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RAPID COMMUNICATION

Daclatasvir Plus Asunaprevir for Chronic HCV Genotype 1b Infection

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All-oral combinations of direct-acting antivirals may improve efficacy and safety outcomes for patients with hepatitis C virus (HCV) infection, particularly those who are poor candidates for current interferon/ribavirin-based regimens. In this open-label, phase 3 study, 135 interferon-ineligible/intolerant and 87 nonresponder patients with chronic HCV genotype 1b infection were enrolled at 24 centers in Japan. Patients received daclatasvir 60 mg once daily plus asunaprevir 100 mg twice daily for 24 weeks. The primary endpoint was sustained virologic response 24 weeks after treatment (SVR_{24}). This study is registered with Clinical Trials.gov (NCT01497834). SVR_{24} was achieved by 87.4% of interferon-ineligible/intolerant patients and 80.5% of nonresponder (null and partial) patients; rates were similar in cirrhosis (90.9%) and noncirrhosis (84.0%) patients, and in patients with *IL28B* CC (84.5%) or non-CC (84.8%) genotypes. Fourteen patients in each group (12.6%) discontinued dual therapy, mainly due to adverse events or lack of efficacy. Nine nonresponder patients received additional treatment with peginterferon/ribavirin per protocol-defined criteria. The rate of serious adverse events was low (5.9%) and varied among patients. The most common adverse events were nasopharyngitis, increased alanine aminotransferase (ALT) and aspartate aminotransferase (AST), headache, diarrhea, and pyrexia. *Conclusion:* Interferon-free, ribavirin-free all-oral therapy with daclatasvir and asunaprevir for 24 weeks is well tolerated and can achieve a high rate of SVR in patients with HCV genotype 1b who were ineligible, intolerant, or had not responded to prior interferon-based therapy. (HEPATOLOGY 2014;59:2083-2091)

Treatment of chronic hepatitis C virus (HCV) infection typically includes a regimen of interferon-based therapy plus ribavirin, with or without a direct-acting antiviral. The efficacy and tolerability of these regimens are not ideal, and there remains a large number of patients for whom these treatments are not acceptable or viable. The addition of direct-acting antivirals can improve treatment outcomes for patients infected with chronic HCV. When combined with peginterferon and ribavirin, the HCV protease inhibitors telaprevir, boceprevir, or simeprevir achieved overall sustained virologic response (SVR) rates ranging from 68% to 89% in treatment-naïve patients with HCV genotype 1 infection.[1-3] Patients with no response to previous peginterferon/ribavirin therapy did not respond as well to this combined regimen, with rates of SVR ranging from 34% to 52%.[3-5] In Japan, patients chronically infected with HCV are older and predominantly infected with HCV genotype 1, both factors which impact response to therapy.[6] For Japanese patients who had no prior response to treatment with peginterferon/ribavirin, telaprevir or

Abbreviations: HCV, hepatitis C virus; LLOQ, lower limit of quantitation; NS, nonstructural; SVR, sustained virologic response; TD, target detected; TND, target not detected.

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simeprevir plus peginterferon and ribavirin provided SVR rates of only 34% or 38-51%, respectively.[3,7] The array of adverse events associated with peginterferon and ribavirin is well known; incremental toxicities associated with the addition of telaprevir to peginterferon and ribavirin included anemia, skin disorders and severe rash, and gastrointestinal-related disorders, while the addition of simeprevir is associated with hyperbilirubinemia due to inhibition of hepatic bilirubin transporters.[3] For patients who cannot tolerate or are not eligible for treatment with interferon-based therapy because of coexisting morbidities, treatment options are few to none. Clearly, the current treatment options are not adequate and an urgent unmet need remains for better treatment regimens for these patient populations.

Daclatasvir is a first-in-class, NS5A replication complex inhibitor with potent pan-genotypic antiviral activity *in vitro* (HCV genotypes 1-6).[8] Asunaprevir is a potent, selective NS3 protease inhibitor with antiviral activity against HCV genotypes 1, 4, 5, and 6 *in vitro*.[9] Both daclatasvir and asunaprevir have demonstrated robust antiviral activity, with no clinically meaningful pharmacokinetic interactions between them when coadministered.[8,10,11] Preliminary phase 2 studies showed potent antiviral effects using daclatasvir and asunaprevir as an all-oral therapy and in combination with a regimen of peginterferon/ribavirin in patients infected with HCV genotype 1 who had not responded to prior therapy.[12,13] We evaluated the safety and antiviral activity of interferon-free, ribavirin-free, all-oral therapy with daclatasvir and asunaprevir in a phase 3 trial involving Japanese patients infected with HCV genotype 1b who are interferon-ineligible/intolerant or nonresponders (null and partial) to interferon-based therapies.

Materials and Methods

Patients. A total of 259 patients were enrolled at 24 centers in Japan from January 5 2012 to March 30 2012. Eligible patients were men and women, 20 to 75 years of age, with chronic HCV genotype 1b infection, an HCV RNA level of 10^5 IU/mL or higher, with a body-mass index of 16 to 35 kg/m^2, and, in up to 10% of enrolled patients, evidence of compensated cirrhosis (Child-Pugh A), as documented either by liver biopsy or discriminated by a previously described algorithm.[14]

Key exclusion criteria included evidence of hepatocellular carcinoma, coinfection with hepatitis B virus or human immunodeficiency virus, or previous exposure to inhibitors of NS5A or NS3 protease. Patients with alanine aminotransferase (ALT) of more than 5 times the upper limit of normal range, total bilirubin of 2 mg/dL or higher, an international normalized ratio of 1.7 or higher, an albumin level 3.5 g/dL or below, and a platelet count of less than 50,000/mm^3 were also excluded.

Patients ineligible for interferon-based therapy, but potentially eligible for enrolment in this study, were treatment-naïve and considered poor candidates for interferon-based therapy because of medical complications including anemia, neutropenia, thrombocytopenia, depression, advanced age ($\geq$65 years), or other conditions deemed not suitable for interferon-based therapy by the investigator, including hypertension, diabetes mellitus, autoimmune disease, and abnormal thyroid function. Patients intolerant to interferon-based therapy had received interferon-based therapy for less than 12 weeks and previously discontinued from therapy due to toxicities associated with interferon or ribavirin. Patients who were null or partial responders to previous peginterferon/ribavirin or interferon-beta/ribavirin therapy were defined as never having attained an undetectable HCV RNA level after at least 12 weeks of therapy. Null responders included patients who never attained at least a 2-log$_{10}$ decrease from baseline in HCV RNA levels at week 12, and partial responders never achieved undetectable HCV RNA levels after 12 weeks of therapy.

Study Design. In this open-label, phase 3 study of two patient cohorts, interferon-ineligible/intolerant and

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nonresponder patients received daclatasvir and asunaprevir for 24 weeks. Patients were followed for an additional 24 weeks after treatment. Daclatasvir was administered orally at a dose of 60 mg once daily, and asunaprevir was administered orally at a dose of 100 mg twice daily. Host *IL28B* genotype was assayed for the rs12979860 single-nucleotide polymorphism by Monogram Biosciences using a real-time polymerase chain reaction (PCR) assay.

Nonresponder patients who met futility criteria, defined as an increase in viral load of at least 1 $\log_{10}$ or confirmed detectable HCV RNA of at least 15 IU/mL on or after week 8, were eligible for addition of peginterferon-alpha/ribavirin to continued treatment with daclatasvir and asunaprevir for an additional 24 weeks at the discretion of the investigator. Interferon-ineligible/intolerant patients were not candidates for interferon-based therapy; therefore, daclatasvir/asunaprevir dual therapy was stopped if futility criteria were met.

Study Oversight. This study was approved by the Institutional Review Board at each participating site and was conducted in compliance with the Declaration of Helsinki, Good Clinical Practice guidelines, and local regulatory requirements. All patients provided written informed consent.

Efficacy Assessments. HCV RNA levels were measured using the Roche COBAS Taqman test with a lower and an upper limit of quantitation of 15 IU/mL and 6.9 $\times$ 10^7 IU/mL, respectively. HCV RNA was measured at screening and at day 1, weeks 1, 2, 4, 6, 8, 10, 12, 16, 20, and 24, and posttreatment at weeks 4, 8, 12, and 24.

Resistance Testing. Patient-derived HCV NS5A and NS3/4A sequence populations were PCR-amplified and sequenced. Patient samples selected for sequencing included all baseline samples and samples from patients with virologic failure.

Safety Assessments. Safety evaluations included reported adverse events and serious adverse events, clinical laboratory tests, physical examinations, and electrocardiograms.

Endpoints. The primary efficacy endpoint was the proportion of patients with HCV RNA <15 IU/mL (target detected [TD] or target not detected [TND]) at 24 weeks after completion of daclatasvir and asunaprevir treatment, including patients who discontinued treatment early. Key secondary endpoints included the proportion of patients with undetectable HCV RNA (TND) at weeks 4 and 12, at the end of treatment, and HCV RNA <15 IU/mL (TD or TND) at 12 weeks after the end of treatment. Safety endpoints included the frequency of serious adverse events, adverse events, discontinuations due to adverse events, and laboratory abnormalities.

Statistical Analysis. Analyses included all patients who received at least one dose of study medications. For virologic response, 2-sided 95% confidence intervals were calculated based on the normal approximation to the binomial distribution. Categorical variables were summarized using counts and percents. Continuous variables were summarized with univariate statistics. Patients with missing data or those who received additional peginterferon/ribavirin therapy were considered failures.

Role of the Funding Source. The study was designed and conducted by the sponsor (Bristol-Myers Squibb/Bristol-Myers KK) in collaboration with the principal investigators. The sponsor collected the data, monitored the study conduct, and performed the statistical analyses. All authors had access to the data and assume responsibility for the accuracy, integrity, and completeness of the reported data and for the fidelity of this report to the trial protocol. The article was prepared by authors employed by Bristol-Myers Squibb, with input from all authors and the assistance of a medical writer employed by Bristol-Myers Squibb. All authors made the decision to submit the article for publication.

Results

Patients. In all, 222 patients received treatment, 135 in the interferon-ineligible/intolerant group (100 medically ineligible for interferon, 35 intolerant to interferon) and 87 in the nonresponder group (48 null responders, 36 partial responders, 3 undetermined) (Fig. 1). Demographic baseline characteristics of patients are shown in Table 1. As expected, when compared with reported demographics from U.S. and European studies, patients were older and a larger proportion were female. Similar to the global population, however, there were more patients with *IL28B* CC genotype in the interferon-ineligible/intolerant population (69.6%) and more patients with *IL28B* non-CC genotype in the nonresponder population (81.6%). Overall, the rate of discontinuations from dual therapy was low (12.6%; 14 patients in each group), and was due primarily to adverse events (nine patients [6.7%] in the interferon-ineligible/intolerant group, two patients [2.3%] in the nonresponder group) and lack of efficacy (four patients [3.0%] in the interferon-ineligible/intolerant group, 11 patients [12.6%] in the nonresponder group).

Virologic Response. HCV RNA levels declined rapidly after initiation of treatment in both groups (Fig. 2). At week 2, the mean decrease in HCV RNA

Table 1. Demographic and Baseline Characteristics of Patients and Their Disease

Characteristic	Interferon-Ineligible/Intolerant n = 135	Nonresponder n = 87	Total N = 222
Age, years			
- Median	64.0	60.0	62.5
- Range	24-75	42-74	24-75
- ≥65 years, n (%)	62 (45.9)	27 (31.0)	89 (40.1)
Male sex, n (%)	38 (28.1)	39 (44.8)	77 (34.7)
IL28B rs12979860 genotype, n (%)			
- CC	94 (69.6)	16 (18.4)	110 (49.5)
- CT	40 (29.6)	66 (75.9)	106 (47.7)
- TT	1 (0.7)	5 (5.7)	6 (2.7)
HCV RNA			
- Mean $\log_{10}$ IU/mL ± SD	6.6 ± 0.58	6.8 ± 0.47	6.6 ± 0.55
- ≥800,000 IU/mL, n (%)	109 (80.7)	80 (92.0)	189 (85.1)
Cirrhosis, n (%)	11 (8.1)	11 (12.6)	22 (9.9)
Response to prior therapy (nonresponders), n (%)			
- Null	NA	48 (55.2)	48 (21.6)
- Partial	NA	36 (41.4)	36 (16.2)
- Other	NA	3 (3.4)*	3 (1.4)
Premedical status (interferon-ineligible/intolerant), n (%)			
- Ineligible-naïve	100 (74.1)	NA	100 (45.0)
• Depression	10 (10.0)	NA	10 (10.0)
• Anemia/neutropenia/thrombocytopenia	44 (44.0)	NA	44 (44.0)
• Other complications requiring medications†	34 (34.0)	NA	34 (34.0)
• Advanced age	12 (12.0)	NA	12 (12.0)
- Intolerant	35 (25.9)	NA	35 (15.8)

*Three patients had insufficient data to be classified as partial or null nonresponders.
†Other complications included hypertension, diabetes mellitus, autoimmune disease, abnormal thyroid function, insomnia, stroke, and psychological.
NA = not applicable.

from baseline was 5.2 $\log_{10}$ IU/mL. Overall, 167/222 patients (75.2%) had undetectable HCV RNA at week 4 during treatment, and 202 patients (91.0%) had undetectable HCV RNA at week 12 on treatment. At 12 weeks after the end of treatment period, 119 (88.1%) interferon-ineligible/intolerant and 70 (80.5%) nonresponder patients had achieved SVR_{12}; by 24 weeks after the end of treatment 118 (87.4%) interferon-ineligible/intolerant and 70 (80.5%) nonresponder patients had achieved SVR_{24} (Table 2). Patients with cirrhosis also achieved high rates of SVR_{24} (20/22, 90.9%). When analyzed by *IL28B* genotype, the response rates were similar for patients with *IL28B* CC genotype (84.5%) and *IL28B* non-CC genotypes (84.8%) (Table 2). Other baseline factors including gender, age, and baseline HCV RNA, did not appear to impact response rates (Table 2).

Virologic Failure. Thirty-four (15.3%) patients (17 each in the interferon-ineligible/intolerant group and nonresponder group) were considered virologic failures. Of patients with undetectable HCV RNA at the end of treatment, 11/129 (8.5%) interferon-ineligible/intolerant patients experienced viral relapse during posttreatment follow-up. Six of 76 patients (7.9%) in the nonresponder group with undetectable HCV RNA at the end of treatment had viral relapse. Two patients in the interferon-ineligible/intolerant group and one patient in the nonresponder group had detectable HCV RNA at the end of treatment. Virologic breakthrough occurred in 4 (3.0%) interferon-ineligible/intolerant patients and in 10 (11.5%) nonresponder patients. At the discretion of the investigators, 9 of the 10 nonresponder patients with virologic breakthrough had additional treatment with peginterferon/ribavirin according to protocol-defined criteria; all nine patients were declared treatment failures in the analysis of the primary endpoint. One of the nine patients who received additional peginterferon/ribavirin responded to treatment with no detectable HCV RNA at follow-up week 24, two patients had HCV RNA detectable at end of treatment, and six patients relapsed.

Of the 34 patients with virologic failure, 29 had resistance-associated substitutions to both daclatasvir (predominantly NS5A-L31M/V-Y93H) and asunaprevir (predominantly NS3-D168 variants) detected at failure. Twenty-two patients with virologic failure had NS5A polymorphisms L31M/V and/or Y93H prior to treatment (Supporting Table 1).

We also investigated the influence of pretreatment resistance-associated variants on efficacy in this study. Pretreatment L31M, Y93H, or linked L31V+Y93H NS5A polymorphisms were detected in 7, 29, and 1 of the 214 patients with available baseline NS5A

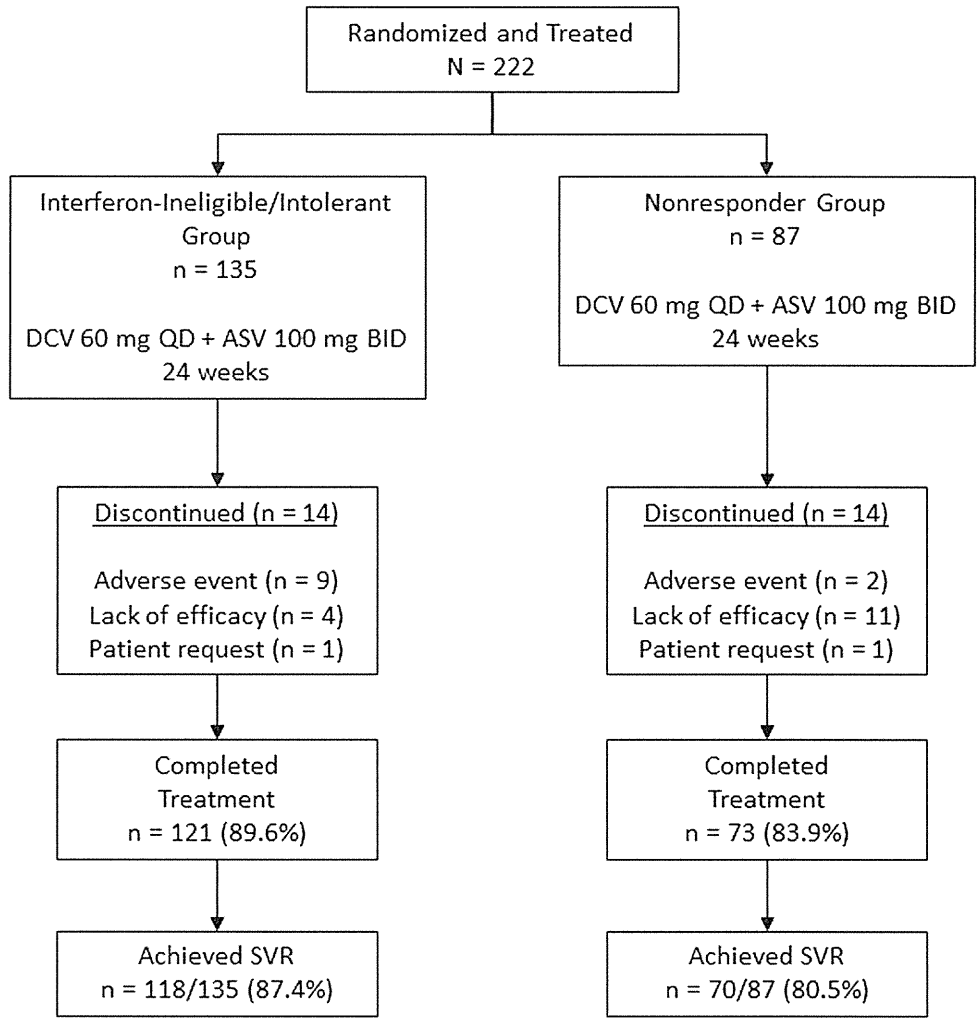

Fig. 1. Patient disposition.

sequences, respectively. Of the 37 patients with L31M/V and/or Y93H at baseline, 11/23 interferon-ineligible/intolerant patients and 4/14 nonresponder patients achieved SVR. The primary asunaprevir resistance-associated variant, NS3-D168E, was present in 2/221 patients with available baseline NS3 sequences; neither of these patients had concomitant NS5A resistance-associated variants. One of these patients achieved SVR; the other relapsed posttreatment.

In comparison with patients who achieved SVR, patients with virologic failure were more likely to have daclatasvir and asunaprevir trough concentrations below their respective median values but within the expected range (Supporting Fig. 1). Most patients with trough concentrations below median values achieved SVR. Treatment compliance, assessed by pill counts and interviews at each study visit, was 83.9% in prior nonresponders and 88.9% in interferon-ineligible/intolerant patients. Across both cohorts, patients with ≥95% compliance in dose and duration of treatment had an SVR_{24} rate of 92.7% (179/193), compared with a 31.0% (9/29) SVR_{24} rate in patients who were <95% compliant (15 out of the 29 patients were discontinued due to the lack of efficacy).

Safety. A total of 194 patients (87.4%) completed 24 weeks of therapy, 121 (89.6%) in the interferon-ineligible/intolerant group and 73 (83.9%) in the nonresponder group. No deaths occurred during the study period. Eleven patients (5.0%) discontinued after 4 to 23 weeks of treatment; 10 discontinued due to ALT and aspartate aminotransferase (AST) elevations and one patient discontinued due to myasthenia gravis, with subsequent detection of preexisting myasthenia gravis-related antibodies.

The most common adverse events were nasopharyngitis, increased ALT and AST, headache, diarrhea, and pyrexia (Table 3). Serious adverse events were reported in 13 (5.9%) patients during treatment. In nine (6.7%) interferon-ineligible/intolerant patients, these events included periarthritis, schizoaffective disorder, myasthenia gravis, myocardial infarction, pyrexia, appendicitis, pyelonephritis,

Table 2. Virologic Outcomes

Virologic Response, n (%) [95% CI]	Interferon-Ineligible/Intolerant n = 135	Nonresponder n = 87	Total N = 222
Week 4,*	114 (84.4)	53 (60.9)	167 (75.2)
	[78.3, 90.6]	[50.7, 71.2]	[69.5, 80.9]
Week 12,*	125 (92.6)	77 (88.5)	202 (91.0)
	[88.2, 97.0]	[81.8, 95.2]	[87.2, 94.8]
Weeks 4 and 12,*	106 (78.5)	48 (55.2)	154 (69.4)
	[71.6, 85.4]	[44.7, 65.6]	[63.3, 75.4]
End of treatment response*	129 (95.6)	76 (87.4)	205 (92.3)
	[92.1, 99.0]	[80.4, 94.3]	[88.8, 95.8]
Sustained virologic response	126 (93.3)	71 (81.6)	197 (88.7)
4 weeks after treatment (SVR_4)†	[89.1, 97.5]	[73.5, 89.7]	[84.6, 92.9]
Sustained virologic response	119 (88.1)	70 (80.5)	189 (85.1)
12 weeks after treatment (SVR_{12})†	[82.7, 93.6]	[72.1, 88.8]	[80.5, 89.8]
Sustained virologic response	118 (87.4)	70 (80.5)	188 (84.7)
24 weeks after treatment (SVR_{24})†	[81.8, 93.0]	[72.1, 88.8]	[79.9, 89.4]
SVR_{24} by subpopulations			
- Null responders	N/A	39/48 (81.3)	39/48 (81.3)
- Partial responders	N/A	28/36 (77.8)	28/36 (77.8)
- Undetermined	N/A	3/3 (100)	3/3 (100)
- Ineligible-naïve	85/100 (85.0)	N/A	85/100 (85.0)
- Intolerant	33/35 (94.3)	N/A	33/35 (94.3)
- Cirrhosis	10/11 (90.9)	10/11 (90.9)	20/22 (90.9)
- Noncirrhosis	108/124 (87.1)	60/76 (78.9)	168/200 (84.0)
- Male	32/38 (84.2)	32/39 (82.1)	64/77 (83.1)
- Female	86/97 (88.7)	38/48 (79.2)	124/145 (85.5)
- Age $<$ 65 years	61/73 (83.6)	47/60 (78.3)	108/133 (81.2)
- Age $\geq$ 65 years	57/62 (91.9)	23/27 (85.2)	80/89 (89.9)
- HCV RNA $<$ 800,000 IU/mL	25/26 (96.2)	6/7 (85.7)	31/33 (93.9)
- HCV RNA $\geq$ 800,000 IU/mL	93/109 (85.3)	64/80 (80.0)	157/189 (83.1)
SVR_{24} by *IL28B* genotype (rs12979860)			
- CC	79/94 (84.0)	14/16 (87.5)	93/110 (84.5)
- CT	38/40 (95.0)	52/66 (78.8)	90/106 (84.9)
- TT	1/1 (100)	4/5 (80)	5/6 (83.3)
Virologic failures			
- Virologic breakthrough	4 (3.0)	10 (11.5)‡	14 (6.3)
- End of treatment detectable	2 (1.5)	1 (1.1)	3 (1.4)
- Relapse (among patients undetectable at end of treatment)	11/129 (8.5)	6/76 (7.9)	17/205 (8.3)

*HCV RNA <LLOQ (<15 IU/mL), target not detected.
†HCV RNA <LLOQ, target detected or target not detected.
‡9/10 patients received additional treatment with peginterferon/ribavirin according to protocol criteria.

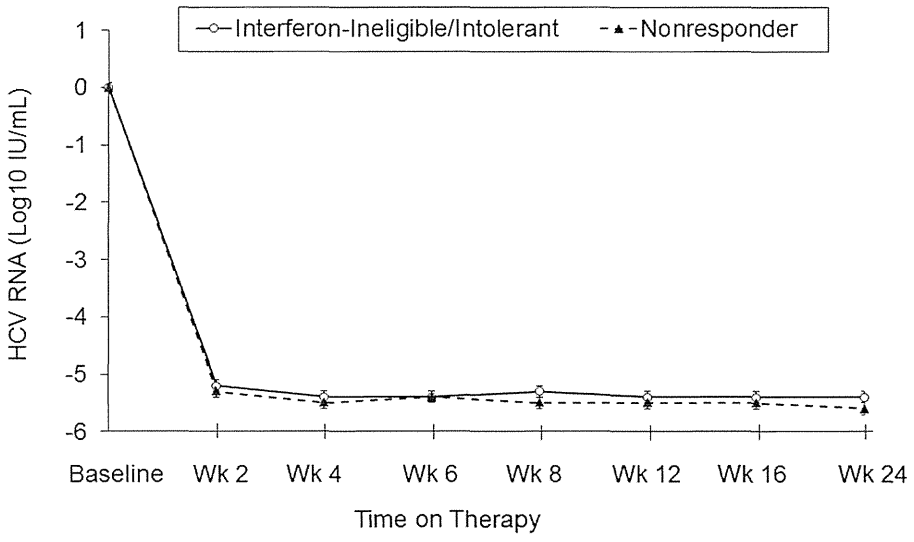

Fig. 2. Mean change in HCV RNA during treatment with daclatasvir and asunaprevir in interferon-ineligible/intolerant and nonresponder patients.

Table 3. Adverse Events and Grade 3-4 Laboratory Abnormalities During the Treatment Period

Event or Laboratory Abnormality, n (%)	Interferon-Ineligible/Intolerant n = 135	Nonresponder n = 87	Total N = 222
Serious adverse events (on treatment)	9 (6.7)	4 (4.6)	13 (5.9)
Adverse event*			
Nasopharyngitis	40 (29.6)	27 (31.0)	67 (30.2)
Increased ALT	24 (17.8)	11 (12.6)	35 (15.8)
Increased AST	18 (13.3)	10 (11.5)	28 (12.6)
Headache	18 (13.3)	17 (19.5)	35 (15.8)
Diarrhea	12 (8.9)	10 (11.5)	22 (9.9)
Pyrexia	12 (8.9)	15 (17.2)	27 (12.2)
Grade 3-4 laboratory abnormality			
Alanine aminotransferase	12 (8.9)	4 (4.6)	16 (7.2)
Aspartate aminotransferase	10 (7.4)	2 (2.3)	12 (5.4)
Hemoglobin	6 (4.4)	1 (1.1)	7 (3.2)
Lymphocytes	5 (3.7)	1 (1.1)	6 (2.7)
Platelets	2 (1.5)	2 (2.3)	4 (1.8)
Bilirubin, total	1 (0.7)	1 (1.1)	2 (0.9)
Neutrophils	0	1 (1.1)	1 (0.5)
Creatinine	1 (0.7)	0	1 (0.5)
Lipase, total	1 (0.7)	0	1 (0.5)

*Adverse events that occurred in more than 10% of patients in any group.

basal cell carcinoma, and hepatocellular carcinoma, respectively; events in four (4.6%) nonresponder patients included second-degree burn, increased liver enzymes, esophageal variceal hemorrhage, and herpes zoster.

ALT and AST elevations were the most frequent adverse events and grade 3/4 laboratory abnormalities (Table 3) and were the basis for 10 of the 11 discontinuations due to adverse events. Two of these 10 patients also had grade 3/4 total bilirubin elevations, but no patient experienced hepatic decompensation. Eight of the 10 patients who discontinued due to ALT/AST elevations (80%) subsequently achieved SVR. For the 16 patients who had grade 3/4 ALT elevations on-treatment, the median time to elevation was ~10 weeks (range 4 to 23 weeks), with rapid reversal in ~2.5 weeks after discontinuation. Most patients with baseline ALT and AST elevations experienced rapid improvement during the first 2 to 4 weeks of treatment, including all patients with grade 3/4 elevations at baseline, with mean decreases at 4 weeks of 43.7 U/L and 35.1 U/L, respectively.

Discussion

Treatment with interferon-based therapy is not an option for many patients with chronic HCV. The findings from this phase 3 study evaluating interferon-free, ribavirin-free, all-oral treatment with daclatasvir and asunaprevir demonstrated high rates of SVR in Japanese patients infected with HCV genotype 1b. Both interferon-ineligible/intolerant and previously treated nonresponder patient groups experienced a rapid reduction in HCV RNA by week 2. The primary endpoint, SVR_{24}, was achieved in 87.4% of patients who were ineligible or intolerant to interferon-based therapies and in 80.5% of patients who had not responded to treatment previously. These high rates of SVR obtained with daclatasvir and asunaprevir represent a significant improvement of cure rates in patient populations typically associated with poor responses to other therapies or with limited therapeutic options. Other factors typically associated with a poor response to therapy, including male gender, high baseline HCV RNA, advanced age, non-CC *IL28B* genotype, and cirrhosis, did not appear to impact response rates, although the number of patients in these subgroups was small.

The response rates in this study were higher than those observed in a phase 3 study evaluating triple therapy with telaprevir and peginterferon/ribavirin in Japanese patients infected with HCV genotype 1 with no response to prior treatment. The SVR rate of nonresponder patients in that study was 34.4%, and safety issues included anemia, severe rash, renal toxicity, and gastrointestinal-related disorders.[7] In a global phase 3 trial, SVR rates ranged from 54% to 59% in partial-responder and 29% to 33% in null-responder patients receiving telaprevir combined with peginterferon/ribavirin.[4] Simeprevir in combination with peginterferon/ribavirin achieved an SVR rate of 38-51% in Japanese nonresponder patients. In the present study, partial-responder and null-responder patients achieved better outcomes (77.8% and 81.3%, respectively), with a much more favorable safety profile. The response rate observed in the ineligible/intolerant group in this study was also notable, especially when considering these patients had no option for curative treatment.

This study was limited to Japanese patients; an ongoing phase 3 study in a similar patient population in the U.S. and Europe will determine whether region-related differences in patient characteristics influence outcomes with this regimen. The results from this phase 3 trial are consistent with the results of a small phase 2a study of Japanese patients treated with daclatasvir and asunaprevir; SVR rates were 64% in peginterferon/ribavirin-ineligible or intolerant patients and 91% in null responder patients.[15] A phase 2b trial combining NS3 (faldaprevir) and NS5B (deleobuvir) inhibitors showed only a 57% SVR in previously untreated patients with HCV genotype-1b infection.[16] In addition, other all-oral regimens earlier in clinical development may provide greater efficacy: in a phase 2

study, SVR rates of 95% to 100% were achieved in treatment-naïve and experienced genotype 1-infected patients treated with sofosbuvir (NS5B inhibitor) in combination with ledipasvir (NS5A inhibitor), with or without ribavirin.[17] The more complex combination of NS3 (ABT-450, plus ritonavir to improve drug exposure), NS5A (ABT-267), and NS5B (ABT-333) inhibitors, with or without ribavirin, achieved SVR rates of 88-96% in treatment-naïve patients and prior null responders with genotype 1 infection. Recent press reports indicate similar results in phase 3 studies with both of these regimens, although full study details are not yet available.[19,20] The combination of daclatasvir and sofosbuvir achieved SVR rates of 88-100% in treatment-naïve patients with genotype 1, 2, or 3 infection, and 95-100% in treatment-experienced patients with genotype 1 infection.[21] However, none of these studies involved patient populations directly comparable to those reported in the present study. Previous experience with HCV regimens indicates that both treatment eligibility and outcomes can vary in relation to variables such as disease stage, patient ethnicity, concomitant medical conditions, and other factors.[3] Further studies of all-oral combinations may provide the evidence needed for optimizing regimen selection on the basis of virologic and patient characteristics.

Response rates at on-treatment week 4 were somewhat higher in the ineligible/intolerant group than in prior nonresponders (84.4% versus 60.9%), but this difference diminished as treatment continued. The early difference in response rates may reflect a reduced contribution of endogenous interferon response in prior nonresponders; the ultimate achievement of an 80.5% SVR rate in this group suggests that such nonresponsiveness can be largely overcome with a potent antiviral regimen.

All-oral treatment with daclatasvir and asunaprevir generally suppressed the enrichment/selection of NS5A and NS3 resistance-associated variants. Virologic failure occurred in 17 patients in each group. Both NS5A and NS3 resistance-associated variants were detected in most patients with virologic failure. There was no apparent association between preexisting NS3 resistance-associated polymorphisms and subsequent virologic outcome. Although more patients with NS5A L31M/V and/or Y93H resistance-associated variants experienced virologic failure, 15/37 of patients with these baseline variants achieved SVR. Thus, pretreatment resistance-associated variants were not absolutely predictive of virologic outcome. Moreover, factors other than resistance, such as lower drug exposure and suboptimal compliance to treatment, likely contributed to treatment failure. The patients with daclatasvir and asunaprevir trough plasma concentrations below median values appeared to be at increased risk of virologic failure (Supporting Fig. 1). Given that patients with $\geq$95% compliance had an SVR_{24} rate of 92.7%, the maintenance of higher compliance is essential for optimizing treatment outcomes.

The rate of premature discontinuation of treatment with daclatasvir and asunaprevir due to adverse events was low. Despite early discontinuation that occurred between weeks 4 and 23, 8 of the 10 patients who discontinued because of elevated levels of ALT and AST achieved SVR_{24}, with rapid reversal of transaminase elevations posttreatment. Although small in number, six patients who achieved SVR were on treatment for 12 weeks or less, suggesting that a shorter treatment period may be possible in some patients. Additionally, baseline elevations of ALT and AST corrected rapidly in most patients after 2 to 4 weeks on treatment, as would be expected with the rapid reduction in HCV RNA levels. The rate of serious adverse events was low and varied among patients, with no consistent pattern of events. The frequency of adverse events was also low, especially compared with historical data in patients receiving a triple regimen with telaprevir and peginterferon/ribavirin that showed a high rate of anemia (91%), pyrexia (85%), and skin disorders (82%).[7]

In conclusion, our findings suggest that 24-week treatment with daclatasvir and asunaprevir provides a highly effective option for patients who currently have no effective treatment options (ineligible or intolerant to interferon-based therapy) and for those patients who did not achieve SVR with prior treatment.

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Contributors: HK, EH, HI, AD, and HM designed the study; EH was the medical lead. HK, YS, KI, JT, YKar, KC, YKaw, AI, KY, KT, NI, KK, TT, NK, and MK recruited patients and obtained the data. HM, HI, EH, and AD analyzed the data. TE and FM provided pharmacokinetic and resistance analyses, respectively. HK, YS, KI, JT, YKar, KC, YKaw, AI, KY, KT, NI, KK, TT, NK, MS, HM, TE, FM, AD,

HI, and EH interpreted study findings. All authors participated in writing the report.

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Supporting Information

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

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

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

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

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

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

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What's new?

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

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

Material and Methods

Subjects

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

Our study was carried out in accordance with the ethical guidelines of the 1975 Declaration of Helsinki and was approved by the Institutional Research Ethics Committees of the authors' institutions. In our study, informed consent was obtained for the use of the samples.

Measurement of MtCK activity

MtCK activity was measured with an immune-inhibition method using two types of anti-MtCK monoclonal antibodies, that is, an anti-uMtCK monoclonal antibody and an anti-sMtCK monoclonal antibody in addition to an anticreatine kinase-M antibody[16] as described previously.[8] JCA-BM8040 (JEOL, Tokyo, Japan) was used as an automatic analyzer. The regression line of this assay was linear up to at least 1,800 U/L. The minimum detection limit was 1.9 U/L. The within-run coefficient variations were 3.1 and 0.8% at the mean MtCK activities of 25.7 and 64.4 U/L, respectively. The between-run coefficient variations were 2.3% for both the mean MtCK activities of 24.0 and 59.5 U/L.

Measurements of other parameters

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

Animals and induction of liver fibrosis

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

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

Quantitative real-time polymerase chain reaction

Total RNA of mouse livers was extracted using TRIZOL reagent (Invitrogen, Carlsbad, CA). One microgram of purified total RNA was transcribed using a Transcriptor First Strand cDNA Synthesis Kit (Roche Diagnostics, Mannheim, Germany). Quantitative real-time polymerase chain reaction (PCR) was performed with a TaqMan Universal Master Mix No AmpErase UNG (Applied Biosystems, Foster City, CA). Mouse uMtCK primers and probe were obtained from Applied Biosystems, TaqMan Gene Expression Assays (Mm00438221_m1). The samples were incubated for 10 min at 95°C, followed by 40 cycles at 95°C for 15 sec and 60°C for 60 sec. The target gene mRNA expression level was relatively quantified to 18S

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

Immunoblot analysis

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

Immunohistochemical analysis

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

Patient follow-up and diagnosis of HCC

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

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

Statistical analysis

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

Table 1. Characteristics of the enrolled chronic hepatitis C patients

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

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

Results

Increased serum MtCK activity in patients with chronic hepatitis C

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

Relationships between serum MtCK activity and various parameters

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

Table 2. Relation between serum MtCK activity and various parameters

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

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

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

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

Increased serum MtCK activity as an independent risk for hepatocarcinogenesis

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

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

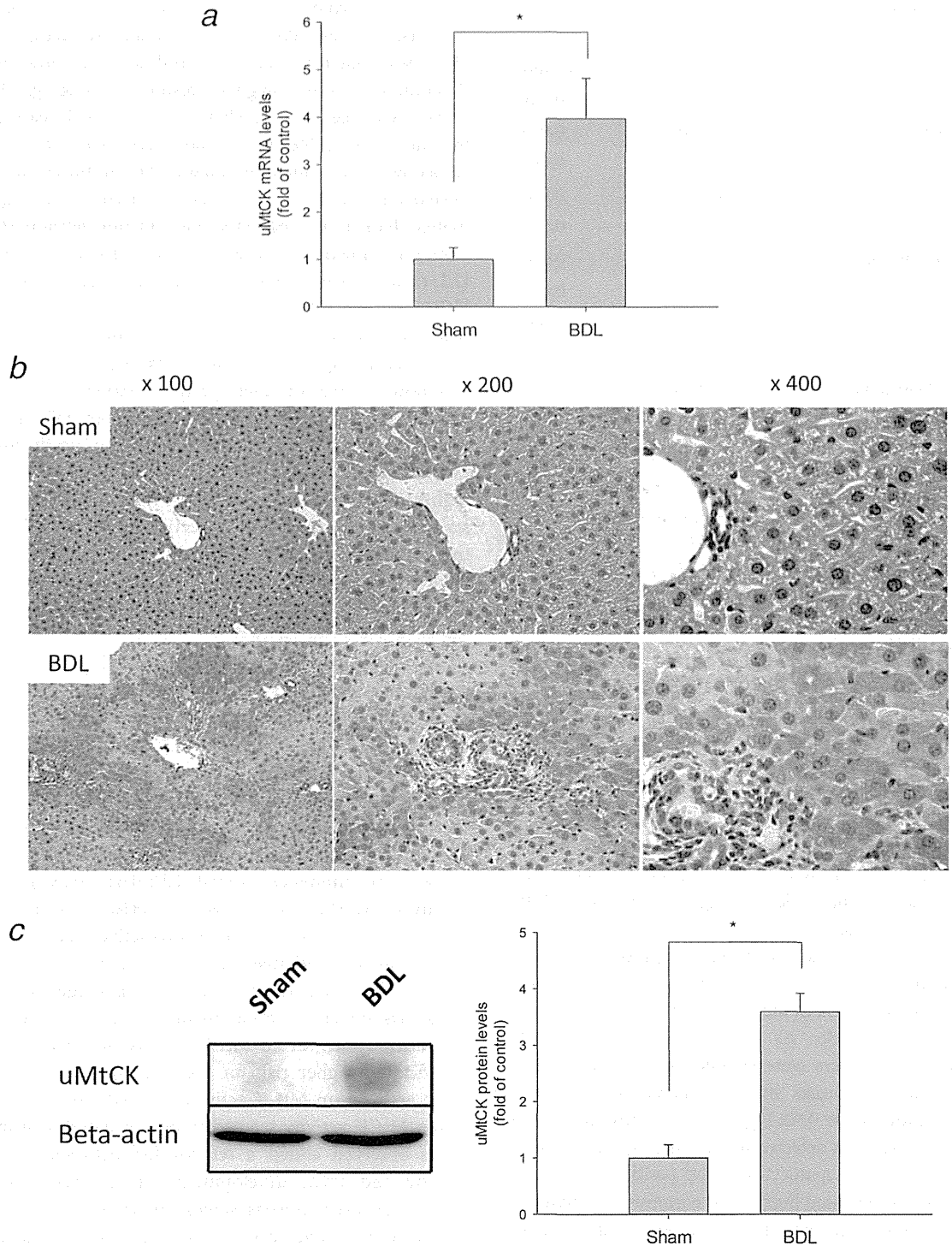

Figure 1. uMtCK mRNA and protein expressions in fibrotic livers induced by bile duct ligation in mice. (*a*) uMtCK mRNA expressions were evaluated by quantitative real-time PCR in the livers of bile duct-ligated and sham-operated mice at 4 weeks after the operation. Results represent a fold of control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

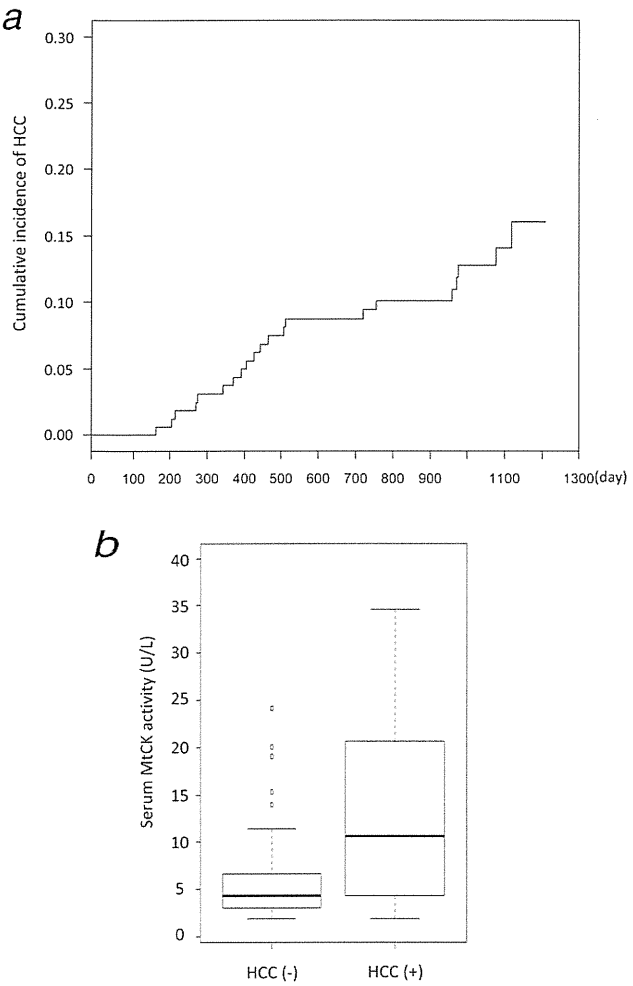

Figure 2. Serum MtCK activity and HCC development in chronic hepatitis C patients. (*a*) Cumulative incidence of HCC in chronic hepatitis C patients. During the mean follow-up period of 2.7 years, HCC developed in 21 patients. The cumulative incidence rates of HCC at 1, 2 and 3 years estimated by the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

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

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

Discussion

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

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

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

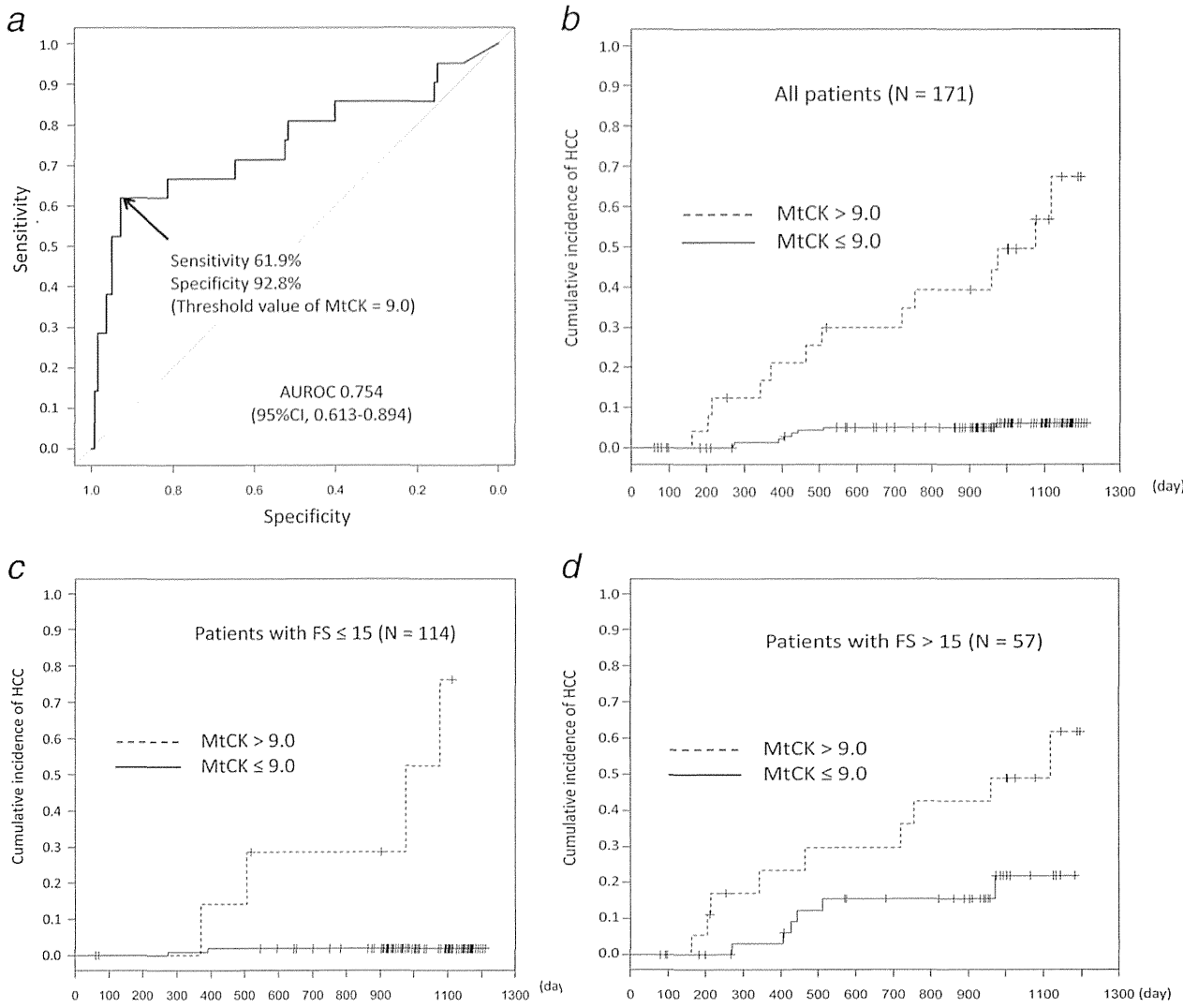

Figure 3. ROC curve showing the overall accuracy of serum MtCK activity for the prediction of HCC development and cumulative incidence of HCC subdivided according to serum MtCK activity in chronic hepatitis C patients. (*a*) ROC curve showing the overall accuracy of serum MtCK activity for the prediction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

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

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

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

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

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

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

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

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

Abbreviations

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

Introduction

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

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

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

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

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

Materials and methods

Patients

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

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

IL28B genotyping

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

Study endpoint

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

Statistical analysis

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