Table V. Multivariate stepwise analysis for factors associated with the early-onset of hepatocellular carcinoma in patients with a serum albumin level of ≥3.5 g/dl.

	Unit	Odds ratio	95% CI	P
HBcAb	Positive	0.59	0.27-1.26	0.169
HBV DNA	Positive	145.18	1.38-15296.61	0.036
Prothrombin time	10	0.76	0.54-1.08	0.109
ALT	10	1.08	0.97-1.21	0.145
Albumin	0.1	1.17	1.01-1.36	0.036
DCP	20	0.99	0.98-1.00	0.037
Platelet count	1	0.92	0.84-1.02	0.107
WBC count	1000	1.64	1.15-2.35	0.006

All P-values were 2-tailed, and a level of <0.05 was considered statistically significant. HBcAb, antibody for hepatitis B core antigen; HBV, hepatitis B virus; ALT, alanine aminotransferase; DCP, des-γ-carboxy prothrombin; WBC, white blood cell.

Table VI. Multivariate stepwise analysis for factors associated with the early-onset of hepatocellular carcinoma in patients with a serum albumin level of <3.5 g/dl.

	Unit	Odds ratio	95% CI	P
HbA1c	1	1.83	0.75-4.47	0.183
HBV DNA	Positive	0.00	0.00-2.96	0.093
AFP	20	1.39	1.01-1.93	0.045

All P-values were 2-tailed, and a level of <0.05 was considered statistically significant. HbA1c, hemoglobin A1c; HBV, hepatitis B virus; AFP, α-fetoprotein.

presence of HBcAb and HBV DNA did not differ between the albumin level of ≥3.5 g/dl and <3.5 g/dl groups (Table IV).

In patients with a serum albumin level of ≥3.5 g/dl, the WBC count and serum levels of albumin and DCP were identified as independent factors associated with the early-onset of HCC (OR 1.64; 95% CI 1.15-2.35; P=0.006, OR 1.17; 95% CI 1.01-1.36; P=0.036, and OR 0.99; 95% CI 0.98-1.00; P=0.037, respectively; Table V). Although the presence of HBcAb was not found to be a significant risk factor for the early-onset of HCC, the presence of HBV DNA was identified as a significant independent risk factor associated with the early-onset of HCC (OR 145.18; 95% CI 1.38-15296.61; P=0.036; Table VI).

In patients with a serum albumin level of <3.5 g/dl, the serum AFP level was the only significant risk factor found to be associated with the early-onset of HCC (Table V). The presence of HBcAb and HBV DNA was not found to be a significant risk factor for the early-onset of HCC.

Discussion

In the overall analysis, the presence of HBV DNA in serum was not identified as a risk factor for the early-onset of HCC in HCV-infected patients. However, a stratification analysis according to a serum albumin level of ≥3.5 g/dl revealed that the presence of HBV DNA was an independent factor for the early-onset of HCC. These findings suggest that occult HBV infection may accelerate hepatocarcinogenesis in HCV-infected patients with a relatively low carcinogenic potential.

Although co-infection of HCV and HBV is thought to synergistically increase the risk of HCC (6), the overall analysis in this study showed that occult HBV infection was not significantly associated with the early-onset of HCC in HCV-infected patients. Similarly, several studies conducted in Asia have also failed to show any significant effect of occult HBV infection in these patients (33-35). Recently, Lok *et al* (36) performed a nested case-control study using a large number of patients enrolled in the HALT-C cohort and reported no significant difference in the prevalence of occult HBV infection between HCC and non-HCC patients with HCV infection. Taken together, these results suggest that occult HBV infection may not be an intensive promoter of HCC development in the presence of a potent carcinogenic factor such as HCV infection.

In contrast with these previous studies and with our own findings for all patients, a stratification analysis according to a serum albumin level of ≥3.5 g/dl showed that occult HBV infection was an independent risk factor for the early-onset of HCC. In patients with occult HBV infection, it is unclear whether a presence of HBV DNA is due to full-length HBV DNA replicated from covalently closed circular DNA in hepatocytes or fragmented HBV DNA integrated into the hepatocyte genome. However, the *HBx* gene is frequently integrated into cellular genes in HCC (37). The HBx protein upregulates the expression of proto-oncogenes including *c-jun*, *c-fos* and *c-myc*, all of which can promote hepatocarcinogenesis (38,39). In addition, albumin plays a crucial role in the development of various diseases, as it is a major antioxidant (19). In cirrhotic patients with a serum albumin level of <3.5 g/dl, branched-chain amino acids increase serum albumin levels, and this subsequently suppresses hepatocarcinogenesis (23,24). In this study, we found a significant association between occult HBV infection and the early-onset of HCC in patients with a serum albumin level of ≥3.5 g/dl, but not in patients with a serum albumin level of <3.5 g/dl. Taken together, these findings suggest that HBV DNA may promote hepatocarcinogenesis in HCV-infected patients with relatively low carcinogenic potential.

Although we designed this study to investigate the effect of HBV DNA on the early-onset of HCC in HCV-infected patients, we found instead that an elevated WBC count is an independent risk factor for the early-onset of HCC in HCV-infected patients. An elevated WBC count may reflect the consequences or underlying pathogenesis of the early-onset of HCC. One possible explanation is aging, because the WBC count declines in old age (40). Alternatively, an elevated WBC count still within the reference range is known to be associated with the development of various malignancies including gastric, colorectal, endometrial and lung cancers (41,42). The WBC count is a well-validated biomarker of inflammation. Chronic inflammation is a possible risk factor for hepatocarcinogenesis as it leads to the activation of receptors for chemokine and advanced glycation-end products (43,44). Another inflammation marker, C-reactive protein, is reported to be a diagnostic and prognostic marker of HCC (45,46). Taken together, these findings suggest that inflammation may promote the early-onset of HCC in HCV-infected patients.

A limitation of this study is that there were only a small number of HBV DNA-positive patients. Previous studies regarding occult HBV infection had a similar limitation (33,47,48). Since occult HBV infection is not frequently seen in HCV-infected patients with HCC, a multicenter study is needed to confirm our findings.

In conclusion, the presence of HBV DNA in serum was not a risk factor for the early-onset of HCC in HCV-infected patients. However, a stratification analysis based on a serum albumin level of ≥3.5 g/dl revealed that presence of HBV DNA in serum was an independent risk factor for the early-onset of HCC. These findings suggest that occult HBV infection may accelerate hepatocarcinogenesis in HCV-infected patients with relatively low carcinogenic potential.

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Efficacy, Safety, and Survival Factors for Sorafenib Treatment in Japanese Patients with Advanced Hepatocellular Carcinoma

Masahito Nakano[a] Masatoshi Tanaka[b] Ryoko Kuromatsu[a] Hiroaki Nagamatsu[c] Kenji Sakata[d] Satoru Matsugaki[e] Masahiko Kajiwara[f] Kunitaka Fukuizumi[g] Nobuyoshi Tajiri[h] Norito Matsukuma[i] Terufumi Sakai[j] Noriyuki Ono[k] Yoichi Yano[l] Hironori Koga[a] Junichi Kurogi[a] Akio Takata[a] Shuji Sumie[a] Manabu Satani[a] Shingo Yamada[a] Takashi Niizeki[a] Hajime Aino[a] Hideki Iwamoto[a] Takuji Torimura[a] Michio Sata[a] for the Kurume Liver Cancer Study Group of Japan

[a]Division of Gastroenterology, Department of Medicine, Kurume University School of Medicine, and [b]Kurume University Medical Center, Kurume, [c]Yame General Hospital, Yame, [d]Ōmuta City Hospital, Ōmuta, [e]Tobata Kyouritsu Hospital, Kitakyushu, [f]Asakura Medical Association Hospital, Asakura, [g]Kyushu Medical Center, Fukuoka, [h]Social Insurance Tagawa Hospital, Tagawa, [i]Kurume Daiichi Social Insurance Hospital, and [j]St. Mary's Hospital, Kurume, [k]Chikugo City Hospital, Chikugo and [l]Saga Social Insurance Hospital, Saga, Japan

Key Words
Sorafenib · Hepatocellular carcinoma · Japanese

Abstract
Background: Sorafenib, an oral multikinase inhibitor, was approved for the treatment of advanced hepatocellular carcinoma (HCC), but has not been adequately evaluated for safety and effectiveness in Japanese patients with advanced HCC. ***Aims:*** The purpose of this study was to prospectively assess the efficacy, safety, and risk factors for survival in patients with advanced HCC treated with sorafenib. ***Methods:*** Between May 2009 and December 2010, 96 Japanese patients with advanced HCC (76 male, 20 female, mean age: 70.4 years) were treated with sorafenib. Eighty-eight patients had Child-Pugh class A, and 8 patients had Child-Pugh class B liver cirrhosis. Barcelona Clinic Liver Cancer stage B and C were found in 64 and 32 patients, respectively. ***Results:*** Twelve patients demonstrated partial response to sorafenib therapy, 43 patients had stable disease, and 33 patients had progressive disease at the first radiologic assessment. The most frequent adverse events leading to discontinuation of sorafenib treatment were liver dysfunction (n = 8), hand-foot skin reaction (n = 7), and diarrhea (n = 4). The median survival time and time to progression were 11.6 and 3.2 months, respectively. By multivariate analysis, des-γ-carboxy prothrombin serum levels and duration of treatment were identified as independent risk factors for survival. ***Conclusions:*** This study showed that sorafenib was safe and useful in Japanese patients with advanced HCC. In addition, this study demonstrated that sorafenib should be administered as a long-term treatment for advanced HCC regardless of therapeutic effect and dosage.

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Introduction

Hepatocellular carcinoma (HCC) is one of the most common malignancies in the world [1–3]. Recent advances in imaging have enabled an increased detection rate for early-stage HCC. By detecting HCC at an early stage, curative therapies, such as hepatic resection, liver transplantation, and radiofrequency ablation, are possible,

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Fax +41 61 306 12 34
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Masahito Nakano, MD
Division of Gastroenterology, Department of Medicine
Kurume University School of Medicine
67 Asahi-machi, Kurume, Fukuoka 830-0011 (Japan)
E-Mail nakano_masahito@kurume-u.ac.jp

which improve patient survival rates [4, 5]. In Japan, transarterial chemoembolization is an important locoregional treatment for patients with unresectable HCC [6]. However, long-term survival remains limited due to high rates of recurrence, even after these curative therapies [7, 8]. In particular, the development of advanced HCC with macroscopic vascular invasion or extrahepatic metastasis greatly reduces survival rates as effective systemic therapies have not been developed to date [9–11].

Recently, sorafenib, an oral multikinase inhibitor, has become available as a new molecular targeted therapy for advanced HCC. The magnitude of the benefit obtained with sorafenib (25–35% decreased risk of death) is similar to that observed with trastuzumab in breast cancer, bevacizumab in colon cancer, or erlotinib in lung cancer [12–14]. Sorafenib has been shown to suppress tumor growth and angiogenesis by inhibiting the Raf/MEK/ERK signaling pathway and by inhibiting receptor tyrosine kinases, such as vascular endothelial growth factor receptor (VEGFR)-1, VEGFR-2, and VEGFR-3, and platelet-derived growth factor receptor-β (PDGFR-β) [15].

The introduction of sorafenib has changed the standard systemic therapy for advanced HCC, as demonstrated by the recent positive results from randomized controlled trials, and this new treatment was approved in Japan in May 2009 [16, 17]. These results, proving the efficacy of molecular targeted therapies for liver cancer, have triggered the search for additional molecular agents to further improve patient survival. However, concerns regarding the development and approval of new molecular targeted therapies, including sorafenib, include the inclusion and exclusion criteria for the trials and frequent adverse events. The SHARP trial was conducted at 121 centers in 21 countries in Europe, North America, South America, and Australasia [16], and 23 centers in China, South Korea, and Taiwan were enrolled in the Asia-Pacific study [18], but no trials have been performed in Japan. Moreover, these studies did not primarily include patients infected with hepatitis C virus (HCV). In Japan, >70% of HCC cases are related to chronic liver disease with HCV infection. Therefore, in this study, we prospectively assessed the efficacy and safety of sorafenib and identified the factors associated with improved survival in Japanese patients with advanced HCC primarily due to HCV infection.

Patients and Methods

Patients

Eligibility criteria for this study were as follows: (1) Eastern Cooperative Oncology Group (ECOG) performance status of 0–1; (2) measurable disease using the Response Evaluation Criteria in Solid Tumors (RECIST); (3) Child-Pugh class A or B; (4) leukocyte count $\geq 2{,}000/mm^3$; (5) platelet count $\geq 50 \times 10^9/l$; (6) hemoglobin level ≥ 8.5 g/dl; (7) serum creatinine level <1.5 mg/dl, and (8) no ascites or encephalopathy. Between May 2009 and December 2010, 96 patients diagnosed with advanced HCC were included in this study. HCC was either confirmed on histology or diagnosed using noninvasive criteria according to the European Association for the Study of Liver. Included patients were treated with sorafenib at 1 of the 12 experienced member institutions of the Kurume Liver Cancer Study Group of Japan: Asakura Medical Association Hospital, Chikugo City Hospital, Kurume Daiichi Social Insurance Hospital, Kurume University Medical Center, Kurume University School of Medicine, Kyushu Medical Center, Ōmuta City Hospital, Saga Social Insurance Hospital, Social Insurance Tagawa Hospital, St. Mary's Hospital, Tobata Kyouritsu Hospital, or Yame General Hospital. The primary outcome of this study was overall survival time. Overall survival time was defined as the time from sorafenib initiation to the date of death or the patient's last follow-up. Relevant data from the patients' clinical records, including history, laboratory results, radiologic findings, histologic results, and survival data, as well as the dosage and adverse events associated with sorafenib therapy, were prospectively collected. The study protocol was approved by University hospital Medical Information Network (UMIN) Center (No. UMIN000007427) and conformed to the guidelines of the 1975 Declaration of Helsinki. Patients were given full information regarding the details of the clinical study, and they provided written informed consent prior to participation in the study.

Diagnosis of Intrahepatic Lesions and Extrahepatic Metastasis

Intrahepatic lesions and vascular invasion were diagnosed using a combination of contrast-enhanced computed tomography (CT), magnetic resonance imaging (MRI), ultrasonography, and digital subtraction angiography. In addition, determination of α-fetoprotein (AFP), *Lens culinaris* agglutinin-reactive fraction of AFP (AFP-L3), and des-γ-carboxy prothrombin (DCP) serum levels was performed up to 1 month prior to treatment. Intra-abdominal metastases were detected on abdominal CT, MRI, and ultrasonography, which were performed to evaluate intrahepatic lesions. Pulmonary lesions were detected on chest radiography or chest CT, which were routinely performed up to 1 month prior to treatment. Additional examinations, such as bone scintigraphy and brain CT or MRI, were indicated when symptoms attributable to extrahepatic metastasis appeared. These examinations were also undertaken when AFP, AFP-L3, or DCP were elevated, and the elevation could not be accounted for by the status of the intrahepatic lesions [11]. Tumor stage was classified according to the Barcelona Clinic Liver Cancer (BCLC) staging classification [19].

Sorafenib Treatment

An initial sorafenib dose of 400 mg was orally administered twice daily. Discontinuation and dose reduction were based on tolerance. Side effects of sorafenib were determined via the National Cancer Institute's Common Terminology Criteria for Adverse Events (CTCAE), version 3.0 [20]. Treatments were discontinued upon development of grade 3 or higher adverse events according to CTCAE classification with the exception of platelet counts and leukocyte counts of $<25 \times 10^9/l$ and $<1{,}500/mm^3$, respectively.

Table 1. Baseline clinical characteristics

Patient characteristics	n
Age, <70/≥70 years	39/57
Sex, male/female	76/20
Etiology, HBV/HCV/both negative	20/59/17
Child-Pugh class, A/B	88/8
BCLC stage, B/C	64/32
AFP, <1,000/≥1,000 ng/ml	62/34
DCP, <1,000/≥1,000 mAU/ml	49/47

HBV = Hepatitis B virus.

Assessment of Tumor Response

To assess tumor response, 4 weeks after beginning the administration of sorafenib and every 4–6 weeks thereafter, an imaging study was performed. Tumor response was evaluated according to the RECIST criteria, version 1.1 [21] as follows: complete response, all measurable lesions disappeared for >4 weeks; partial response (PR), the sum of the diameters of the largest target lesions decreased by >30% and there was no development of a new lesion for >4 weeks; progressive disease (PD), the sum of the largest diameters increased by >20% or a new lesion appeared, and stable disease, neither PR nor PD was seen [22]. Cancer in patients who died before their first radiographic assessment was classified as PD. The time to radiologic progression was defined as the time from sorafenib initiation to disease progression. Data from patients who died without tumor progression were censored. The disease control rate was defined, on the basis of independent radiologic review, as the percentage of patients whose best-response RECIST rating of complete response, PR, and stable disease was maintained for at least 30 days after the first demonstration of that rating.

Statistical Analysis

Baseline patient characteristics were analyzed using descriptive statistical methods. Survival curves were calculated via the Kaplan-Meier method. Univariate survival curves were compared using the log-rank test. A p value <0.05 was considered statistically significant. All analyses were performed using the statistical software package SPSS (IBM, Armonk, N.Y., USA). The Cox proportional hazards model was used to evaluate the interaction between baseline characteristics and the effect of sorafenib on overall survival.

Results

Patient Characteristics

There were 76 male (79%) and 20 female (21%) patients, with a mean age of 70.4 (range 33–87) years (table 1). Chronic HCV infection was the predominant cause of liver disease (n = 59; 61%), followed by chronic hepatitis B virus infection (n = 20; 21%). Eighty-eight (92%) patients had Child-Pugh class A, and 8 (8%) patients had Child-Pugh class B liver cirrhosis. With respect to tumor stage, 64 (67%) patients had stage B disease and 32 (33%) patients had stage C disease, according to the BCLC staging classification [19]. The most frequent sites of extrahepatic metastases were the lung (n = 41), bone (n = 14), and lymph nodes (n = 12). Prior to sorafenib therapy, 88 (92%) patients had been treated with surgical, loco-regional, or pharmacologic therapies. Of these 88 patients, 48 received transcatheter arterial infusion chemoembolization, 34 received hepatic arterial infusion chemotherapy, 25 underwent hepatic resection, and 23 patients underwent radiofrequency ablation.

Overall Response and Efficacy

The mean duration of oral treatment was 4.2 (range 0.1–16.2) months, and the mean follow-up duration was 6.4 (range 0.1–16.2) months. Forty (42%) patients died during the observation period, whereas 56 (58%) patients were alive at the end of the follow-up period. At the first radiologic assessment, 12 (13%) patients showed PR, 43 (45%) patients showed stable disease, and 33 (34%) patients showed PD; 8 (8%) patients had no follow-up radiologic evaluation and were not included in further analysis.

Treatment Compliance

Performance status was used to determine initial sorafenib dose at the discretion of each chief physician. Fifty-eight patients with a performance status of 0 started treatment with 800 mg sorafenib daily and 38 patients with a performance status of 1 began with a 400-mg daily dose of sorafenib. Dose reduction was necessary in 40 patients during treatment. By December 2010, the end of the follow-up period, 71 patients had discontinued treatment. The reasons for discontinuation were adverse events (36 patients), radiologic and symptomatic progression (27 patients), and deterioration in performance status (8 patients). The mean duration of treatment, prior to discontinuation, was 3.5 (range 0.1–15.5) months.

Treatment-Related Toxicities

Hand-foot skin reaction (HFSR) was the most troublesome adverse event in our series, occurring in 49 (51%) patients. Other frequent toxicities included diarrhea (n = 23; 24%), alopecia (n = 13; 14%), liver dysfunction (n = 13; 14%), and fatigue (n = 11; 11%). The most frequent adverse events leading to discontinuation of sorafenib treatment were HFSR (n = 7; 7%), diarrhea (n = 4; 4%), and liver dysfunction [n = 8; 8%; 7 patients with Child-Pugh class A disease (8%) and 1 with Child-Pugh class B (13%)]. In par-

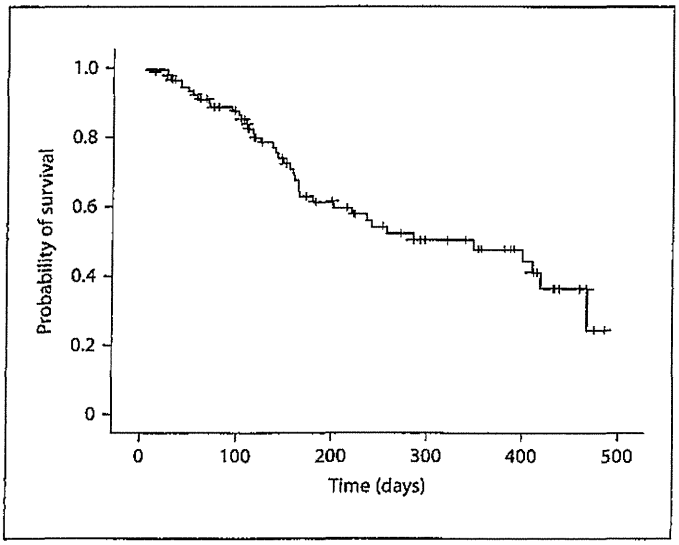


Fig. 1. Cumulative survival of 96 patients with advanced HCC treated with sorafenib. The MST of these patients was 11.6 months. The 1-year survival rate was 48%.

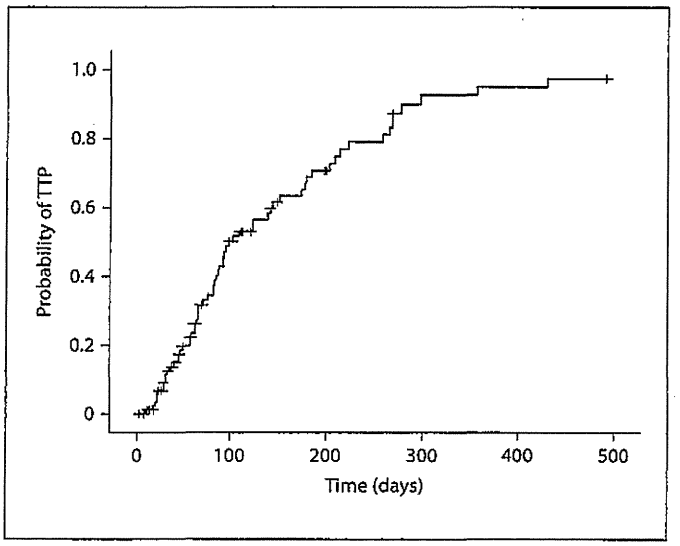


Fig. 2. Cumulative progression of 96 patients with advanced HCC treated with sorafenib. The median TTP of these patients was 3.2 months.

Table 2. Univariate and multivariate analyses of survival in patients with HCC

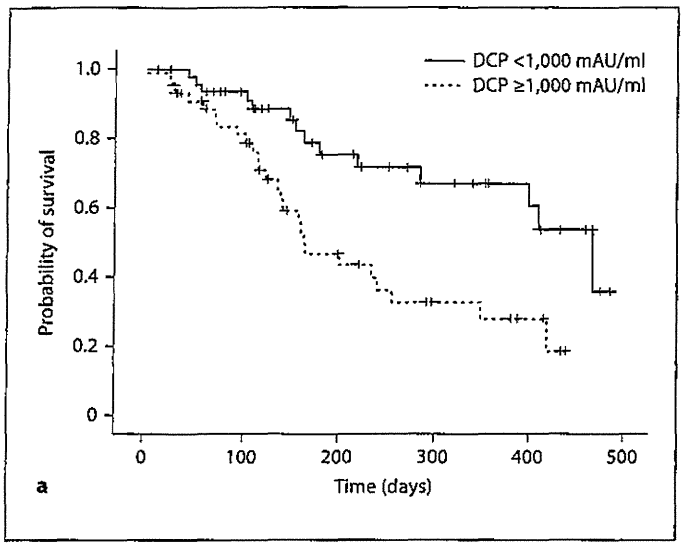
	Univariate		Multivariate	
	HR (95% CI)	p value	HR (95% CI)	p value
Age (≥70 years)	1.091 (0.581–2.050)	0.786		
Sex (male)	0.670 (0.320–1.403)	0.288		
Child-Pugh class (B)	2.273 (0.868–5.952)	0.094		
AFP (≥1,000 ng/ml)	1.953 (1.046–3.647)	0.036		
DCP (≥1,000 mAU/ml)	2.723 (1.394–5.316)	0.003	2.722 (1.369–5.412)	0.004
Daily average dosage (≥400 mg)	0.970 (0.503–1.870)	0.927		
Daily average dosage (≥600 mg)	1.042 (0.556–1.954)	0.898		
Duration of treatment (≥30 days)	0.403 (0.199–0.816)	0.012	0.407 (0.196–0.848)	0.016
Therapeutic effect (PD)	1.876 (0.991–3.549)	0.053		

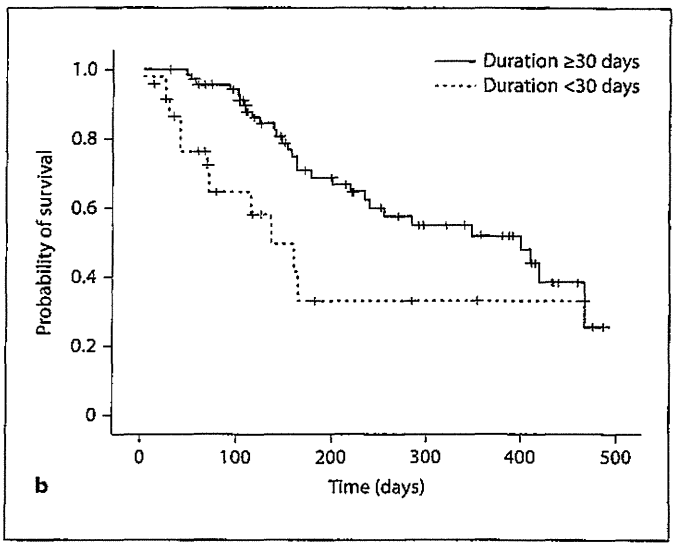
HR = Hazard ratio; 95% CI = 95% confidence interval.

ticular, interstitial pneumonia (n = 1; 1%) and tumor lysis syndrome (n = 1; 1%) were serious adverse events. The single case of interstitial pneumonia resulted in death.

Survival and Factors Associated with Outcome

The cumulative survival curve of 96 patients is shown in figure 1. The median survival time (MST) was 11.6 (range 0.1–16.2) months, with a 1-year survival rate of 48%. The median time to progression (TTP) was 3.2 (range 0.1–16.2) months (fig. 2). Cox proportional hazards regression analysis was performed to identify independent factors associated with survival (table 2). The results of univariate analysis showed that AFP serum level (≥1,000 ng/ml, p = 0.036), DCP serum level (≥1,000 mAU/ml, p = 0.003), and duration of treatment (>30 days, p = 0.012) were significant risk factors adversely impacting survival. Multivariate analysis showed that DCP serum level (≥1,000 mAU/ml, HR 2.722, 95% CI 1.369–5.412, p = 0.004) and duration of treatment (>30 days, HR 0.407, 95% CI 0.196–0.848, p = 0.016) were independent risk factors for decreased survival. Cumulative survival curves, plotted for DCP serum level and duration of treatment, are shown in figure 3.





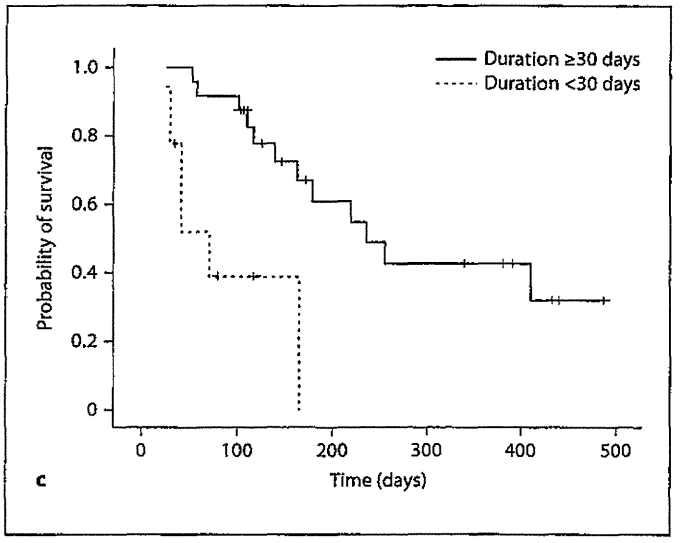


Fig. 3. **a** Cumulative survival of patients grouped by serum DCP levels. The MSTs of the group with DCP >1,000 and <1,000 mAU/ml were 5.4 and 15.6 months, respectively ($p = 0.0023$). **b** Cumulative survival of patients grouped by duration of treatment. The MSTs of >30 and <30 days of treatment were 13.3 and 4.5 months, respectively ($p = 0.0091$). **c** Cumulative survival of patients with PD grouped by duration of treatment. The MSTs with >30 and <30 days of treatment were 7.8 and 2.4 months, respectively ($p = 0.0008$).

Discussion

Sorafenib, an oral multikinase inhibitor, has recently become available as a new molecular targeted therapy for advanced HCC. A significant survival benefit and good tolerance was demonstrated with sorafenib treatment for patients with advanced HCC in 2 randomized phase III placebo-controlled trials [16, 18]. Consequently, sorafenib has become the standard treatment for advanced HCC in the United States, Europe, and many other countries, including Japan. This study prospectively assessed the efficacy and safety of sorafenib and identified the factors associated with survival in Japanese patients with advanced HCC. In this study, the TTP and MST of Japanese patients receiving sorafenib were 3.2 and 11.6 months, respectively. TTP in this study was shorter than that observed in the SHARP trial (5.5 months) and was similar to that observed in the Asia-Pacific study (2.8 months) [16, 18]. However, the MST in the current study was longer than that observed in the Asia-Pacific study (6.5 months) and was similar to that observed in the SHARP trial (10.7 months) [16, 18]. Compared with these 2 previous studies, the time between TTP and MST was longer in the current study, though the reason for this is unclear.

An exploratory multivariate analysis with the use of a Cox proportional hazards model identified 2 baseline patient characteristics that were prognostic indicators for overall survival: duration of treatment and serum DCP

level. In contrast, therapeutic effect and dosage of sorafenib were not significant risk factors adversely affecting survival in this study. In the SHARP trial and the Asia-Pacific study, administration of sorafenib was continued until the occurrence of both radiologic and symptomatic progression, or the occurrence of either unacceptable adverse events or death [16, 18]. In the current study, neither radiologic nor symptomatic progression were criteria for discontinuation. The difference in the discontinuation criteria may explain the gap between TTP and MST in this study. Even with tumor progression, the patients who continued on sorafenib may have had better survival potential compared to the patients in whom sorafenib was discontinued (fig. 3c). Therefore, this study suggests that sorafenib should be administered long-term in patients with advanced HCC independent of therapeutic effect and dosage.

Previous studies reported that for patients with HCC, high serum DCP levels are associated with vascular invasion, metastasis, and tumor recurrence [23]. Hypoxia has been reported to induce epithelial mesenchymal transition or cytoskeletal changes. Indeed, hypoxic stimulation induced hepatoma cell lines (HepG2 or PLC/PRF/5 cells) to undergo epithelial-to-fibroblastoid conversion or epithelial mesenchymal transition, and these cells produced DCP [23]. Therefore, DCP as an HCC tumor marker is more useful in larger tumors which are likely to be exposed to hypoxia during tumor development [23]. Thus, it is suggested that higher serum DCP levels represent a more advanced state of HCC, and, as a result, lead to reduced survival rates.

In this study, disease classification at the first radiologic assessment was PR for 12 (13%) patients, stable disease for 43 (45%) patients, and PD for 33 (34%) patients. Notably, the proportion of patients with PR in our study was higher compared to the SHARP trial (2%) and the Asia-Pacific study (3.3%). It is not clear why there appears to be a higher rate of PR in Japanese patients. Previous studies suggested that there may be racial differences in terms of gene mutations that may affect sorafenib treatment [24, 25]. Lynch et al. [26] reported that patients with non-small-cell lung cancer have specific mutations in the *EGFR* gene, which correlate with clinical responsiveness to the tyrosine kinase inhibitor gefitinib. Therefore, it is suggested that Japanese patients with advanced HCC may be more sensitive to sorafenib than Western and other Asian populations. To investigate the possible differences in the therapeutic effects of sorafenib, further studies with larger patient populations will be needed.

Treatment-related adverse events were a substantial issue impacting the continuation of treatment with sorafenib. In this study, although the overall incidence of treatment-related adverse events was high (90%), events were primarily controlled with medical treatment and/or sorafenib dose reductions. Adverse events leading to discontinuation of treatment included liver dysfunction (8%), HFSR (7%), and diarrhea (4%), which are commonly associated with sorafenib [27, 28]. However, in the SHARP trial, the overall incidence of treatment-related adverse events was 80% in the sorafenib group, and the most frequent adverse events leading to discontinuation of sorafenib treatment were gastrointestinal events (6%), fatigue (5%), and liver dysfunction (5%) [16]. HFSR is particularly well known as an early adverse event [29–31] associated with sorafenib therapy and the severity of HFSR depends on the duration of treatment, dosage, and accumulation of the drug [32]. Further effort put towards the effective control of adverse effects and management of sorafenib dosing, with a priority given to facilitating long-term administration, will lead to the most effective therapy for patients with HCC. Moreover, hepatic reserve is important for hepatic extraction and metabolism of sorafenib. In this study, liver dysfunction necessitating suspension or discontinuation of sorafenib occurred with similar frequency in patients with Child-Pugh class B and Child-Pugh class A disease. This result suggests that sorafenib can be used in patients with Child-Pugh class B, as well as in patients with Child-Pugh class A disease.

In conclusion, sorafenib was a safe and effective therapy in Japanese patients with advanced HCC. In addition, duration of treatment and serum level of DCP were independent risk factors negatively impacting survival in this study. The results of this study indicate that sorafenib should be administered as a long-term treatment for advanced HCC in patients regardless of therapeutic effect and dosage.

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肝炎から肝癌までのわが国の動向

鳥村　拓司　　佐田　通夫

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特集／肝炎から肝硬変・肝癌まで

肝炎から肝癌までのわが国の動向

鳥村　拓司①②　佐田　通夫①②

はじめに

わが国における慢性肝疾患の原因としてはB型肝炎とC型肝炎ウイルスに起因するものが全体の80%以上を占めると推測される。近年，これらのウイルス性肝疾患は感染に対する防御体制が進歩したことに加え，核酸アナログ製剤，インターフェロン製剤，プロテアーゼ阻害剤などが相次いで開発，改良されたことから治療方法が様変わりし，それに伴い治療成績も大きく改善してきた。一方，肝細胞癌による死亡は1975年から年々増加の一途をたどっていた。このうち女性患者の死亡は現在も相変わらず増加傾向にあるものの男性患者の死亡数が減少し始めたため，全体としては2002年をピークに近年ではやや減少に転じている[1]。肝細胞癌の原因となる背景肝疾患に関しては，B型肝炎ウイルスによる肝疾患の占める割合は変わらないが，C型肝炎ウイルスの占める割合が低下傾向にあり，変わって，B，C型肝炎ウイルス陰性の肝細胞癌が増加傾向にある[2]。肝細胞癌の治療に関しては，2009年に本邦でも認可された分子標的治療薬であるソラフェニブを用いた治療成績がいくつかの施設で公表され，分子標的治療薬の長所・問題点が次第に明らかにされつつある。

本稿では本邦におけるウイルス性慢性肝疾患，肝細胞癌の動向を紹介する。

I．B型肝炎

B型肝炎は全世界で約4億人の感染者が存在しており，特にアジア地域は高浸淫地帯である。本邦では1985年に始まった母子感染防止事業によりB型肝炎ウイルス感染者は減少しているが，それでも未だに推計で約150万人の感染者が存在すると考えられている。その多くは無症候性キャリアであるが，約7万人程度が慢性肝炎，3万人が肝硬変や肝細胞癌を患っていると推定されている。

B型肝炎ウイルスは遺伝子の全塩基配列の8%以上の相違によりA型からJ型（I型はC型の亜型）までの9種類の遺伝子型に分類されており，さらに4～8%の塩基配列の違いにより34のサブタイプが存在している。これら遺伝子型やサブタイプには地域特性があるばかりでなく，臨床像の違い，治療に対する反応性の違いなどにも関連していることが明らかとなってきた。たとえば，欧米にはGenotype A，Dが多く存在し，本邦を含めたアジアにはGenotype B，Cが多く分布している。このうちGenotype Aは急性肝炎から遷延化，慢性化しやすいが肝発癌の頻度は低いと言われている。またインターフェロン治療に対する感受性が高く，時にはHBs抗原のセロコンバージョンも起こることが報告されている。Genotype DはGenotype Aに比べてインターフェロン治療に対する感受性が低いと言われている。Genotype Bのうち日本型であるBjは予後良好であり，ほとんどのヒトは無症候性キャリアで一生を終えるがプレコア領域（1,896番目）に変異が入ると劇症化することがある。アジア型のBaは肝発癌のリスクが高いと言われている。Genotype Cは急性肝炎からの慢性化は希でありインターフェロン治療に対する感受性も低いと報告されている。さらに肝発癌のリスクが高く予後不良とされている。

現在，B型肝炎の治療は主にインターフェロンと核酸アナログ製剤で行われている。若年者においてはHBe抗原からHBe抗体へのセロコ

①久留米大学医学部内科学講座消化器内科部門
②久留米大学先端癌治療研究センター肝癌部門

ンバージョンを起こし薬剤の中止が期待できるインターフェロン療法が主体となる。2011年9月からペグインターフェロンα2aの48週間治療がHBe抗原陽性者のみならず陰性者に対しても可能となった。国内第Ⅱ/Ⅲ相試験の結果からすると，HBe抗原陽性例では35歳未満が治療の良い適応であり，HBe抗原陰性症例では35歳以上でもHBV DNA量が7.0 log copy/ml未満であれば治療効果が期待できると考えられる。現在本邦で認可されている核酸アナログ製剤はラミブジン，アデホビル，エンテカビルである。ラミブジンは耐性株の出現頻度が高いため耐性が出現しなくてもエンテカビルへの切り替えが推奨されている。また，耐性が出現した場合はアデホビルとの併用治療が行われる。エンテカビルは耐性株の出現率が低いためHBe抗原のセロコンバージョンの可能性の低い比較的年配者に対する治療の第一選択となっている。本邦では治験が始まった段階のテノホビルはアデホビルに似た構造を持っているが腎毒性がアデホビルに比べて少ないために大量投与が可能であり強い抗ウイルス効果が期待されている。AASLDやEASLではすでにエンテカビルと並んで第一選択薬として位置づけされている[3]。今後，本邦でもエンテカビルに対し耐性が生じた症例などに対する有効性を評価する必要がある。

母子感染の予防などによりHBV感染者は本邦において減少したが，国際交流の増加や性交渉の多様化によりGenotype Aによる水平感染が増加しており今後Genotype Aの成人感染からの慢性化の増加が問題となっている。さらに，B型肝炎の高浸淫地帯であるアジア諸国では未だにラミブジンが第一選択として使用されていることが多く，今後ラミブジン耐性株の本邦への流入が危惧される。これらの事に対処するためにもユニバーサルワクチンの導入は重要な検討課題と思われる。

Ⅱ．C　型　肝　炎

本邦における一般献血者のHCV抗体陽性率は1～2％であり，わが国には約150万～200万人のHCV感染者が存在すると言われている。HCV感染者の年齢分布をみると高齢者になるにつれて感染率が高くなる傾向がある。急性肝炎の発生に関しては，HCVのスクリーニングが導入されてから輸血後肝炎の発生はほとんどなく散発性の肝炎の発生率も低く横ばいである。C型慢性肝炎に対する治療は長らくペグインターフェロンとリバビリン併用療法が主力であったが本邦に多いGenotype 1b型，高ウイルス量の症例ではSVR率は約50％に留まっていた。ことに，*IL28B*のSNPがminor typeの症例ではSVRが得られにくいとされていた[4]。この為，HCVに直接作用するDAA（direct antiviral agents）と呼ばれる薬剤の開発が進み2011年11月に第一世代のプロテアーゼ阻害剤であるテラプレビルが認可された。この薬剤にペグインターフェロンとリバビリンを加えた3剤併用12週間＋ペグインターフェロンとリバビリン2剤併用12週間で治療が開始され，Genotype 1b型，高ウイルス量の症例でSVR率が73％に向上した。しかし，従来からのペグインターフェロンとリバビリンによる副作用に加えテラプレビル自体の貧血，皮膚症状，腎機能障害などの重篤な副作用発現の報告があり，治療完遂に難渋する症例も少なくない。この様な問題点を克服するために，第二世代のプロテアーゼ阻害剤にペグインターフェロンとリバビリンを併用した臨床試験が多数国内外で進行中である。第二世代のプロテアーゼ阻害剤の特徴としては，テラプレビルと比べ治療効果は大きくは変わらないものの，強い副作用が少ないことである。これらのDAAはここ1～2年の間に認可される予定である。しかし，これらの治療法は何れもインターフェロンを併用する治療法であるため，*IL28B*のSNPがminor typeの症例などインターフェロンに不応の症例の治癒効果が大幅に改善されることは期待しにくい。この問題点を解決するためgenotype 1b，高ウイルス症例に対しNS5A阻害剤であるダクラタスビルとプロテアーゼ阻害剤のアスナプレビルの併用試験がペグインターフェロン，リバビリン併用無効例21例とIFN治療が行えない患者22例に行われた。SVRは77％，副作用中止例は高ビリルビン血症1名，トランスアミナーゼ上昇2例という結果であった[5]。今後様々なDAAの組み合わせによる臨床試験が行われ，近い将来高齢者にも安全に施行できる抗ウイルス療法が確立されC型肝炎患者の減少が現実のものとなるこ

とが期待される。

Ⅲ. 肝　細　胞　癌

本邦における肝細胞癌による死亡は男性が女性に比べ約2倍多いという特徴があり，1975年以降年々増加し2002年には人口10万人において27.5人まで達した。2002年以降女性は依然増加傾向にあるものの男性は2002年をピークに減少に転じ，全体でも2007年には人口10万人において26.6人と減少した。肝発癌の原因となる背景肝疾患はHCVによる慢性肝疾患が約70%，HBVによる慢性肝疾患が10%強を占める。背景肝疾患別の肝細胞癌患者数はHBV由来の例は変化なく，HCV由来の例が減少し，HBV，HCV陰性の症例が増加傾向にある。このうち多くは非アルコール性脂肪性肝炎などによると考えられている。1995年の調査では肝発癌の好発年齢は男性が60歳台前半，女性は男性に比べやや発癌年齢が高い傾向にあったが，その後の調査で発癌年齢は徐々に高齢化する傾向にあることが明らかとなった[6]。

肝細胞癌はハイリスク　グループを設定しやすい腫瘍であり，われわれは定期的に腹部超音波検査，CTスキャン，MRIなどの画像診断とAFP，AFP-L3，PIVKA-Ⅱなどの腫瘍マーカーを定期的に検査することで肝細胞癌の早期発見に努めてきた[7]。このサーベイランス　システムは肝細胞癌の早期発見に効果を発揮してきたが，超音波造影剤のソナゾイドとMRIの造影剤であるGd-EOB-DTPA（プリモビスト）が各々2007年，2008年に認可された。このうちソナゾイドによる超音波検査は肝細胞癌のスクリーニング，ステージング，局所再発の局在診断，Bモード超音波検査で認識できない結節の局在診断などに有用であると言われている。一方，Gd-EOB-DTPA MRI検査は従来のMRI検査やCTスキャンに比べて肝内の結節性病変の検出に効果を発揮する。今後スクリーニングに従来の画像診断に加えこれら新たな検査法を組み込むことでさらに肝細胞癌の早期発見が可能となることが期待される。

本邦における肝細胞癌の治療は基本的には2009年に改訂された“科学的根拠に基づく肝癌診療ガイドライン”に基づいて行われることが多い[8]。このうち根治的治療は外科的切除（移植）の他，内科的治療としては主にラジオ波焼灼療法で行われている。外科的切除（移植）もラジオ波焼灼療法も導入されてから時間がたっておりその適応基準や治療成績はほぼ出そろった感がある。2009年の日本肝臓学会で行われたコンセンサスミーティングでは腫瘍径2cm以下の肝細胞癌はラジオ波焼灼療法を選択するという意見が多かった。一方，3cm前後の単発の腫瘍で肝予備能がChild-Pugh class Aであれば外科的切除を推奨する意見が多かった。ラジオ波焼灼療法と外科的切除に関しては現在無作為前向き比較試験（SURF試験）が全国で進行中でありこの結果の解析により，よりエビデンスに基づいた治療法の選択が可能になると考えられる。

肝移植に関しては脳死肝移植ではミラノ基準を遵守すべきであるという意見が多いのに対し，生体肝移植では必ずしもミラノ基準にこだわる必要がないとの考えが多いようである。レシピエントの背景肝に関してはChild-Pugh class Cに限定すべきという考えが支配的であり，これはChild-Pugh class A/Bの段階では，移植以外の治療法で対応可能との考えを反映しているものと思われる。

根治不能な進行肝細胞癌に対する治療は脈管浸潤がない場合は肝動脈化学塞栓術（TACE）がエビデンスのある治療法として世界中で広く行われている。門脈などへ高度に腫瘍が浸潤した症例やTACEができないほどの肝内多発症例などに対しては本邦ではシスプラチン＋5-FUもしくはインターフェロン皮下注射＋5-FUによる肝動注化学療法（HAIC）が行われている。いずれのHAICも奏効率は30～40%で，予後もほぼ同等である。しかし，HAICは本邦において積極的に行われているが，治療効果を対照群と比較した報告がないため欧米では受け入れられていないのが現状である。2007年に公表されたSHARP試験[9]の結果により分子標的治療薬であるソラフェニブの進行肝細胞癌に対する有用性が証明され，本邦にも2009年に臨床での使用が認可された。現時点でのソラフェニブの治療対象症例は肝予備能がChild-Pugh class AでTACEやHAICが不応もしくは不能になった症例や肝外転移を認める症例である。SHARP試験以来症例の蓄積が進み，ソラフェニブ単独治

療では予後延長の効果がわずか3ヵ月であること，副作用が多く，こまめな薬剤の減量が必要なことなど様々な問題点も明らかとなってきた。今後しばらくはソラフェニブに勝る新規分子標的治療薬が認可される予定がないため，既存の治療法とソラフェニブとを組み合わせた治療法に更なる予後改善効果を期待したい。現在進行中のTACEとソラフェニブとの併用療法（TACTICS）やHAICとの併用療法（SILIUS）の結果次第で今後進行肝細胞癌の治療法が大きく変わる可能性がある。

おわりに

B型肝炎は母子感染の予防方法が確立・実行されていることでウイルス保菌者の数が今後激減していくことが予想される。さらに，核酸アナログ製剤の登場により病勢のコントロールが容易になり肝硬変による肝不全や肝発癌に至る症例も減少することが予想される。同様に，C型肝炎も感染予防の進歩とインターフェロン製剤，プロテアーゼ阻害剤の出現，さらに近い将来認可されるであろうポリメラーゼ阻害剤など薬剤の進歩およびインターフェロンを用いないプロテアーゼ阻害剤とNS5A阻害剤併用療法の開発によるウイルス駆除の効率が改善され患者数は減少の一途をたどることが予想される。このため肝細胞癌に関しても本邦では自然に減少することが考えられるが，現在肝細胞癌に苦しんでいる患者や発癌直前の患者も数多くいることも事実である。また，海外においては，今後しばらくの間患者数が増加し続けることが予想される。このため，ソラフェニブに既存の治療法を併用した治療法の効果を早く検証し，エビデンスのある治療法として確立することが我々の責務と考える。

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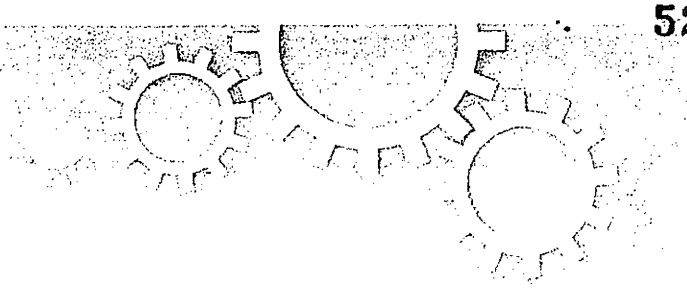

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Correspondence and requests for materials should be addressed to S.K. (skojima@riken.jp)

HCV NS3 protease enhances liver fibrosis via binding to and activating TGF-β type I receptor

Kotaro Sakata[1,2,3], Mitsuko Hara[1], Takaho Terada[4,5], Noriyuki Watanabe[6], Daisuke Takaya[4,7], So-ichi Yaguchi[8], Takehisa Matsumoto[4,7], Tomokazu Matsuura[9], Mikako Shirouzu[4,7], Shigeyuki Yokoyama[4,5], Tokio Yamaguchi[10], Keiji Miyazawa[8], Hideki Aizaki[6], Tetsuro Suzuki[11], Takaji Wakita[6], Masaya Imoto[2] & Soichi Kojima[1]

[1]Micro-signaling Regulation Technology Unit, RIKEN Center for Life Science Technologies, Saitama 351-0198, Japan, [2]Department of Biosciences and Informatics, Faculty of Science and Technology, Keio University, Kanagawa 223-8522, Japan, [3]Drug Discovery Laboratory, Wakunaga Pharmaceutical Co., Ltd., Hiroshima 739-1195, Japan, [4]RIKEN Systems and Structural Biology Center, Kanagawa 230-0045, Japan, [5]RIKEN Structural Biology Laboratory, Kanagawa 230-0045, Japan, [6]Department of Virology II, National Institute of Infectious Diseases, Tokyo 162-8640, Japan, [7]Division of Structural and Synthetic Biology, RIKEN Center for Life Science Technologies, Kanagawa 230-0045, Japan, [8]Department of Biochemistry, Interdisciplinary Graduate School of Medicine and Engineering, University of Yamanashi, Yamanashi 409-3898, Japan, [9]Department of Laboratory Medicine, the Jikei University School of Medicine, Tokyo 105-8461, Japan, [10]RIKEN Program for Drug Discovery and Medical Technology Platforms, Saitama 351-0198, Japan, [11]Department of Infectious Diseases, Hamamatsu University School of Medicine, Shizuoka 431-3192, Japan.

Viruses sometimes mimic host proteins and hijack the host cell machinery. Hepatitis C virus (HCV) causes liver fibrosis, a process largely mediated by the overexpression of transforming growth factor (TGF)-β and collagen, although the precise underlying mechanism is unknown. Here, we report that HCV non-structural protein 3 (NS3) protease affects the antigenicity and bioactivity of TGF-β2 in $(CAGA)_9$-Luc CCL64 cells and in human hepatic cell lines via binding to TGF-β type I receptor (TβRI). Tumor necrosis factor (TNF)-α facilitates this mechanism by increasing the colocalization of TβRI with NS3 protease on the surface of HCV-infected cells. An anti-NS3 antibody against computationally predicted binding sites for TβRI blocked the TGF-β mimetic activities of NS3 *in vitro* and attenuated liver fibrosis in HCV-infected chimeric mice. These data suggest that HCV NS3 protease mimics TGF-β2 and functions, at least in part, via directly binding to and activating TβRI, thereby enhancing liver fibrosis.

Viruses sometimes take over the host cell machinery by mimicking host cell proteins. This strategy infers survival, infection, and replication advantages to the virus[1,2], which may thereby contribute to the development of human disease.

Chronic hepatitis C virus (HCV) infection is one of the major causes of liver fibrosis, cirrhosis, and hepatocellular carcinoma[3,4]. However, the molecular mechanism by which HCV induces liver fibrosis is not fully understood. An estimated 130–170 million people worldwide are infected with HCV[5]. HCV, classified in the genus *Hepacivirus* of the family *Flaviviridae*, is a positive-strand RNA virus with an approximately 9.6-kb viral genome encoding structural (core, E1, and E2) and non-structural proteins (p7, NS2, NS3, NS4A, NS4B, NS5A, and NS5B)[6]. Of these proteins, NS3 is a member of the serine protease family that cleaves the HCV polyprotein to generate mature viral proteins that are required for viral replication[7].

Liver fibrosis, a common feature of chronic liver diseases, is caused by the excessive accumulation of extracellular matrix (ECM) proteins, including collagen. Transforming growth factor (TGF)-β, the most potent fibrogenic cytokine, is produced in its high molecular weight latent form and partly activated through the proteolytic cleavage of its propeptide region, termed latency associated protein (LAP), by serine proteases, plasmin, and plasma kallikrein[8,9]. The resultant active TGF-β signals via TGF-β type I (TβRI) and type II receptors (TβRII), inducing the phosphorylation of Smad2/3, which then binds to Smad4 and forms a complex that enters the cell nucleus. This complex acts as a transcription factor that controls the expression of target genes, including collagen and TGF-β itself, by binding to the DNA elements containing the minimal Smad-binding element, CAGA box[10].

Because the LAPs of TGF-β2 and -β3 have sequences that share partially homology with the NS3 cleavage site between NS3 and NS4A of HCV[7], we speculated that NS3 might activate TGF-β2 and/or TGF-β3 via the proteolytic cleavage of their LAP portions. We found, however, that NS3 protease DID NOT directly activate latent TGF-β2/3. Instead, it mimicked TGF-β2 and induced TGF-β signaling by binding and activating TβRI, leading to the induction of fibrogenic genes. This pathway was enhanced in the presence of an inflammatory cytokine, tumor necrosis factor (TNF)-α, as TNF-α increased the expression of TβRI. Furthermore, we found that NS3 colocalized with TβRI on the surface of an HCV-infected hepatoma cell line, and we observed direct binding between recombinant NS3 and TβRI. These phenomena were reproduced in chimeric mice transplanted with human hepatocytes that had been infected with HCV. These data suggest a novel mechanism by which HCV induces liver fibrosis.

Results

HCV NS3 protease exerted TGF-β mimetic activity via TβRI. To confirm whether HCV NS3 protease might induce the activation of latent TGF-β2, bacterially expressed recombinant NS3 (Supplementary Fig. S1) was incubated with conditioned medium obtained from HEK293T cells transiently overexpressing latent TGF-β2, and the concentration of active TGF-β2 in the reaction mixtures were measured by ELISA. Although the addition of NS3 increased active TGF-β2 concentrations in a dose-dependent manner, these increases were not time-dependent (Supplementary Fig. S2). Instead, we found that NS3 protease itself reacted with TGF-β2 in a dose-dependent manner, as determined by ELISA (Fig. 1A). Next, to assess whether NS3 could induce the bioactivity of TGF-β via TβRI, and whether its activity was dependent on protease activity, we performed a luciferase reporter assay with the TGF-β-responsive $(CAGA)_9$-Luc reporter in CCL64 cells. NS3 demonstrated TGF-β mimetic activity, which was alleviated in the presence of TβRI kinase inhibitors (SB-431542 and LY-364947) in a dose-dependent manner (Fig. 1B). In contrast, an NS3 protease inhibitor, VX-950 (telaprevir), did not affect luciferase activity (Fig. 1B). An unrelated protein with almost the same molecular weight as NS3, HLA class II histocompatibility antigen, DM α chain (HLA-DMA), as well as a carrier-free, tag-control sample, did not exert TGF-β mimetic activity, thus demonstrating the specificity of NS3 (Supplementary Fig. S3). Additionally, an anti-TGF-β2 antibody that detected NS3 in the TGF-β2 ELISA did not inhibit luciferase activity (Supplementary Fig. S4).

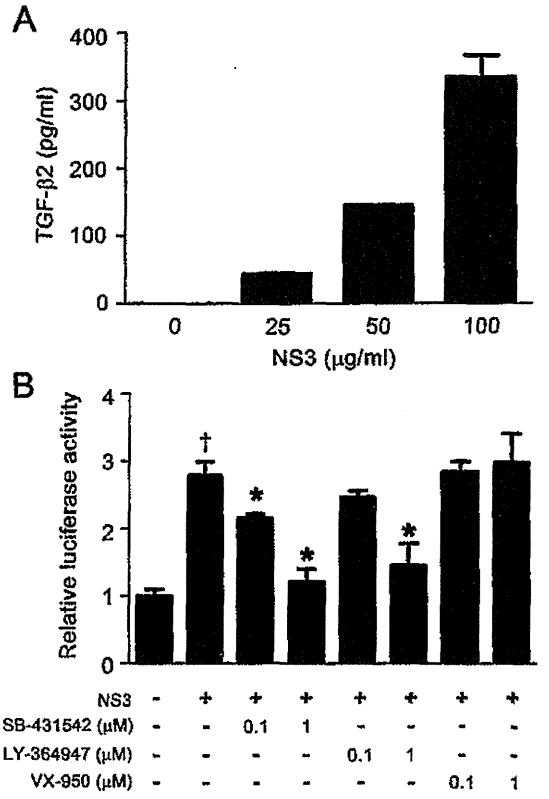

Figure 1 | HCV NS3 protease exerted TGF-β mimetic activity via the type I receptor. (A) TGF-β2 antigenicity of NS3. The indicated concentrations of recombinant NS3 protease were used in the TGF-β2 ELISA assays. (B) TGF-β mimetic activity of NS3 and its suppression by TβRI kinase inhibitors. $(CAGA)_9$-Luc CCL64 cells were stimulated with 100 μg/ml of recombinant NS3 protease for 24 hours, with or without the indicated concentration of TβRI kinase inhibitor or the NS3 protease inhibitor VX-950 (telaprevir). After 24 hours, the cells were harvested and luciferase activity measured. †$p < 0.05$ compared with untreated control cells, *$p < 0.05$ compared with NS3-treated cells without any inhibitors. The data are shown as the mean ± SD (n = 3), and representative results from three independent experiments with similar results are shown.

NS3 stimulated collagen production in hepatic cells, which was augmented by TNF-α. We examined the effect of NS3 on the expression of TGF-β1 and collagen α1 (I) in the human hepatic stellate cell line LX-2. Treatment with NS3 for 12 hours significantly increased both TGF-β1 (1.6-fold) and collagen α1 (I) (1.4-fold) expression in these cells (Fig. 2A). On the contrary, NS3 did not affect the expression of these genes in the normal hepatic cell line Hc. The pretreatment of the cells with tumor necrosis factor-α (TNF-α) enhanced increased TGF-β1 and collagen α1 (I) expression mediated by NS3 and was also accompanied by an increase in TGF-β receptor expression (Fig. 2B). Further increases in TβRI expression were not observed by combination treatment with TNF-α, suggesting that TNF-α increased TβRI expression, which may have enhanced the TGF-β mimetic activity of NS3 in these cells. Furthermore, Smad3 phosphorylation was also induced by NS3 in Hc cells that had been pretreated with TNF-α (Fig. 2D). A similar cooperativity between TNF-α and NS3 protease was not observed in LX-2 cells (Fig. 2C).

Interaction between NS3 and TβRI on the surface of HCV-infected HCC cells. NS3 was immunostained on the surface of HCV-infected Huh-7.5.1 cells both with and without permeabilization. In contrast, an ER marker, calnexin, was only positive after the permeabilization of the cells (Fig. 3A). To examine whether NS3 that was localized to the surface of HCV-infected Huh-7.5.1 cells interacted with TβRI, we performed co-immunostaining (Fig. 3B) and in situ proximity ligation assay (PLA) (Fig. 3C) using antibodies against NS3 and TβRI. Both results showed that NS3 was colocalized and formed a complex with TβRI on the cell surface. Because LX-2 cells (hepatic stellate cells) are not infected with HCV, the data were not recorded. We also co-cultured Huh-7.5.1 infected with HCV and LX-2 cells and examined them using *in situ* PLA. However, the interaction between NS3 protease and TβRI was not observed on the surface of LX-2 cells. Furthermore, we performed co-immunoprecipitation assays using recombinant NS3 and the extracellular domain of TβRI and TβRII. As shown in Figure 3D, FLAG-tagged NS3 bound to TβRI and TβRII, whereas FLAG-tag alone failed to interact with TGF-β receptors (Fig. 3D and Supplementary Fig. S5).

Docking simulation using the Katchalski-Katzir algorithm predicted that NS3 interacts with TβRI at three sites, T22-S42, T76-P96, and G120-S139, in NS3 and F55-M70, I72-V85, and C86-Y99 in TβRI, respectively (Fig. 3E, Table 1, and Supplementary Fig. S6). The predicted binding site peptides, particularly the peptide derived from site 3, completely blocked the interaction between NS3 and

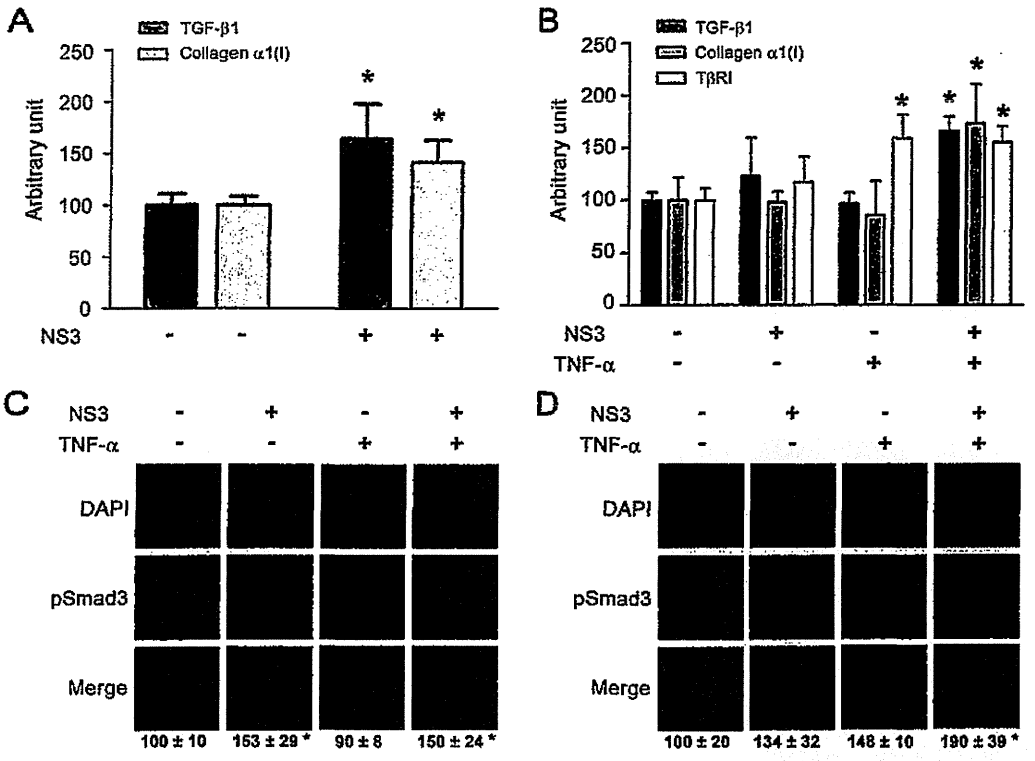

Figure 2 | Cooperativity between NS3 and TNF-α in the stimulation of TGF-β1, collagen α1(I), and TβRI expression. (A) Effect on TGF-β1 and collagen α1(I) mRNA expression in LX-2 cells. The cells were stimulated with 50 μg/ml of NS3 for 12 hours. Total cellular RNA was isolated and reverse transcribed to cDNA, and real-time PCR was performed as described in the Methods section. $*p < 0.05$ compared with untreated control cells. (B) Effect of pretreatment with TNF-α on the stimulation of expression of TGF-β1, collagen α1(I), and TβRI by NS3 protease in HC cells. Following the pretreatment of the cells with 20 ng/ml TNF-α for 12 hours, they were stimulated with 25 μg/ml NS3 for 12 hours, and mRNA expression was measured as described above. $*p < 0.05$ compared with untreated cells. The data are shown as the mean ± SD (n = 3). (C and D) The effect of pretreatment with TNF-α on the stimulation of phosphorylation of Smad3 by NS3 protease in LX-2 cells (C) and Hc cells (D). After the cells were treated with 20 ng/ml TNF-α for 12 hours and 25 μg/ml NS3 for another 12 hours, they were fixed, and immunofluorescent staining was performed as described in the Methods section. The experiments were performed in duplicate. The relative fluorescence intensities of phospho-Smad3 (% of untreated control cells) in 4 randomly selected fields from each dish were calculated with ZEN software and are shown as the mean ± SD. The results are representative of three independent experiments with similar results.

TβRI in the immunoprecipitation experiment (Supplementary Fig. S7A). Antibodies produced to these predicted binding sites within both NS3 and TβRI decreased the TGF-β mimetic activity of NS3 in $(CAGA)_9$-Luc CCL64 cells (Fig. 3F–H). Furthermore, the anti-NS3 antibody inhibited HCV-induced Smad3 phosphorylation (Supplementary Fig. S7B).

Anti-NS3 antibody prevented liver fibrosis in HCV-infected chimeric mice. To test our hypothesis that NS3 exerts TGF-β mimetic activity, thereby causing liver fibrosis, we examined whether the anti-NS3 antibody could prevent liver fibrosis in HCV-infected human hepatocyte-transplanted chimeric mice. The anti-NS3 antibody significantly prevented hepatic collagen accumulation in the mice (Fig. 4A) and decreased the mRNA expression of both TGF-β1 and collagen α1 (I) (Fig. 4B and 4C). There was no significant change in the serum levels of human albumin and HCV RNA during treatment with the anti-NS3 antibody (Supplementary Fig. S8A and S8B).

Discussion

Several groups have studied the molecular mechanisms by which HCV induces liver fibrosis and have reported the following: (i) HCV core protein activates the TGF-β1 promoter via the MAPK pathway in core protein-expressing human hepatocellular carcinoma HepG2 cells[11]; (ii) recombinant core protein upregulates the expression of fibrogenic genes in the human hepatic stellate cell line LX-2 via the toll-like receptor 2 [12] and the obese receptor[13]; and (iii) NS3 protease induces TGF-β1 production in NS3-overexpressing human hepatoma Huh-7 cells[14]. Our data show that NS3 protease mimics TGF-β2 and directly exerts its activity, at least in part, via binding to and activating TβRI, thereby enhancing liver fibrosis. The following experiments should be carried out in the future: effect of NS3 on TβRI phosphorylation, the expression of TGF-β2, TGF-β3, and other TGF-β responsive genes, such as plasminogen activator inhibitor-1, a tissue inhibitor of metalloproteinase-1, and α-smooth muscle actin, to further validate the TGF-β mimetic activity of NS3.

HCV NS3 is a chimera of a helicase and serine protease, which cleaves not only the junction between NS3-4A, NS4A-4B, NS4B-5A, and NS5A-5B for viral polyprotein processing, which is essential to the viral lifecycle, but also the toll-interleukin-1 receptor domain-containing, adaptor-inducing beta interferon, and mitochondrial antiviral signaling protein, which results in the disruption of innate immune responses[7,15]. An NS3 protease inhibitor, telaprevir, which was approved by the FDA in 2011, has been used in triple combination therapy with the current standard treatment of PEGylated interferon and ribavirin[16]. Telaprevir did not inhibit TGF-β mimetic activity in a $(CAGA)_9$-Luc reporter gene assay (Fig. 1C), suggesting that the TGF-β mimetic activity of NS3 is independent of its protease activity.

Much interest has centered on the fact that extraordinarily high concentrations of NS3 protease, up to 100 μg/ml, could exist in

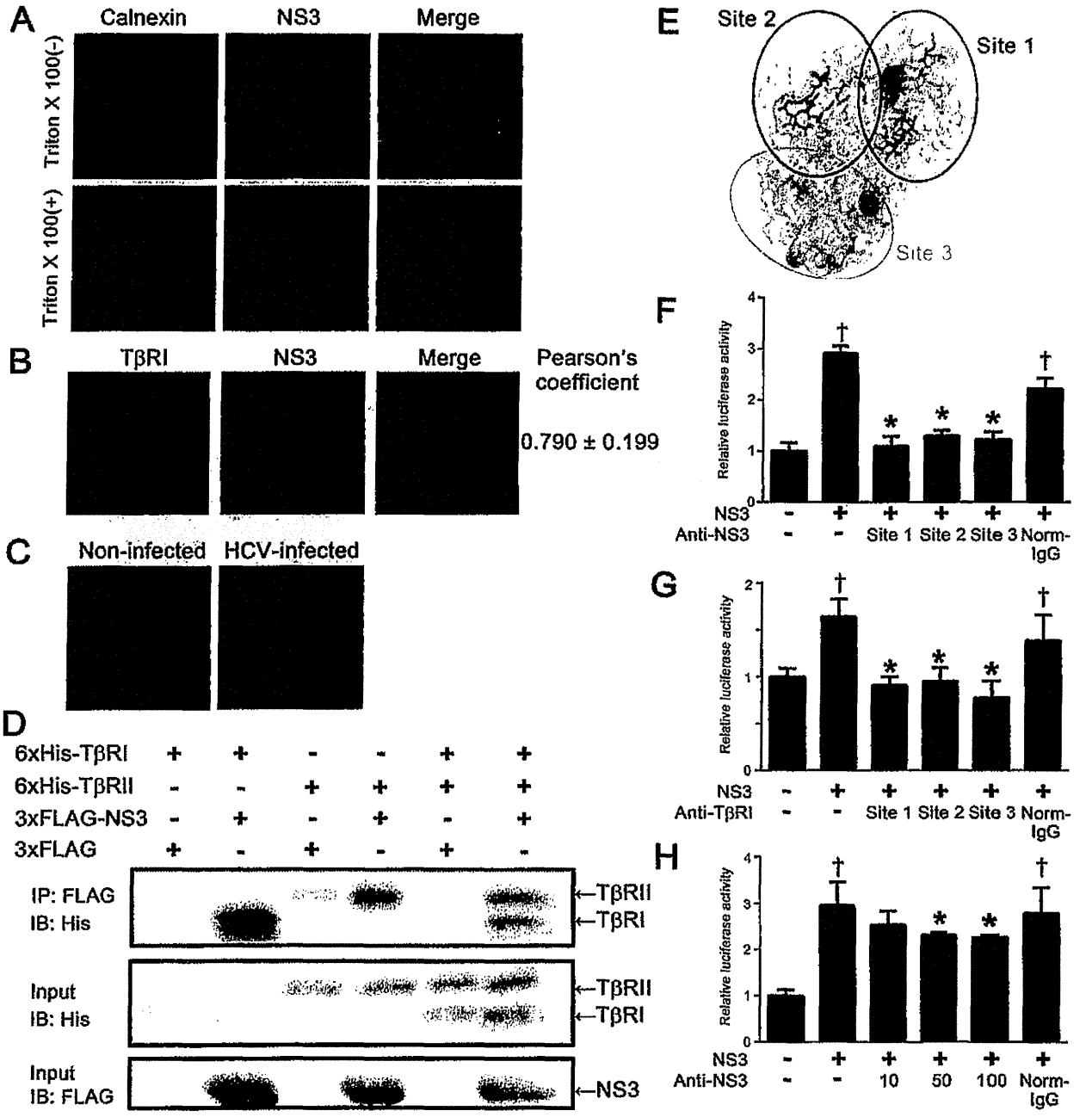

Figure 3 | NS3 protease colocalized and directly interacted with TβRI on the surface of HCV-infected cells. (A) The detection of NS3 protease on the surface of HCV-infected Huh-7.5.1 cells. The cells were fixed, followed ± by permeabilization with Triton-X 100, and then stained with DAPI, anti-NS3 antibody, and anti-calnexin antibody. (B) The colocalization of NS3 protease with TβRI in HCV-infected Huh7.5.1 cells. The cells were fixed and stained with DAPI, anti-NS3 antibody, and anti-TβRI antibody, as described in the Methods section. Pearson's colocalization coefficient values were obtained from 4 randomly selected fields using the ZEN software. The results are shown as the mean ± SD and are representative of three independent experiments with similar results. (C) The detection of NS3-TβRI proximity by in situ PLA in HCV-infected Huh-7.5.1 cells. The red dots indicate interactions between NS3 protease and TβRI, and the nuclei were identified by DAPI staining. (D) The physical interaction of NS3protease with TβRI and TβRII. FLAG-tagged NS3protease was incubated with 6xHis-tagged TβRI and/or TβRII and immunoprecipitated. The coprecipitated proteins were visualized by immunoblotting using anti-His antibody. The gels were run under the same experimental conditions. Cropped blots are shown (full-length blots are presented in Supplementary Fig. S5). (E) The structural overview of the NS3protease. The indicated colored amino acids (site 1, red; site 2, magenta; and site 3, cyan) show the important residues within the putative binding sites to TβRI, and the sequences are presented in Table 1. TGF-β mimetic activity of NS3 was inhibited in the presence of either anti-NS3 polyclonal antibodies against the predicted binding sites of TβRI (F), or anti-TβRI polyclonal antibodies against predicted binding sites of NS3 (G), and anti-NS3 monoclonal antibody against predicted binding site 3 of TβRI (H). Luciferase activities in $(CAGA)_9$-Luc CCL64 cells were measured as before. Normal mouse IgG (Norm-IgG) was used as a negative control. The data are shown as the mean ± SD. †$p < 0.05$ compared with untreated control cells, *$p < 0.05$ compared with NS3-treated cells without any antibodies. Representative results from three independent experiments with similar results are shown.

Table 1 | The amino acid sequences of predicted binding sites between NS3 protease and TβRI

	NS3 protease	TβRI
Site 1	TGRDKNQVEGEVQVVSTATQS	FVSVTETTDKVIHNSM
Site 2	TNVDQDLVGWPAPPGARSLTP	TAEIDLIPRDRPFV
Site 3	GDNRGSLLSPRPVSYLKGSS	CAPSSKTGSVTITTY

The underlined letters denote the putative contact residues.

proximity to a TGF-β receptor. This line of inquiry led us to identify the cooperativity between NS3 and TNF-α, although the cooperative effect was maximal at one fourth this concentration of NS3. Serum levels of TNF-α in chronic hepatitis C patients are known to be significantly higher than those in healthy subjects[17,18]. We showed that TNF-α increased the susceptibility of cells to NS3 by enhancing the expression of TβRI, thereby further increasing the levels of profibrogenic genes (Fig. 2B). Various hepatic cell lines expressed different levels of TβRI, and there appeared to be a threshold in the level of TβRI that enabled cells to produce collagen mRNA upon stimulation with NS3. In particular, Hc cells expressed levels of TβRI below this predicted threshold (Supplementary Fig. S9). Consistent with our findings, carbon tetrachloride has recently been reported to induce acute liver injury, specifically significant liver fibrosis with inflammation, in transgenic mice expressing the full-length HCV polyprotein[19].

We documented the colocalization of NS3 and TβRI on the cell surface of HCV JFH-1-infected Huh-7.5.1 cells (Fig. 3). The results of co-immunoprecipitation and in situ PLA studies supported this conclusion. In future studies, we intend to use mutagenesis experiments of the predicted binding site and competition assays using NS3 and TGF-β in $(CAGA)_9$-Luc CCL64 cells to determine the mechanism of NS3 and TβRI binding. However, at present, how NS3 is released to the extracellular milieu remains to be elucidated. One possibility is that NS3 leaks passively from injured hepatocytes, as is the case for alanine aminotransferase and aspartate aminotransferase. Another possibility is that NS3 is secreted from HCV-infected cells via the Golgi complex. A recent report showed that nonstructural protein (NS) 1 of the dengue virus (DENV) and West Nile virus (WNV) is secreted from DENV- and WNV-infected cells through the Golgi complex following expression in association with the endoplasmic reticulum. Like HCV, these viruses are also members of the family *Flaviviridae*[20].

Zhang et al.[21] identified antibodies against NS3 in the serum of chronic hepatitis C patients and suggested that extracellular NS3 may be present in such cases. However, it remains unclear whether the concentration of HCV NS3 is as high as in our in vitro experiments. Although DENV NS1 has been reportedly detected at high levels (up to 50 μg/ml) in the serum of DENV-infected patients[22], further study is warranted to determine the serum or tissue NS3 concentrations in patients with chronic hepatitis C.

In this study, we generated polyclonal and monoclonal anti-NS3 antibodies that block the NS3-TβRI interaction. All anti-NS3 and anti-TβRI polyclonal antibodies generated against the predicted binding sites almost completely blocked TGF-β mimetic activity. This finding was likely due to steric hindrance by these antibodies or a requirement of binding at all three sites for signal transduction by NS3. The monoclonal antibody is a powerful tool that can be used to explore our working hypothesis that NS3 enhances liver fibrosis via the TGF-β receptor *in vivo*. We showed that the anti-NS3 monoclonal antibody generated against a predicted binding site to TβRI ameliorated liver fibrosis in HCV-infected human hepatocyte transplanted chimeric mice (Fig. 4A–C). The control of fibrosis after the eradication of the virus determines the prognosis, including the likelihood of progression to tumorigenesis. Therefore, the NS3 antibody

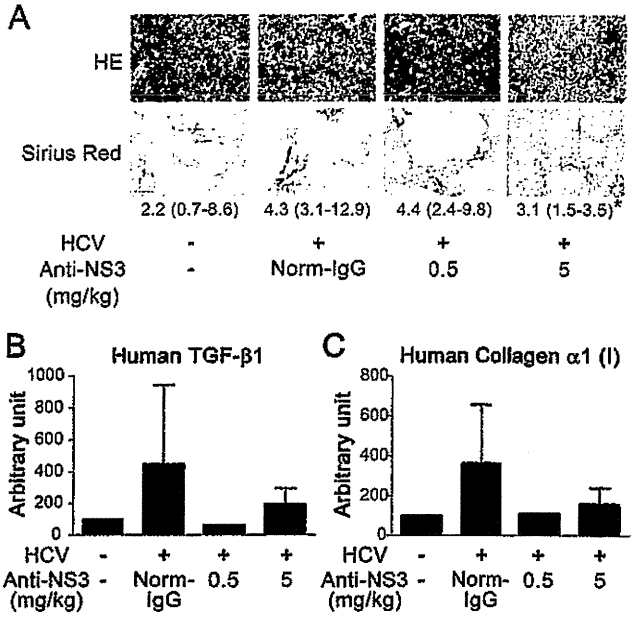

Figure 4 | Anti-NS3 antibody attenuated liver fibrosis in the HCV-infected chimeric mice. (A) Staining of liver sections. Paraffin sections were prepared from the livers of HCV-infected chimeric mice 16 weeks after HCV inoculation, and stained with hematoxylin and eosin (upper panels) and Sirius Red (lower panels). An anti-NS3 antibody was administered at the indicated doses, and normal mouse IgG (Norm-IgG) was administered at a dose of 5 mg/kg. For each group, the median ratios in Sirius Red positive/total area (%) from 6 randomly selected fields are shown, with the range in parentheses. $*p < 0.05$ compared with HCV-infected mice without anti-NS3 antibody. Scale bar = 100 μm. The representative result from 6 randomly selected fields is shown. (B) and (C) Hepatic mRNA expression in HCV-infected chimeric mice. Total RNA was isolated from the livers of these mice and reverse transcribed to cDNA, and real-time PCR was performed as described in the Methods section to quantitate the expression of human TGF-β1 expression (B) and human collagen α1 (I) (C). The data are shown as the mean ± SD, and representative results from two independent experiments with similar results are shown.

against the TβRI binding site might have a clinical benefit in HCV patients with cirrhosis after combination therapy.

In conclusion, we demonstrated for the first time that HCV NS3 protease serves as a novel TGF-β receptor ligand and enhances liver fibrosis. This phenomenon might be beneficial to the virus, as TGF-β signals suppress host immunity. Our results provide elucidation regarding the molecular mechanism by which HCV induces liver fibrosis.

Methods

Materials. SB-431542 and LY-364947 were purchased from Sigma-Aldrich (St. Louis, MO). Recombinant human TNF-α was purchased from R&D systems, Inc. (Minneapolis, MN). Anti-NS3 antibody and anti-calnexin antibody were purchased from Abcam (Cambridge, UK). Anti-TβRI antibody and anti-phospho-Smad3 antibody were purchased from Santa Cruz Biotechnology (Santa Cruz, CA) and Immuno-Biological Laboratories (Gunma, Japan), respectively. Anti-Flag M2 antibody and anti-His antibody were purchased from Sigma (St. Louis, MO). Anti-NS3 antibodies and anti-TβRI antibodies against predicted binding sites were provided by the BioMatrix Research Institute (Chiba, Japan).

Cell culture. $(CAGA)_9$-Luc CCL64 cells were kindly provided by Prof. Hideaki Kakeya (Kyoto University, Kyoto, Japan), the hepatic stellate cell line LX-2 was kindly provided by Prof. Norifumi Kawada (Osaka City University, Osaka, Japan), and the human hepatoma cell line Huh-7.5.1 were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, penicillin, and streptomycin. HC cells, a normal human hepatocyte cell line purchased from Cell Systems (Kirkland, WA), were cultured in CS-C complete medium (Kirkland, WA).