines, and evaluated each category as an event. We estimated the association between hazard for adverse reactions and the number of cumulative exposures to contrast media. We also evaluated subsequent contrast media injections and adverse reactions.

Results: There were 23,684 contrast media injections in 1,729 patients. One hundred and thirty-two patients were excluded because they were given no contrast media during the study period. Adverse reactions occurred in 196 (0.83%) patients. The cumulative incidence at 10th, 20th, and 30th examination was 7.9%, 15.2%, and 24.1%, respectively. Presence of renal impairment was found to be one of risk factors for adverse reactions. The estimated hazard of overall adverse reaction gradually decreased until around 10th exposure and rose with subsequent exposures. The estimated hazard of hypersensitivity showed V-shaped change with cumulative number of exposures. The estimated hazard of physiologic reaction had a tendency toward decreasing and that of other reaction had a tendency toward increasing. Second adverse reaction was more severe than the initial in only one among 130 patients receiving subsequent injections.

Conclusion: Repeated exposures to iodinated contrast media increase the risk of adverse reaction.

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Introduction

Hepatocellular carcinoma (HCC) is the sixth most prevalent cancer and the third most frequent cause of cancer-related death [1] and the incidence of HCC is increasing over the last decade [2,3]. Patients with HCC receive repeated contrast media (CM) injections, not only for diagnosis but also for surveillance of recurrence after initial complete treatment by surgery or local ablation [4]. This is because the residual liver tissue is usually already damaged by chronic liver disease and intrahepatic recurrence is very frequent [5].

The non-ionic CM have lower osmolality and tend to have fewer side effects, while retaining satisfactory radiographic opacification [6,7], and are thus have already completely replaced the older ionic higher osmolality contrast media for intravascular use. Previous studies have reported the rate of adverse reactions (ARs) to non-ionic CM to be from 1 to 4% [8–10]. The severity ranges from mild symptoms, such as