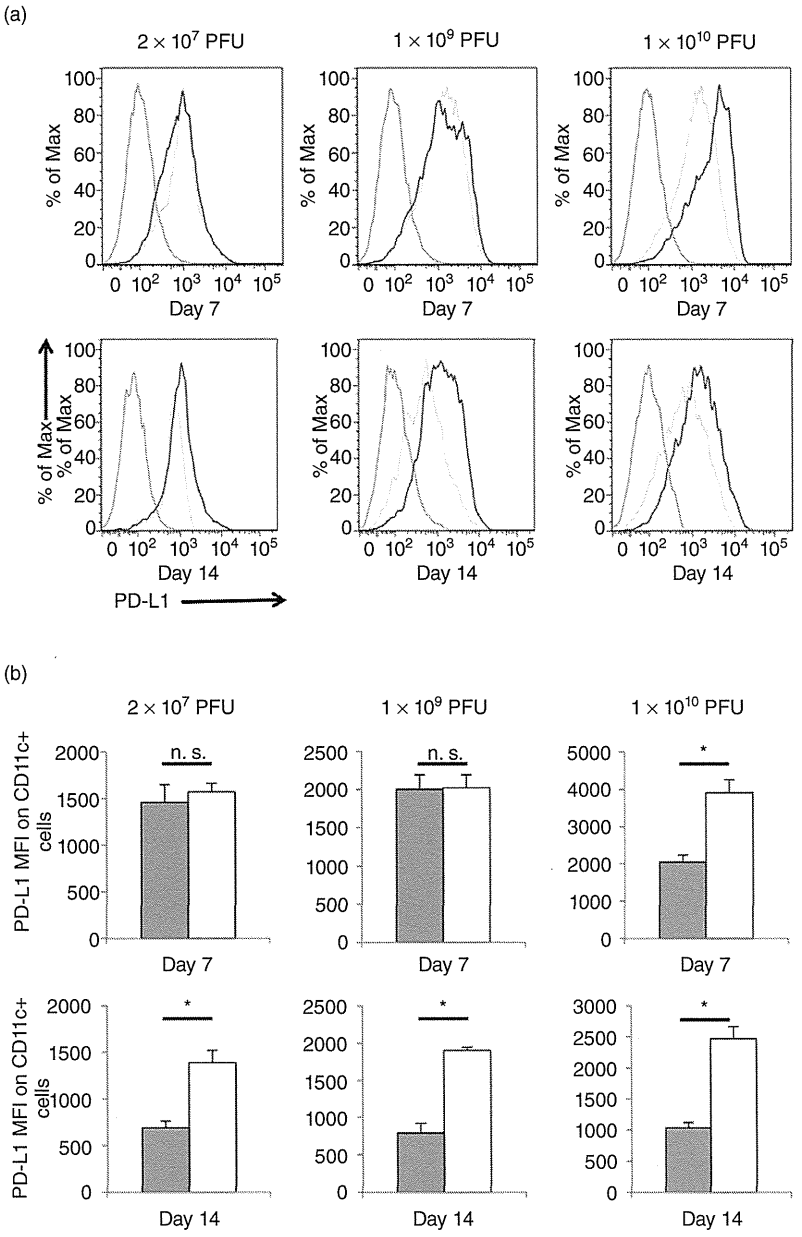

Figure 5 PD-L1 expression in the liver of core (+) and core (−) mice. Core (+) and core (−) mice were injected with 2×10^7, 1×10^9 and 1×10^{10} plaque-forming units (PFU) of adenovirus (Ad)-hepatitis C virus (HCV)-NS3 or Adψ5 control vector. (a) PD-L1 expression by intrahepatic antigen-presenting cells (APC) from core (+) and core (−) mice infected with Ad-HCV-NS3. The % of Max is the number of cells in each sample divided by the number of cells in the sample that contains the largest number of cells. (b) The median fluorescence index (MFI) expression of PD-L1 by intrahepatic $CD11c^+$ leukocyte from core (+) and core (−) mice infected with Ad-HCV-NS3 (*$P < 0.05$; n.s., not statistically significant). (c) PD-L1 expression by intrahepatic APC from core (+) and core (−) mice infected with Ad-HCV-NS3 or Adψ5 control vector. (d) The MFI expression of PD-L1 by intrahepatic $CD11c^+$ leukocyte from core (+) and core (−) mice infected with Ad-HCV-NS3 or Adψ5 control vector (n.s., not statistically significant). (a) ——, isotype; ·····, core (−); ——, core (+); (b) ■, core (−); □, core (+); (c) ——, isotype; ·····, Adψ5; ——, Ad-NS3; (d) ■, Adψ5; □, Ad-NS3.

in Ag-specific intrahepatic CD8 T cells. These results indicated that high infectious dose and the presence of HCV core gene were strongly involved in ineffective CD8 T-cell responses.

Recently, a novel mechanism of T-cell dysfunction was demonstrated in a murine model of chronic LCMV infection.[24] It was found that the expression of PD-1 was upregulated on dysfunctional LCMV-specific CD8 T cells in mice.[24] *In vivo* blockade of PD-1/PD-L1 interaction restored the functions of LCMV-specific CD8 T cells and reduced the viral titer.[24] More recently, other inhibitory receptors such as Tim-3 have also been studied as the factors that can cause T-cell impairments in chronic viral infections.[25] These influential discoveries led to

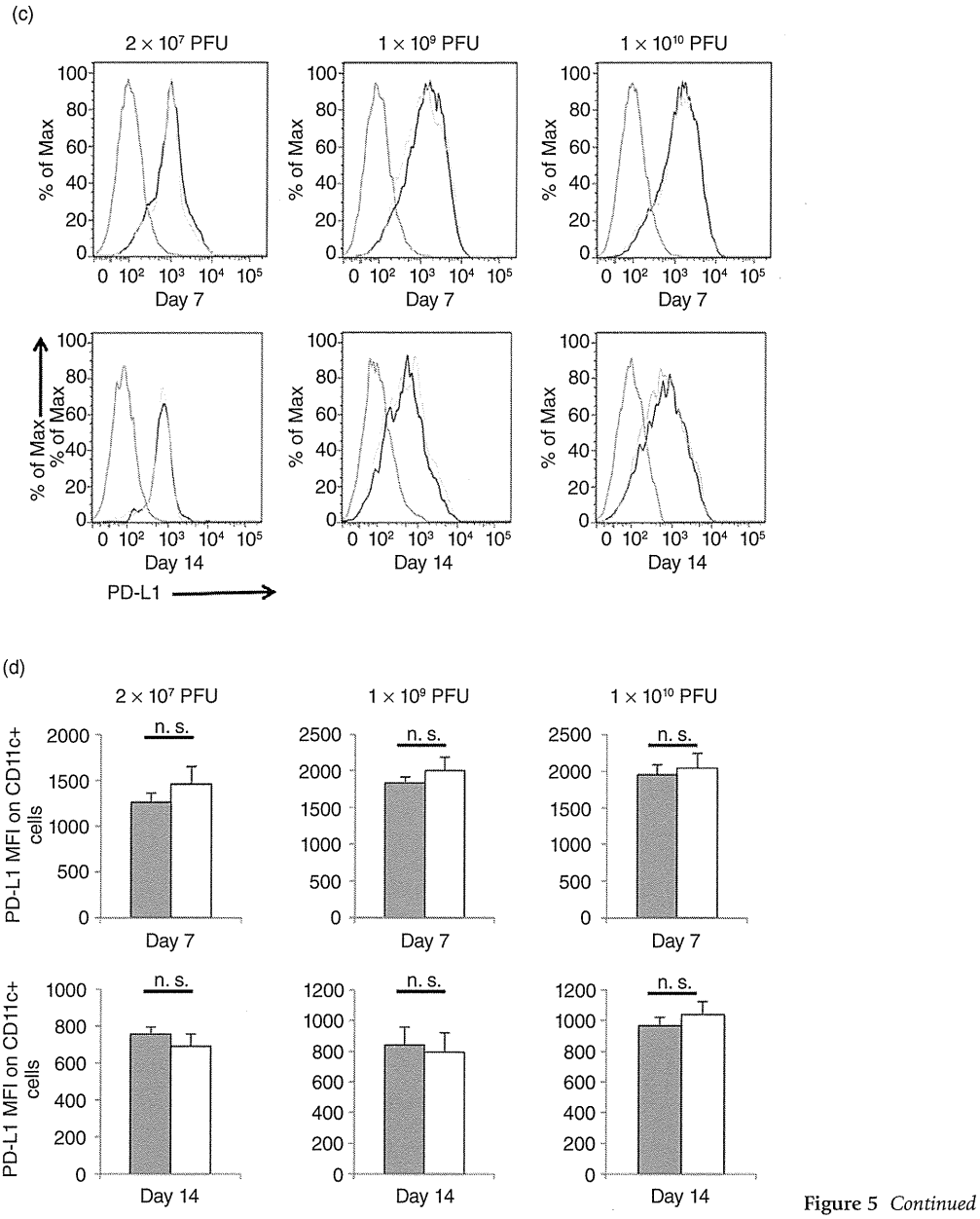

Figure 5 *Continued*

extensive investigations of inhibitory receptors in the regulation of T cells in human chronic viral infections.[25,26]

Chronic HCV infection in humans is characterized by CD8 T-cell exhaustion and dysfunction.[27] As in chronic LCMV infection, the expression of PD-1 is similarly upregulated on the virus-specific CD8 T cells in chronic HCV infection, and HCV-specific PD-1^{high} T cells are functionally impaired.[28–30] Also, Tim-3 is overexpressed on HCV-specific dysfunctional CD8 T cells.[25] In addition, a blockade of PD-1/PD-L1 or Tim-3/galectin9 (Gal9) interaction restores T-cell functions such as proliferation, cytolytic activity and cytokine (IFN-γ and tumor necrosis factor-α) production.[25,28–30] As was

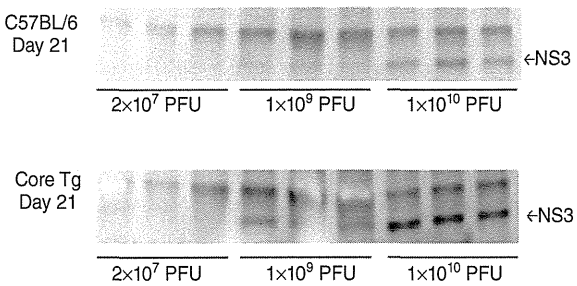

Figure 6 Persisting hepatitis C virus (HCV)-NS3 antigen detection was performed on the liver sections isolated 21 days post-infection. Liver sections were analyzed by IP-western blot assay using anti-FLAG antibody.

mentioned above, it has been reported that increased expression of inhibitory receptors is associated with the impaired HCV-specific CD8 T cells observed in chronic HCV patients. However, the underlying mechanisms for HCV-mediated impaired CD8 T-cell responses have yet to be determined. Based on our finding that lower level of activation and higher levels of expression of regulatory molecules, Tim-3 and PD-1, by intrahepatic CD8 T cells and higher levels of expression of PD-L1 by intrahepatic APC were observed in core (+) mice in comparison with core (−) mice, it is possible that HCV core-induced T-cell dysfunction is one of the viral factors that contributes to impaired CD8 T-cell responses as seen in chronic HCV patients. Our speculation is in accordance with the study by Lukens *et al.*[31]

Suppression of CTL responses via highly expressed Ag was found in chronic HCV infection. Inverse relationships between HCV viral titer and HCV-specific T cells have been reported.[7,32,33] In this study, we found higher levels of expressions of PD-L1 by intrahepatic APC and an impaired intrahepatic CD8 T-cell response in high infectious dose setting. Moreover, we found a significant inverse correlation between the percentages of IFN-γ-producing cells and expression of regulatory molecules in Ag-specific intrahepatic CD8 T cells. It is likely that the PD-1/PD-L1 or Tim-3/Gal9 pathway play a major inhibitory role in our model. High-dose Ad-HCV NS3 infection may inhibit the NS3-specific CD8 T-cell responses not at the induction phase but at the effector phase because Ag-specific-MHC tetramer$^+$ T cells were observed, and most Ag-specific MHC tetramer$^+$ T cells was anergic to PMA/ionophore stimulation and these T cells expressed PD-1 and Tim-3. The role of PD-1/PD-L1 as mechanism for liver tolerance has been well established. PD-1 expression by T cells has been shown to inhibit intrahepatic antiviral immune responses at the effector phase.[34–36]

Hepatitis C virus infection affects approximately 170 million people in the world and is a major global health problem because infected individuals can develop liver cirrhosis and hepatocellular carcinoma. Pegylated interferon and ribavirin therapy, although beneficial in approximately half of treated patients, are expensive and associated with significant side-effects.[37] In this clinical context, there is an urgent need for the development of a therapeutic and/or prophylactic HCV vaccine.[38] Because HCV infects only humans and chimpanzees, it is difficult to evaluate effective therapeutic vaccine candidates. Recently, as a small animal model for HCV infection study, chimeric humanized mouse harboring a human hepatocyte and hematolymphoid system was established by xenotransplantation technique.[39,40] The xenograft model provides a unique opportunity for HCV vaccine development. However, the generation of this chimeric humanized mouse requires advanced technical skills and the scarcity of adequate human primary material remains a significant logistical challenge.[41,42] Our model showed in the present study is easy to create, and it has Ag-specific T-cell exhaustion and Ag persistent in the liver seen in chronic HCV patients. These features suggest that this system is useful for therapeutic HCV vaccine development.

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REFERENCES

1 Shepard CW, Finelli L, Alter MJ. Global epidemiology of hepatitis C virus infection. *Lancet Infect Dis* 2005; **5**: 558–67.
2 Kamal SM. Acute hepatitis C: a systematic review. *Am J Gastroenterol* 2008; **103**: 1283–97.
3 Alter HJ, Seeff LB. Recovery, persistence, and sequelae in hepatitis C virus infection: a perspective on long-term outcome. *Semin Liver Dis* 2000; **20**: 17–35.
4 Grüner NH, Gerlach TJ, Jung MC *et al.* Association of hepatitis C virus-specific CD8+ T cells with viral clearance in acute hepatitis C. *J Infect Dis* 2000; **181**: 1528–36.

5 Chang KM, Rehermann B, McHutchison JG *et al.* Immunological significance of cytotoxic T lymphocyte epitope variants in subjects chronically infected by the hepatitis C virus. *J Clin Invest* 1997; **100:** 2376–85.
6 Lechmann M, Woitas RP, Langhans B *et al.* Decreased frequency of HCV core-specific peripheral blood mononuclear cells with type 1 cytokine secretion in chronic hepatitis. *Can J Hepatol* 1999; **31:** 971–8.
7 Rehermann B, Chang KM, McHutchison JG *et al.* Differential cytotoxic T-lymphocyte responsiveness to the hepatitis B and C viruses in chronically infected patients. *J Virol* 1996; **70:** 7092–102.
8 Wedemeyer H, He XS, Nascimbeni M *et al.* Impaired effector function of hepatitis C virus specific CD8+ T cells in chronic hepatitis C virus infection. *J Immunol* 2002; **169:** 3447–58.
9 Zinkernagel RM, Hengartner H. Regulation of the immune response by antigen. *Science* 2001; **293:** 251–3.
10 Moskophidis D, Lechner F, Pircher H, Zinkernagel RM. Virus persistence in acutely infected immunocompetent mice by exhaustion of antiviral cytotoxic effector T cells. *Nature* 1993; **362:** 758–61.
11 Wherry EJ, Blattman JN, Murali-Krishna K, van der Most R, Ahmed R. Viral persistence alters CD8 T-cell immunodominance and tissue distribution and results in distinct stages of functional impairment. *J Virol* 2003; **77:** 4911–27.
12 Zajac AJ, Blattman JN, Murali-Krishna K *et al.* Viral immune evasion due to persistence of activated T cells without effector function. *J Exp Med* 1998; **188:** 2205–13.
13 Wherry EJ, McElhaugh MJ, Eisenlohr LC. Generation of CD8 T cell memory in response to low, high, and excessive levels of epitope. *J Immunol* 2002; **168:** 4455–61.
14 Eisen-Vandervelde AL, Waggoner SN, Yao ZQ, Cale EM, Hahn CS, Hahn YS. Hepatitis C virus core selectively suppresses interleukin-12 synthesis in human macrophages by interfering with AP-1 activation. *J Biol Chem* 2004; **279:** 43479–86.
15 Watanabe T, Bertoletti A, Tanoto TA. PD1/PD-L1 pathway and T-cell exhaustion in chronic hepatitis virus infection. *J Viral Hepat* 2010; **17:** 453–8.
16 Yao ZQ, Eisen-Vanderveld A, Waggoner SN, Cale EM, Hahn YS. Direct binding of hepatitis C virus core to gC1qR on CD4+ and CD8+ T cells leads to impaired activation of Lck and Akt. *J Virol* 2004; **78:** 6409–19.
17 Yao ZQ, Nguyen DT, Hiotellis AI, Hahn YS. Hepatitis C virus core protein inhibits human T lymphocyte responses by a complement-dependent regulatory pathway. *J Immunol* 2001; **167:** 5264–72.
18 Cavanaugh VL, Guidotti LG, Chisari FV. Inhibition of hepatitis B virus replication during adenovirus and cytomegalovirus infections in transgenic mice. *J Virol* 1998; **72:** 2630–7.
19 von Freyend MJ, Untergasser A, Arzberger S *et al.* Sequential control of hepatitis B virus in a mouse model of acute, self-resolving hepatitis B. *J Viral Hepat* 2011; **18:** 216–26.
20 Moriya K, Yotsuyanagi H, Shintani Y *et al.* Hepatitis C virus core protein induces hepatic steatosis in transgenic mice. *J Gen Virol* 1997; **78:** 1527–31.
21 Koike K, Moriya K, Ishibashi K *et al.* Sialadenitis histologically resembling Sjogren syndrome in mice transgenic for hepatitis C virus envelope genes. *Proc Natl Acad Sci U S A* 1997; **94:** 233–6.
22 Kolykhalov AA, Agapov EV, Blight KJ, Mihalik K, Feinstone SM, Rice CM. Transmission of hepatitis C by intrahepatic inoculation with transcribed RNA. *Science* 1997; **277:** 570–4.
23 Frelin L, Alheim M, Chen A *et al.* Low dose and gene gun immunization with a hepatitis C virus nonstructural (NS) 3 DNA-based vaccine containing NS4A inhibit NS3/4A-expressing tumors in vivo. *Gene Ther* 2003; **10:** 686–99.
24 Barber DL, Wherry EJ, Masopust D *et al.* Restoring function in exhausted CD8 T cells during chronic viral infection. *Nature* 2006; **439:** 682–7.
25 Golden-Mason L, Palmer BE, Kassam N *et al.* Negative immune regulator Tim-3 is overexpressed on T cells in hepatitis C virus infection and its blockade rescues dysfunctional CD4+ and CD8+ T cells. *J Virol* 2009; **83:** 9122–30.
26 Sharpe AH, Wherry EJ, Ahmed R, Freeman GJ. The function of programmed cell death 1 and its ligands in regulating autoimmunity and infection. *Nat Immunol* 2007; **8:** 239–45.
27 Spangenberg HC, Viazov S, Kersting N *et al.* Intrahepatic CD8+ T-cell failure during chronic hepatitis C virus infection. *Hepatology* 2005; **42:** 828–37.
28 Golden-Manson L, Palmer B, Klarquist J, Mengshol JA, Castelblanco N, Rosen HR. Upreglation of PD-1 expression on circulating and intrahepatic hepatitis C virus-specific CD8+ T cells associated with reversible immune dysfunction. *J Virol* 2007; **81:** 9249–58.
29 Penna A, Pilli M, Zerbini A *et al.* Dysfunction and functional restoration of HCV-specific CD8 responses in chronic hepatitis C virus infection. *Hepatology* 2007; **45:** 588–601.
30 Radziewicz H, Ibegbu CC, Fernandez ML *et al.* Liver-infiltrating lymphocytes in chronic human hepatitis C virus infection display an exhausted phenotype with high levels of PD-1 and low levels of CD127 expression. *J Virol* 2007; **81:** 2545–53.
31 Lukens JR, Cruise MW, Lassen MG, Hahn YS. Blockade of PD-1/B7-H1 interaction restores effector CD8+ T cell responses in a hepatitis C virus core murine model. *J Immunol* 2008; **180:** 4875–84.
32 Sreekumar R, Gonzalez-Koch A, Maor-Kendler Y *et al.* Early identification of recipient with progressive histologic recurrence of hepatitis C after liver transplantation. *Hepatology* 2000; **32:** 1125–30.

33 Sugimoto K, Ikeda F, Standanlick J, Frederick A, Alter HJ, Chang KM. Suppression of HCV-specific T cells without differential hierarchy demonstrated ex vivo in persistent HCV infection. *Hepatology* 2003; **38:** 1437–48.
34 Isogawa M, Furuichi Y, Chisaki FV. Oscillating CD8+ T cell effector functions after antigen recognition in the liver. *Immunity* 2005; **23:** 53–63.
35 Iwai Y, Terawaki S, Ikegawa M, Okazaki T, Honjo T. PD-1 inhibits antiviral immunity at the effector phase in the liver. *J Exp Med* 2003; **198:** 39–50.
36 Maier H, Isogawa M, Freeman GJ, Chisari FV. PD-1:PD-L1 interactions contribute to the functional suppression of virus-specific CD8+ T lymphocytes in the liver. *J Immunol* 2007; **178:** 2714–20.
37 Pawlotsky JM. Therapy of hepatitis C: from empiricism to eradication. *Hepatology* 2006; **43:** S207–20.
38 Callendret B, Walker C. A siege of hepatitis: immune boost for viral hepatitis. *Nature Med* 2011; **17:** 252–3.
39 Legrand N, Ploss A, Balling R *et al.* Humanized mice for modeling human infectious disease: challenges, progress, and outlook. *Cell Host Microb* 2009; **6:** 5–9.
40 Robinet E, Baumert TF. A first step towards a mouse model for hepatitis C virus infection containing a human immune system. *J Hepatol* 2011; **55:** 718–20.
41 Kimura K, Kohara M. An experimental mouse model for hepatitis C virus. *Exp Anim* 2011; **60:** 93–100.
42 Ploss A, Rice CM. Towards a small animal model for hepatitis C. *EMBO Rep* 2009; **10:** 1220–7.

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

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

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

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

*B.U. and K.E. contributed equally to this work

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

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

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

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

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

Material and Methods

Materials

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

Cells and cell culture

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

Transgenic mice

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

Quantitative real-time PCR

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

ASB9 transfection

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

Immunoblot analysis

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

uMtCK siRNA transfection

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

Cell membrane integrity and proliferation assays

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

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

Cell migration and invasion assays

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

Patients and measurement of MtCK activity

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

This study was performed in accordance with the ethical guidelines of the 1975 Declaration of Helsinki and was approved by the Institutional Research Ethics Committees of the authors' institutions. A written informed consent was obtained for the use of the samples in this study.

Statistical analysis

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

Results

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

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

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

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

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

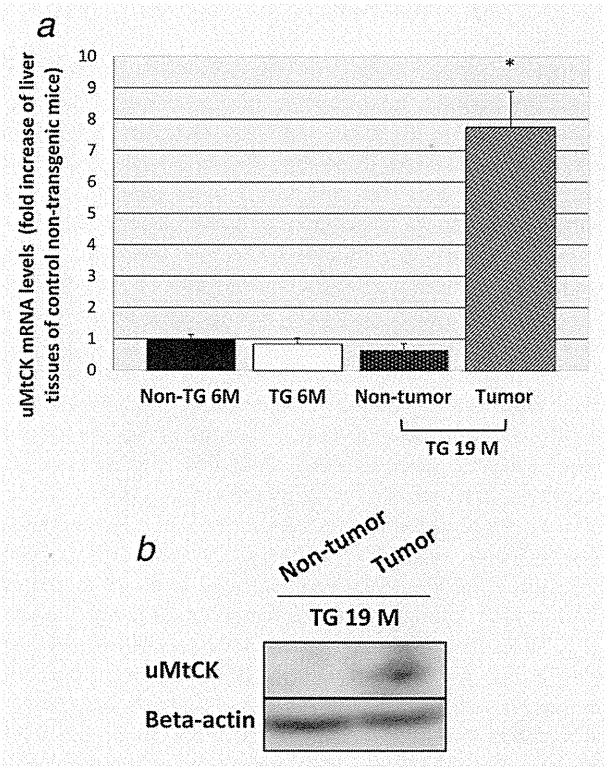

Figure 1. uMtCK mRNA and protein levels in liver tissues of the control non-transgenic, HCV core gene transgenic mice. (*a*) uMtCK mRNA levels were examined by real-time PCR in liver tissues of the control non-transgenic mice (Non-TG) at 6 months of age ($n = 4$), and HCV core gene transgenic mice (TG)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

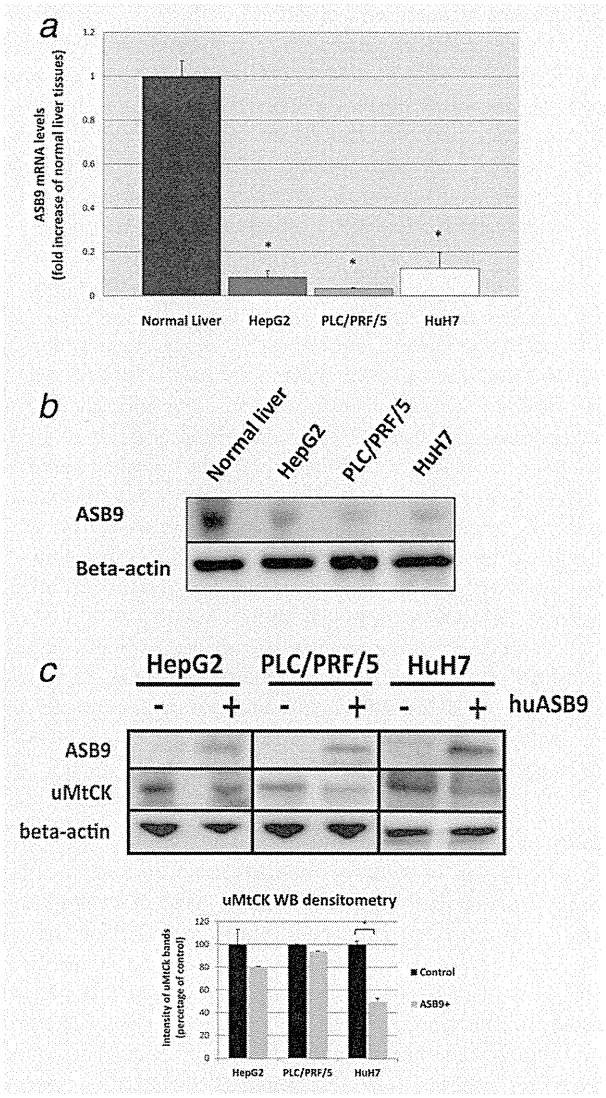

Figure 2. ASB9 expression and the effect of ASB9 transfection on uMtCK protein levels in HCC cells. ASB9 mRNA (*a*) and protein (*b*) levels in HepG2, PLC/PRF/5 and HuH7 cells were examined by real-time PCR and immunoblot analysis, respectively. As a positive control for ASB9 mRNA and protein expressions,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

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

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

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

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

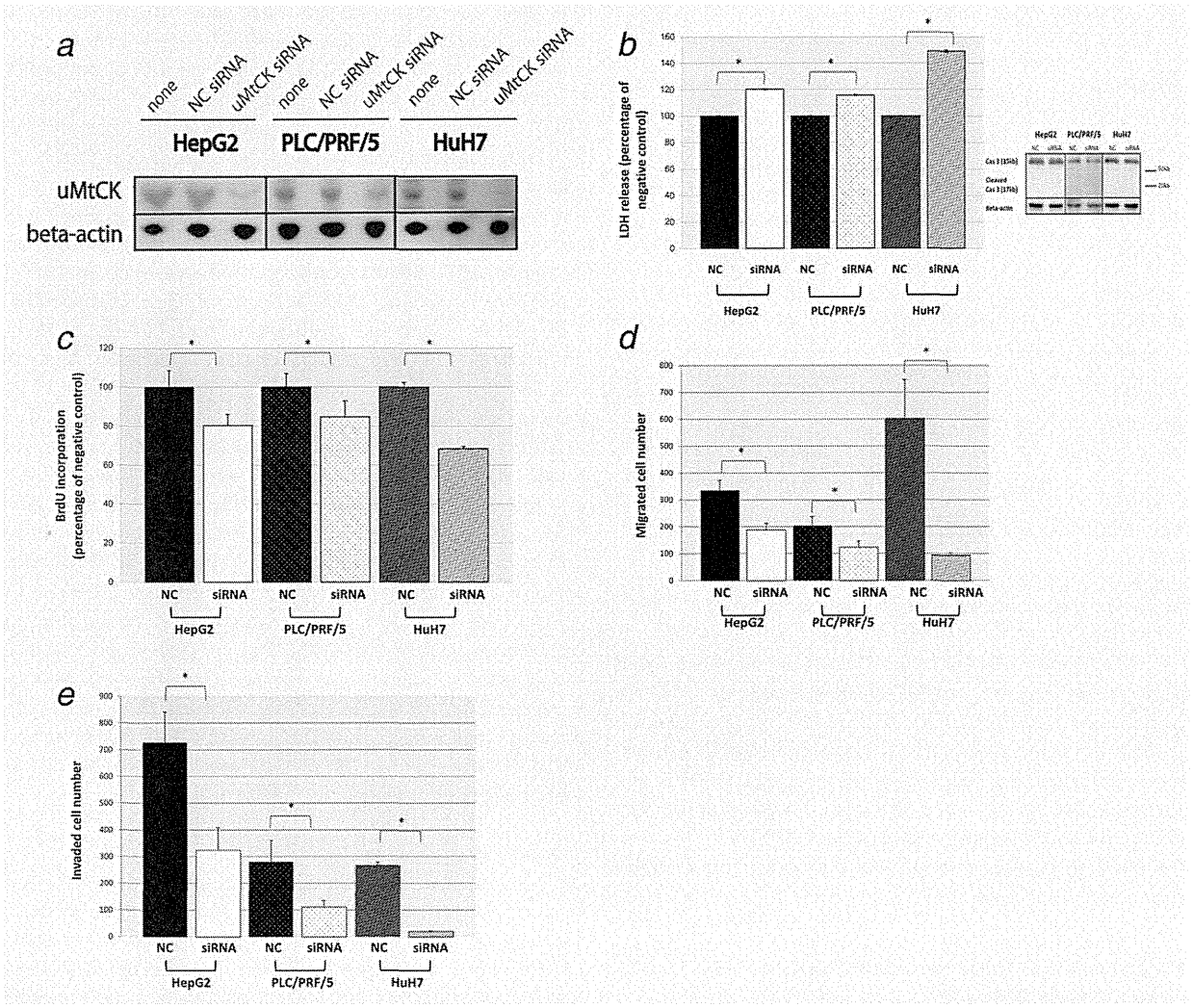

Figure 3. Increase in cell death and reduction in proliferation, migration and invasion by reduced uMtCK expression with siRNA in HCC cell lines. Human HCC cell lines, HepG2, PLC/PRF/5 and HuH7 cells, were transfected with 20 nM uMtCK siRNA or universal negative control, and uMtCK levels were examined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

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

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

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

Table 1. Baseline characteristics

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

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

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

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

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

Discussion

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

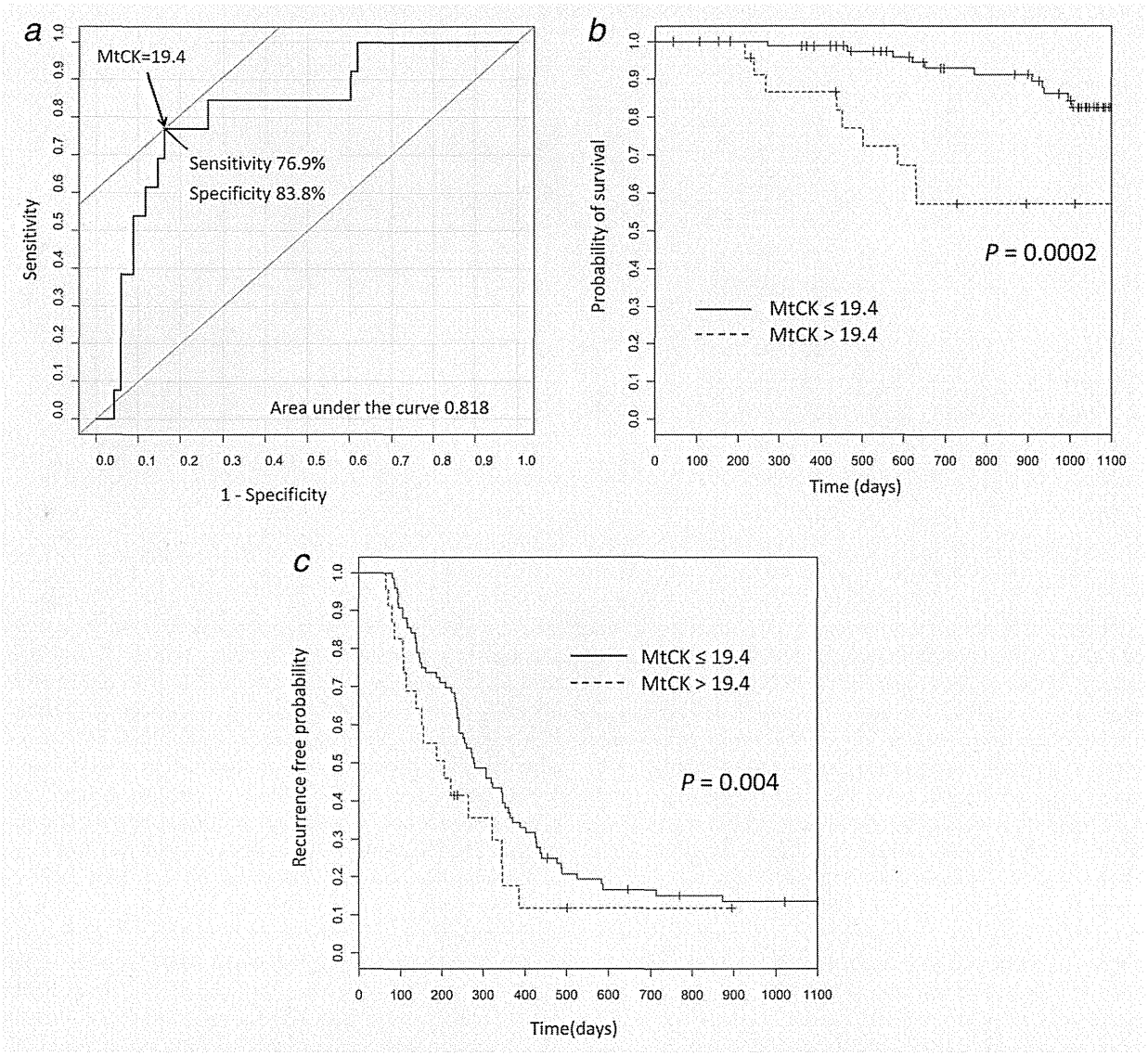

Figure 4. (*a*) ROC curve showing the overall accuracy of serum MtCK activity for discriminating between survivors and deceased patients. The arrow identifies the best cutoff value (*i.e.*, 19.4 U/L) of serum MtCK activity. Kaplan–Meier survival (*b*) and recurrence (*c*) curve of the studied patients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

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

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

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

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

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

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

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

References

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

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

Results of the Tokyo Trial of Prevention of Post-ERCP Pancreatitis with Risperidone-2: a multicenter, randomized, placebo-controlled, double-blind clinical trial (CME)

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Background: Our previous study suggested that a combination of ulinastatin and risperidone reduced post-ERCP pancreatitis (PEP) compared with ulinastatin alone.

Objective: The aim of this study was to evaluate the efficacy of risperidone alone for prevention of PEP.

Design: A multicenter, randomized, placebo-controlled, double-blind clinical trial.

Setting: Two academic hospitals and 5 referral hospitals in Tokyo and Saitama, Japan.

Patients: Patients undergoing therapeutic or interventional-diagnostic ERCP.

Intervention: The patients were randomized to receive 2 mg of oral risperidone or oral placebo at 0.5 to 2 hours before ERCP.

Main Outcome Measurements: The primary endpoint was the incidence of PEP. Secondary endpoints were the incidence of hyperenzymemia and enzyme levels (amylase, pancreatic amylase, lipase). Risk factors for PEP were evaluated.

Results: We initially enrolled 500 patients in the study (250 in the risperidone group and 250 in the placebo group), but 17 (11 in the risperidone and 6 in the placebo group) were excluded after randomization. PEP developed in 24 patients (10.0%) in the risperidone group and 21 patients (8.6%) in the placebo group ($P = .587$). Serum amylase levels at 3 hours after ERCP were lower in the risperidone group ($P = .007$ in a single test of hypothesis, significance removed by Bonferroni correction for multiple testing). In multivariate analysis, a small papilla of Vater, total procedure time ≥ 40 minutes, and stenosis of the intrahepatic duct were significantly associated with PEP.

Limitations: Multiplicity of study centers and a relatively wide time range of drug administration time.

Conclusion: Risperidone did not show a benefit in prevention of PEP in this trial. (Clinical trial registration number: NCT000004592.) (Gastrointest Endosc 2013;78:842-50.)

Abbreviations: 5-HT, 5-hydroxytryptamine; p-amylase, pancreatic amylase; PEP, post-ERCP pancreatitis.

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Acute pancreatitis is a common and important complication of ERCP. The incidence of post-ERCP pancreatitis (PEP) is about 10%.[1-6] Various pharmacologic agents have been tested for prevention of PEP, but there is currently no effective preventive treatment other than nonsteroidal anti-inflammatory drugs. In the 2010 Guidelines of the European Society of Gastrointestinal Endoscopy, routine rectal administration of 100 mg of diclofenac or indomethacin is recommended.[7] Elmunzer et al[8] showed that rectal administration of indomethacin reduced the incidence of PEP, but the majority of patients (82%) in the study had suspected sphincter of Oddi dysfunction, and it is uncertain whether indomethacin is effective in other patient populations.

In a previous multicenter, randomized, controlled trial we found that infusion of ulinastatin (150,000 U) before ERCP significantly reduced the incidence of PEP.[1] Ulinastatin is an intrinsic trypsin inhibitor, which inhibits various enzymes such as alpha-chymotrypsin, lipase, amylase, elastase, and carboxylase. However, the incidence did not differ significantly in subgroup analysis of a high-risk group. Yoo et al[9] also have shown that ulinastatin does not decrease the incidence of PEP. In addition, ulinastatin is not approved for use in the United States. Therefore, there is a need for a drug for prevention of PEP in high-risk patients that is effective, safe, and available globally.

Serotonin (5-hydroxytryptamine [5-HT]) activates pancreatic enzyme secretion, and 5-HT is associated with development and aggravation of acute pancreatitis.[10] Risperidone is a 5-HT_{2A} antagonist that ameliorates cerulein-induced edematous pancreatitis in mice and attenuates serum interleukin-6 levels, amylase and lipase levels, platelet counts, histologic alterations, and mortality in a diet-induced mouse model of necrotic pancreatitis.[10,11] In our multicenter, randomized, phase II, non-placebo–controlled Tokyo P3R (Tokyo Trial of Prevention of Post-ERCP Pancreatitis with Risperidone) study[12] comparing risperidone plus ulinastatin combination therapy with ulinastatin alone, the incidence of PEP did not differ significantly between the two groups. However, post-ERCP levels of amylase, pancreatic amylase (p-amylase), and lipase were significantly lower in the risperidone plus ulinastatin group.

These results suggest that risperidone might have an effect on prevention of PEP. No adverse events related to risperidone occurred in the Tokyo P3R study,[12] and risperidone is used globally to treat schizophrenia; thus, the safety of the drug has been established. With this background, the aim of the current study was to evaluate the efficacy of risperidone alone for prevention of PEP.

Take-home Message

- Risperidone did not show a benefit in the prevention of post-ERCP pancreatitis compared with placebo.
- Risperidone was associated with observed reduced serum pancreatic amylase levels 3 hours after ERCP, but this change lacked statistical significance.

METHODS

Study design

A multicenter, prospective, randomized, placebo-controlled, double-blind clinical trial was conducted in 2 academic hospitals and 5 referral hospitals in Tokyo and Saitama, Japan. The protocol was approved by the institutional review board at each institution. This study was registered with the University Hospital Medical Information Network (UMIN) Clinical Trials Registry (No. 000004592).

Patients

The participants were patients scheduled to undergo a first therapeutic ERCP and/or invasive diagnostic ERCP (brushing or biopsy of the bile or pancreatic duct, intraductal US, or pancreatic juice aspiration). Patients were excluded if they met any of the following criteria: (1) age <20 or >79 years; (2) active acute pancreatitis; (3) disseminated intravascular coagulation, sepsis, or acute circulatory failure; (4) severe underlying disease of the heart, lung, liver, kidney, endocrine system, or GI tract; (5) pregnant or breast-feeding women; (6) treatment with risperidone; (7) psychosomatic disorder, depression, Parkinson's disease, or Parkinsonism; (8) known sensitivity to risperidone; (9) treatment in the preceding 2 weeks with a 5-HT_2 receptor inhibitor, epinephrine, protease-inhibitor, diclofenac, octreotide, or somatostatin; and (10) severe chronic pancreatitis. All patients provided written informed consent before the start of the study.

Study protocol

Eligible patients were randomly assigned to receive 2 mg of risperidone or placebo orally 0.5 to 2 hours before ERCP. The placebo was made in the pharmaceutical department in the Tokyo University Hospital by Y.Y. and K.N. Randomization was performed by a minimization technique (Zelen's method) by using a computer-generated list, with stratification according to study center and main indication for ERCP (bile duct or pancreatic duct). Patients also were stratified by age (<70 vs ≥ 70 years), sex, and body mass index (<25 vs ≥ 25 kg/m^2); age and female sex have been considered as risk factors for PEP,[13-15] whereas obesity may be associated with the severity of PEP. ERCP was performed in the standard manner. Before the procedure, patients were sedated with diazepam and pethidine hydrochloride or meperidine. Hyoscine N-butyl bromide or glucagon was administered to obtain temporary paralysis of the stomach and duodenum. After ERCP, each endoscopist recorded the ERCP findings and interventions in a Web-based database. Serum amylase, p-amylase, lipase, C-reactive protein, and

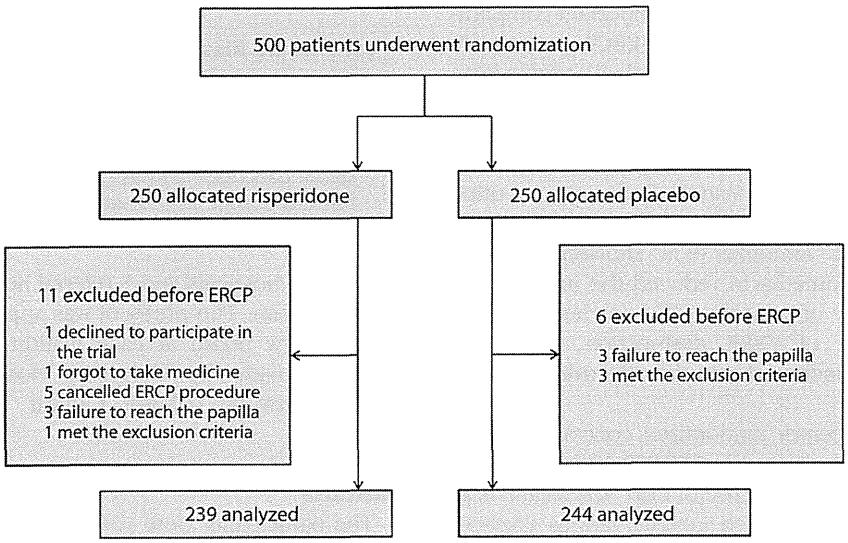

Figure 1. Flow diagram of the study.

blood counts were measured before and 3 hours and 18 hours after ERCP. All patients were hospitalized at least 1 night for the evaluation of abdominal symptoms at 3 hours and 18 hours after ERCP, which is routine clinical practice in Japan. If patients were discharged within 48 hours after ERCP, they were contacted by telephone to assess their symptoms. Treatment group allocation was blinded to all investigators and patients until the study was completed.

Definitions

Hyperenzymemia was defined as amylase, pancreatic amylase, or lipase levels >3 times the upper limit of normal at 18 hours after ERCP. The diagnosis of PEP was based on consensus criteria: new onset of abdominal pain lasting for at least 24 hours after ERCP with serum amylase levels at 3 hours and/or 18 hours after ERCP >3 times the upper limit of normal.[2] PEP was graded according to the duration of treatment for pancreatitis. Mild PEP required treatment for ≤3 days, moderate PEP required fasting and treatment for 4 to 10 days, and severe PEP required treatment for >10 days, intensive care, or surgical intervention.[2] Difficult cannulation was defined as a time of >10 minutes required to cannulate into the desired duct. A small papilla of Vater was defined as <3 mm and/or the absence of a caudal fold.

Study outcome

The primary endpoint was the incidence of PEP. The secondary endpoints were the incidence of hyperenzymemia and pancreatic enzyme levels (amylase, pancreatic amylase, lipase). Risk factors for PEP also were analyzed.

Statistical analysis

In our previous randomized, controlled trial, the incidence of pancreatitis after therapeutic ERCP was 12.4% in the placebo group.[1] Therefore, the incidences of PEP were projected to be 10% in the placebo group and 3% in the risperidone group. With these hypothetical rates, calculations indicated that 420 patients (210 per group) were needed to give 80% power with testing by the χ^2 test ($\alpha = 0.05$). Thus, to allow for some unexpected attrition, the required sample size was determined to be 500 patients (250 per group). All continuous variables are reported as the mean with the 95% confidence interval. Categorical variables were compared by the χ^2 test or the Fisher exact test and continuous variables by the t test or Welch t test, as appropriate. To identify risk factors for PEP, univariate logistic regression analysis was first performed. Factors with $P < .05$ in univariate analysis were then included in multivariate logistic regression analysis. The analysis included multiple testing of outcome data from individual patients. The results from the multivariate analysis to determine factors associated with post-ERCP pancreatitis are taken as the primary definitive findings. Preliminary univariate tests were conducted to indicate possible variables to be included in the multivariate analysis. There is no correction of the P values from these statistical tests because these were exploratory examinations of data that were intended to highlight differences. The results of other hypothesis tests relating to secondary outcomes are used for descriptive purposes only. Significant results were subjected to Bonferroni correction, based on the total number of primary and secondary hypothesis tests. For these secondary hypothesis tests, the uncorrected P values are presented

TABLE 1. Baseline characteristics of patients

Characteristic	Risperidone (n = 239)	Placebo (n = 244)	*P* value
Female sex, no. (%)	93 (38.9)	95 (38.9)	.996
Age, mean (range), y	65 (23-79)	65 (28-79)	.492
Body mass index, mean (range), kg/m^2	22.8 (12-29)	22.7 (12.6-38.8)	.628
Main indication for ERCP			
Bile duct, no. (%)	211 (88.3)	216 (88.5)	.934
Pancreatic duct, no. (%)	28 (11.5)	28 (11.7)	.934
Indication for ERCP			
Common bile duct stone, no. (%)	97 (40.6)	107 (43.9)	.467
Gallbladder stone, no. (%)	70 (29.3)	71 (29.1)	.963
Bile duct cancer, no. (%)	17 (7.1)	16 (6.6)	.809
Pancreatic cancer, no. (%)	33 (13.8)	42 (17.2)	.302
IPMN, no. (%)	10 (4.2)	13 (5.3)	.555
Cholangitis, no. (%)	30 (12.6)	34 (13.9)	.166
Cholecystitis, no. (%)	11(4.6)	9 (3.7)	.614
Previous pancreatitis, no. (%)	11 (4.6)	11 (4.5)	.960
Other, no. (%)*	30 (12.6)	19 (7.8)	.082
ASA Physical Status Classification System, class 1, no. (%)	97 (40.6)	99 (40.6)	.998

IPMN, Intraductal papillary mucinous neoplasm; *ASA,* American Society of Anesthesiologists.
*Other include gallbladder cancer, hilar cholangiocarcinoma, cancer of the ampulla of Vater, primary sclerosing cholangitis, autoimmune pancreatitis, and sphincter of Oddi dysfunction.

TABLE 2. Incidences of pancreatitis, hyperenzymemia, and abdominal pain

	Risperidone (n = 239)	Placebo (n = 244)	*P* value
Pancreatitis, no. (%)	24 (10.0)	21 (8.6)	.587
Severity of pancreatitis, no.			.789
Mild	15	13	
Moderate	9	8	
Severe	0	0	
Hyperpancreatic amylasemia, no. (%)	76 (31.8)	83 (34.0)	.604
Hyperlipasemia	90 (37.7)	88 (36.1)	.742
Abdominal pain	51 (21.3)	48 (19.7)	.650

along with the effect of Bonferroni corrections for multiple testing when the correction removed nominal statistical significance found in a single statistical test. Statistical significance was indicated by a P value $< .05$. All analyses were performed by using JMP version 9 (SAS Institute Inc, Cary, NC).

RESULTS

Patients

Between March 2011 and October 2012, 500 patients were enrolled in the study and allocated equally to the risperidone and placebo groups, respectively (Fig. 1). After randomization, 17 patients were excluded from the final analysis (11 in the risperidone group, 6 in the placebo group) because of declining to participate in the trial (1 in the risperidone group), not taking the drug (1 in the risperidone group), cancellation of ERCP (5 in the risperidone group), failure to reach the papilla (3 in the risperidone group, 3 in the placebo group), and meeting the exclusion criteria after randomization (1 in the risperidone group, 3 in the placebo group). There were no significant differences in baseline characteristics and ERCP procedures and findings between the 2 groups (Table 1). A pancreatic stent was placed in 16 patients in the risperidone group and 18 in the placebo group.

TABLE 3. Serum levels of pancreatic enzymes before and after ERCP*

		Risperidone (n = 239)	Placebo (n = 244)	*P* value
Amylase (IU/L), mean (CI)	Before	84 (70-97)	93 (79-106)	.350
	3h	187 (151-223)	257 (221-293)	.007
	18h	323 (264-382)	324 (265-382)	.989
Pancreatic amylase (IU/L), mean (CI)	Before	50 (38-63)	57 (44-70)	.481
	3h	126 (91-161)	186 (152-221)	.016
	18h	269 (213-325)	256 (200-313)	.754
Lipase (IU/L), mean (CI)	Before	218 (48-118)	93 (58-127)	.698
	3h	346 (215-477)	528 (399-658)	.052
	18h	433 (323-543)	434 (325-542)	.993

CI, Confidence interval.
*Because of Bonferroni correction for multiple testing of data from individual patients for these secondary outcomes, each of the instances of nominal $P < .05$ significance in a single statistical test in the table has this significance removed by the correction.

TABLE 4. Other adverse events

	Risperidone (n = 239)	Placebo (n = 244)
Bleeding after endoscopic sphincterotomy	2	2
Cholangitis	3	3
Cholecystitis	1	1
Hemobilia	2	0
Aspiration pneumonia	0	2
Obstructive jaundice (edema of papilla of Vater)	1	0
Retroperitoneal perforation	1	1
Other	3	0

PEP

PEP occurred in 45 of 483 patients (9.3%), including 24 in the risperidone group and 21 in the placebo group (10.0% vs 8.5%; $P = .587$) (Table 2). No patients developed severe PEP, and the severity of PEP did not differ significantly between the two groups. The incidence of PEP also did not differ between the two groups in intention to treat analysis (9.6% vs 8.5%; $P = .659$).

Hyperenzymemia, pancreatic enzyme levels, and abdominal pain

The levels of amylase, pancreatic amylase, and lipase before ERCP were not significantly different in the risperidone and placebo groups. The incidence of hyperenzymemia also did not differ significantly between the two groups (Table 2). However, serum amylase and p-amylase levels at 3 hours after ERCP were observed to be lower in the risperidone group compared with the placebo group (nominally significantly lower in a single test of hypothesis; however, this significance was removed by Bonferroni correction for multiple testing) (Table 3). Similar results were obtained in the intention-to-treat analysis. No significant difference in abdominal pain was observed between the two groups.

Serum C-reactive protein levels and white blood cell counts

Serum C-reactive protein levels and white blood cell counts before ERCP were not significantly different in the risperidone and placebo groups. Serum C-reactive protein levels and white blood cell counts at 18 hours after ERCP also were not significantly different in the risperidone and placebo groups.

Other complications and adverse events

Complications developed in 22 patients, including 13 in the risperidone group and 9 in the placebo group (5.4% vs 3.7%; $P = .356$) (Table 4). No patients had adverse events related to risperidone, including no cases with malignant syndrome or dyskinesia.

Risk factors for PEP

In univariate analysis, a small papilla of Vater, difficult cannulation, acinarization, stenosis of the intrahepatic bile duct, and a total procedure time of ≥ 40 minutes were identified as risk factors (Table 5). In multivariate analysis, a small papilla of Vater, total procedure time ≥ 40 minutes, and stenosis of the intrahepatic duct remained significant