

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

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

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

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

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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.<sup>1</sup> Furthermore, the worldwide incidence of HCC has increased over several decades, and HCC has recently received considerable attention as a common cause of mortality.<sup>2</sup> 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.<sup>3,4</sup> 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.<sup>5</sup> 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.<sup>4,6</sup> Recurrence occurs almost in 70% of patients with HCC of the first occurrence within 5 years.<sup>7</sup> 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.<sup>8</sup> 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.<sup>9,10</sup> However, its effect is rather limited, just extending median survival from 7.9 months to 10.7 months in patients with advanced HCC.<sup>10</sup> 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.<sup>11</sup> Although other potential markers such as desgamma-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.<sup>11-14</sup> 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 cholangiocarcinoma.<sup>15</sup>

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.<sup>16</sup> 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.<sup>16</sup> Then, we have further observed the higher expression of uMtCK mRNA in HCC cell lines than in normal human liver tissues.<sup>16</sup> 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.<sup>17-19</sup> In contrast, uMtCK was down-regulated in oral squamous cell carcinoma,<sup>20</sup> and sarcomeric MtCK was

also down-regulated during sarcoma development in leg muscle in mice.<sup>21</sup> 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.<sup>22,23</sup> 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.<sup>24</sup> One of ASB family protein, ASB9, was found to interact with brain type of creatine kinase, leading to its degradation.<sup>25</sup> Recently, uMtCK was found to be another ASB9 target.<sup>26</sup> 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.<sup>16</sup> 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.<sup>22</sup> 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<sup>TM</sup> 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'-CCTGGCATCAGGCTCTTTC-3' and 5'-ACCCCTGGCTGATGAGGTTTC-3'<sup>27</sup>; human beta-actin: 5'-GGGTCAGAAGGATTCTATG-3' and 5'-CCTTAATGTCACGCACGATTT-3'.<sup>26</sup> 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<sup>-ΔΔCt</sup> 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'-GAGCGGCCGCTAAGATGTAGGAGAACTGTTT-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<sup>TM</sup> Protease Inhibitor Cocktail (Thermo Fisher

Scientific). Immunoblot analysis was performed as previously described,<sup>28</sup> 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 venter instructions (Integrated DNA Technologies, IA). The human uMtCK-specific RNA duplex used was 5'-UGAAGCACACCACGGAUCU-3' and 3'-ACUUCGUGUGGUGCCUAGA-5',<sup>29</sup> negative control RNA duplex, 5'-CGUUAUACGCGUAUAAUACGCGUAT-3' and 3'-CAGCAAUUAGCGCAUUAUUUUGCGCAUA-5' (Integrated DNA Technologies). The transfection was performed using Lipofectamine Plus<sup>TM</sup> (Invitrogen) as described.<sup>29</sup>

### 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  $2.5 \times 10^5$  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 venter's instruction (BD, NJ). Cells transfected with uMtCK siRNA or universal negative control were cultured for 24 hr, then  $2 \times 10^4$  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.<sup>16</sup> 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.<sup>30,31</sup> Prior to the treatment of HCC, serum MtCK activity was measured<sup>16</sup> with an immuno-inhibition method using the two types of anti-MtCK monoclonal antibodies.<sup>32</sup> 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.<sup>33</sup> 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 (Math-Soft, 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

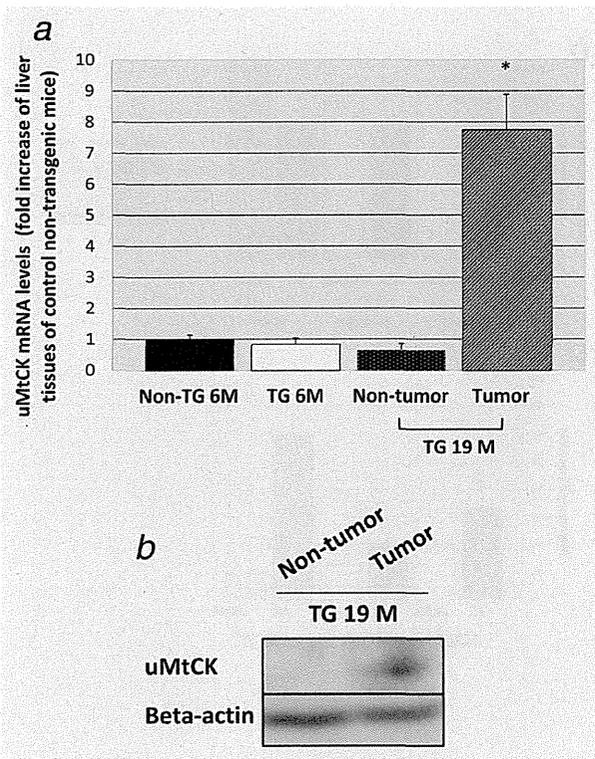
Mutations of mitochondrial DNA have been reported to be involved in hepatocarcinogenesis in humans.<sup>34,35</sup> Furthermore, in a mouse model for hepatocarcinogenesis, oxidative stress was shown to lead to loss of mitochondrial integrity in

the liver and ultimately hepatocarcinogenesis.<sup>23</sup> 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.<sup>22</sup> 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,<sup>23</sup> and ultimately HCC develops at 19 months of age without apparent inflammation or fibrosis in the liver.<sup>22</sup>

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<sup>22</sup> as well as loss of mitochondrial integrity.<sup>23</sup> 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. 1a). 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. 1b). 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.<sup>16</sup> To study whether ASB9 could regulate uMtCK protein levels in these HCC cells, we first measured ASB9 mRNA expression in those cells. Figure 2a demonstrates the low ASB9 mRNA expression in HCC cell lines, contrasting with high uMtCK mRNA expression levels in those cells.<sup>16</sup> 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. 2b). 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

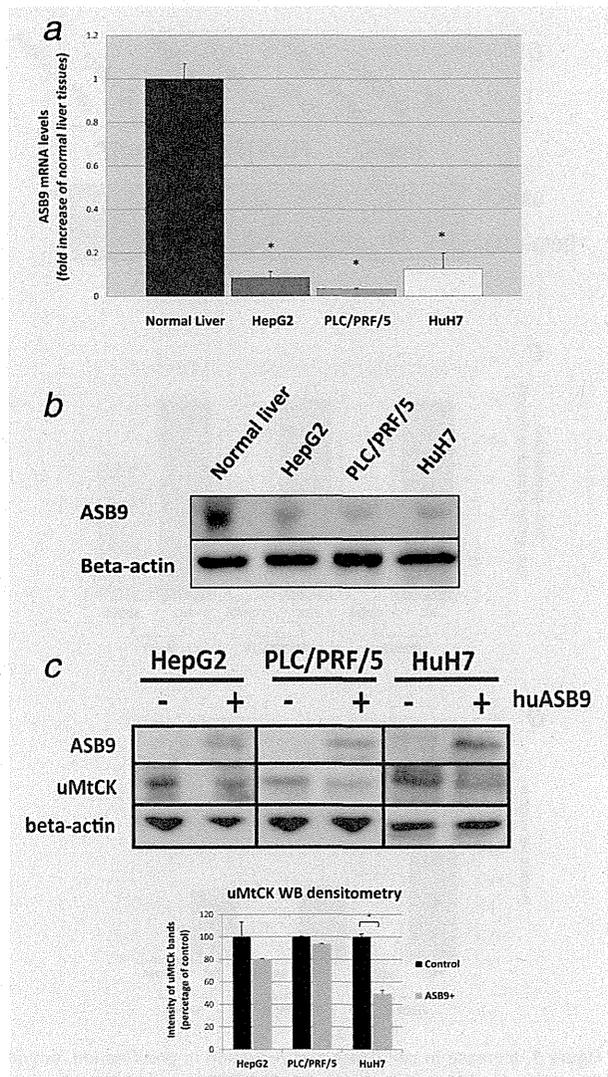


**Figure 1.** uMtCK mRNA and protein levels in liver tissues of the control non-transgenic, HCV core gene transgenic mice. (a) uMtCK mRNA levels were examined by real-time PCR in liver tissues of the control non-transgenic mice (Non-TG) at 6 months of age ( $n = 4$ ), and HCV core gene transgenic mice (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.

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. 2c). These results suggest a functional interaction of ASB9 with uMtCK may lead to degradation of uMtCK protein in HCC cell lines, as previously described.<sup>26</sup>

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

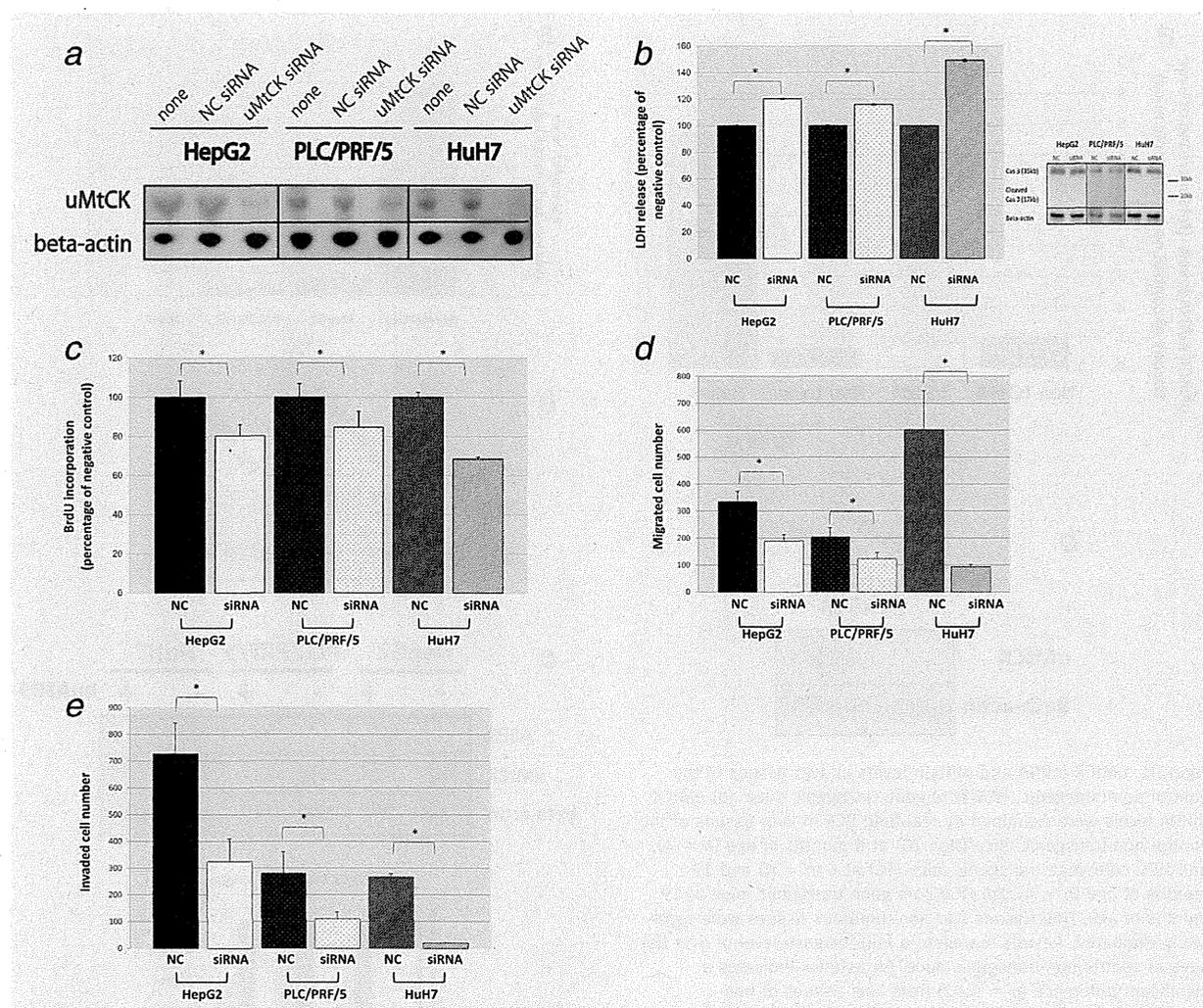
To inhibit high uMtCK expression in HepG2, PLC/PRF/5 and HuH7 cells,<sup>16</sup> isoform-specific siRNA was chosen as described<sup>29</sup> and successfully silenced target protein expression; the results from immunoblot analysis of untransfected and transfected cell lysates with universal negative control and uMtCK siRNA are shown in Figure 3a. As expected, in



**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.

all HCC cell lines transfected with uMtCK siRNA, the expression levels of uMtCK were clearly reduced at 36 hr after transfection (Fig. 3a).

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. 3b). 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. 3b), 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. 3c). 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 3d, silencing of uMtCK decreased migration rate by 44.1% in HepG2, by 40.0% in

Table 1. Baseline characteristics

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

<sup>1</sup>Data were expressed as mean ± SD (range).

<sup>2</sup>Data were expressed as number (%).

<sup>3</sup>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. 3e). Collectively, high expression of uMtCK may contribute to active migration and invasion of HCC cells.

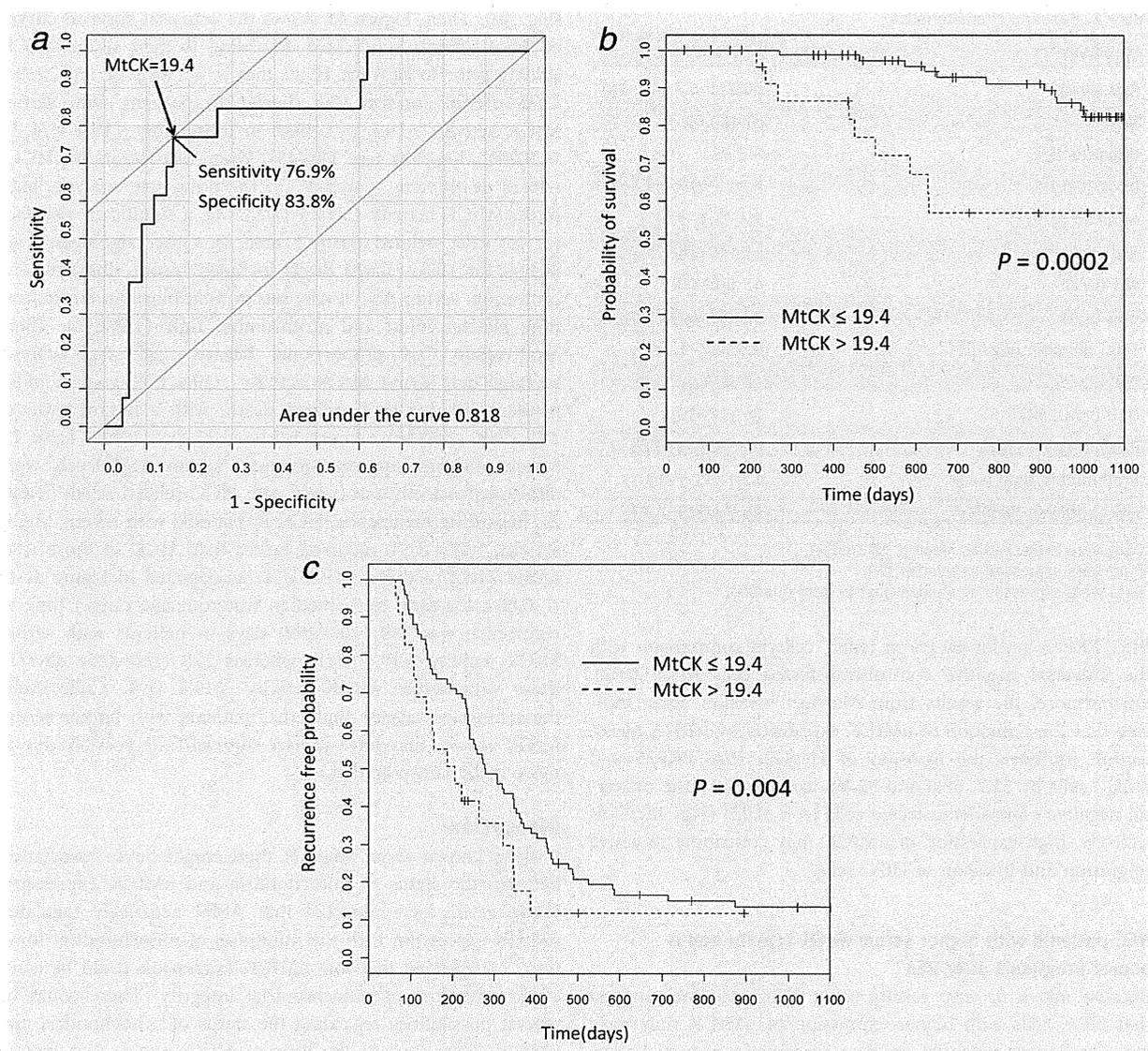
#### HCC patients with higher serum MtCK activity had a poorer prognosis after RFA

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.<sup>16</sup> 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. 4a). Then, Figure 4b shows the actuarial survival curves of these patients subdivided according to their serum MtCK activity prior to RFA for HCC, that is,  $\leq 19.4$  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  $\leq 19.4$  U/L ( $p = 0.0002$ ; log-rank test; Fig. 4b). 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  $\leq 19.4$  U/L, as depicted in Figure 4c ( $p = 0.004$ ; 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  $\leq 19.4$  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,<sup>26</sup> 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.<sup>26</sup> 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.<sup>36</sup> 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.<sup>22,23</sup> 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,  $\leq 19.4$  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.<sup>26</sup> 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,<sup>37</sup> whereas ASB9 mRNA expression is reportedly abundant.<sup>26</sup> 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,<sup>27</sup> 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

Parameter	Univariate		Multivariate	
	HR (95% CI)	<i>p</i> value	HR (95% CI)	<i>p</i> 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<sup>29</sup> and breast cancer cells.<sup>17</sup> 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.<sup>37</sup> 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.<sup>37,38</sup> Overexpression of uMtCK in different Hodgkin-derived cell lines has been described as a marker for poor prognosis.<sup>39</sup> 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.<sup>40</sup> 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.<sup>16</sup> 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,<sup>16</sup> 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,<sup>33</sup> 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.<sup>33</sup> 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.

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OPEN ACCESS



## ORIGINAL ARTICLE

# Hepatitis C virus kinetics by administration of pegylated interferon- $\alpha$ in human and chimeric mice carrying human hepatocytes with variants of the *IL28B* gene

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## ABSTRACT

**Objective** Recent studies have demonstrated that genetic polymorphisms near the *IL28B* gene are associated with the clinical outcome of pegylated interferon  $\alpha$  (peg-IFN- $\alpha$ ) plus ribavirin therapy for patients with chronic hepatitis C virus (HCV). However, it is unclear whether genetic variations near the *IL28B* gene influence hepatic interferon (IFN)-stimulated gene (ISG) induction or cellular immune responses, lead to the viral reduction during IFN treatment.

**Design** Changes in HCV-RNA levels before therapy, at day 1 and weeks 1, 2, 4, 8 and 12 after administering peg-IFN- $\alpha$  plus ribavirin were measured in 54 patients infected with HCV genotype 1. Furthermore, we prepared four lines of chimeric mice having four different lots of human hepatocytes containing various single nucleotide polymorphisms (SNP) around the *IL28B* gene. HCV infecting chimeric mice were subcutaneously administered with peg-IFN- $\alpha$  for 2 weeks.

**Results** There were significant differences in the reduction of HCV-RNA levels after peg-IFN- $\alpha$  plus ribavirin therapy based on the *IL28B* SNP rs8099917 between TT (favourable) and TG/GG (unfavourable) genotypes in patients; the first-phase viral decline slope per day and second-phase slope per week in TT genotype were significantly higher than in TG/GG genotype. On peg-IFN- $\alpha$  administration to chimeric mice, however, no significant difference in the median reduction of HCV-RNA levels and the induction of antiviral ISG was observed between favourable and unfavourable human hepatocyte genotypes.

**Conclusions** As chimeric mice have the characteristic of immunodeficiency, the response to peg-IFN- $\alpha$  associated with the variation in *IL28B* alleles in chronic HCV patients would be composed of the intact immune system.

## INTRODUCTION

Hepatitis C is a global health problem that affects a significant portion of the world's population. The WHO estimated that, in 1999, 170 million hepatitis C virus (HCV)-infected patients were present worldwide, with 3–4 million new cases appearing per year.<sup>1</sup>

## Significance of this study

### What is already known on this subject?

- Genetic polymorphisms near the *IL28B* gene are associated with a chronic HCV treatment response.
- HCV-infected patients with the *IL28B* homozygous favourable allele had a more rapid decline in HCV kinetics in the first and second phases by peg-IFN- $\alpha$ -based therapy.
- During the acute phase of HCV infection, a strong immune response among patients with the *IL28B* favourable genotype could induce more frequent spontaneous clearance of HCV.

### What are the new findings?

- In chronically HCV genotype 1b-infected chimeric mice that have the characteristic of immunodeficiency, no significant difference in the reduction in serum HCV-RNA levels and the induction of antiviral hepatic ISG by the administration of peg-IFN- $\alpha$  was observed between favourable and unfavourable human hepatocyte *IL28B* genotypes.
- By comparison of serum HCV kinetics between human and chimeric mice, the viral decline in both the first and second phases by peg-IFN- $\alpha$  treatment was affected by the variation in *IL28B* genotypes only in chronic hepatitis C patients.

### How might it impact on clinical practice in the foreseeable future?

- The immune response according to *IL28B* genetic variants could contribute to the first and second phases of HCV-RNA decline and might be critical for HCV clearance by peg-IFN- $\alpha$ -based therapy.

The standard therapy for hepatitis C still consists of pegylated interferon- $\alpha$  (peg-IFN- $\alpha$ ), administered once weekly, plus daily oral ribavirin for 24–48 weeks in countries where protease inhibitors are not available.<sup>2</sup> This combination therapy is quite successful in patients with HCV genotype 2 or 3

infection, leading to a sustained virological response (SVR) in approximately 80–90% of patients treated; however, in patients infected with HCV genotype 1 or 4, only approximately half of all treated individuals achieved a SVR.<sup>3–4</sup>

Host factors were shown to be associated with the outcome of the therapy, including age, sex, race, liver fibrosis and obesity.<sup>5</sup> Genome-wide association studies have demonstrated that genetic variations in the region near the interleukin-28B (*IL28B*) gene, which encodes interferon (IFN)- $\lambda$ 3, are associated with a chronic HCV treatment response.<sup>6–10</sup> Furthermore, it was demonstrated that genetic variations in the *IL28B* gene region are also associated with spontaneous HCV clearance.<sup>11–12</sup>

Interestingly, a recent report showed the effect of genetic polymorphisms near the *IL28B* gene on the dynamics of HCV during peg-IFN- $\alpha$  plus ribavirin therapy in Caucasian, African American and Hispanic individuals;<sup>13</sup> HCV-infected patients with the *IL28B* homozygous favourable allele had a more rapid decline of HCV in the first phase, which is associated with the inhibition of viral replication as well as the second phase associated with immuno-destruction of viral-infected hepatocytes.<sup>14</sup> However, it is unknown how a direct effect by the *IL28B* genetic variation, such as the induction of IFN-stimulated genes (ISG) or cellular immune responses, would influence the viral kinetics during IFN treatment. Over recent periods, engineered severe combined immunodeficient (SCID) mice transgenic for urokinase-type plasminogen activator (uPA) received human hepatocyte transplants (hereafter referred to as chimeric mice)<sup>15–17</sup> and are suitable for experiments with hepatitis viruses in vivo.<sup>18–19</sup> We have also reported that these chimeric mice carrying human hepatocytes are a robust animal model to evaluate the efficacy of IFN and other anti-HCV agents.<sup>20–21</sup>

The purpose of this study was to reveal the association between genetic variations in the *IL28B* gene region and viral decline during peg-IFN- $\alpha$  treatment in patients with HCV, and to clarify the association between different *IL28B* alleles of human hepatocytes in chimeric mice and the response to peg-IFN- $\alpha$  without immune response. These studies will elucidate whether the immune response by the *IL28B* genetic variation affects the viral kinetics during peg-IFN- $\alpha$  treatment.

## MATERIALS AND METHODS

### Patients

Fifty-four Japanese patients with chronic HCV genotype 1 infection at Nagasaki Medical Center and Nagoya City University were enrolled in this study (table 1). Patients received peg-IFN- $\alpha$ 2a (180  $\mu$ g) or 2b (1.5  $\mu$ g/kg) subcutaneously every

week and were administered a weight-adjusted dose of ribavirin (600 mg for <60 kg, 800 mg for 60–80 kg, and 1000 mg for >80 kg daily), which is the recommended dosage in Japan. Patients with other hepatitis virus infection or HIV coinfection were not included in the study. The study protocol conformed to the ethics guidelines of the 1975 Declaration of Helsinki as reflected by earlier approval by the institutions' human research committees.

### Laboratory tests

Blood samples were obtained before therapy, as well as on day 1 and at weeks 1, 2, 4, 8 and 12 after the start of therapy and were analysed for the HCV-RNA level by the commercial Abbott Real-Time HCV test with a lower limit of detection of 12 IU/ml (Abbott Molecular Inc., Des Plaines, Illinois, USA). Genetic polymorphism in the *IL28B* gene (rs8099917), a single nucleotide polymorphism (SNP) recently identified to be associated with treatment response,<sup>6–8</sup> was tested by the TaqMan SNP genotyping assay (Applied Biosystems, Foster City, California, USA).

### HCV infection of chimeric mice with the liver repopulated for human hepatocytes

SCID mice carrying the uPA transgene controlled by an albumin promoter were injected with  $5.0\text{--}7.5 \times 10^5$  viable hepatocytes through a small left-flank incision into the inferior splenic pole, thereafter chimeric mice were generated. The chimeric mice were purchased from PhoenixBio Co, Ltd (Hiroshima, Japan).<sup>17</sup> Human hepatocytes with the *IL28B* homozygous favourable allele, heterozygous allele or homozygous unfavourable allele were imported from BD Biosciences (San Jose, California, USA) (table 2). Murine serum levels of human albumin and the body weight were not significantly different among four chimeric mice groups, providing a reliable comparison for anti-HCV agents.<sup>22</sup> Three different serum samples were obtained from three chronic HCV patients (genotype 1b).<sup>21–22</sup> Each mouse was intravenously infected with serum sample containing  $10^5$  copies of HCV genotype 1b. Administration of peg-IFN- $\alpha$ 2a (Pegasys; Chugai Pharmaceutical Co., Ltd., Tokyo, Japan) at the dose formulation (30  $\mu$ g/kg) was consecutively applied to each mouse on days 0, 3, 7 and 10 (table 3).

### HCV-RNA quantification

HCV-RNA in mice sera (days 0, 1, 3, 7 and 14) was quantified by an in-house real-time detection PCR assay with a lower quantitative limit of detection of 10 copies/assay, as previously reported.<sup>21</sup>

### Quantification of IFN-stimulated gene-expression levels

For analysis of endogenous ISG levels, total RNA was isolated from the liver using the RNeasy RNA extraction kit (Qiagen, Valencia, California, USA) and complementary DNA synthesis was performed using 2.0  $\mu$ g of total RNA (High Capacity RNA-to-cDNA kit; Applied Biosystems). Fluorescence real-time PCR analysis was performed using an ABI 7500 instrument (Applied Biosystems) and TaqMan Fast Advanced gene expression assay (Applied Biosystems). TaqMan Gene Expression Assay primer and probe sets (Applied Biosystems) are shown in the supplementary information (available online only). Relative amounts of messenger RNA, determined using a FAM-Labeled TaqMan probe, were normalised to the endogenous RNA levels of the housekeeping reference gene, glyceraldehyde-3-phosphate dehydrogenase. The delta Ct method ( $2^{-(\Delta\Delta C_t)}$ ) was used for quantitation of relative mRNA levels and fold induction.<sup>23–24</sup>

**Table 1** Characteristics of 54 patients infected HCV genotype 1

	<i>IL28B</i> SNP rs8099917		p Value
	TT (n=34)	TG (n=19) + GG (n=1)	
Age (years)	55.6 $\pm$ 10.1	54.7 $\pm$ 11.3	0.746
Gender (male %)	70	50	0.199
Body mass index (kg/m <sup>2</sup> )	24.6 $\pm$ 3.1	24.7 $\pm$ 3.3	0.870
Viral load at therapy (log IU/ml)	6.0 $\pm$ 0.7	5.8 $\pm$ 0.8	0.357
SVR rate (%)	50	11	0.012
Serum ALT level (IU/l)	100.3 $\pm$ 80.8	79.3 $\pm$ 45.0	0.226
Platelet count ( $\times 10^4/\mu$ l)	17.1 $\pm$ 9.0	16.5 $\pm$ 5.8	0.771
Fibrosis (F3+4 %)	42	40	0.877

HCV, hepatitis C virus; SNP, single nucleotide polymorphism; SVR, sustained virological response.

## Hepatology

**Table 2** Four lines of uPA/SCID mice from four different lots of human hepatocytes (donor) containing various SNP around the *IL28B* gene

uPA/SCID mice	Donor	Race	Age	Gender	rs8103142	rs12979860	rs8099917
PXB mice	A	African American	5 Years	Male	CC	TT	TG
	B	Caucasian	10 Years	Female	CC	TT	TG
	C	Hispanic	2 Years	Female	TT	CC	TT
	D	Caucasian	2 Years	Male	TT	CC	TT

PXB mice; urokinase-type plasminogen activator/severe combined immunodeficiency (uPA/SCID) mice repopulated with approximately 80% human hepatocytes. SCID, severe combined immunodeficient; SNP, single nucleotide polymorphism.

### Statistical analyses

Statistical differences were evaluated by Fisher's exact test or the  $\chi^2$  test with the Yates correction. Mice serum HCV-RNA and intrahepatic ISG expression levels were compared using the Mann-Whitney U test. Differences were considered significant if p values were less than 0.05.

## RESULTS

### Characteristics of the study patients

Genotypes (rs8099917) TT, TG and GG were detected in 34, 19 and one patient infected with HCV genotype 1, respectively. SVR rates were significantly higher in HCV patients with genotype TT than in those with genotype TG/GG (50% vs 11%,  $p=0.012$ ). The initial HCV serum load was comparable between genotypes TT and TG/GG ( $6.0\pm 0.7$  vs  $5.8\pm 0.8$  log IU/ml). There were no significant differences in sex (male%, 70% vs 50%), age ( $55.6\pm 10.1$  vs  $54.7\pm 11.3$  years), serum alanine aminotransferase level ( $100.3\pm 80.8$  vs  $79.3\pm 45.0$  IU/L), platelet count ( $17.1\pm 9.0$  vs  $16.5\pm 5.8\times 10^4/\mu\text{l}$ ) and fibrosis stages (F3/4%, 42% vs 40%) between HCV patients with the favourable (rs8099917 TT) and unfavourable (rs8099917 TG/GG) *IL28B* genotypes (table 1).

### Changes in serum HCV-RNA levels in patients treated by peg-IFN- $\alpha$ plus ribavirin

Figure 1 shows the initial change in the serum HCV-RNA level for 14 days after peg-IFN- $\alpha$  plus ribavirin therapy in patients infected with HCV genotype 1 based on the genetic polymorphism near the *IL28B* gene. The immediate antiviral response (viral drop 24 h after the first IFN injection) was significantly higher in HCV patients with genotype TT than genotype TG/GG ( $-1.08$  vs  $-0.39$  log IU/ml,  $p<0.001$ ). Figure 2 also shows the subsequent change in the serum HCV-RNA reduction after peg-IFN- $\alpha$  plus ribavirin therapy in patients infected with HCV genotype 1.

Similarly, during peg-IFN- $\alpha$  plus ribavirin therapy, a statistically significant difference in the median reduction in serum HCV-RNA levels was noted according to the genotype (TT vs TG/GG). The median reduction in the serum HCV-RNA levels (log IU/ml) at 1, 2, 4, 8 and 12 weeks between genotypes TT and TG/GG was as follows:  $-1.58$  vs  $-0.62$ ,  $p<0.001$ ;  $-2.35$  vs  $-0.91$ ,  $p<0.001$ ;  $-3.48$  vs  $-1.56$ ,  $p<0.001$ ;  $-4.53$  vs  $-2.37$ ,  $p<0.01$ ;  $-4.93$  vs  $-2.86$ ,  $p<0.001$ . Furthermore, the initial first-phase viral decline slope per day (Ph1/day) and subsequent second-phase viral decline slope per week (Ph2/week) in TT genotype were significantly higher than in genotype TG/GG (Ph1/day  $0.94\pm 0.83$  vs  $0.38\pm 0.40$  log IU/ml,  $p<0.001$ ; Ph2/week  $0.08\pm 0.06$  vs  $0.04\pm 0.03$  log IU/ml,  $p<0.001$ ) (figure 3).

### Changes in serum HCV-RNA levels in chimeric mice treated by peg-IFN- $\alpha$

In order to clarify the association between *IL28B* alleles of human hepatocytes and the response to peg-IFN- $\alpha$ , we prepared four lines of uPA/SCID mice and four different lots of human hepatocytes containing various rs8099917, rs8103142 and rs12979860 SNPs around the *IL28B* gene (table 2). The chimeric mice were inoculated with serum samples from each HCV-1b patient, and then HCV-RNA levels had increased and reached more than  $10^6$  copies/ml in all chimeric mice sera at 2 weeks after inoculation. After confirming the peak of HCV-RNA in all chimeric mice, they were subcutaneously administered with four times injections of the bolus dose of peg-IFN- $\alpha$ 2a for 2 weeks (table 3). Figure 4 shows the change in the serum HCV-RNA levels for 14 days during IFN injection into chimeric mice transplanted with *IL28B* favourable or unfavourable human hepatocyte genotypes. On peg-IFN- $\alpha$  administration, no significant difference in the median reduction in HCV-RNA levels in the serum A-infected<sup>22</sup> chimeric mice sera was observed between favourable ( $n=7$ ) and unfavourable

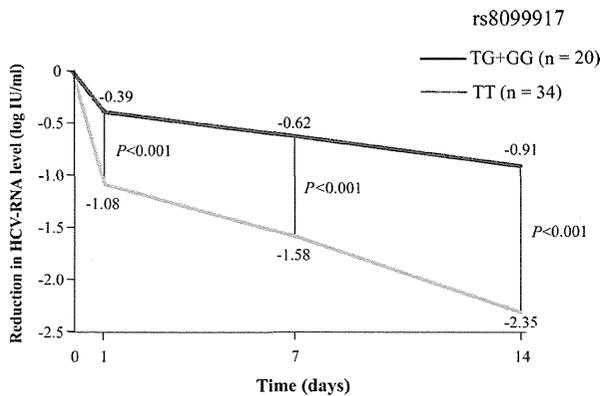
**Table 3** Dosage and time schedule of pegIFN- $\alpha$ 2a\* treatment for HCV genotype 1b infected chimeric mice

Donor hepatocyttest	No of chimeric mice	Inoculum	Test compound	Dose			
				Level ( $\mu\text{g}/\text{kg}$ )	Concentration ( $\mu\text{g}/\text{ml}$ )	Volume (ml/kg)	Frequency
A	3	Serum A	Peg-IFN- $\alpha$ 2a	30	3	10	Day 0, 3, 7, 10
B	4	Serum A	Peg-IFN- $\alpha$ 2a	30	3	10	Day 0, 3, 7, 10
C	3	Serum A	Peg-IFN- $\alpha$ 2a	30	3	10	Day 0, 3, 7, 10
D	3	Serum A	Peg-IFN- $\alpha$ 2a	30	3	10	Day 0, 3, 7, 10
A	2	Serum B	Peg-IFN- $\alpha$ 2a	30	3	10	Day 0, 3, 7, 10
C	2	Serum B	Peg-IFN- $\alpha$ 2a	30	3	10	Day 0, 3, 7, 10
A	2	Serum C	Peg-IFN- $\alpha$ 2a	30	3	10	Day 0, 3, 7, 10
C	2	Serum C	Peg-IFN- $\alpha$ 2a	30	3	10	Day 0, 3, 7, 10

\*Pegasys; Chugai Pharmaceutical Co., Ltd., Tokyo, Japan.

†The *IL28B* genetic variation of the donor hepatocytes was indicated in table 2.

HCV, hepatitis C virus; peg-IFN- $\alpha$ , pegylated interferon  $\alpha$ .



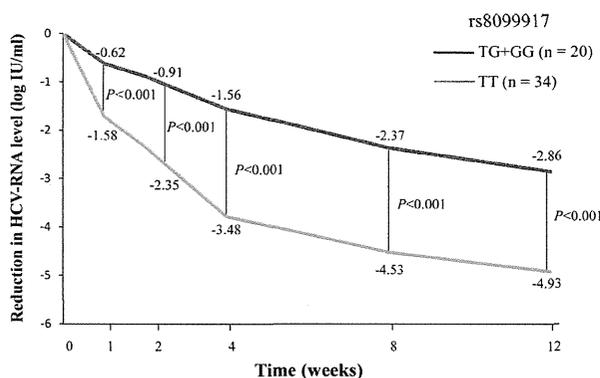
**Figure 1** Rapid reduction of median hepatitis C virus (HCV)-RNA levels (log IU/ml) at 1, 7 and 14 days between *IL28B* single nucleotide polymorphisms rs8099917 genotype TT (n=34) and TG/GG (n=20) in HCV genotype 1-infected patients treated with peg-IFN- $\alpha$  plus ribavirin.

(n=6) *IL28B* genotypes on days 1, 3, 7 and 14 (-1.2 vs -1.3, -1.4 vs -1.4, -1.8 vs -1.7, and -2.3 vs -1.9 log copies/ml) (figure 4A). Moreover, we prepared two additional serum samples from the other HCV-1b patients (serum B and C)<sup>21</sup> to confirm the influence of *IL28B* genotype in early viral kinetics during IFN treatment. After establishing persistent infection with new HCV-1b strains in all chimeric mice, they were also administered four times injections of the bolus dose of peg-IFN- $\alpha$ 2a for 2 weeks (figure 4B,C). In a similar fashion, no significant difference in HCV-RNA reduction in chimeric mice sera was observed between favourable and unfavourable *IL28B* genotypes.

#### Expression levels of ISG in chimeric mice livers

Because chimeric mice have the characteristic of severe combined immunodeficiency, the viral kinetics in chimeric mice sera during IFN treatment could be contributed by the innate immune response of HCV-infected human hepatocytes. Therefore, ISG expression levels in mice livers transplanted with human hepatocytes were compared between favourable and unfavourable *IL28B* genotypes (figure 5).

As shown in figure 5A, ISG expression levels in mice livers were measured at 8 h and 24 h after IFN treatment. The levels



**Figure 2** Weekly reduction of median hepatitis C virus (HCV)-RNA levels (log IU/ml) at 1, 2, 4, 8 and 12 weeks between *IL28B* single nucleotide polymorphisms rs8099917 genotype TT (n=34) and TG/GG (n=20) in HCV genotype 1-infected patients treated with pegylated interferon  $\alpha$  plus ribavirin.

of representative antiviral ISG (eg, myxovirus resistance protein A, oligoadenylate synthetase 1, RNA-dependent protein kinase) and other ISG for promoting antiviral signalling (eg, Toll-like receptor 3, retinoic acid-inducible gene 1) were significantly induced at least 8 h after treatment, and prolonged at 24 h. No significant difference in ISG expression levels in HCV-infected livers was observed between favourable and unfavourable *IL28B* genotypes. The other inoculum for persistent infection of HCV-1b also demonstrated no significant difference in ISG expression levels between favourable and unfavourable *IL28B* genotypes (figure 5B,C). Interestingly, IFN- $\lambda$  expression levels by treatment of peg-IFN- $\alpha$  were significantly induced in HCV-infected human hepatocytes harbouring the favourable *IL28B* genotype (figure 5 A-C).

#### DISCUSSION

Several recent studies have demonstrated a marked association between the chronic hepatitis C treatment response<sup>6-9</sup> and SNP (rs8099917, rs8103142 and rs12979860) near or within the region of the *IL28B* gene, which affected the viral dynamics during peg-IFN- $\alpha$  plus ribavirin therapy in Caucasian, African American and Hispanic individuals.<sup>13</sup>

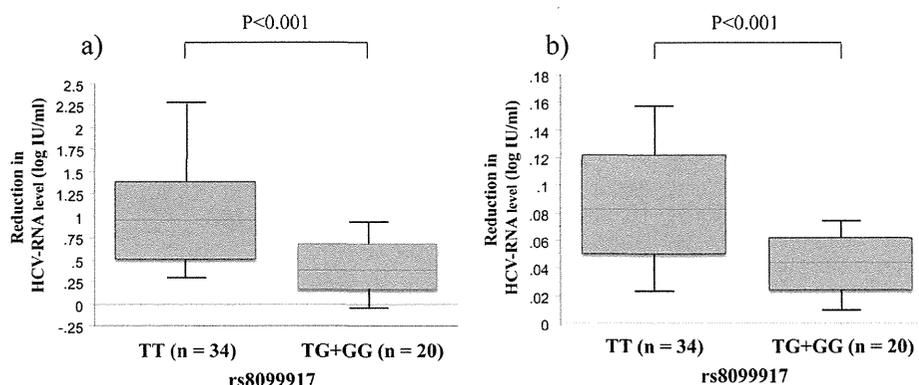
It has been reported that when patients with chronic hepatitis C are treated by IFN- $\alpha$  or peg-IFN- $\alpha$  plus ribavirin, HCV-RNA generally declines after a 7-10 h delay.<sup>25</sup> The typical decline is biphasic and consists of a rapid first phase lasting for approximately 1-2 days during which HCV-RNA may fall 1-2 logs in patients infected with genotype 1, and subsequently a slower second phase of HCV-RNA decline.<sup>26</sup> The viral kinetics had a predictive value in evaluating antiviral efficacy.<sup>14</sup> In this study, biphasic decline of the HCV-RNA level during peg-IFN- $\alpha$  treatment was observed in both patients and chimeric mice infected with HCV genotype 1; however, in the first and second phases of viral kinetics, a difference between *IL28B* genotypes was observed only in HCV-infected patients; a more rapid decline in serum HCV-RNA levels after administering peg-IFN- $\alpha$  plus ribavirin was confirmed in patients with the TT genotype of rs8099917 compared to those with the TG/GG genotype.

On the other hand, in-vivo data using the chimeric mouse model showed no significant difference in the reduction of HCV-RNA titers in mouse serum among four different lots of human hepatocytes containing *IL28B* favourable (rs8099917 TT) or unfavourable (rs8099917 TG) genotypes, which was confirmed by the inoculation of two additional HCV strains. These results indicated that variants of the *IL28B* gene in donor hepatocytes had no influence on the response to peg-IFN- $\alpha$  under immunosuppressive conditions, suggesting that the immune response according to *IL28B* genetic variants could contribute to the first and second phases of HCV-RNA decline and might be critical for HCV clearance by peg-IFN- $\alpha$ -based therapy.

Two recent studies indeed revealed an association between the *IL28B* genotype and the expression level of hepatic ISG in human studies.<sup>27 28</sup> Quiescent hepatic ISG before treatment among patients with the *IL28B* favourable genotype have been associated with sensitivity to exogenous IFN treatment and viral eradication; however, it is difficult to establish whether the hepatic ISG expression level contributes to viral clearance independently or appears as a direct consequence of the *IL28B* genotype. Another recent study addressed this question and the results suggested that there is no absolute correlation with the *IL28B* genotype and hepatic expression of ISG.<sup>29</sup> Our results on the hepatic ISG expression level in immunodeficient chimeric mice also suggested that no significant difference in ISG expression levels was observed between favourable and unfavourable

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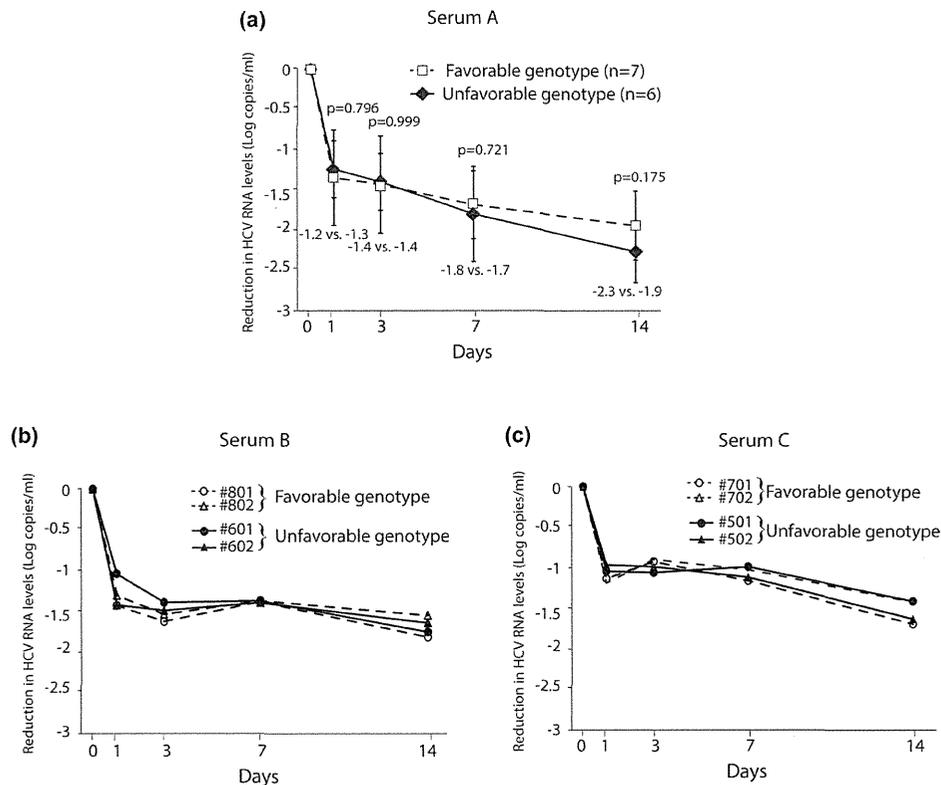
**Figure 3** (A) The first-phase viral decline slope per day (Ph1/day) and (B) second-phase viral decline slope per week (Ph2/week) in hepatitis C virus (HCV) genotype 1-infected patients treated with pegylated interferon  $\alpha$  plus ribavirin. The lines across the boxes indicate the median values. The hash marks above and below the boxes indicate the 90th and 10th percentiles for each group, respectively.



*IL28B* genotypes. However, these results were not consistent with a previous report using chimeric mice that the favourable *IL28B* genotype was associated with an early reduction in HCV-RNA by ISG induction.<sup>30</sup> The reasons for the discrepancy might depend on the dose and type of IFN treatment, as well as the time point when ISG expression was examined in the liver. In addition, although IFN- $\lambda$  transcript levels measured in peripheral blood mononuclear cells or liver revealed inconsistent results in the context of an association with the *IL28B* genotype,<sup>7, 8</sup> our preliminary assay on the *IL28A*, *IL28B* and *IL29* transcripts in the liver first indicated that the induction of IFN- $\lambda$  on peg-IFN- $\alpha$  administration could be associated with the *IL28B* genotype. Therefore, the induction of IFN- $\lambda$  followed by immune response

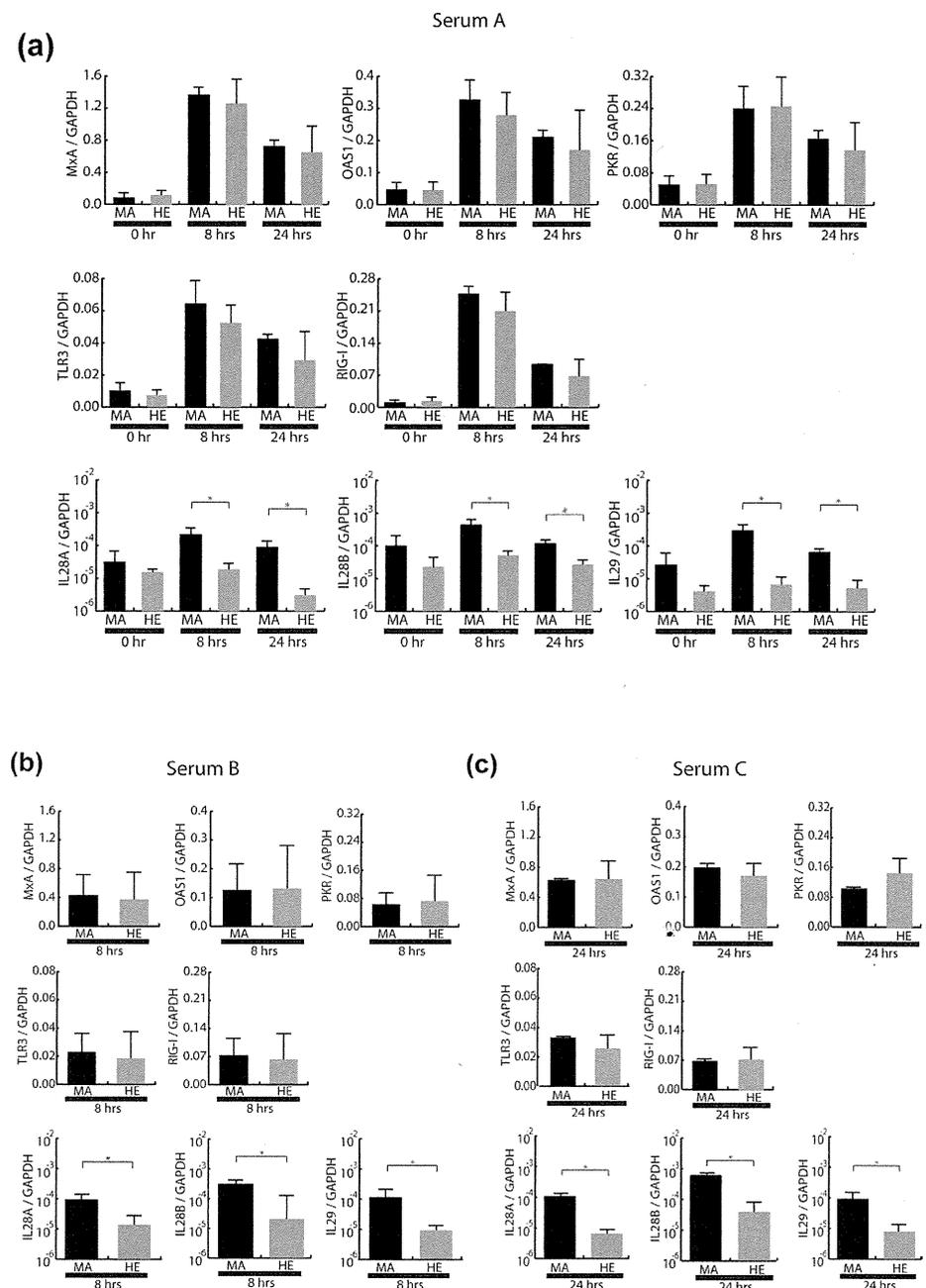
might contribute to different viral kinetics and treatment outcomes in HCV-infected patients, because no difference was found in chimeric mice without immune response.

It has also been reported that the mechanism of the association of genetic variations in the *IL28B* gene and spontaneous clearance of HCV may be related to the host innate immune response.<sup>11</sup> Interestingly, participants with seroconversion illness with jaundice were more frequently rs8099917 homozygous favourable allele (TT) than other genotypes (32% vs 5%,  $p=0.047$ ). This suggests that a stronger immune response during the acute phase of HCV infection among patients with the *IL28B* favourable genotype would induce more frequent spontaneous clearance of HCV.



**Figure 4** Median reduction of hepatitis C virus (HCV)-RNA levels (log copies/ml) after administering pegylated interferon  $\alpha$  to chimeric mice having human hepatocytes containing various single nucleotide polymorphisms around the *IL28B* gene as favourable (rs8099917 TT) and unfavourable (rs8099917 TG) genotypes. Data are represented as mean+SD. Chimeric mice infected with a) serum A (n=7; favourable genotype, n=6; unfavourable genotype), (B) serum B (n=2, each genotype), and (C) serum C (n=2, each genotype). All serum samples were obtained from HCV-1b patients.

**Figure 5** Intrahepatic interferon (IFN)-stimulated gene (ISG) expression levels in the pegylated interferon  $\alpha$  (peg-IFN- $\alpha$ )-treated chimeric mice having human hepatocytes containing homozygous favourable allele (rs8099917 TT; MA) and heterozygous unfavourable allele (rs8099917 TG; HE) were measured and expressed relative to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) messenger RNA. Data are represented as mean  $\pm$ SD. (A) Time kinetics of ISG after administration of the peg-IFN- $\alpha$  in serum A-infected chimeric mice (n=3, each genotype). Comparison of ISG expression levels at (B) 8 h in serum B-infected mice and (C) 24 h in serum C-infected mice after administering peg-IFN- $\alpha$  (n=3, each genotype). Predesigned real-time PCR assay of IL28B transcript purchased from Applied Biosystems can be cross-reactive to IL28A transcript. \*p<0.05. MxA, myxovirus resistance protein A; OAS1, oligoadenylate synthetase 1; PKR, RNA-dependent protein kinase; RIG-I, retinoic acid-inducible gene 1; TLR3, Toll-like receptor 3.



Taking into account both the above results in acute HCV infection and our results conducted on chimeric mice that have the characteristic of immunodeficiency, it is suggested that the response to peg-IFN- $\alpha$  associated with the variation in *IL28B* alleles in chronic hepatitis C patients would be composed of the intact immune system.

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**Contributors** YT and MM conceived the study. TW and FS and YT conducted the study equally. TW and FS coordinated the analysis and manuscript preparation. All the authors had input into the study design, patient recruitment and management or mouse management and critical revision of the manuscript for intellectual content. TW, FS and YT contributed equally.

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**Competing interests** None.

**Patient consent** Obtained.

**Ethics approval** This study was conducted with the approval of each ethics committee at the Nagoya City University and Nagasaki Medical Center (see supplementary information, available online only).

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## Hepatitis C virus kinetics by administration of pegylated interferon- $\alpha$ in human and chimeric mice carrying human hepatocytes with variants of the *IL28B* gene

Tsunamasa Watanabe, Fuminaka Sugauchi, Yasuhito Tanaka, et al.

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# Application of a Glycoproteomics-Based Biomarker Development Method: Alteration in Glycan Structure on Colony Stimulating Factor 1 Receptor as a Possible Glycobiomarker Candidate for Evaluation of Liver Cirrhosis

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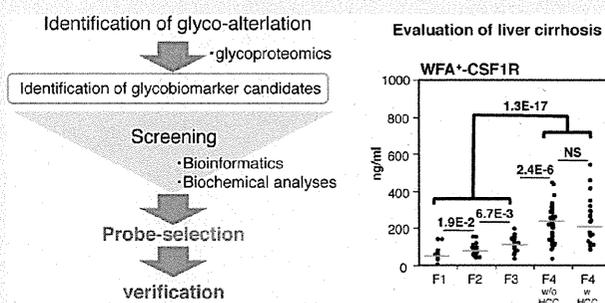
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## Supporting Information

**ABSTRACT:** The importance of diagnosis and therapies for liver cirrhosis (LC) is indisputable. Thus, a reliable method for monitoring the progression of liver fibrosis and resultant LC is urgently needed. Previously, using a lectin-assisted glycoproteomic method, we identified 26 serum glycoproteins as promising glycobiomarker candidates for monitoring the progression of liver diseases. In this study, we identified colony stimulating factor 1 receptor (CSF1R) as a promising LC marker candidate and then established *Wisteria floribunda* agglutinin (WFA)-reactive CSF1R (WFA<sup>+</sup>-CSF1R) as a novel possible glycobiomarker candidate by utilizing a glycoproteomics-based strategy. The serum level of WFA<sup>+</sup>-CSF1R in patients with hepatitis C virus (HCV)-infected liver disease was measured by an antibody-lectin sandwich ELISA. In a proof-of-concept experiment of the strategy preceding to future clinical studies, LC patients showed a high serum WFA<sup>+</sup>-CSF1R level in selected samples ( $P = 1.3 \times 10^{-17}$ ). This result suggests WFA<sup>+</sup>-CSF1R is a possible biomarker candidate for evaluation of LC. Our results verified feasibility of this strategy for glycobiomarker development.

**KEYWORDS:** glycobiomarker, glycoprotein, glycoproteomics, liver cirrhosis, colony stimulating factor 1 receptor, lectin array, glycan alteration



## INTRODUCTION

Glycosylation plays an important role in regulating the properties of proteins and lipids on the cell surface. Glycan profiles change significantly along with the cell-autonomous changes or the changes in the pericellular surroundings. Cancer-related structural modifications of glycans, such as sialylation, multiple branching polylactosamine elongation and fucosylation are caused by cellular malignant transformation.<sup>1–4</sup> Additionally, expression levels of sialyl Lewis x antigens are elevated in a hypoxic environment.<sup>5</sup> Many test kits used for clinical diagnosis of cancer, such as the kits for the tumor markers CA19-9 and CA125, have been developed based on the observed alterations in the glycan structures. Currently, certain types of cancers are diagnosed by measuring levels of specific serum proteins. However, sometimes these serum proteins are secreted from the background lesions of inflammatory diseases associated with cancer or from other physiologically associated organs.<sup>6</sup> This observation suggests

that these serum proteins are not good biomarkers for cancer diagnosis as their increased levels might not always reflect the cancer itself. Therefore, to find a disease-specific biomarker, it would be more advantageous to monitor alterations in the glycan structures as an indicator of the disease status rather than measuring the quantitative changes in the protein. Indeed, several serum glycobiomarkers have been developed as possible diagnostic or prognostic indices for liver diseases. For example, the *Lens culinaris* agglutinin (LCA)-reactive  $\alpha$ -fetoprotein (AFP) AFP-L3 is a more specific biomarker for the diagnosis of hepatocellular carcinoma (HCC) than the AFP.<sup>7</sup> Similarly, *Aleuria aurantia* lectin (AAL)-reactive serum proteins (e.g., complement component 3) have been proposed as the diagnostic marker for early HCC,<sup>8</sup> and a multilectin-based assay for the serum  $\alpha$ 1-acid glycoprotein (AGP) has been

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