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Impact of IL28B Genetic Variation on HCV-Induced Liver Fibrosis, Inflammation, and Steatosis: A Meta-Analysis

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Abstract

Background & Aims: IL28B polymorphisms were shown to be strongly associated with the response to interferon therapy in chronic hepatitis C (CHC) and spontaneous viral clearance. However, little is known about how these polymorphisms affect the natural course of the disease. Thus, we conducted the present meta-analysis to assess the impact of IL28B polymorphisms on disease progression.

Methods: A literature search was conducted using MEDLINE, EMBASE, and the Cochrane Library. Integrated odds ratios (OR) were calculated with a fixed-effects or random-effects model based on heterogeneity analyses.

Results: We identified 28 studies that included 10,024 patients. The pooled results indicated that the rs12979860 genotype CC was significantly associated (vs. genotype CT/TT; OR, 1.122; 95%CI, 1.003–1.254; P = 0.044), and that the rs8099917 genotype TT tended to be (vs. genotype TG/GG; OR, 1.126; 95%CI, 0.988–1.284; P = 0.076) associated, with an increased possibility of severe fibrosis. Both rs12979860 CC (vs. CT/TT; OR, 1.288; 95%CI, 1.050–1.581; P = 0.015) and rs8099917 TT (vs. TG/GG; OR, 1.324; 95%CI, 1.110–1.579; P = 0.002) were significantly associated with a higher possibility of severe inflammation activity. Rs8099917 TT was also significantly associated with a lower possibility of severe steatosis (vs. TG/GG; OR, 0.580; 95%CI, 0.351–0.959; P = 0.034), whereas rs12979860 CC was not associated with hepatic steatosis (vs. CT/TT; OR, 1.062; 95%CI, 0.415–2.717; P = 0.901).

Conclusions: IL28B polymorphisms appeared to modify the natural course of disease in patients with CHC. Disease progression seems to be promoted in patients with the rs12979860 CC and rs8099917 TT genotypes.

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Introduction

Hepatitis C virus (HCV) infection is a major cause of chronic hepatitis, liver cirrhosis, and hepatocellular carcinoma (HCC) [1]. In epidemiological studies of chronic HCV infection, age, duration of infection, alcohol consumption, coinfection with human immune deficiency virus, low CD4 count, male gender, and HCV genotype 3 have been shown to be associated with histological activity [2–7]. Although these factors explain part of the extreme variability seen in the progression of fibrosis among HCV-infected patients, they do not completely account for the differences. Genetic host factors have long been suspected to play a role in chronic hepatitis C (CHC) [8–10]. Two genome-wide association studies recently reported the susceptible loci for the progression of liver cirrhosis [11,12].

Currently, patients with CHC are treated with a combination of peg-interferon (peg-IFN) and ribavirin [13,14]. Telaprevir and boceprevir, two protease inhibitors, were recently approved for patients with genotype 1 in combination with peg-IFN and ribavirin. This combination has been shown to lead to substantial improvement in the sustained virologic response rate [15,16]. Genetic variations near the interleukin 28B (IL28B) gene, encoding type III IFN-λ3, were shown to be strongly associated with the response to peg-IFN and ribavirin treatment in patients with CHC [17–20] and with spontaneous clearance of HCV [21]. Host immune cells produce IFN and other cytokines in response to viral infection. In response to HCV, cellular sensors detect the double-stranded RNA via retinoic acid-inducible gene-I and toll-like receptor 3 and activate a pathway to produce antiviral cytokines, including alpha and beta IFNs that trigger an antiviral response to eradicate the virus [22,23].

Polymorphisms of genes involved in innate immunity are likely to influence the strength and nature of this defense system [24]. Moreover, IL28B polymorphisms were shown to be associated with lipid metabolism [25]. Thus, this genetic factor is thought to influence the natural course of HCV infection including liver fibrosis, inflammation activity, or steatosis. However, associations between IL28B polymorphisms and the state of background liver disease (fibrosis, inflammation activity, or steatosis) in patients with CHC remain controversial. Single studies may have limited statistical power to detect the modest effects of IL28B polymorphisms on disease progression.

Thus, we conducted the present meta-analysis to integrate the results of eligible studies and provide statistically reliable evidence of the role of IL28B polymorphisms in patients with CHC.

Materials and Methods

2.1 Search strategy

An electronic search was conducted in MEDLINE, EMBASE, and the Cochrane Library for articles published prior to 30 April, 2012. Search terms included *IL28B*, *IL28*, *IL-28B*, *interleukin-28B*, *interleukin 28B*, *rs12979860*, and *rs8099917*. The search was limited to the English language.

2.2 Inclusion criteria

A study was included in the current analysis if it satisfied the following criteria: (1) It evaluated the associations between IL28B polymorphisms (rs12979860 or rs8099917) and liver fibrosis, inflammation activity, or steatosis. We also included studies that evaluated fibrosis or inflammation activity using the aminotransferase platelet ratio index or ALT. (2) It provided sufficient published data for estimating odds ratios (OR) with 95% confidence intervals (CIs). In case of multiple studies based on the same population, we selected the study with the largest number of participants. A study was excluded if (1) it dealt only with co-infection of HCV and human immunodeficiency virus, (2) it dealt only with patients with a specific condition such as a comorbid disease (e.g., thalassemia) or status after liver transplantation, or (3) it only used a recessive hereditary model (rs12979860 CC + CT vs. TT, or rs8099917 TT +TG vs. GG).

2.3 Data extraction

Two authors (M.S. and M.K.) independently screened titles and abstracts for potential eligibility and full texts for final eligibility. Disagreements were resolved by consultation with a third author (R.T.). The following information was extracted or calculated from each study: first author, year of publication, country of origin, ethnicity, sex, HCV genotype, and background liver information (fibrosis, inflammation activity, or steatosis) for each genotype. The analysis was based on the dominant model (CC vs. CT and TT in rs12979860; TT vs. TG and GG in rs8099917).

2.4 Definition

In some studies, mild or severe fibrosis or inflammation activity was not defined. To compare results among studies on these outcomes, we defined Ishak level F4 to F6; METAVIR, Ludwig Batts, and Inuyama level F3 to F4; and Knodell histology activity index as severe fibrosis. We also defined METAVIR A2 to A3 as severe inflammation activity.

2.5 Statistical analysis

The association of liver fibrosis, inflammation activity, or steatosis with the IL28B genotype in patients with CHC was assessed by summary ORs and corresponding 95% CIs. Heterogeneity among studies was examined with I^2 statistics interpreted as the proportion of total variation contributed by between-study variation [26]. If there was no or low statistical heterogeneity among studies ($I^2<50\%$ and $P>0.05$), the ORs and 95% CIs were calculated by the fixed-effects model. Otherwise, the random-effects model was adopted. When significant heterogeneity was observed, we performed a meta-regression analysis to investigate relationships between the effect of IL28B polymorphisms on liver fibrosis, inflammation activity, or steatosis; and continuous variables (proportion of patients with genotype 1 or 4 virus infection, proportion of males; and proportion of Caucasian, African-American, and Asian patients) to explore the possible reason for heterogeneity between studies [27,28]. To check for publication bias, we used the linear regression approach described by Egger et al. [29]. All calculations were performed using Comprehensive Meta-Analysis software (Biostat, Englewood, NJ).

Results

3.1 Characteristics of articles

Figure 1 shows the literature search and study selection procedures. A total of 471 potentially relevant publications up to 30 April, 2012, were initially identified through MEDLINE, EMBASE, and the Cochrane Library, 443 of which were excluded because they did not meet our inclusion criteria. Therefore, 28 studies involving a total number of 10,024 patients were included in the meta-analysis. Study characteristics are shown in Table 1. There were 5616 males and 3974 females, and the sex was not reported in the remaining 434 patients (1 study). Nineteen studies (7542 patients) evaluated liver fibrosis according to rs12979860 polymorphism and 16 studies (5052 patients) according to rs8099917 polymorphism; four studies (2301 patients) evaluated inflammation activity according to rs12979860 polymorphism and eight studies (2904 patients) according to rs8099917 polymorphism; and four studies (962 patients) evaluated steatosis according to rs12979860 polymorphism and five studies (1308 patients) according to rs8099917 polymorphism.

3.2 Fibrosis

For rs12979860, the between-study heterogeneity was not significant ($I^2 = 25\%$, $P = 0.147$); thus, the fixed-effects model was applied. The pooled results indicated that IL28B rs12979860 genotype CC was associated with an increased possibility of severe fibrosis (OR, 1.122; 95%CI, 1.003–1.254; $P = 0.044$) (Fig. 2-a). For rs8099917, there was no or low heterogeneity ($I^2 = 31\%$, $P = 0.111$), and IL28B rs8099917 genotype TT tended to be associated with a higher possibility of severe fibrosis; however, the difference did not reach statistical significance (OR, 1.126; 95%CI, 0.988–1.284; $P = 0.076$) (Fig. 2-b). Egger's test showed no evidence for publication biases for either rs12979860 ($P = 0.839$) or rs8099917 ($P = 0.342$). When restricted to studies in which only treatment-naïve patients were included, 12 studies (5865 patients) according to rs12979860 polymorphism and eight studies (3333 patients) according to rs8099917 polymorphism were extracted. The between-study heterogeneities were not significant for rs12979860 ($I^2 = 0\%$, $P = 0.615$) and rs8099917 ($I^2 = 16\%$, $P = 0.304$). For rs12979860, fixed-effect model analyses showed a higher probability of severe fibrosis in genotype CC (OR, 1.184; 95%CI, 1.040–1.348; $P = 0.010$) (Fig. 2-c), and for rs8099917, genotype TT tended to be associated with a higher possibility of severe fibrosis; however, the difference was not statistically significant (OR, 1.154; 95%CI, 0.985–1.351; $P = 0.076$) (Fig. 2-d). Egger's test showed no evidence of publication bias ($P = 0.394$ for rs12979860 and $P = 0.295$ for rs8099917).

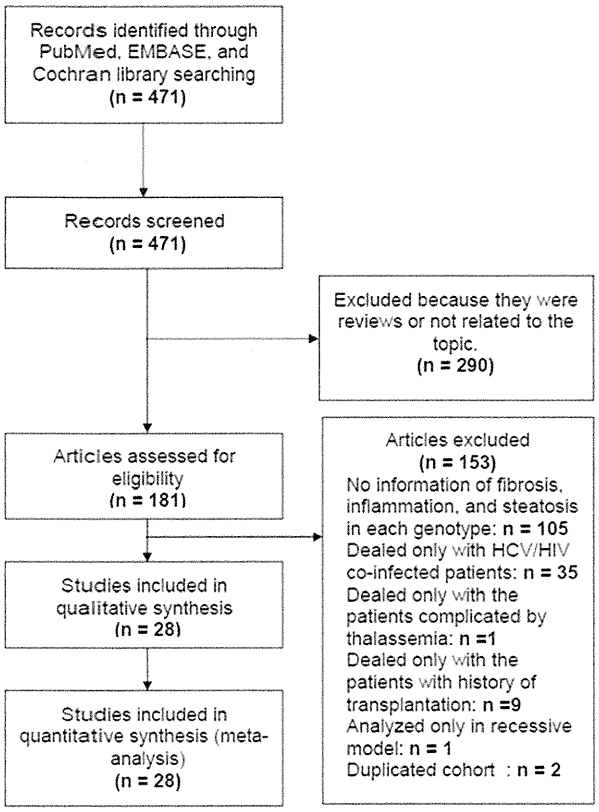

Figure 1. Literature search and study selection process. Twenty-eight individual studies that met all of the inclusion and exclusion criteria.
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3.3 Inflammation activity

The between-study heterogeneity was not significant ($I^2 = 35\%$, $P = 0.204$) for rs12979860. In the fixed-effects model, the pooled results indicated that IL28B rs12979860 genotype CC was associated with a higher possibility of severe inflammation activity (OR, 1.288; 95%CI, 1.050–1.581; $P = 0.015$) (Fig. 3-a). For rs8099917, there was no or low heterogeneity ($I^2 = 0\%$, $P = 0.598$), and IL28B rs8099917 genotype TT was also associated with a higher possibility of severe inflammation activity (OR, 1.324; 95%CI, 1.110–1.579; $P = 0.002$) (Fig. 3-b). Egger's test showed no evidence of publication biases for rs12979860 ($P = 0.448$) and rs8099917 ($P = 0.531$). When restricted to studies in which only treatment-naïve patients were included, three studies (2192 patients) according to rs12979860 polymorphism and two studies (1769 patients) according to rs8099917 polymorphism were extracted. Significant heterogeneities were found for rs12979860 ($I^2 = 53\%$, $P = 0.120$); thus, the random-effect model was applied. The pooled results indicated that IL28B rs12979860 genotype was not associated with inflammatory activity (OR, 1.340; 95%CI, 0.938–1.916; $P = 0.108$) (Fig. 3-c). For rs8099917, the between-study heterogeneity was not significant ($I^2 = 0\%$, $P = 0.585$). In the fixed-effects model, genotype TT tended to be associated with a higher possibility of severe inflammation activity (OR, 1.217; 95%CI, 0.978–1.515; $P = 0.079$) (Fig. 3-d). Egger's test showed no evidence of publication bias in rs12979860 ($P = 0.646$). For rs8099917, Egger's test was not applicable because only 2 studies were included. We also performed a meta-regression analysis for rs12979860 because significant heterogeneities were observed. Table 2 shows the results of these meta-regression analyses. Significant correlation was observed between rs12979860 polymorphisms and the proportion of patients with genotype 1 or 4 virus (slope, 2.992 ± 1.497; $P = 0.046$).

3.4 Steatosis

Significant heterogeneities were found for rs12979860 ($I^2 = 86\%$, $P<0.001$) and rs8099917 ($I^2 = 52\%$, $P = 0.082$); thus, we applied the random-effects model for this outcome. The pooled results indicated that IL28B rs12979860 genotype CC was not associated with hepatic steatosis (OR, 1.062; 95%CI, 0.415–2.717, $P = 0.901$) (Fig. 4-a), whereas rs8099917 TT was significantly associated with a lower possibility of severe steatosis (OR, 0.580; 95%CI, 0.351–0.959; $P = 0.034$) (Fig. 4-b). Egger's test showed no evidence of publication biases for rs12979860 ($P = 0.238$) or rs8099917 ($P = 0.182$). We also performed a meta-regression analysis because significant heterogeneities were observed. Table 3 shows the results of these meta-regression analyses. In terms of the effect of rs12979860 on steatosis, significant correlations were observed between the proportion of patients with genotype 1 or 4 virus (slope, -4.947 ± 1.086; $P<0.001$), the proportion of Caucasian patients (slope, 7.361 ± 1.569; $P<0.001$), and the proportion of African-American patients (slope, -8.996 ± 1.918; $P<0.001$). We also observed a significant correlation between the effect of rs8099917 polymorphism on steatosis and the proportion of male patients (slope, 6.225 ± 2.530; $P = 0.014$) (Fig. 5). Finally, we observed significant correlations between rs8099917 polymorphisms and the proportion of patients with genotype 1 or 4 virus (slope, -2.704 ± 1.277; $P = 0.034$), the proportion of Caucasian patients (slope, 1.168 ± 0.422; $P = 0.006$), and the proportion of Asian patients (slope, -1.049 ± 0.398; $P = 0.008$). When restricted to studies in which only treatment-naïve patients were included, two studies (495 patients) according to rs12979860 polymorphism and four studies (812 patients) according to rs8099917 polymorphism were extracted. The between-study heterogeneities were not significant for rs12979860 ($I^2 = 0\%$, $P = 0.823$) and rs8099917 ($I^2 = 41\%$, $P = 0.166$). For rs12979860, fixed-effect model analyses showed that rs12979860 genotype CC was significantly associated with a higher possibility of severe steatosis (OR, 1.708; 95%CI, 1.047–2.787; $P = 0.032$) (Fig. 4-c), whereas rs8099917 TT was significantly associated with a lower possibility of severe steatosis (OR, 0.675; 95%CI, 0.474–0.960; $P = 0.026$) (Fig. 4-d). Egger's test showed no evidence of publication bias in rs8099917 ($P = 0.554$). For rs12979860, Egger's test was not applicable because only 2 studies were included.

Discussion

In the present study, we evaluated the association between IL28B polymorphisms and the background liver disease (fibrosis, inflammation activity, or steatosis) in patients with CHC. The rs12979860 CC genotype was significantly associated with a higher probability of severe fibrosis (Fig. 2-c), and the rs8099917 TT genotype tended to be associated with a higher possibility of severe fibrosis (Fig. 2-d). The accumulation of liver inflammation promotes liver fibrosis, and these polymorphisms are associated with the effect of IFN-based treatment; therefore, past treatment might alter the results. Thus, we also analyzed studies involving only patients without a history of IFN-based treatment; however, the results were not changed.

The rs12979860 CC and rs8099917 TT genotypes were also associated with a higher possibility of severe inflammation activity. Genetic variations near the IL28B gene were originally reported as

Table 1. Main characteristics of all studies included in the meta-analysis.

First author (year)	Ref.	Population ethnicity, region	IL-28B SNP rsID, Allele	Outcome measure F(Fibrosis) A(Activity) S(Steatosis)	Patients*			HCV genotype	Genotype for patients rs12979860		Genotype for patients rs8099917	
					Male	Female	Total		CC	CT/TT	TT	TG/GG
Abe (2010)	[48]	Asian, Japan	rs8099917 T/G	F, A: Inuyama	212	152	364	1/2			265	99
Honda (2010)	[49]	Asian, Japan	rs8099917 T/G	F, A: Inuyama	58	33	91	1			60	31
Lotrich (2010)	[50]	Mixed (African-American/Caucasian), USA	rs12979860 C/T	F: Ishak	101	32	133	1/2	57	76		
Monte (2010)	[51]	Caucasian, Spain	rs12979860 C/T	F: Scheuer	166	117	283	1–4	129	154		
Thompson (2010)	[52]	Mixed (African-American/Caucasian/Asian/Hispanic), USA	rs12979860 C/T	F: METAVIR	986	642	1628	1	538	1090		
Bochud (2011)	[53]	Caucasian, Switzerland	rs12979860 C/T rs8099917 T/G	F: Ishak, A: ALT S: Histological finding	163	79	242	1–3	90	150	150	92
Dill MT (2011)	[54]	Caucasian, Switzerland	rs12979860 C/T rs8099917 T/G	F, A: METAVIR	30	79	109	1–4	33	96	52	57
Fabris (2011)	[44]	Caucasian, Italy	rs12979860 C/T	F: Ishak	N.A	N.A	434	1–4	133	301		
Falleti (2011)	[55]	Caucasian, Italy	rs12979860 C/T	F: Ishak	357	272	629	1–4	205	424		
Kurosaki (2011)	[56]	Asian, Japan	rs8099917 T/G	F: METAVIR S: Histological finding	250	246	496	1			269	106
Lagging (2011)	[57]	Caucasian, Sweden	rs12979860 C/T rs8099917 T/G	F: Ishak S: Histological finding	169	83	252	1–4	93	159	153	99
Lin (2011)	[58]	Asian, Taiwan	rs12979860 C/T rs8099917 T/G	F: METAVIR	123	68	191	1	171	20	170	21
Lindh (2011)-1	[59]	Mixed (Caucasian/Asian), Sweden	rs12979860 C/T rs8099917 T/G	F: Batts Ludwig	67	43	110	1	38	72	66	44
Lindh (2011)-2	[60]	Caucasian, Sweden	rs12979860 C/T	F: Ishak	204	137	341	2/3	150	191		
Marabita (2011)	[61]	Caucasian, Italy	rs12979860 C/T rs8099917 T/G	F: Ishak	129	118	247	1–4	88	159	131	116
Miyamura (2011)	[62]	Asian, Japan	rs8099917 T/G	F, A: Inuyama	37	42	79	1			53	26
Moghaddam(2011)	[63]	Caucasian, Norway	rs12979860 C/T rs8099917 T/G	F: APRI score	166	115	281	3	129	152	201	80
Rueda (2011)	[64]	Caucasian, Spain	rs12979860 C/T	F, A: Scheuer	246	177	423	1–4	83	184		
Tillman (2011)	[35]	Mixed (African-American/Caucasian/Asian), USA	rs12979860 C/T rs8099917 T/G	S: Histological finding	215	110	325	1	88	237	97	67
Yu (2011)	[65]	Asian, Taiwan	rs8099917 T/G	F: Knodell and Scheuer	264	218	482	2			315	34
Asahina (2011)	[66]	Asian, Japan	rs12979860 C/T rs8099917 T/G	F: Inuyama	28	60	88	1	54	34	54	34

Table 1. Cont.

First author (year)	Ref.	Population ethnicity, region	IL-28B SNP rsID, Allele	Outcome measure F(Fibrosis) A(Activity) S(Steatosis)	Patients*			HCV genotype	Genotype for patients rs12979860		Genotype for patients rs8099917	
					Male	Female	Total		CC	CT/TT	TT	TG/GG
Bochud (2012)	[47]	Caucasian, Switzerland	rs12979860 C/T rs8099917 T/G	F, A: METAVIR	870	657	1527	1–4	534	993	855	672
Mach (2012)	[67]	Slav: Poland	rs12979860 C/T	F: Batts Ludwig	82	60	142	1	38	104		
Miyashita (2012)	[68]	Asian, Japan	rs8099917 T/G	F, A: Desmet	88	132	220	1/2			155	63
Ohnishi (2012)	[69]	Asian, Japan	rs8099917 T/G	S: Histological finding	83	70	153	1			116	37
Rembeck (2012)	[70]	Caucasian, Sweden	rs12979860 C/T	F: Ishak	199	140	339	2/3	144	179		
Tolmane (2012)	[71]	Caucasian, Latvia	rs12979860 C/T	F: Knodell histology activity index S: Histological finding	84	58	142	1–3	41	80		
Toyoda (2012)	[72]	Asian, Japan	rs8099917 T/G	F, A: METAVIR	139	133	272	1			187	59

*Patients included in the original study.
Thus, patients without information regarding IL28B polymorphism were also included.
APRI, aminotransferase platelet ratio index.
doi:10.1371/journal.pone.0091822.t001

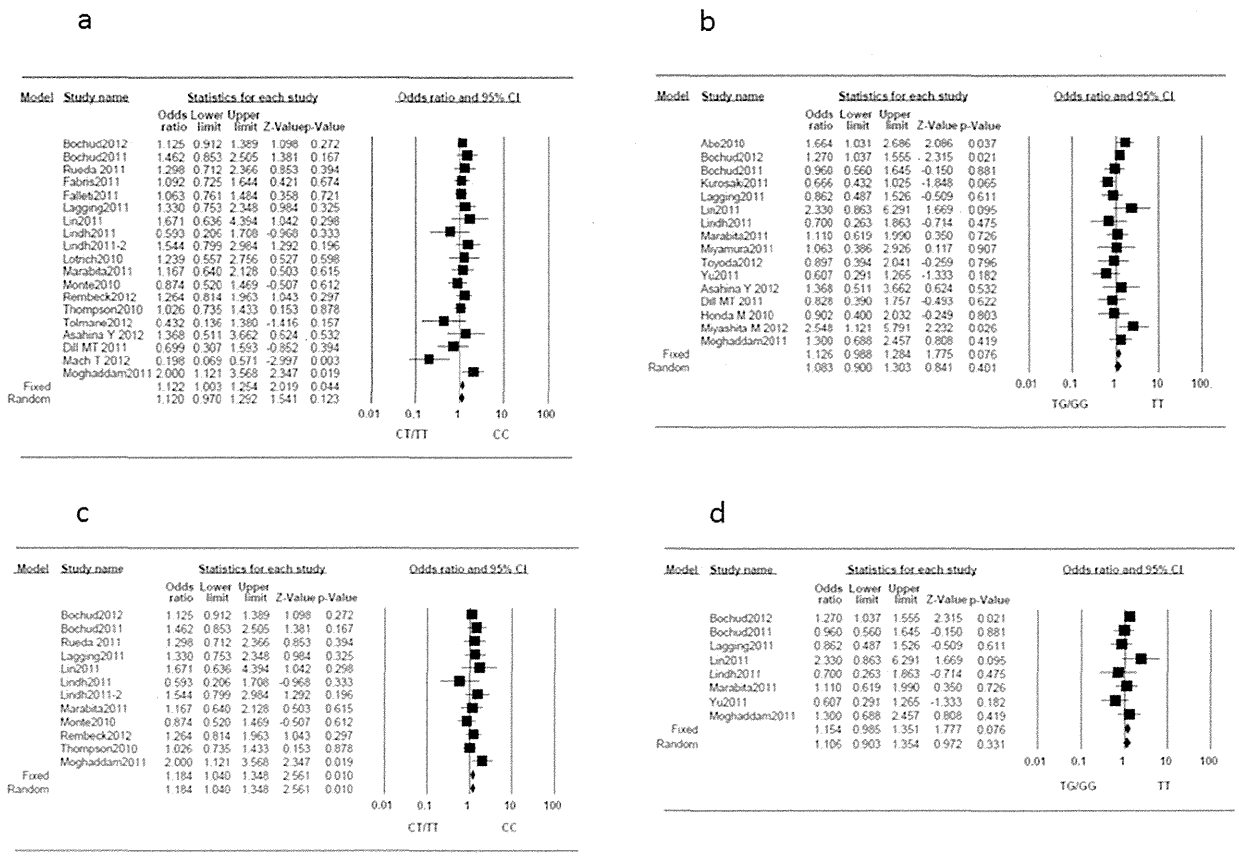

Figure 2. Forest plot of the IL28B genotypes and the risk of severe fibrosis. (a) rs12979860 in all patients, (b) rs8099917 in all patients, (c) rs12979860 in treatment-naïve patients, and (d) rs8099917 in treatment-naïve patients.
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strong predictors of a sustained viral response [17–20] or spontaneous clearance of HCV [21]. The level of IL28B gene transcripts is reportedly higher in patients homozygous for the IFN responsive allele [18,19]. Therefore, in patients with the rs12979860 CC and rs8099917 TT genotype, IL28B production, which induces expression of interferon-stimulated genes, including some inflammatory cytokines, was thought to be increased. This may be the underlying cause of the higher inflammation activity and progressed fibrosis in patients with the IFN responsive allele. In analysis with the studies involving only patients without a history of IFN-based treatment, rs12979860 CC and rs8099917 TT genotypes were associated with higher possibility of having severe inflammation activity; however, the differences did not reach to the significant level. Only three studies according to rs12979860 polymorphism and two studies according to rs8099917 polymorphism were included when restricted to studies with only treatment-naïve patients, and may be underpowered to detect the effects of IL28B polymorphisms on inflammation activity. The further analyses with larger sample are needed to confirm this association. Additionally, meta-regression analysis showed that the effect of the rs12979860 polymorphism was influenced by viral genotype distribution. This result may imply a different influence of rs12979860 polymorphism on immune response according to viral genotype in treatment-naïve patients.

IL28B polymorphisms were also shown to be associated with lipid metabolism [25]. In the present study, the rs8099917 TT genotype was significantly associated with a lower possibility of severe steatosis. This association still remained statistically significant after we restricted to studies in which only treatment-naïve patients were included. The lower hepatic steatosis in patients with the IFN responsive allele could be explained by a more efficient export of lipids from hepatocytes. Higher interferon expression was shown to lead to suppression of lipoprotein lipase, which would result in decreased conversion of VLDL to LDL and subsequent higher steatosis [30–33]. The difference in IL28B expression might cause an aberration of lipid metabolism in patients with CHC. We found no significant association of rs12979860 with steatosis. And when we restricted to treatment-naïve patients, rs12979860 CC genotype was significantly associated with a higher possibility of severe steatosis. Previous studies have shown that racial differences or viral genotypes make a difference in the effects of rs12979860 and rs8099917 polymorphisms [34,35]. This may explain the discrepancy between the effect of rs12979860 and rs8099917 on hepatic steatosis. However, only four studies (962 patients) were included in the analysis of rs12979860; or when it comes to the studies with only treatment-naïve patients, only two studies (495 patients) were extracted. Thus, we should not make any definite conclusion on this matter right now. Further studies with larger sample sizes are needed to identify their exact correlation.

According to the meta-regression analysis, the effect of rs8099917 polymorphisms on steatosis became smaller with the

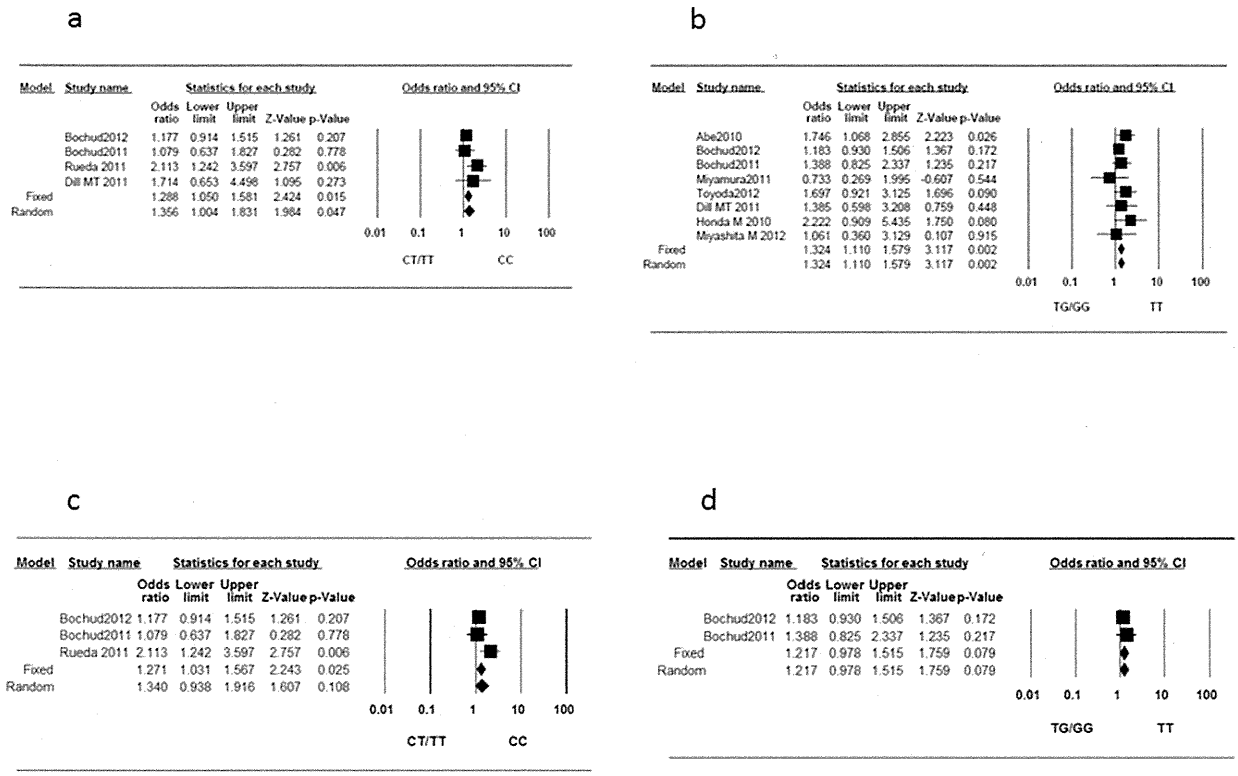

Figure 3. Forest plot of the IL28B genotypes and the risk of severe inflammation activity. (a) rs12979860 and (b) rs8099917. (c) rs12979860 in treatment-naïve patients, and (d) rs8099917 in treatment-naïve patients.
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increase in the male proportion (Fig. 5), suggesting that a sexual dimorphism might be involved in the effect of rs8099917 polymorphisms on the liver fat content. Although the present study cannot explain the interaction between the polymorphism and sex, immune systems responding to IFN are reportedly controlled by estrogenic sex hormones [36,37]. Differences in IL28B expression mediated by sex hormones could be a possible mechanism for the sexual dimorphism in the effect of rs8099917 polymorphisms on liver steatosis.

The rs738409 genotype within the patatin-like phospholipase domain containing 3 locus was also reported to be associated with hepatic steatosis in patients with CHC [38–40]. Notably, previous meta-analysis evaluating the effect of patatin-like phospholipase domain containing 3 polymorphisms on steatosis also reported a

Table 2. Meta-regression analysis between each continuous variable among the studies (only treatment- naïve patients were included) and the effect (log odds ratio) of IL28B polymorphisms on inflammation activity.

Variables	Slope*	Standard error	P-value
Proportion of patients with genotype 1 or 4 virus, per 1% increase			
rs12979860	**2.992**	**1.497**	**0.046**
Proportion of male patients, per 1% increase			
rs12979860	**−2.963**	**5.802**	**0.610**
Proportion of Caucasian patients, per 1% increase			
rs12979860†	**–**	**–**	**–**
Proportion of African-American patients, per 1% increase			
rs12979860†	**–**	**–**	**–**
Proportion of Asian patients, per 1% increase			
rs12979860†	**–**	**–**	**–**

*Positive (negative) slope values indicate that the proportions of patients with the rs12979860 CC genotype with severe inflammation activity are increasing (decreasing) as the values of each contentious variable (proportions of genotype 1 or 4 virus, male, or each race) is increasing.
†We could not perform meta-regression analyses for these outcomes because only caucasian patients were included in all 3 studies included in this analysis.
doi:10.1371/journal.pone.0091822.t002

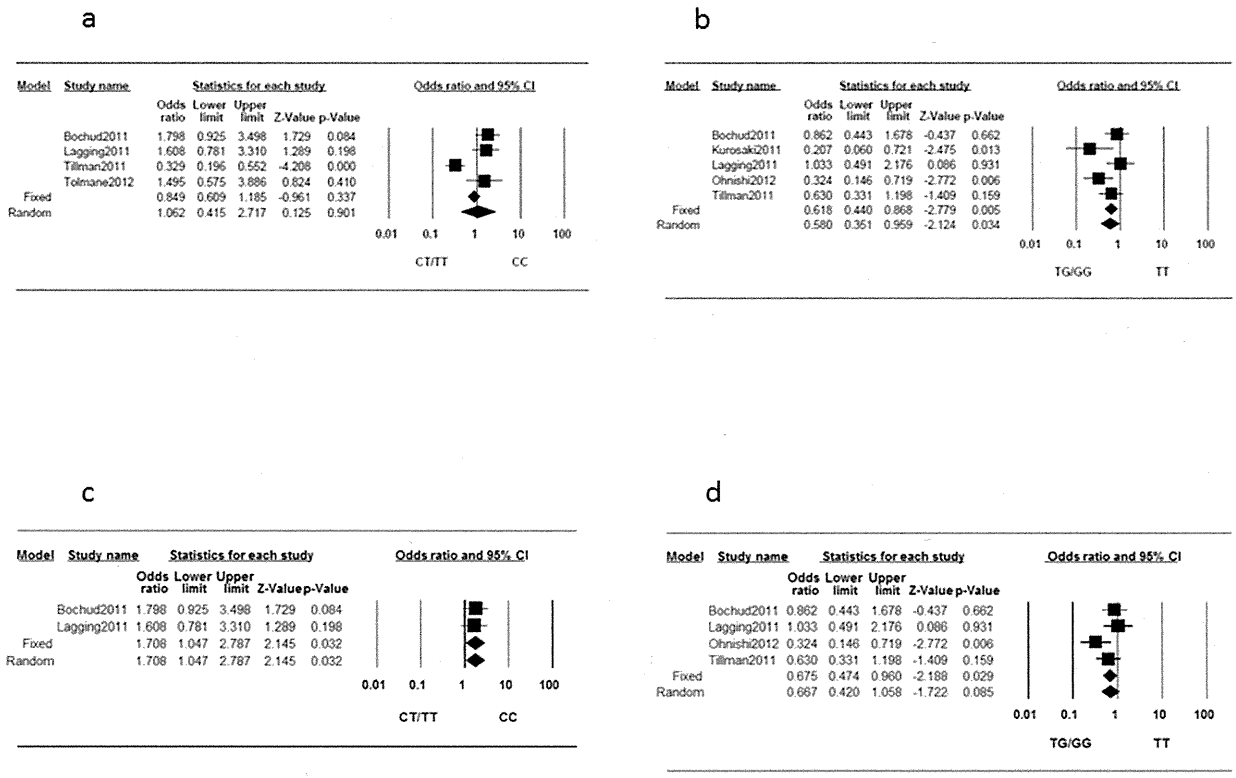

Figure 4. Forest plot of the IL28B genotypes and the risk of hepatic steatosis. (a) rs12979860 and (b) rs8099917. (c) rs12979860 in treatment-naïve patients, and (d) rs8099917 in treatment-naïve patients.
doi:10.1371/journal.pone.0091822.g004

Table 3. Meta-regression analysis between each continuous variable among the studies and the effect (log odds ratio) of IL28B polymorphisms on steatosis.

Variables	Slope*	Standard error	P-value
Proportion of patients with genotype 1 or 4 virus, per 1% increase			
rs12979860	−4.947	1.086	<0.001
rs8099917	−2.704	1.277	0.034
Proportion of male patients, per 1% increase			
rs12979860	−2.899	16.577	0.861
rs8099917	6.225	2.530	0.014
Proportion of Caucasian patients, per 1% increase			
rs12979860	7.361	1.569	<0.001
rs8099917	1.168	0.422	0.006
Proportion of African-American patients, per 1% increase			
rs12979860	−8.996	1.918	<0.001
rs8099917	0.142	2.147	0.947
Proportion of Asian patients, per 1% increase			
rs12979860†	–	–	–
rs8099917	−1.049	0.398	0.008

*Positive (negative) slope values indicate that the proportions of patients with the rs12979860 CC or rs8099917 TT genotypes with severe steatosis are increasing (decreasing) as the values of each contentious variable (proportions of genotype 1 or 4 virus, male, or each race) is increasing.
†We could not perform a meta-regression analysis for this outcome because only one patient was included in the corresponding studies.
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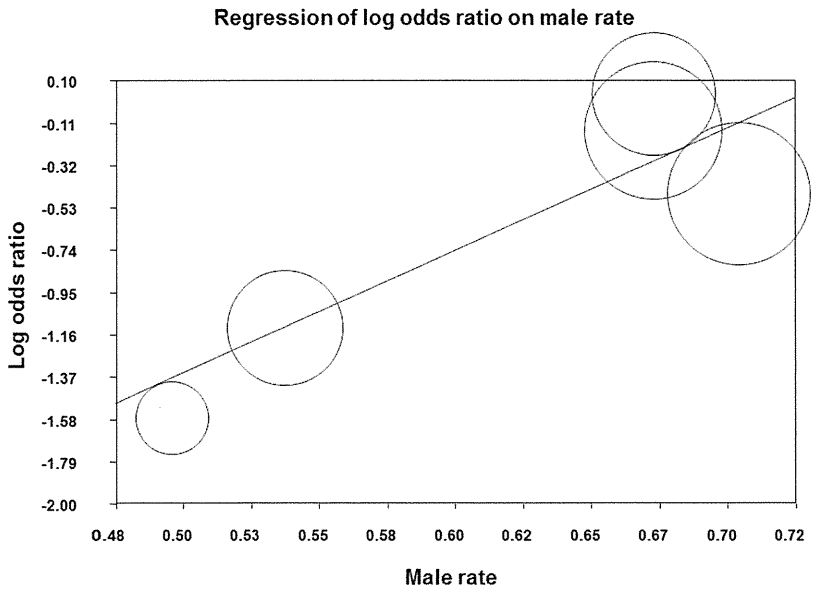

Figure 5. Meta-regression plot for log odds ratios in rates of patients with severe hepatic steatosis by proportion of males (%) in rs8099917.
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negative correlation between the male proportion and the effect of rs738409 on the liver fat content in nonalcoholic fatty liver disease [41]. Interestingly, the meta-regression analysis in the present study showed that the effect of the IL28B (rs12979860 and rs8099917) polymorphisms on steatosis was also influenced by racial and viral genotype distributions.

In the present study, we included studies that did not report the associations between IL28B genotypes and background liver diseases as study outcomes, but provided raw data that allowed us to calculate the OR for each outcome, which may have minimized potential publication bias. In fact, no publication bias was observed in the present study. The Human Genome Epidemiology Network highlighted the necessity of meta-analysis before evidence for a particular association can be regarded as strong [42]. The impact of IL28B genotypes on the disease progression found in the present meta-analysis may provide clinically important information in the follow-up of patients with CHC. The effect of IL28B polymorphisms on hepatocarcinogenesis, which is also crucial information in the HCC screening of patients with CHC, remains controversial [43–47]. Further analysis with larger sample sizes may be needed to elucidate the exact effect of IL28B polymorphisms on hepatocarcinogenesis.

A potential limitation of this study is inter-study variability in the outcome measure and the definition of "severe" among studies, where some discrepancies among studies exist. The studies without a pathological diagnosis, using laboratory data as surrogates, were also included. These studies may have diminished the accuracy of our research results concerning liver disease severity.

In conclusion, the present study highlighted the impact of IL28B polymorphisms on liver fibrosis, inflammation activity, and steatosis in patients with CHC. Disease progression appeared to be promoted in patients with rs12979860 CC or rs8099917 TT genotypes. The current findings may provide clinically important information in the follow-up of patients with CHC.

Supporting Information

Checklist S1 PRISMA 2009 Checklist.
(DOC)

Acknowledgments

The English in this document has been checked by at least two professional editors, both native speakers of English. For a certificate, please see: http://www.textcheck.com/certificate/IWcYpT.

Author Contributions

Conceived and designed the experiments: MS RT NK. Performed the experiments: MS MK RT. Analyzed the data: MS RT. Contributed reagents/materials/analysis tools: MS. Wrote the paper: MS RT HY. Critical revision of manuscript: NF MT KK.

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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

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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

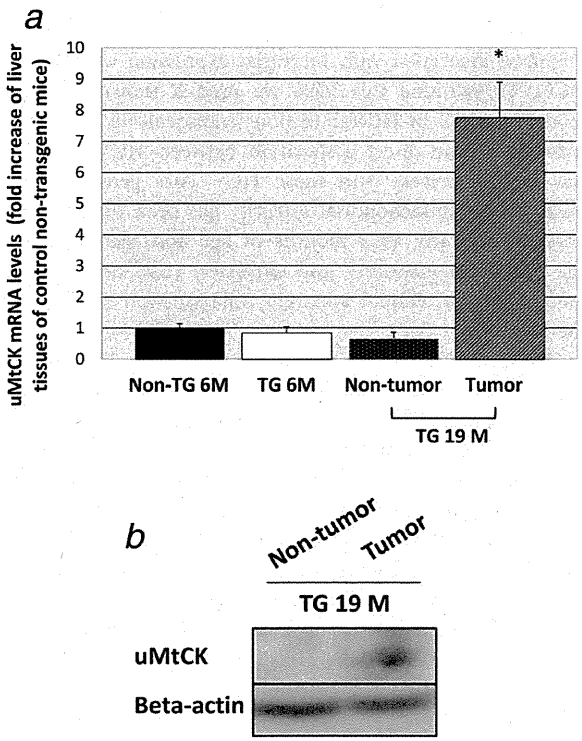

Figure 1. uMtCK mRNA and protein levels in liver tissues of the control non-transgenic, HCV core gene transgenic mice. (*a*) uMtCK mRNA levels were examined by real-time PCR in liver tissues of the control non-transgenic mice (Non-TG) at 6 months of age ($n = 4$), and HCV core gene transgenic mice (TG)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

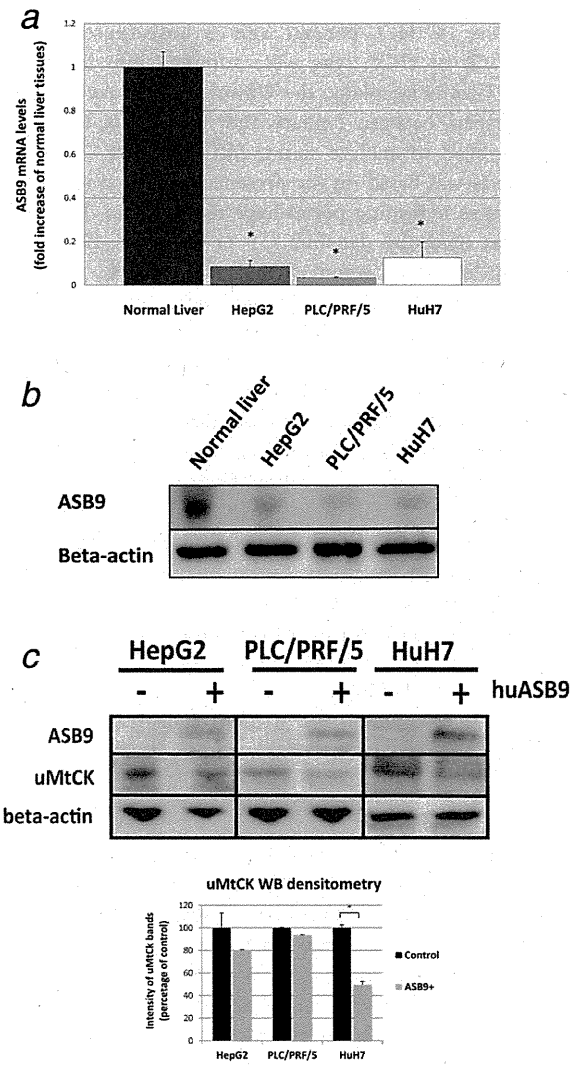

Figure 2. ASB9 expression and the effect of ASB9 transfection on uMtCK protein levels in HCC cells. ASB9 mRNA (*a*) and protein (*b*) levels in HepG2, PLC/PRF/5 and HuH7 cells were examined by real-time PCR and immunoblot analysis, respectively. As a positive control for ASB9 mRNA and protein expressions, 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

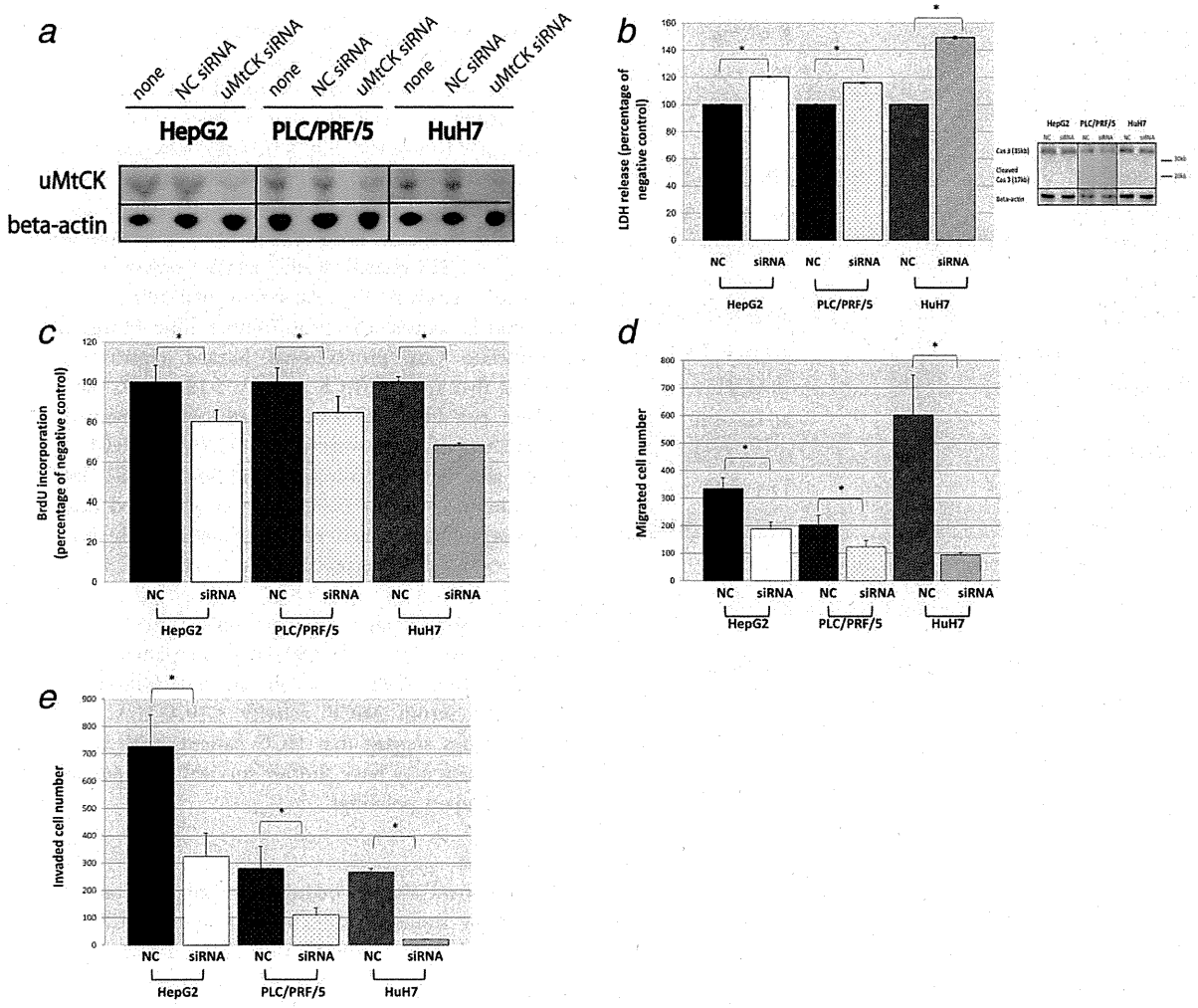

Figure 3. Increase in cell death and reduction in proliferation, migration and invasion by reduced uMtCK expression with siRNA in HCC cell lines. Human HCC cell lines, HepG2, PLC/PRF/5 and HuH7 cells, were transfected with 20 nM uMtCK siRNA or universal negative control, and uMtCK levels were examined 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

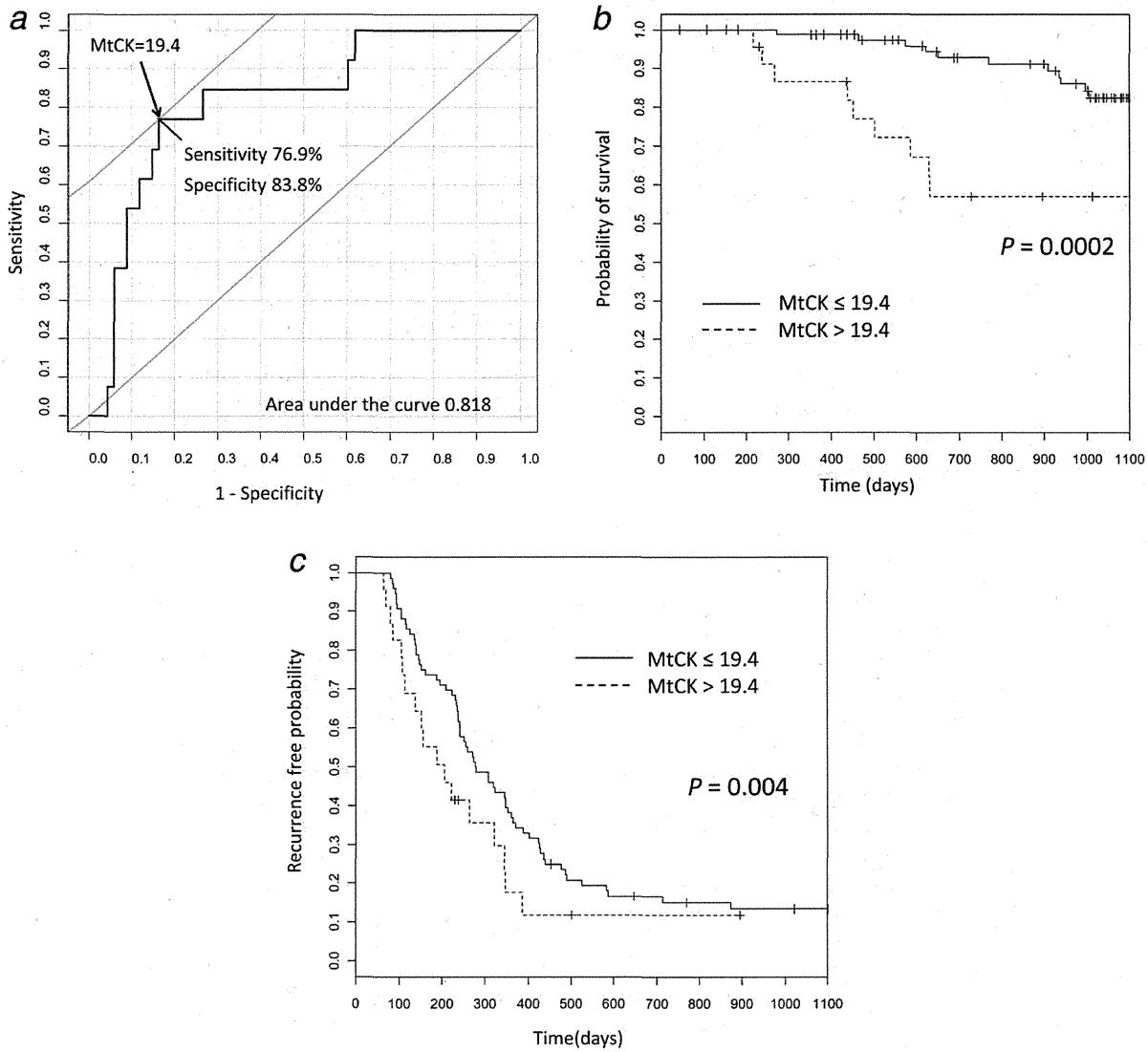

Figure 4. (*a*) ROC curve showing the overall accuracy of serum MtCK activity for discriminating between survivors and deceased patients. The arrow identifies the best cutoff value (*i.e.*, 19.4 U/L) of serum MtCK activity. Kaplan–Meier survival (*b*) and recurrence (*c*) curve of the studied patients 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.