hypothesis is supported by the demonstration that plasma VEGF concentrations increased shortly after treatment with TACE.[24-26] It is believed that these increases in plasma VEGF concentration are related to the induction of tissue hypoxia.[27] However, the peak time point of VEGF elevation during sorafenib administration was different from that previously reported in TACE, in which a transient elevation of VEGF was observed within 7 days after TACE.[24-26] This observed difference may be related to the continuous induction of hypoxia by sorafenib administration.

It is noteworthy that, in our study, decreases in plasma VEGF observed within 8 weeks of sorafenib administration were associated with better OS. One possible reason for this association may be that the decrease in VEGF concentrations reflects a decrease in the number of tumor cells secreting VEGF. An association between changes in VEGF concentrations and disease progression was observed in a previous study of an anti-VEGF antibody, bevacizumab, in patients with advanced HCC.[23] In that study, plasma VEGF-A concentrations decreased from baseline in all patients after 8 weeks of bevacizumab therapy and increased to near baseline levels in 5 of 6 patients at the time of disease progression. Unfortunately, plasma VEGF-A levels after 8 weeks of bevacizumab in that study were available for only 8 of 46 patients who were enrolled the study, and plasma VEGF-A levels after 4 weeks were not evaluated. In our study, all patients were evaluated before and every 4 weeks after starting sorafenib. Moreover, we demonstrated the usefulness of plasma VEGF concentrations at 8 weeks and not at 4 weeks. Zhu et al[28] reported that plasma levels of VEGF and placental growth factor increased after cediranib, a pan-VEGFR tyrosine kinase inhibitor monotherapy for advanced HCC. In that study, progression-free survival was correlated inversely with baseline levels of VEGF, soluble VEGFR2 (sVEGFR2), and basic fibroblast growth factor and with on-treatment levels of basic fibroblast growth factor and insulin-like growth factor-1; and progression-free survival was directly associated with on-treatment levels of interferon-γ. Because changes of VEGF concentrations during therapy were not identified as a prognostic factor in the study by Zhu et al, biomarkers that predict prognosis may be different among different types of tyrosine kinase inhibitors. Jayson et al[29] reported that plasma VEGF-A in patients who received bevacizumab was potentially predictive and prognostic in metastatic breast, gastric, and pancreatic cancers; however, it was only prognostic (and not predictive) in metastatic colorectal cancer, nonsmall cell lung cancer, and renal cell carcinoma. In our study, we measured plasma VEGF concentrations and not plasma VEGF-A concentrations. Sorafenib is a multikinase inhibitor, whereas bevacizumab is a humanized monoclonal antibody that recognizes and blocks VEGF-A expression. Further studies to evaluate the clinical usefulness of determining VEGF and VEGF-A concentrations during sorafenib therapy are necessary in various cancers. Although the precise mechanism underlying the association between serial changes in VEGF and disease progression is unclear, the findings of the current study are extremely valuable for clinical practice in predicting the prognosis of patients who receive treatment with sorafenib.

Llovet et al[5] studied plasma biomarkers as predictors of outcome in patients with advanced HCC. They measured plasma biomarkers in 491 patients at baseline and in 305 patients after 12 weeks in a phase 3, randomized, controlled trial (the SHARP trial). Those authors concluded that angiopoietin-2 and VEGF were independent predictors of survival in patients with advanced HCC and that none of the tested biomarkers significantly predicted response to sorafenib. In our study, by measuring plasma VEGF monthly, we demonstrated that the changes 8 weeks after starting sorafenib were important for predicting OS.

It has been reported that modified RECIST guidelines are useful for predicting efficacy and prognosis after patients with advanced HCC receive treatment with sorafenib.[30] However, modified RECIST can only be used for typical hypervascular HCC, and not for atypical HCC, including poorly differentiated HCC and diffuse-type HCC. Moreover, the percentage of patients in our study who had PD was only 11.1% (9 of 63 patients), and the objective response rate (CR + PR vs SD) could not predict OS, suggesting that using only modified RECIST guidelines was insufficient for predicting OS in most patients who received sorafenib (non-PD patients). Therefore, it is important to identify a predictive biomarker for those patients who can expect long survival during sorafenib therapy, although their radiologic findings may not be categorized as objective responses.

From this point of view, decreases in VEGF observed in non-PD patients at week 8 may identify patients who have a favorable prognosis. According to our results, the median survival of patients who had a VEGF decrease was extremely good at 31.0 months, and we demonstrated that a VEGF decrease, but not modified RECIST or AFP, was the only significant post-therapeutic factor associated with favorable survival after sorafenib administration (Table 3). In our study, all

patients who had both a VEGF decrease and an AFP response survived during the observation period (median, 19.7 months). Taken together, the combination of a plasma VEGF decrease, an AFP response, and modified RECIST is useful for predicting an extremely favorable prognosis.

This study had a few limitations. The first was our subanalysis of consecutive patients. However, the median survival for the 23 excluded patients who were available for estimation was equivalent to that of the included patients (16.8 months); therefore, it is unlikely that selection bias affected our results. The second limitation is that we measured only plasma VEGF concentrations. In previous studies, many factors, including VEGF-A, short VEGF-A isoform, sVEGFR1, sVEGFR2, sVEGFR3, angiopoietin-2, and insulin-like growth factor-2, were evaluated as biomarkers. However, to our knowledge, this is the first clinical study to demonstrate the early dynamic changes in plasma VEGF concentrations in patients who received sorafenib. Finally, the number of patients in this study was relatively small to make recommendations to physicians. Our results indicated that patients who have decreased VEGF concentrations at 8 weeks have a favorable prognosis, regardless of their radiologic findings. However, further studies with a larger number of patients will be necessary to propose new recommendations.

In conclusion, changes in plasma VEGF concentrations during sorafenib treatment are dynamic in patients with advanced HCC, and an observed decrease in the plasma VEGF concentration 8 weeks after starting sorafenib is associated significantly with favorable OS. Today, because many clinical trials of new molecular-targeted agents for HCC are being conducted, it is necessary for hepatologists and oncologists to determine the time when alternative agents should be started as a second or third line of treatment. Our results have potentially important clinical implications for physicians and may influence their decisions regarding a treatment strategy for advanced HCC in individual patients.

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CONFLICT OF INTEREST DISCLOSURES

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REFERENCES

1. World Health Organization, International Agency for Research on Cancer (IARC); Boyle P, Levin B, eds. World Cancer Report 2008. Lyon, France: IARC Press; 2008.
2. European Association for the Study of the Liver, European Organisation for Research and Treatment of Cancer. EASL-EORTC Clinical Practice Guidelines: management of hepatocellular carcinoma. *J Hepatol.* 2012;56:908-943.
3. Llovet JM, Ricci S, Mazzaferro V, et al. SHARP Investigators Study Group. Sorafenib in advanced hepatocellular carcinoma. *N Engl J Med.* 2008;359:378-390.
4. Cheng AL, Kang YK, Chen Z, et al. Efficacy and safety of sorafenib in patients in the Asia-Pacific region with advanced hepatocellular carcinoma: a phase III randomized, double-blind, placebo-controlled trial. *Lancet Oncol.* 2009;10:25-34.
5. Llovet JM, Pena CE, Lathia CD, Shan M, Meinhardt G, Bruix J. Plasma biomarkers as predictors of outcome in patients with advanced hepatocellular carcinoma. *Clin Cancer Res.* 2012;18: 2290-3000.
6. Lencioni R, Llovet JM. Modified RECIST (mRECIST) assessment for hepatocellular carcinoma. *Semin Liver Dis.* 2010;30:52-60.
7. Shao YY, Lin ZZ, Hsu C, Shen CH, Cheng AL. Early alpha-fetoprotein response predicts treatment efficacy of antiangiogenic systemic therapy in patients with advanced hepatocellular carcinoma. *Cancer.* 2010;116:4590-4596.
8. Kuzuya T, Asahina Y, Tsuchiya K, et al. Early decrease in α-fetoprotein, but not des-γ-carboxy prothrombin, predicts sorafenib efficacy in patients with advanced hepatocellular carcinoma. *Oncology.* 2011;81:251-258.
9. Personeni N, Bozzarelli S, Pressiani T, et al. Usefulness of alpha-fetoprotein response in patients treated with sorafenib for advanced hepatocellular carcinoma. *J Hepatol.* 2012;57:101-107.
10. Raoul JL, Bruix J, Greten TF, et al. Relationship between baseline hepatic status and outcome, and effect of sorafenib on liver function: SHARP trial subanalyses. *J Hepatol.* 2012;56:1080-1088.
11. Hora C, Romanque P, Dufour JF. Effect of sorafenib on murine liver regeneration. *Hepatology.* 2011;53:577-586.
12. Kudo M, Izumi N, Kokudo N, Matsui O, Sakamoto M, Makuuchi M. Management of hepatocellular carcinoma in Japan: consensus-based clinical practice guidelines proposed by the Japan Society of Hepatology (JSH) 2010 updated version. *Dig Dis.* 2011;29:339-364.
13. Bruix J, Sherman M; American Association for the Study of Liver Diseases. Management of hepatocellular carcinoma: an update. *Hepatology.* 2011;53:1020-1022.
14. El-Assal ON, Yamanoi A, Soda Y, et al. Clinical significance of microvessel density and vascular endothelial growth factor expression in hepatocellular carcinoma and surrounding liver: possible involvement of vascular endothelial growth factor in the angiogenesis of cirrhotic liver. *Hepatology.* 1998;27:1554-1562.
15. Yamaguchi R, Yano H, Iemura A, Ogasawara S, Haramaki M, Kojiro M. Expression of vascular endothelial growth factor in human hepatocellular carcinoma. *Hepatology.* 1998;28:68-77.
16. Yoshiji H, Kuriyama S, Yoshii J, et al. Synergistic effect of basic fibroblast growth factor and vascular endothelial growth factor in murine hepatocellular carcinoma. *Hepatology.* 2002;35:834-842.
17. Tamesa T, Iizuka N, Mori N, et al. High serum levels of vascular endothelial growth factor after hepatectomy are associated with poor prognosis in hepatocellular carcinoma. *Hepatogastroenterology.* 2009; 56:1122-1126.
18. Hu J, Xu Y, Shen ZZ, et al. High expressions of vascular endothelial growth factor and platelet-derived endothelial cell growth factor

predict poor prognosis in alpha-fetoprotein-negative hepatocellular carcinoma patients after curative resection. *J Cancer Res.* 2009;135: 1359-1367.
19. Poon RT, Lau C, Pang R, Ng KK, Yuen J, Fan ST. High serum vascular endothelial growth factor levels predict poor prognosis after radiofrequency ablation of hepatocellular carcinoma: importance of tumor biomarker in ablative therapies. *Ann Surg Oncol.* 2007;14: 1835-1845.
20. Schoenleber SJ, Kurtz DM, Talwalkar JA, Rober LR, Gores GJ. Prognostic role of vascular endothelial growth factor in hepatocellular carcinoma: systemic review and meta-analysis. *Br J Cancer.* 2009; 100:1385-1392.
21. Kaseb AO, Hanbali A, Cotant M, Hassan MM, Wollner I, Philip PA. Vascular endothelial growth factor in the management of hepatocellular carcinoma: a review of literature. *Cancer.* 2009;115: 4895-4906.
22. Kaseb A, Hassan M, Lin E, Xiao L, Kumar V, Morris J. V-CLIP: integrating plasma vascular endothelial growth factor into a new scoring system to stratify patients with advanced hepatocellular carcinoma for clinical trials. *Cancer.* 2011;117:2478-2488.
23. Siegel AB, Cohen EI, Ocean A, et al. Phase II trial evaluating the clinical and biologic effects of bevacizumab in unresectable hepatocellular carcinoma. *J Clin Oncol.* 2008;26:2992-2998.
24. Li X, Feng GS, Zheng CS, Zhuo CK, Liu X. Expression of plasma vascular endothelial growth factor in patients with hepatocellular carcinoma and effect of transcatheter arterial chemoembolization therapy on plasma vascular endothelial growth factor level. *World J Gastroenterol.* 2004;10:2878-2882.
25. Suzuki H, Mori M, Kawaguchi C, Adachi M, Miura S, Ishii H. Serum vascular endothelial growth factor in the course of transcatheter arterial embolization of hepatocellular carcinoma. *Int J Oncol.* 1999; 14:1087-1090.
26. Shim JH, Park JW, Kim JH, et al. Association between increment of serum VEGF and poor prognosis after transcatheter arterial chemoembolization in hepatocellular carcinoma patients. *Cancer Sci.* 2008;99:2037-2044.
27. von Marschall Z, Cramer T, Hocker M, Finkenzeller G, Wiedenmann B, Rosewicz S. Dual mechanism of vascular endothelial growth factor upregulation by hypoxia in human hepatocellular carcinoma. *Gut.* 2001;48:87-96.
28. Zhu AX, Ancukiewicz M, Duda DG, et al. Efficacy, safety, pharmacokinetics, and biomarkers of cediranib monotherapy in advanced hepatocellular carcinoma: a phase II study. *Clin Cancer Res.* 2013; 19:1557-1566.
29. Jayson GC, de Haas S, Delmar P, et al. Evaluation of plasma VEGF-A as a potential predictive pan-tumour biomarker for bevacizumab. Abstract 804. Paper presented at: 2011 European Multidisciplinary Cancer Congress; Stockholm, Sweden; September 23-27, 2011.
30. Edeline J, Boucher E, Rolland Y, et al. Comparison of tumor response by Response Evaluation Criteria in Solid Tumors (RECIST) and modified RECIST in patients treated with sorafenib for hepatocellular carcinoma. *Cancer.* 2012;118:147-156.

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ORIGINAL ARTICLE—LIVER, PANCREAS, AND BILIARY TRACT

Inhibition of hepatocellular carcinoma by PegIFNα-2a in patients with chronic hepatitis C: a nationwide multicenter cooperative study

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Abstract
Background We investigated whether the administration of maintenance doses of interferon prevented hepatocellular carcinoma (HCC) in patients with chronic hepatitis C.
Methods Study 1: A multicenter, retrospective, cooperative study was carried out to determine whether long-term administration of low-dose peginterferon alpha-2a (PegIFNα-2a) prevented HCC development in patients with chronic hepatitis C. In total, 594 chronic hepatitis C patients without a history of HCC were enrolled and treated with 90 μg PegIFNα-2a administered weekly or bi-weekly for at least 1 year. Study 2: HCC developed in 16 of 99 additional patients without PegIFNα-2a treatment during 3.8 years of observation. A propensity-matched control study was then carried out to compare the incidence of

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HCC between the 59 patients who received low-dose PegIFNα-2a (PegIFNα-2a group) and 59 patients who did not receive PegIFNα-2a treatment (control group), matched for sex, age, platelet count, and total bilirubin levels.

Results Study 1: HCC developed in 49 patients. The risk of HCC was lower in patients with undetectable hepatitis C virus RNA, ≤40 IU/L alanine aminotransferase (ALT), or ≤10 ng/L alpha-fetoprotein (AFP) 24 weeks after the start of therapy. Study 2: The incidence of HCC was significantly lower in the PegIFNα-2a group than in the control group.

Conclusions Low-dose and long-term maintenance administration of PegIFNα-2a decreased the incidence of HCC in patients with normalized ALT and AFP levels at 24 weeks compared with patients without normal ALT and AFP levels.

Keywords Chronic hepatitis C · Hepatocellular carcinoma · Peginterferon

Introduction

Hepatocellular carcinoma (HCC), the sixth most common cancer worldwide, often develops because of long-term hepatitis B or C virus infection [1, 2]. In particular, chronic hepatitis C and hepatic cirrhosis increase the risk of HCC; the annual incidence of tumor development in such patients may be as high as 2–4 % [3–5]. The incidence of HCC decreases in patients who achieve a sustained virological response (SVR) to interferon (IFN) treatment, although the incidence remains high in non-SVR patients [6–9]. A detailed analysis of HCC development revealed that chronic hepatitis C patients aged 65 years or more, especially those with advanced fibrosis of the liver, were at an increased risk of developing HCC [10]. For patients 65 years or older with advanced liver fibrosis, the dose of ribavirin is often reduced or the agent is discontinued, resulting in lower SVR rates in those with discontinuation of ribavirin. Establishing an effective treatment strategy for preventing the development of HCC is important for these high-risk patients.

Factors related to the development of HCC have been analyzed in patients who did not achieve an SVR even after IFN treatment; advanced fibrosis of the liver and high levels of serum alanine aminotransferase (ALT), and alpha-fetoprotein (AFP) are risk factors for HCC development [11, 12]. A randomized controlled trial was conducted in Western countries to determine whether combined peginterferon and ribavirin treatment with weekly administration of 90 μg peginterferon alpha-2a (PegIFNα-2a) could prevent HCC in non-responders. A 3.5-year follow up showed that administration of a maintenance dose of PegIFNα-2a did not reduce tumor incidence in these patients [13]. However, after 8.5 years of observation, the incidence of HCC was decreased among those in the PegIFNα-2a group with cirrhosis [14]. Meanwhile, Bruix et al. [15] reported that maintenance therapy with PegIFNα-2b did not prevent HCC in chronic hepatitis C patients with cirrhosis. In Japan, long-term low-dose administration of natural IFN has been reported to decrease the incidence of HCC [16]. In light of these conflicting results, investigations should be carried out in a large number of patients with chronic hepatitis C to resolve the question of whether IFN treatment prevents the development of HCC.

We carried out a multicenter retrospective cooperative study of patients with chronic hepatitis C to determine whether those treated with 90 μg PegIFNα-2a without ribavirin had a reduced incidence of HCC compared with those not treated with IFN.

Patients and methods

Study 1: analysis of risk factors for HCC in patients treated with long-term low-dose-PegIFNα-2a

In total, at 21 hepatitis centers throughout Japan, 743 patients with hepatitis C who had received 90 μg of PegIFNα-2a therapy weekly or bi-weekly for 1 year or more without having received the full dose (180 μg) since December 2003 were examined retrospectively for the development of HCC. The end of enrollment in this study was the end of December 2008 and the end of follow up was the end of December 2010. Patients with a history of HCC before the start of therapy and those with a therapy period of less than 48 weeks were excluded, leaving 594 patients who had undergone long-term administration of PegIFNα-2a for analysis. At the 21 centers involved in this

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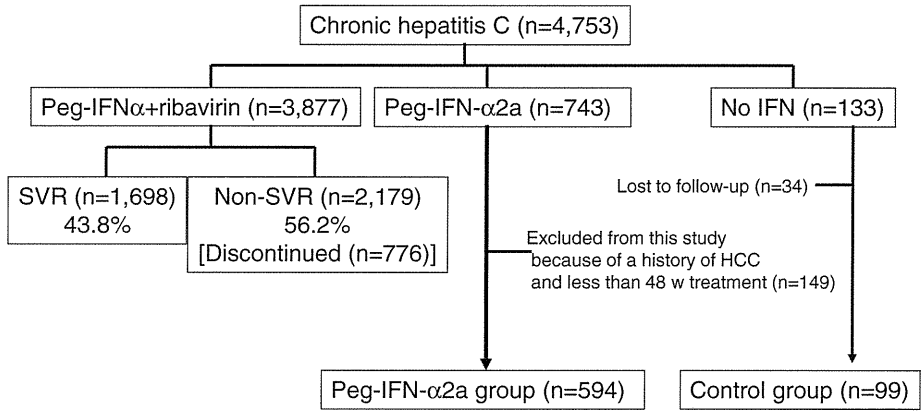

Fig. 1 Flow diagram of the patients' enrollment in the study. *Peg-IFNα* pegylated interferon α, *SVR* sustained viral response, *HCC* hepatocellular carcinoma, *w* week

study, 4,753 patients with chronic hepatitis C had been treated; Peg-IFN and ribavirin combination treatment had been administered to 3,877 patients, 743 patients had received Peg-IFN alone, and 133 patients had not agreed to receive IFN (a flow diagram of the enrollment of patients in this study is shown in Fig. 1). In the patients with Peg-IFN and ribavirin combination treatment, the SVR rate was 43.8 %; SVR was not achieved in 2,179 patients, and in 776 of these patients, the combination therapy was discontinued owing to adverse events or the patient's choice. Patients who failed to achieve an SVR were not included in this study, because the incidence of HCC is known to be reduced even in non-responders to IFN [17].

The backgrounds of the 594 patients studied are shown in Table 1. Findings from the liver biopsies of the patients were classified according to international standards [18]. Long-term PegIFNα-2a treatment is approved by the Japanese Medical Insurance system. Written informed consent was obtained from all patients prior to participation in this study. The study design was approved by the regional ethics committees of the 21 centers involved in this study, including the Musashino Red Cross Hospital, in accordance with the Helsinki Declaration. The 743 patients treated with PegIFNα-2a alone were not indicated for Peg-IFNα and ribavirin combination therapy because of anemia or heart disease. The 133 patients who did not agree to receive IFN served as the control group (see Fig. 1). A large proportion of the 594 study patients had advanced fibrosis of the liver and active inflammation. A dose of 90 μg PegIFNα-2a was administered to 512 and 82 patients weekly and biweekly, respectively, according to the patients' wishes. There were no significant differences between the weekly and biweekly groups in the patients' background data (data not shown).

The median duration of follow up in the PegIFNα-2a group was 1,273 days (range 228–2,768 days) and HCC was observed in 49 of the 594 patients (Table 1). Pre-treatment and on-treatment factors associated with the development of HCC were analyzed by Student's *t*-test, the Mann–Whitney *U*-test, and the χ^2 test (Table 2). Independent factors for the development of HCC were assessed by multivariate analysis using logistic regression. The

Table 1 Background data of patients treated with PegIFNα-2a ($n = 594$)

	$n = 594$
Age (years)	61.7 ± 11.7
Sex (male/female)	258/336
BMI	23.2 ± 3.3
Genotype (1/2)	443/151
Diagnosis (ASC/CH/LC)	4/460/130
History of excess alcohol consumption (≥60 g/day; yes/no)	118/376
Fibrosis (F0, 1, 2/F3, 4)	443/151
Inflammatory activity (A0, 1/A2, 3)	469/125
Diabetes mellitus (no/yes)	499/95
LDL cholesterol (mg/dL)	94.2 ± 31.1
Fasting blood sugar (mg/dL)	106.3 ± 28.5
White blood cell count (/mm^3)	4,360 ± 1,470
Red blood cell count (×10^6/μL)	423.8 ± 56.4
Hemoglobin (g/dL)	13.3 ± 1.8
Platelet count (×10^3/μL)	137 ± 56
Albumin (g/dL)	4.0 ± 0.5
Total bilirubin (mg/dL)	0.8 ± 0.6
AST (IU/L)	65.8 ± 47.8
ALT (IU/L)	72.1 ± 68.0
Gamma-GTP (IU/L)	55.2 ± 51.3
Esophageal varices (no/yes)	344/31
Alpha fetoprotein (ng/L)	6.9 (4.2–13.8)
Once weekly or biweekly PegIFNα-2a	512:82
Baseline HCV RNA (KIU/mL)	1,024 (73–2,130)
Development of HCC (no/yes)	545/49

PegIFN pegylated interferon, *BMI* body mass index, *ASC* asymtomatic carrier, *CH* chronic hepatitis, *LC* liver cirrhosis, *LDL* low-density lipoprotein, *AST* aspartate aminotransferase, *ALT* alanine aminotransferase, *GTP* guanosine triphosphate, *HCV* hepatitis C virus, *HCC* hepatocellular carcinoma

Values are means ± SD, with ranges in parentheses

incidence of HCC was analyzed according to the ALT, AFP, and hepatitis C virus (HCV) RNA levels 24 weeks after the start of PegIFNα-2a administration by using the Kaplan–Meier method. The risk of HCC was analyzed, using the Kaplan–Meier method, only in the non-responders with detectable HCV RNA during PegIFNα-2a administration by dividing them according to the ALT and AFP levels 24 weeks after the start of therapy. The incidence of HCC was compared between the patients with ALT levels of <41 IU/L and those with levels of ≥41 IU/L, and between patients with serum AFP levels of <10 ng/L and those with levels of ≥10 ng/mL at 24 weeks after starting treatment, because at most of the centers participating in the this study, the upper normal range of serum ALT is set at 40 IU/L, and the most significant difference in the incidence of HCC was observed between the PegIFNα-2a and control group with the cut-off serum ALT set at 41 IU/L and cutoff serum AFP set at 10 ng/mL, 24 weeks after starting treatment. The HCV RNA level was measured using the Amplicor Monitor method with a lower detection limit of 50 IU/L (Roche Diagnostics, Tokyo, Japan). A history of excess alcohol consumption was determined as >60 g alcohol per day in order to exclude alcoholic liver disease.

An asymptomatic carrier was defined as a patient with a serum ALT level within the normal range and minimal inflammation or fibrosis in the biopsied tissues of the liver. Chronic hepatitis was defined as mild-to-severe fibrosis of the liver according to liver biopsy [18]. The diagnosis of liver cirrhosis was based on the results of histological examination of the biopsied liver tissues.

Study 2: incidence of HCC in the PegIFNα-2a therapy and non-administration (control) groups in comparison with propensity-matched controls

Ninety-nine of the 133 chronic hepatitis C patients who had not received IFN were examined as controls; patients in this group received liver-protective agents such as glycyrrhizin or were untreated, and the group was observed for more than 1 year. None of the individuals in the control groups had received IFN alone or PegIFNα and ribavirin combination treatment. They were treated for a median of 1,395 days (range 75–6,556 days). Fifty-nine of these patients underwent liver biopsy before the treatment and were considered the control group for the propensity-matched study. For the propensity-matched study, 59 patients were selected from the PegIFNα-2a group according to their age, sex, platelet count, and total bilirubin levels, which had been identified as independent pretreatment risk factors for the development of HCC in Study 1. The rates of HCC were analyzed using the Kaplan–Meier method, and the risk of HCC was analyzed particularly in patients with advanced fibrosis of the liver (F3 and F4).

Table 2 Comparison of HCC and non-HCC patients with long-term PegIFNα-2a administration ($n = 594$)

	Patients with or without development of HCC		*p* value
	With HCC ($n = 49$)	Without HCC ($n = 545$)	
Pretreatment parameters			
Age (years)	63.8 ± 1.7	61.3 ± 0.5	<0.05
Sex (male/female)	32/17	226/319	<0.01
BMI	24.0 ± 0.5	23.1 ± 0.2	n.s.
Genotype (1/2)	47/6	397/148	n.s.
History of excess alcohol consumption (≥60 g/day; yes/no)	11/38	107/338	n.s.
Fibrosis (F0, 1, 2/F3, 4)	25/24	418/127	<0.001
Inflammatory activity (A0, 1/A2, 3)	7/42	462/83	<0.001
Diabetes mellitus (no/yes)	38/11	461/84	n.s.
LDL cholesterol (mg/dL)	88.2 ± 9.0	94.7 ± 2.6	n.s.
White blood cell count (/mm^3)	4,355 ± 210	4,360 ± 64	n.s.
Red blood cell count (×10^6/μL)	420.8 ± 8.1	424.1 ± 2.6	n.s.
Hemoglobin (g/dL)	13.6 ± 0.3	13.3 ± 0.1	n.s.
Platelet count (×10^3/μL)	106 ± 8	140 ± 2	<0.001
Albumin (g/dL)	3.8 ± 0.1	4.0 ± 0.1	<0.001
Total bilirubin (mg/dL)	1.2 ± 0.1	0.8 ± 0.1	<0.001
AST (IU/L)	78.1 ± 6.8	64.6 ± 2.1	n.s.
ALT (IU/L)	72.8 ± 9.7	72.0 ± 2.9	n.s.
Gamma-GTP (IU/L)	68.7 ± 7.5	53.9 ± 2.3	n.s.
Alpha fetoprotein (ng/L)	17.1 (4.4–36.8)	16.7 (4.1–23.1)	n.s.
Esophageal varices	29.0 % (9/31)	6.4 % (22/344)	<0.01
On-treatment parameters			
ALT (IU/L)	59.4 ± 5.7	44.6 ± 1.8	<0.05
Alpha fetoprotein (ng/L)	9.8 (4.6–17.4)	5.5 (3.7–11.1)	<0.01
HCV RNA level (KIU/mL)	236 (<0.5–2,210)	21 (<0.5–1,780)	<0.05

n.s. not significant

Statistical analysis

Categorical data were compared using the χ^2 test or Fisher's exact test. The distributions of continuous variables were analyzed using Student's *t*-test and the Mann–Whitney *U*-test for two groups. Multivariate analysis was

conducted using logistic regression. The cumulative incidence curve was determined using the Kaplan–Meier method and differences between groups were assessed by the log-rank test. For all methods, the level of significance was set at $p < 0.05$. Multivariate analysis of the risk of HCC was carried out using the Cox proportional hazard model. Statistical analyses were performed using the Statistical Package for the Social Sciences software version 11.0 (SPSS, Chicago, IL, USA). In Study 1, age, sex, platelet count, and total bilirubin levels were identified as independent factors for the development of HCC; therefore, these factors were selected for the propensity-matched control study (Study 2) in which 59 patients from the PegIFNα-2a group were included.

Results

Study 1

We analyzed the factors involved in the development of HCC in patients who received 90 μg PegIFNα-2a weekly or biweekly for more than a year. The incidence of HCC did not differ significantly between the groups treated with PegIFNα-2a weekly and biweekly (34 of 512 vs. 15 of 82, respectively). As shown in Table 2, univariate analysis revealed statistically significant differences in the pretreatment parameters including age, sex, fibrosis of the liver, platelet count, albumin level, and total bilirubin, between patients who developed HCC and those who did not. Endoscopy was carried out in 375 patients, and esophageal varices were noted in 31 of them. The incidence of HCC was higher in patients with esophageal varices than in those without varices [29.0 % (9 of 31) vs. 6.4 % (22 of 344)]. Assessment of on-treatment factors by univariate analysis revealed statistically significant differences in serum ALT, AFP, and HCV RNA levels 24 weeks after the start of PegIFNα-2a maintenance treatment (Table 2).

Multivariate analysis including pretreatment parameters revealed that age, sex, fibrosis of the liver, platelet count, and total bilirubin were independent risk factors for HCC development (Table 3). Multivariate analysis including on-treatment parameters identified ALT levels of ≥41 IU/L and AFP levels of ≥10 ng/L 24 weeks after the start of the PegIFNα-2a therapy as independent risk factors for HCC development (Table 3).

The incidence of HCC was significantly lower in patients with ALT levels of ≤40 IU/L than in those with ALT levels of ≥41 IU/L 24 weeks after the start of observation (Fig. 2). The incidence of HCC was also significantly lower in patients with AFP concentrations of <10 ng/mL at 24 weeks after the start of observation than in those with AFP concentrations of ≥10 ng/mL (Fig. 3). The dose of PegIFNα-2a was reduced to 45 μg in 16 patients because of neutropenia and thrombocytopenia. In addition, PegIFNα-2a was discontinued in 18 patients because of adverse events, including depression (7 patients), interstitial pneumonitis (3 patients), thrombocytopenia (3 patients), neutropenia (1 patient), itching (1 patient), and ascites (3 patients). No statistically significant differences were found between the patients with reduced dosage or treatment interruption and those without treatment modifications with respect to overall survival, HCC incidence, ascites formation, variceal bleeding, hepatic encephalopathy, and 2-point increases in the Child-Pugh score. No patients underwent liver transplantation.

Table 3 Independent risk factors for HCC development in patients treated with 90 μg PegIFNα-2a weekly or bi-weekly, evaluated by multivariate analysis (logistic regression analysis)

	Multivariate analysis		
	Odds ratio	95 % Confidence interval (CI)	*p*
Age (years) (every 5 years)	2.24	1.76–9.33	<0.005
Sex (male/female)	3.16	1.56–10.7	<0.005
Fibrosis (F3, 4/F0, 1, 2)	1.69	1.18–5.2	<0.01
Platelet count (<120 × 10^3/μL vs. ≥120 × 10^3/μL)	3.24	1.44–27.6	<0.01
Total bilirubin (mg/dL)	1.59	1.09–2.58	<0.05
ALT (at 24 weeks) (≥41 vs. <40 IU/L)	2.49	1.51–8.28	<0.05
AFP (at 24 weeks) (≥10 vs. <10 ng/L)	3.78	1.92–11.8	<0.01

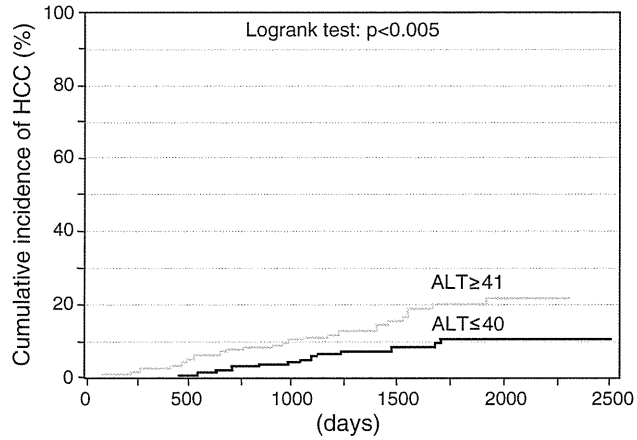

Fig. 2 Comparison of HCC rates in patients administered with PegIFNα-2a ($n = 594$) with respect to alanine aminotransferase (*ALT*) levels 24 weeks after the start of therapy. *Black line* patients with ALT ≥41 IU/L in the first 24 weeks, *gray line* patients with ALT ≤40 IU/L in the first 24 weeks

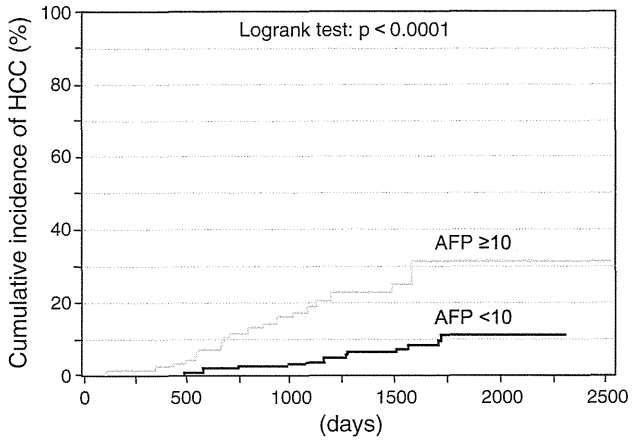

Fig. 3 Comparison of HCC rates in patients administered PegIFNα-2a ($n = 594$) with respect to alpha-fetoprotein (*AFP*) levels in the first 24 weeks after the start of therapy. *Black line* patients with AFP ≥10 ng/mL at 24 weeks, *gray line* patients with AFP <10 ng/mL at 24 weeks

Study 2

We compared the incidence of HCC between 59 patients in the control group and the same number of patients in the PegIFNα-2a group using the matched-pair test. The backgrounds of the patients are shown in Table 4. The PegIFNα-2a group had higher rates of advanced fibrosis (F3 and F4) and active inflammation (A2 and A3). No other differences were found between the two groups, except for the white blood cell count (Table 4).

Development of HCC was observed in 2 patients in the PegIFNα-2a group and 8 in the control group. The incidence of HCC was compared between the two groups, using the Kaplan–Meier method. The incidence of HCC in the PegIFNα-2a group was significantly lower than that in the control group (log-rank test, $p = 0.0187$; Fig. 4). Among the patients with advanced fibrosis of the liver (F3 and F4), those in the PegIFNα-2a group had a lower incidence of HCC than those in the control group. The independent risk factors for the development of HCC were analyzed using the stepwise Cox proportional hazard model. Only PegIFNα-2a administration and age were identified as independent risk factors for the development of HCC (Table 5).

Discussion

The number of HCC cases resulting from HCV infection continues to increase worldwide [19]. To date, IFN therapy is the most effective preventive measure against HCC in patients with chronic hepatitis C; furthermore, the incidence of HCC is reduced in patients who achieve an SVR to IFN [6–9] Therefore, achieving an SVR is the most effective approach for reducing the risk of developing HCC. In Japan, the incidence of HCC is elevated in older patients with hepatitis C. Corroborating this finding, the results of a Japanese study show a higher risk of HCC in patients aged 65 years and more [10]. Therefore, prevention of HCC in aged patients is an important challenge.

In the present multicenter, cooperative, retrospective study conducted in Japan, the incidence of HCC was reduced in patients who received 90 μg PegIFNα-2a weekly or biweekly and had AFP values of <10 ng/mL and ALT values of ≤40 IU/L 24 weeks after the start of the treatment. The results of the matched case–control study of the PegIFNα-2a group and the non-IFN control group show that the incidence of HCC was significantly lower in the PegIFNα-2a group than in the control group, especially in patients with advanced fibrosis of the liver (F3 and F4). However, there could have been a selection bias between

Table 4 Backgrounds of the patients in the propensity-matched control study (PegIFNα-2a group, $n = 59$; control group, $n = 59$)

	PegIFNα-2a group ($n = 59$)	Control group ($n = 59$)	p value
Age (years)	60.5 ± 13.0	63.3 ± 10.5	n.s.
Gender (male/female)	24/35	25/34	n.s.
BMI	22.9 ± 3.6	22.9 ± 3.4	n.s.
Genotype (1/2)	49/10	46/13	n.s.
History of excess alcohol consumption (60 g/day; yes/no)	10/49	4/55	n.s.
Fibrosis (F0, 1, 2/F3, 4)	37/22	43/16	<0.05
Development of HCC (F0–2/F3, 4)	1/1	1/7	n.s.
Inflammatory activity (A0,1/A2, 3)	19/40	30/29	<0.05
Diabetes mellitus (no/yes)	57/2	56/3	n.s.
LDL cholesterol (mg/dL)	95.3 ± 23.8	117.0 ± 4.2	n.s.
White blood cell count (/mm^3)	4,260 ± 1,239	5,193 ± 2,078	<0.05
Red blood cell count ($\times 10^{-4}$/μL)	430 ± 57.8	441 ± 44.9	n.s.
Hemoglobin (g/dL)	13.6 ± 1.5	13.6 ± 1.9	n.s.
Platelet count ($\times 10^{-3}$/μL)	14.5 ± 5.7	15.8 ± 5.7	n.s.
Albumin (g/dL)	4.1 ± 0.5	4.1 ± 0.4	n.s.
Total bilirubin (mg/dL)	0.7 ± 0.5	0.9 ± 0.7	n.s.
AST (IU/L)	58.3 ± 47.7	49.7 ± 26.6	n.s.
ALT (IU/L)	63.6 ± 68.7	58.0 ± 39.2	n.s.
Gamma-GTP (IU/L)	78.3 ± 81.3	55.3 ± 75.1	n.s.
Baseline alpha-fetoprotein (AFP) (ng/L)	7.2 (4.3–14.2)	7.7 (3.9–13.8)	n.s.
Baseline HCV RNA level (KIU/mL)	1,230 (24–3,870)	1,024 (38–3,110)	n.s.

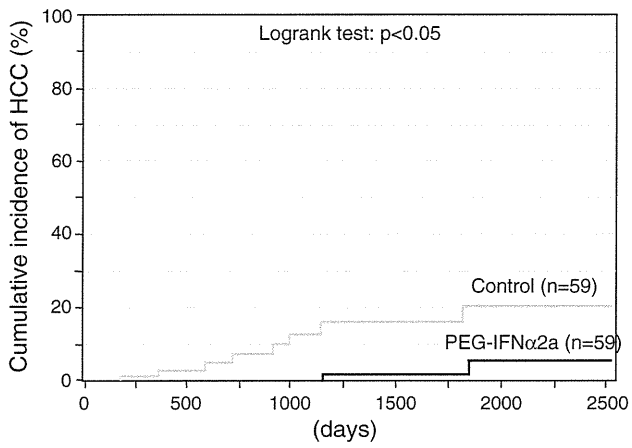

Fig. 4 Comparison of HCC rates between the long-term PegIFNα-2a administration group ($n = 59$) and non-administration group ($n = 59$) in the propensity-matched control study (Kaplan–Meier log-rank test, $p = 0.019$)

Table 5 Risk factors for HCC in the propensity-matched control study (Cox proportional hazard model)

Variables	Risk ratio	95 % CI	p value
PegIFN versus control	0.17	0.03–0.75	<0.05
Age (every 1 year)	1.12	1.02–1.25	<0.05
Fibrosis (F3, 4 vs. F0, 1, 2)	1.70	0.75–4.16	n.s.
Platelet count (every 10 × 10^3/μL)	0.89	0.73–1.09	n.s.
Albumin (every 1.0 g/dL)	0.80	0.10–6.68	n.s.
On-treatment AFP (<10 vs. ≥10 ng/L)	4.07	0.59–40.12	n.s.

the PegIFNα-2a group and the control group (patients who did not agree to receive IFN treatment), because this was a retrospective and non-randomized study. However, concordant with the findings of the HALT-C study [14], the present results show that PegIFNα-2a inhibits the development of HCC in patients with advanced fibrosis of the liver.

Recent studies show that polymorphisms in the host *IL28B* gene are important factors in the response to PegIFNα and ribavirin combination therapy [20, 21]. However, the mechanism of IL28B involvement in the response to PegIFNα and ribavirin has not been elucidated completely. A recent report has shown that IL28B is a significant factor in the development of HCC as well as in the response to IFN therapy [22]. Further studies are warranted to analyze the relationship between IL28B and inhibition of the development of HCC by PegIFNα in chronic hepatitis C.

Risk factors for the development of HCC have been discussed previously. Increased intrahepatic fat is involved in the development of HCC in chronic hepatitis C patients [23, 24]. In addition, diabetes-associated fat disorder [25, 26], hepatic iron overload [27], advanced fibrosis, older age, and fatty deposits in the liver are risk factors for HCC development [4]. Therefore, it is important to establish strategies to mitigate these risk factors to prevent the development of HCC and thus improve the outcomes of hepatitis C patients.

IFN therapy after HCC treatment is reported to inhibit the recurrence of tumors [28, 29], and a meta-analysis has revealed a trend toward inhibition of the recurrence of HCC [30, 31]. The prevention of HCC is an important issue that needs to be addressed to improve the survival of chronic hepatitis C patients. The findings of the present study and the HALT-C trial [14] indicate the effectiveness of long-term administration of maintenance IFN for preventing the development of HCC in chronic hepatitis C patients without an SVR. Improvement in ALT levels is also known to be an important predictor for the prevention of HCC [32]. A low AFP value during IFN administration is also recognized as a significant indicator of a lower risk of HCC [33, 34]. Recently, Osaki et al. [35] reported that a decrease of serum AFP during treatment with IFN was associated with a reduced incidence of HCC. Taking these findings and our own together, we conclude that maintenance administration of low-dose PegIFNα-2a weekly or biweekly to non-SVR patients with chronic hepatitis C decreases the incidence of HCC, especially in patients whose serum ALT and AFP levels are within the normal range 24 weeks after the start of treatment. The preventive effects of IFN against the development of HCC without elimination of the virus may be associated with its anti-carcinogenic effects [16, 35]; however, the precise mechanism should be investigated.

The limitations of the present study are that it is retrospective and multicentric; therefore, potentially there may have been a selection bias. However, the reduction of the rate of development of HCC by maintenance administration of PegIFNα-2a in the patients in whom serum ALT and AFP levels were within the normal ranges 24 weeks after the start of treatment may be attributable to the anticarcinogenic effects of IFN without elimination of the virus.

Conclusion

The incidence of HCC was lower in non-SVR patients with chronic hepatitis C who were administered with maintenance low-dose PegIFNα-2a; especially in those whose serum ALT and AFP levels were within the normal ranges 24 weeks after the start of treatment.

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Conflict of interest Namiki Izumi received lecture fees from Chugai Co. and MSD Co. in 2011.

References

1. Parkin DM, Bray F, Ferlay J, Pisani P. Global cancer statistics, 2002. CA Cancer J Clin. 2005;55:74–108. doi:10.3322/canjclin.55.2.74.
2. Llovet JM, Burroughs A, Bruix J. Hepatocellular carcinoma. Lancet. 2003;362:1907–17. doi:10.1016/S0140-6736(03)14964-1.
3. Kiyosawa K, Sodeyama T, Tanaka E, Gibo Y, Yoshizawa K, Nakano K, et al. Interrelationship of blood transfusion, non-A, non-B hepatitis and hepatocellular carcinoma: analysis by detection of antibody to hepatitis C virus. Hepatology. 1990;12:671–5. doi:10.1002/hep.1840120409.
4. Namiki I, Nishiguchi S, Hino K, Suzuki F, Kumada H, Itoh T, et al. Management of hepatitis C; Report of the consensus meeting at the 45th annual meeting of the Japan Society of Hepatology (2009). Hepatol Res. 2010;40:347–68. doi:10.1111/j.1872-034X.2010.00642.x.
5. Tanaka Y, Hanada K, Mizokami M, Yeo AE, Shin JW, Gojobori T, et al. A comparison of the molecular clock of hepatitis C virus in the United States and Japan predicts that hepatocellular carcinoma incidence in the United States will increase over the next two decades. Proc Natl Acad Sci USA. 2002;99:11584–9. doi:10.1073/pnas.242608099.
6. Ikeda K, Saitoh S, Arase Y, Chayama K, Suzuki Y, Kobayashi M, et al. Effect of interferon therapy on hepatocellular carcinoma in patients with chronic hepatitis type C: a long-term observation study of 1,643 patients using statistical bias correction with proportional hazard analysis. Hepatology. 1999;29:1124–30.
7. Imai Y, Kawata S, Tamura S, Yabuuchi I, Noda S, Inada M, et al. Relation of interferon therapy and hepatocellular carcinoma in patients with chronic hepatitis C. Ann Intern Med. 1998;129:94–9.
8. Bruno S, Stroffolini T, Colombo M, Bollani S, Benveguu L, Mazzella G, et al. Sustained virological response to interferon-alpha is associated with improved outcome in HCV-related cirrhosis: a retrospective study. Hepatology. 2007;45:579–87. doi:10.1002/hep.21492.
9. Veldt BJ, Heathcote EJ, Wedemeyer H, Reichen J, Hofmann WP, Zeuzem S, et al. Sustained virological response and clinical outcomes in patients with chronic hepatitis C and advanced fibrosis. Ann Intern Med. 2007;147:677–84.
10. Asahina Y, Tsuchiya K, Tamaki N, Hirayama I, Tanaka T, Sato M, et al. Effect of aging on risk for hepatocellular carcinoma in chronic hepatitis C virus infection. Hepatology. 2010;52:518–27. doi:10.1002/hep.23691.
11. Amarapurkar D, Han KH, Chan HL, Ueno Y, Asia-Pacific working party on prevention of hepatocellular carcinoma. Application of surveillance programs for hepatocellular carcinoma in the Asia-Pacific Region. J Gastroenterol Hepatol. 2009;24:955–61. doi:10.1111/j.1440-1746.2009.05805.x.
12. Tamura Y, Yamagiwa S, Aoki Y, Kurita S, Suda T, Ohkoshi S, et al. Serum alpha-fetoprotein levels during and after interferon therapy and the development of hepatocellular carcinoma in patients with chronic hepatitis C. Dig Dis Sci. 2009;54:2530–7.
13. Di Bisceglie AM, Shiffman ML, Everson GT, Lindsay KL, Everhart JE, Wright EC, et al. Prolonged therapy of advanced chronic hepatitis C with low-dose peginterferon. N Engl J Med. 2008;359:2429–41. doi:10.1056/NEJMoa0707615.
14. Lok AS, Everhart JE, Wright EC, Di Bischeglie AM, Kim HY, Stering RK, et al. Maintenance peginterferon therapy and other factors associated with hepatocellular carcinoma in patients with advanced hepatitis C. Gastroenterology. 2011;140:840–9. doi:10.1053/j.gastro.2010.11.050.
15. Bruix J, Poynard T, Colombo M, Schiff E, Burak K, Heathcote EJ, et al. Maintenance therapy with peginterferon alfa-2b does not prevent hepatocellular carcinoma in cirrhotic patients with chronic hepatitis C. Gastroenterology. 2011;140:1990–9. doi:10.1053/j.gastro.2010.11.050.
16. Arase Y, Ikeda K, Suzuki F, Suzuki Y, Kobayashi M, Akuta N, et al. Prolonged-interferon therapy reduces hepatocarcinogenesis in aged-patients with chronic hepatitis C. J Med Virol. 2007;79:1095–102. doi:10.1002/jmv.20866.
17. Poynard T, Moussali J, Ratziu V, Regimberu C, Opolan P. Effects of interferon therapy in "non-responder" patients with chronic hepatitis C. J Hepatol. 1999;31S:178–83. doi:10.1016/S0168-8278(99)80397-3.
18. Desmet VJ, Gerber M, Hoofnagle JH, Manns M, Scheuer P. Classification of chronic hepatitis: diagnosis, grading and staging. Hepatology. 1994;19:1513–20. doi:10.1016/0270-9139(94)90250-X, doi:10.1002/hep.1840190629.
19. Kanwal F, Hoang T, Kramer JR, Asch SM, Goetz MB, Zeringue A, et al. Increasing prevalence of HCC and cirrhosis in patients with chronic hepatitis C virus infection. Gastroenterology. 2011;140:1182–8. doi:10.1053/j.gastro.2010.12.032.
20. Ge D, Fellay J, Thompson AJ, Simon JS, Shianna KV, Urban TJ, et al. Genetic variation in IL28B predicts hepatitis C treatment-induced viral clearance. Nature. 2009;461:399–401. doi:10.1038/nature08309.
21. Tanaka Y, Nishida N, Sugiyama M, Kurosaki M, Matsuura K, Sakamoto N, et al. Genome-wide association of IL28B with response to pegylated interferon-alpha and ribavirin therapy for chronic hepatitis C. Nature. 2009;41:1105–9.
22. Fabris C, Falleti E, Cussigh A, Bitetto D, Fontanini E, Bignulin S, et al. IL-28B rs 12979860 C/T allele distribution in patients with liver cirrhosis: role in the course of chronic viral hepatitis and the development of HCC. J Hepatol. 2011;54:716–22. doi:10.1016/j.jhep.2010.07.019.
23. Kurosaki M, Hosokawa T, Matsunaga K, Hirayama I, Tanaka T, Sato M, et al. Hepatic steatosis in chronic hepatitis C is a significant risk factor for developing hepatocellular carcinoma independent of age, sex, obesity, fibrosis stage and response to interferon therapy. Hepatol Res. 2010;40:870–7. doi:10.1111/j.1872-034X.2010.00692.x.
24. Koike K. Steatosis, liver injury, and hepatocarcinogenesis in hepatitis C viral infection. J Gastroenterol. 2009;44(Suppl 19):82–8. doi:10.1007/s00535-008-2276-4.
25. Veldt BJ, Chen W, Heathcote EJ, Wedemeyer H, Reichen J, Hofman WP, et al. Increased risk of hepatocellular carcinoma among patients with hepatitis C cirrhosis and diabetes mellitus. Hepatology. 2008;47:1856–62. doi:10.1002/hep.22251.
26. Lai MS, Hsieh MS, Chiu YH, Chen TH. Type 2 diabetes and hepatocellular carcinoma: a cohort study in high prevalence area of hepatitis virus infection. Hepatology. 2006;43:1295–302. doi:10.1002/hep.21208.
27. Furutani T, Hino K, Okuda M, Gondo T, Nishina S, Kitase A, et al. Hepatic iron overload induces hepatocellular carcinoma in transgenic mice expressing the hepatitis C virus polyprotein. Gastroenterology. 2006;130:2087–98. doi:10.1053/j.gastro.2006.02.060.
28. Kubo S, Nishiguchi S, Hirohashi K, Tanaka H, Shuto T, Kinoshita H. Randomized clinical trial of long-term outcome after resection of hepatitis C virus-related hepatocellular carcinoma by

postoperative interferon therapy. Br J Surg. 2002;89:418–22. doi: 10.1046/j.0007-1323.2001.02054.x.
29. Kudo M, Sakaguchi Y, Chung H, Hatanaka K, Hagiwara S, Ishikawa E, et al. Long-term interferon maintenance therapy improves survival in patients with HCV-related hepatocellular carcinoma after curative radiofrequency ablation. A matched case–control study. Oncology. 2007;72(Suppl 1):132–8. doi: 10.1159/000111719.
30. Singal AK, Freeman DH Jr, Anand BS. Meta-analysis: interferon improves outcomes following ablation or resection of hepatocellular carcinoma. Aliment Pharmacol Ther. 2010;32:851–8. doi:10.1111/j.1365-2036.2010.04414.x.
31. Miyake Y, Takaki A, Iwasaki Y, Yamamoto K. Meta-analysis: interferon-alpha prevents the recurrence after curative treatment of hepatitis C virus-related hepatocellular carcinoma. J Viral Hepat. 2010;17:287–92. doi:10.1111/j.1365-2893.2009.01181.x.
32. Arase Y, Ikeda K, Suzuki F, Suzuki Y, Kobayashi M, Akuta N, et al. Interferon-induced prolonged biochemical response reduces hepatocarcinogenesis in hepatitis C virus infection. J Med Virol. 2007;79:1485–90. doi:10.1002/jmv.20925.
33. Nomura H, Kashiwagi Y, Hirano R, Tanimoto H, Tsutsumi N, Higashi M, et al. Efficacy of low dose long-term interferon monotherapy in aged patients with chronic hepatitis C genotype 1 and its relation to alpha-fetoprotein: a pilot study. Hepatol Res. 2007;37:490–7. doi:10.1111/j.1872-034X.2007.00073.x.
34. Chen TM, Huang PT, Tsai MH, Lin LF, Liu CC, Ho KS, et al. Predictors of alpha-fetoprotein elevation in patients with chronic hepatitis C, but not hepatocellular carcinoma, and its normalization after pegylated interferon alfa 2a-ribavirin combination therapy. J Gastroenterol Hepatol. 2007;22:669–75. doi: 10.1111/j.1440-1746.2007.04898.x.
35. Osaki Y, Ueda Y, Marusawa H, Nakajima J, Kimura T, Kita R, et al. Decrease in alpha-fetoprotein levels predicts reduced incidence of hepatocellular carcinoma in patients with hepatitis C virus infection receiving interferon therapy: a single center study. J Gastroenterol. 2012;47:444–51.

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Hepatitis C Virus NS4B Protein Targets STING and Abrogates RIG-I–Mediated Type I Interferon-Dependent Innate Immunity

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Hepatitis C virus (HCV) infection blocks cellular interferon (IFN)-mediated antiviral signaling through cleavage of Cardif by HCV-NS3/4A serine protease. Like NS3/4A, NS4B protein strongly blocks IFN-β production signaling mediated by retinoic acid–inducible gene I (RIG-I); however, the underlying molecular mechanisms are not well understood. Recently, the stimulator of interferon genes (STING) was identified as an activator of RIG-I signaling. STING possesses a structural homology domain with flaviviral NS4B, which suggests a direct protein-protein interaction. In the present study, we investigated the molecular mechanisms by which NS4B targets RIG-I–induced and STING-mediated IFN-β production signaling. IFN-β promoter reporter assay showed that IFN-β promoter activation induced by RIG-I or Cardif was significantly suppressed by both NS4B and NS3/4A, whereas STING-induced IFN-β activation was suppressed by NS4B but not by NS3/4A, suggesting that NS4B had a distinct point of interaction. Immunostaining showed that STING colocalized with NS4B in the endoplasmic reticulum. Immunoprecipitation and bimolecular fluorescence complementation (BiFC) assays demonstrated that NS4B specifically bound STING. Intriguingly, NS4B expression blocked the protein interaction between STING and Cardif, which is required for robust IFN-β activation. NS4B truncation assays showed that its N terminus, containing the STING homology domain, was necessary for the suppression of IFN-β promoter activation. NS4B suppressed residual IFN-β activation by an NS3/4A-cleaved Cardif (Cardif1-508), suggesting that NS3/4A and NS4B may cooperate in the blockade of IFN-β production. *Conclusion*: NS4B suppresses RIG-I–mediated IFN-β production signaling through a direct protein interaction with STING. Disruption of that interaction may restore cellular antiviral responses and may constitute a novel therapeutic strategy for the eradication of HCV. (HEPATOLOGY 2013;57:46-58)

Type I interferon (IFN) plays a central role in eliminating hepatitis C virus (HCV) both under physiological conditions and when used as a therapeutic intervention.[1-3] In experimental acute-resolving HCV infection in chimpanzees, numerous IFN-related genes are expressed during clinical course of infection.[4] Viruses are recognized by cellular innate immune receptors, such as toll-like receptors, and a family of RIG-I–like receptors, such as retinoic-acid-inducible gene I (RIG-I) and melanoma-differentiation-associated gene 5 (MDA-5); host antiviral responses are then activated, resulting in the

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BiFC, bimolecular fluorescence complementation; CARD, caspase recruitment domain; DAPI, 4',6-diamidino-2-phenylindole; dsRNA, double-stranded RNA; ER, endoplasmic reticulum; FACL4, fatty acid-CoA ligase, long chain 4; HCV, hepatitis C virus; IFN, interferon; IKKε, IκB kinase ε; IRF-3, interferon-regulatory factor 3; ISRE, interferon-stimulated response element; MAM, mitochondria-associated ER membrane; mKG, monomeric Kusabira-Green; PDI, protein disulphide-isomerase; pIRF-3, phosphorylated IRF3; poly(dA:dT), poly(deoxyadenylic-deoxythymidylic) acid; RIG-I, retinoic acid–inducible gene I; siRNA, small interfering RNA; SOCS, suppressor of cytokine signaling; STAT1, signal transducer and activator of transcription protein-1; STING, stimulator of interferon genes; TBK1, TANK binding kinase 1.

**These authors contributed equally to this work.*

production of cytokines such as type I and type III IFNs.[5] RIG-I is activated through recognition of short double-strand RNA (dsRNA) or triphosphate at the 5′ end of dsRNA as pathogen-associated molecular patterns,[6,7] forming a homo-oligomer that binds with the caspase recruitment domain (CARD) of Cardif (also known as MAVS, VISA, or IPS-1).[8-11] Cardif subsequently recruits TANK binding kinase 1 (TBK1) and IκB kinase ϵ (IKKϵ) kinases, which catalyze phosphorylation and activation of IFN regulatory factor-3 (IRF-3).[12] Activation of TBK1 and IKKϵ results in the phosphorylation of IRF-3 or IRF-7, translocation to the nucleus, and induction of IFN-β mRNA transcription.

Several HCV proteins can block host cellular antiviral responses. HCV core protein blocks IFN signaling by interacting with signal transducer and activator of transcription protein-1 (STAT1).[13] The core protein also induces expression of suppressor of cytokine signaling-1 (SOCS1) and SOCS3, and blocks Janus kinase–STAT signaling.[14,15] A well-elucidated immune evasion strategy of HCV involves NS3/4A serine protease and its ability to inhibit host IFN signal pathways. Gale and colleagues[11,16,17] revealed that NS3/4A protease cleaves Cardif at Cys-508 resulting in dislocation of Cardif from mitochondria, and blocks downstream signaling of IFN-β production. On the other hand, Baril et al.[18] reported that Cardif was still able to form a homo-oligomer and to activate downstream IFN production signaling despite delocalization from the mitochondria. These reports suggest that homo-oligomerization of Cardif, and not mitochondrial anchorage, is essential for the activation of downstream IFN signaling and that other virus-derived molecules may cooperate with NS3/4A to abrogate the signaling of IFN production.

We reported previously that HCV-NS4B, as well as NS3/4A, inhibited RIG-I and Cardif-mediated interferon-stimulated response element (ISRE) activation, while TBK1- and IKKϵ-mediated ISRE activation were not suppressed.[19] These results indicate that NS4B suppresses IFN production signaling by targeting Cardif or other unknown signaling molecules between the level of Cardif and TBK1/IKKϵ.

Recently, a stimulator of interferon genes (STING, also known as MITA/ERIS/MPYS/TMEM173) was identified as a positive regulator of RIG-I–mediated IFN-β signaling.[20-23] STING is a 42-kDa protein localized predominantly in the endoplasmic reticulum (ER) that binds RIG-I, Cardif, TBK1, and IKKϵ. STING is thought to act as a scaffold for Cardif/TBK1/IRF-3 complex upon viral infection.[22] It has been reported that NS4B of yellow fever virus, which is a member of the flaviviridae family of viruses, inhibits STING activation probably through a direct molecular interaction.[24] These reports have led us postulate that HCV-NS4B may also inhibit RIG-I dependent IFN signaling through association with STING.

In the present study, we further investigated the molecular mechanisms by which HCV-NS4B protein inhibits RIG-I–mediated IFN expression signaling. We demonstrated that HCV-NS4B specifically binds STING, blocks the molecular interaction between STING and Cardif, and suppresses the RIG-I–like receptor–induced activation of IFN-β production signaling.

Materials and Methods

Plasmids. The ΔRIG-I and RIG-IKA plasmids express constitutively active and inactive RIG-I, respectively.[5] Full-length Cardif (Cardif) and CARD-truncated Cardif (ΔCARD) plasmids were provided by J. Tschopp.[11] Plasmids expressing STING were provided by G. N. Barber.[20] Plasmids expressing HCV NS3/4A, NS4B, and truncated NS4B have been described.[25] Plasmid pIFNβ-Fluc was provided by R. Lin.[26]

Cell Culture. HEK293T and Huh7 cells were maintained in Dulbecco's modified minimal essential medium (Sigma) supplemented with 2 mM L-glutamine and 10% fetal calf serum at 37°C with 5% CO_2.

HCV Replicon Constructs and HCV-JFH1 Cell Culture. An HCV subgenomic replicon plasmid, pRep-Feo, expressed fusion protein of firefly luciferase and neomycin phosphotransferase.[27,28] Huh7 cells were transfected by Rep-Feo RNA, cultured in the presence of 500 μg/mL of G418, and a cell line that stably expressed Feo replicon was established. For HCV cell culture, the HCV-JFH1 strain was used.[29,30]

Antibodies. Antibodies used were anti–IRF-3 (FL-425, Santa Cruz Biotechnology), anti-HA (Invitrogen), anti-myc (Invitrogen), mouse anti-PDI (Abcam),

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rabbit anti-PDI (Enzo Life Science), anti-Flag (Sigma Aldrich), anti-Cardif (Enzo Life Science), anti-phospho–IRF-3 (Ser396, Millipore), anti-monomeric Kusabira-Green C- or N-terminal fragment (MBL), and anti-FACL4 (Abgent).

Luciferase Reporter Assay. IFN-β reporter assays were performed as described.[19,31] The plasmids pIFN-β-Fluc and pRL-CMV were cotransfected with NS3/4A or NS4B, and ΔRIG-I, Cardif, STING or poly(deoxyadenylic-deoxythymidylic) acid [poly(dA:dT)] (Invivogen). RIG-IKA, ΔCARD, and pcDNA3.1, respectively, were used as controls. Luciferase assays were performed 24 hours after transfection by using a 1420 Multilabel Counter (ARVO MX PerkinElmer) and Dual Luciferase Assay System (Promega). Assays were performed in triplicate, and the results are expressed as the mean ± SD.

Immunoblotting. Preparation of total cell lysates was performed as described.[19,28] Protein was separated using NuPAGE 4%-12% Bis/Tris gels (Invitrogen) and blotted onto an Immobilon polyvinylidene difluoride membrane. The membrane was immunoblotted with primary followed by secondary antibody, and protein was detected by chemiluminescence.

Immunoprecipitation Assay. HEK-293T or Huh7 cells were transfected with plasmids as indicated. Twenty-four hours after transfection, cellular proteins were harvested and immunoprecipitation assays were performed using an Immunoprecipitation Kit according to the manufacturer's protocol (Roche Applied Science). The immunoprecipitated proteins were analyzed by immunoblotting.

Indirect Immunofluorescence Assay. Cells seeded onto tissue culture chamber slides were transfected with plasmids as indicated. Twenty-four hours after transfection, the cells were fixed with cold acetone and incubated with primary antibody and subsequently with Alexa488- or Alexa568-labeled secondary antibodies. Mitochondria were stained by MitoTracker (Invitrogen). Cells were visualized using a confocal laser microscope (Fluoview FV10, Olympus).

BiFC Assay. Expression plasmids of NS4B, Cardif, or STING that was fused with N- or C-terminally truncated monomeric Kusabira-Green (mKG) were constructed by inserting polymerase chain reaction–amplified fragments encoding NS4B, Cardif, or STING, respectively, inserted into fragmented mKG vector (Coral Hue Fluo-Chase Kit; MBL). HEK293T cells were transfected with a complementary pair of mKG fusion plasmids. Twenty-four hours after transfection, fluorescence-positive cells were detected and counted by flow cytometry, or observed by confocal laser microscopy.

Small Interfering RNA Assay. Nucleotide sequences of STING-targeted small interfering RNAs (siRNAs) were as follows: (1) 5′-gcaacagcatctatgagcttctggagaac-3′, (2) 5′- gtgcagtgagccagcggctgtatattctc;-3′, (3) 5′-gctggcat ggtcatattacatcggatatc-3′.[22] Stealth RNAi Negative Control Duplex (Medium GC Duplex, Invitrogen) was used. Forty-eight hours after siRNA transfection, expression levels of STING were detected by immunoblotting.

Statistical Analyses. Statistical analyses were performed using unpaired, two-tailed Student t test. $P < 0.05$ were considered to be statistically significant.

Results

NS4B Suppressed RIG-I, Cardif, and STING-Mediated Activation of IFN-β Expression Signaling. First, we performed a reporter assay using a luciferase reporter plasmid regulated by native IFN-β promoter. Consistent with our previous study,[19] overexpression of NS4B, as well as NS3/4A, inhibited the IFN-β promoter activation that was induced by ΔRIG-I and Cardif, respectively (Fig. 1A). We next studied whether NS4B targets STING and inhibits RIG-I pathway–mediated activation of IFN-β production. Expression of NS4B protein significantly suppressed STING-mediated activation of the IFN-β promoter reporter, whereas expression of NS3/4A showed no effect on STING-induced IFN-β promoter activity (Fig. 1A). To study whether NS4B blocks the STING-mediated DNA-sensing pathway, we performed a reporter assay using a luciferase reporter plasmid cotransfection with poly(dA:dT), which is a synthetic analog of B-DNA and has been reported to induce STING-mediated IFN-β production and NS4B. NS4B significantly blocked poly(dA:dT)-induced IFN-β promoter activation, suggesting that NS4B may block STING signaling in the DNA-sensing pathway (Fig. 1A).

Activation of RIG-I signaling induces phosphorylation of IRF-3, which is a hallmark of IRF-3 activation.[32] Thus, we examined the effects of NS3/4A and NS4B expression on phosphorylation of IRF-3 by immunoblotting analysis. As shown in Fig. 1B, overexpression of ΔRIG-I, Cardif, or STING in HEK293T cells increased levels of phosphorylated IRF-3 (pIRF-3). Expression of NS4B impaired the IRF-3 phosphorylation that was induced by ΔRIG-I, Cardif, or STING. NS3/4A also blocked production of pIRF-3 induced by ΔRIG-I or Cardif. Intriguingly, NS3/4A did not block STING-induced pIRF-3 production. These results demonstrate that both NS3/4A and

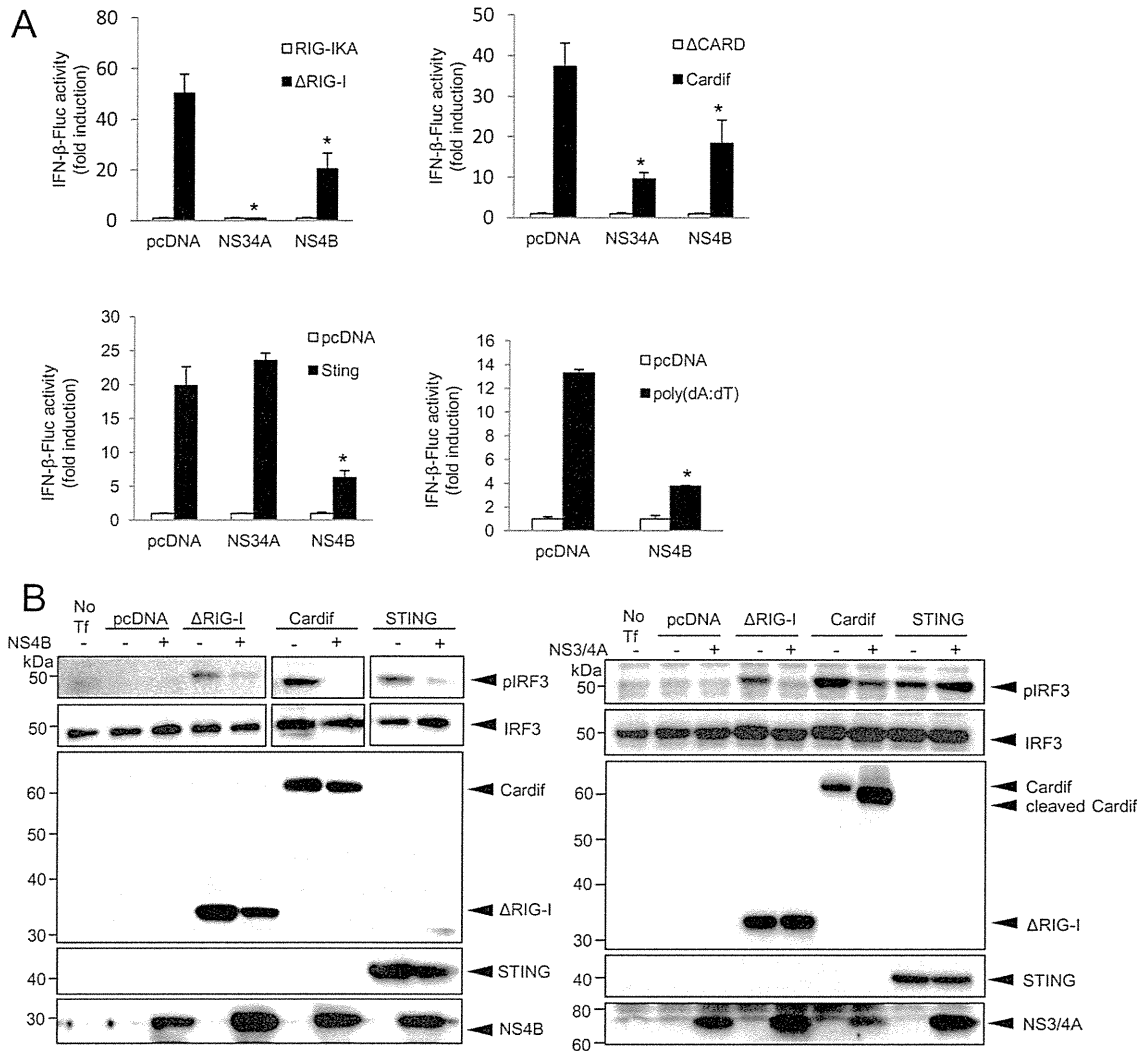

Fig. 1. NS4B suppressed IFN-β signaling mediated by RIG-I, Cardif, or STING. (A) Plasmids expressing ΔRIG-I, Cardif, or STING or poly(dA:dT) as well as NS3/4A or NS4B were cotransfected with pIFN-β-Fluc and pRL-CMV into HEK293T cells. After 24 hours, dual luciferase assays were performed. Plasmids expressing RIG-IKA, ΔCARD, or an empty plasmid (pcDNA) were used as a corresponding negative control. The experiments were performed more than three times and yielded consistent results. The *y* axis indicates relative IFN-β-Fluc activity. Assays were performed in triplicate and error bars indicate mean ± SD. $^*P < 0.05$. (B) HEK293T cells were cotransfected with indicated plasmids. On the day after transfection, the cells were lysed and immunoblot analyses were performed. No Tf, transfection-negative controls. pIRF-3 and IRF-3, phosphorylated and total IRF-3, respectively.

NS4B suppress RIG-I–mediated IFN-β production, but they do so by targeting different molecules in the signaling pathway.

Subcellular Localization of NS4B, Cardif, and STING. We next studied the subcellular localization of NS4B following its overexpression and measured the colocalization of NS4B with Cardif and STING in both HEK293T cells and Huh7 cells by indirect immunofluorescence microscopy. NS4B was localized predominantly in the ER, which is consistent with previous reports[33] (Fig. 2A). Cardif was localized in mitochondria but did not colocalize with the ER-resident host protein disulphide-isomerase (PDI). Interestingly, Cardif and NS4B colocalized partly at the boundary of the two proteins, although their original localization was different (Fig. 2A,C). STING was localized predominantly in the ER[20,21] (Fig. 2B,D). STING colocalized partly with Cardif, which is consistent with a previous report by Ishikawa and Barber[20] (Fig. 2B,D). In cells cotransfected with NS4B and STING expression plasmids, NS4B colocalized precisely with STING (Fig. 2B,D). To examine the region of NS4B-STING interaction, we next observed the two proteins by performing staining for them along with mitochondria-associated ER membrane (MAM), which is a physical association with mitochondria[34] and has been reported the site of Cardif-STING association.[24] Both NS4B and STING were adjacent to and partially colocalized

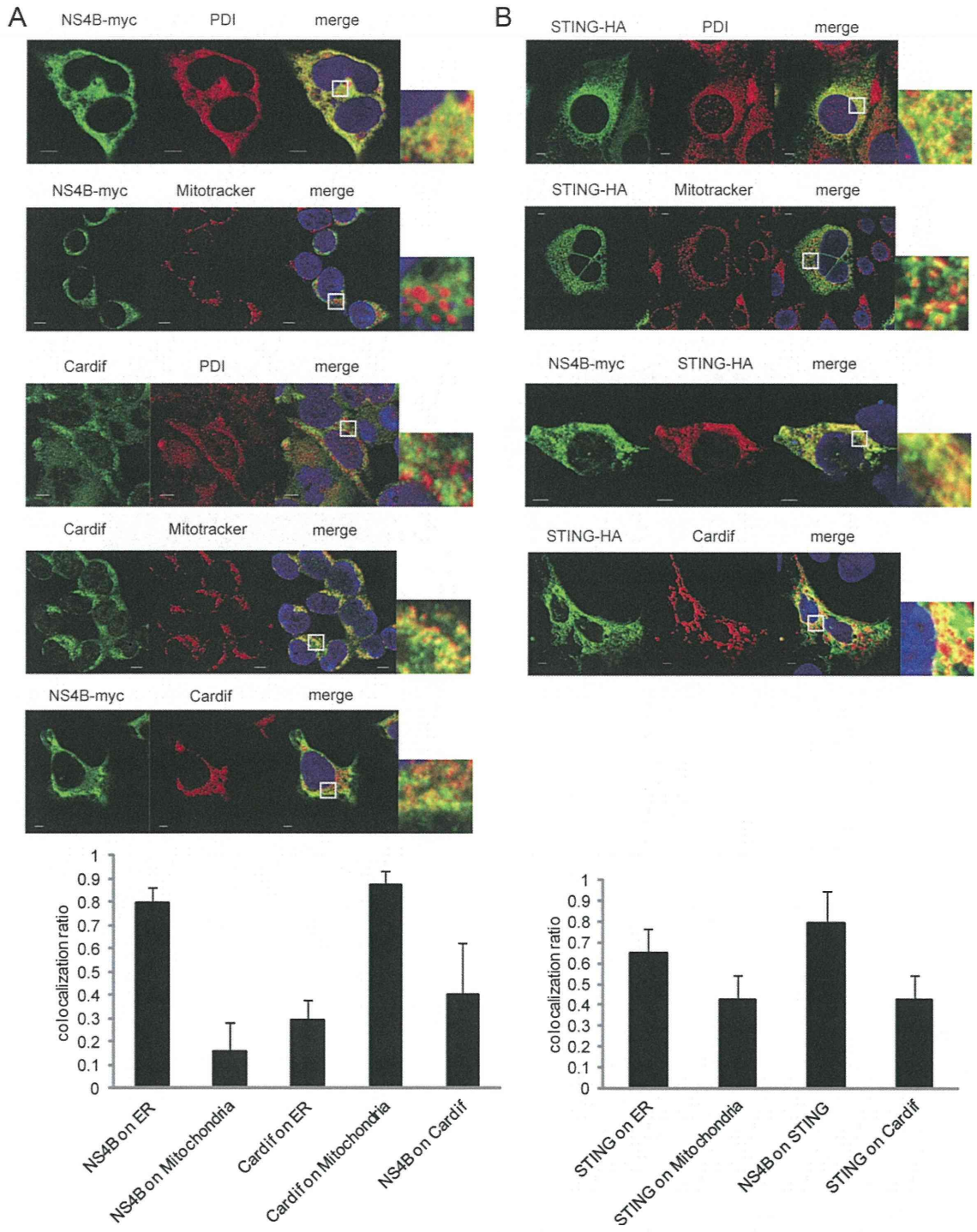

Fig. 2. Subcellular localization of NS4B, Cardif, and STING. (A-D) Subcellular localization of NS4B, Cardif, and STING in 293T (A,C) and Huh7 (B,D) cells. (A,C) NS4B-myc (first, second, and fifth panels of A and third panel of C) was transfected, and 24 hours later the cells were fixed and immunostained with anti-myc. In the third, fourth, and fifth panels of A, and the first and second panels of C, endogenous Cardif was detected with anti-Cardif antibody. ER was immunostained with anti-PDI antibody (first and third panels of A and first panel of C). Mitochondria were stained using Mitotracker (second and fourth panels of A and second panel of C). Nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI). (B,D) STING-HA (all panels) and NS4B-myc (third panels) were transfected, and after 24 hours the cells were fixed and immunostained with anti-HA or anti-myc, respectively. In the fourth panels, endogenous Cardif was detected with anti-Cardif antibody. ER was immunostained with anti-PDI antibody (first panels). Mitochondria were stained using Mitotracker (second panels). Nuclei were stained with DAPI. (E) NS4B-myc and STING-HA were transfected into Huh7 cells and after 24 hours the cells were fixed and immunostained with anti-HA, anti-myc, and anti-FACL4 (MAM) antibody. Cells were visualized by confocal microscopy. Scale bars indicate 5 μm. In each microscopic image, the grade of protein colocalization in a single cell was quantified and is shown in the graphs at the bottom of each panel. Values are shown as the average colocalization ratio in 8 cells. Error bars indicate the mean + SD.

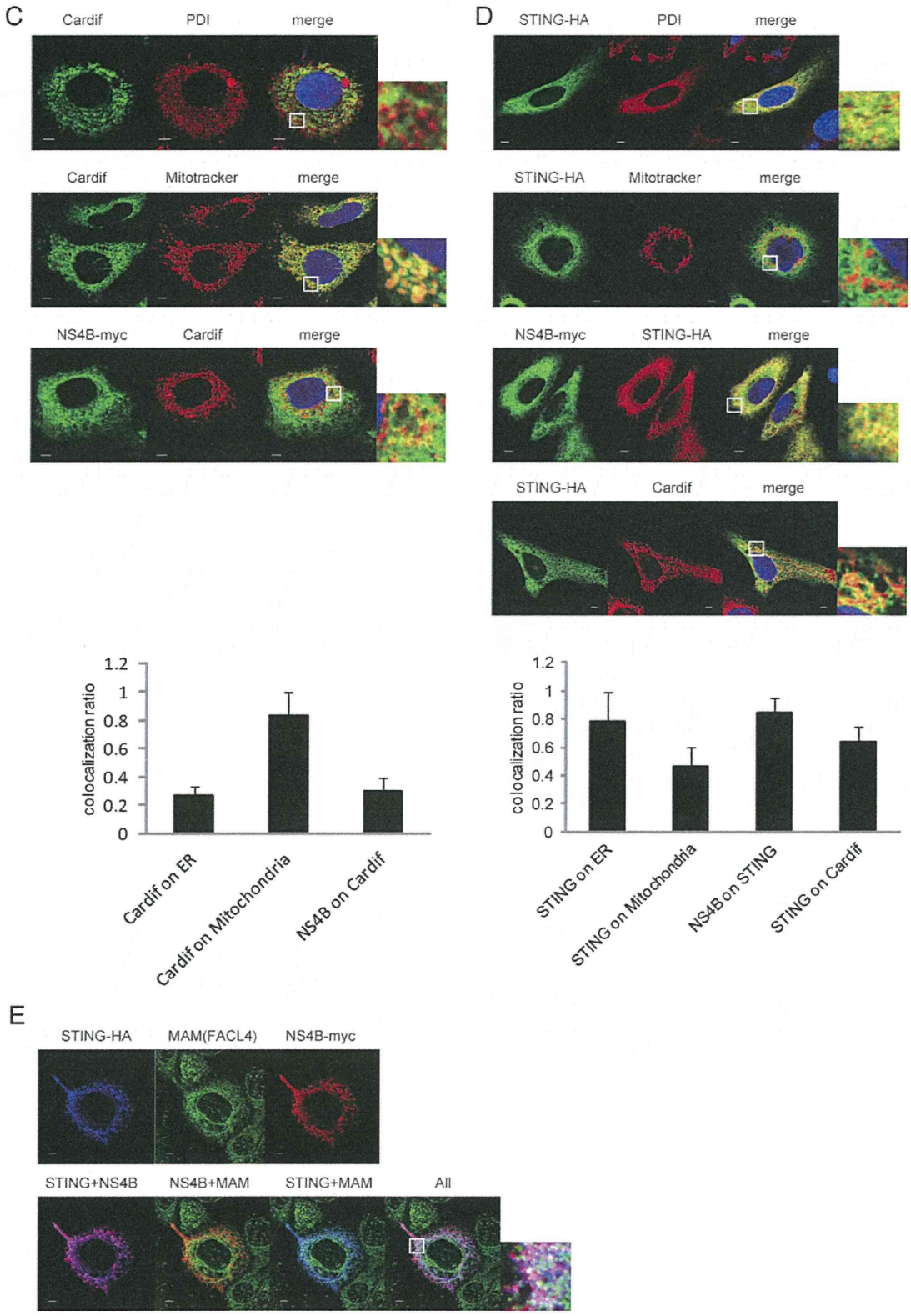

Fig. 2. Continued

with fatty acid-CoA ligase long chain 4 (FACL4), which is a MAM marker protein[35,36] (Fig. 2E). These findings suggest that NS4B might interact with STING on MAM more strongly than with Cardif.

Protein-Protein Interaction Between NS4B, Cardif, and STING. Knowing that NS4B was colocalized strongly with STING and only partly with Cardif, we next analyzed direct protein-protein interactions

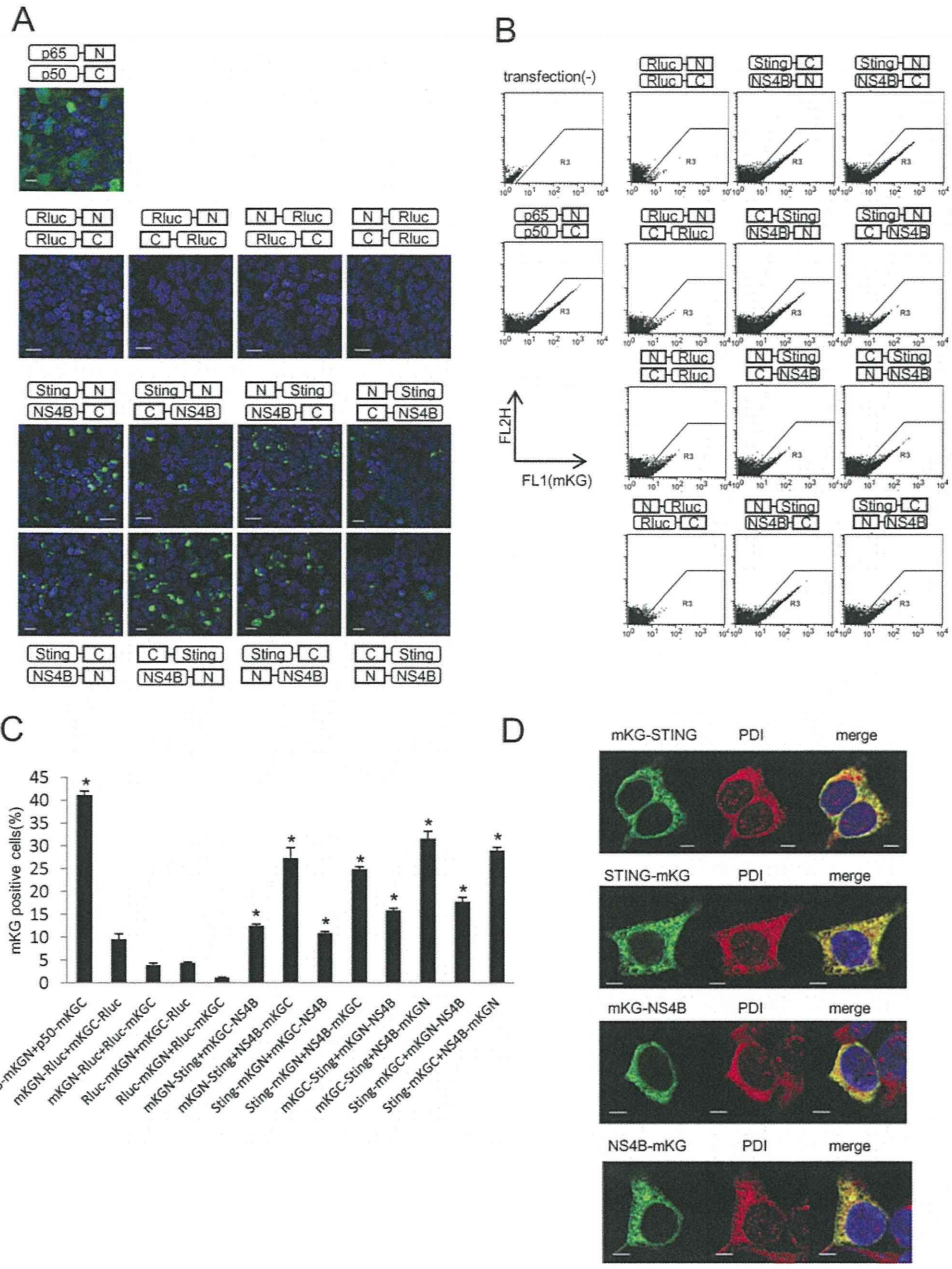

Fig. 3. BiFC assays of STING and NS4B. The complementary pairs of N- or C-terminally mKG-fused NS4B and STING expression plasmids were cotransfected in HEK293T cells. After 24 hours, the cells were fixed and observed by confocal microscopy (A) or subjected to flow cytometry to measure mKG-emitted fluorescence (BiFC signal) and to count BiFC signal-positive cells (B,C). Plasmids expressing p65-mKGN and p50-mKGC individually were used as a BiFC-positive control and plasmids expressing N- or C-terminally mKG fused Rluc were used as a negative control. The letters N and C denote complimentary N- and C-terminal fragments of mKG, respectively. Assays were performed in triplicate and error bars indicate the mean ± SD. Scale bars indicate 10 μm (A). *$P < 0.05$ compared with corresponding negative controls. (D) Plasmids expressing mKG fragment-fused STING or NS4B were transfected in HEK293T cells. After 24 hours, the cells were fixed and immunostained with anti-mKG and anti-PDI (ER) antibody. Nuclei were stained with DAPI. Cells were observed by confocal microscopy. Scale bars = 5 μm.

between NS4B, Cardif, and STING. To detect those interactions in living cells, we performed BiFC assays.[37,38] We constructed NS4B, Cardif, and STING expression plasmids that were N- or C-terminally fused with truncated mKG proteins, respectively. First, we cotransfected several different pairs of NS4B and STING expression plasmids that were fused with complementary pairs of N- or C-terminally truncated mKG. Strong fluorescence by mKG complexes (BiFC signal) was detected in all pairs of cotransfections, suggesting significant molecular interaction (Fig. 3A). In flow cytometry, all pairs of NS4B- and STING-mKG fusion proteins were positive for strong BiFC signal (Fig. 3B). The percentages of cells positive for BiFC signal were significantly higher in STING-mKG and NS4B-mKG fusion complexes than in corresponding controls (Fig. 3C). These results demonstrate that HCV-NS4B and STING proteins interact with each other strongly and specifically in cells. Fluorescence microscopy indicated that N- and C-terminal fusion of mKG onto NS4B and STING did not affect subcellular localization (Fig. 3D).

We next studied the molecular interaction between NS4B and Cardif by BiFC assay using NS4B and Cardif fusion plasmids that were tagged with complementary pairs of truncated mKG. Weak fluorescence was detected in cells transfected with the pairs N-Cardif and NS4B-C, N-Cardif and C-NS4B, C-Cardif and

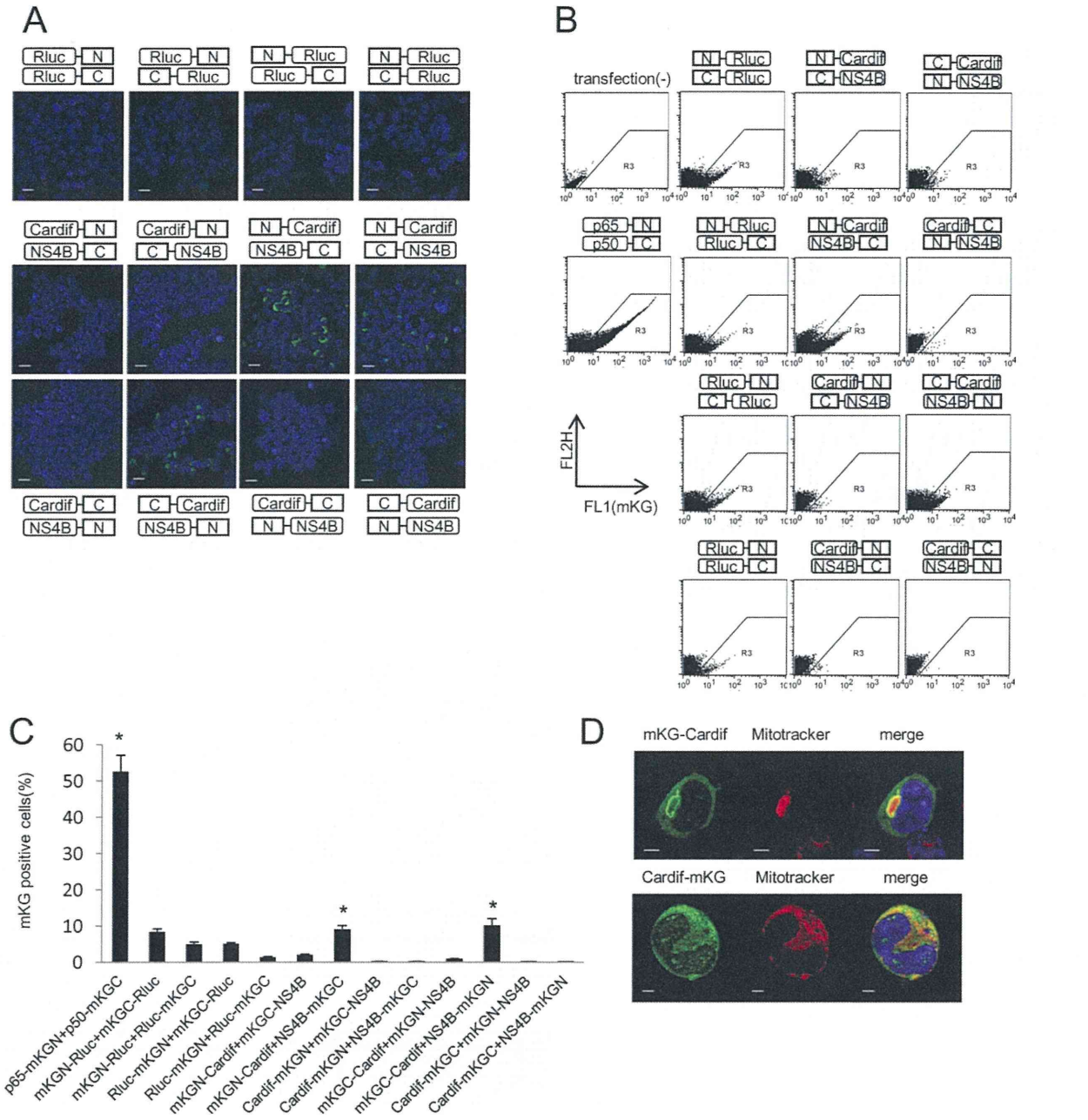

Fig. 4. BiFC assays of Cardif and NS4B. The complementary pairs of N- or C-terminally mKG-fused NS4B and Cardif expression plasmids were cotransfected in HEK293T cells. After 24 hours, the cells were fixed and observed by confocal microscopy (A) or subjected to flow cytometry to measure mKG-emitted fluorescence (BiFC signal) and to count BiFC signal-positive cells (B,C). Plasmids expressing p65-mKGN and p50-mKGC individually were used as a BiFC-positive control and plasmids expressing N- or C-terminally mKG-fused Rluc were used as a negative control. The letters N and C denote complimentary N- and C-terminal fragments of mKG, respectively. Assays were performed in triplicate, and error bars indicate the mean ± SD. Scale bars indicate 10 μm (A). *$P < 0.05$ compared with corresponding negative controls. (D) Plasmids expressing mKG fragment-fused STING or NS4B were transfected in HEK293T cells. After 24 hours, the cells were fixed and immunostained with anti-mKG antibody. Mitochondria were stained using Mitotracker, and nuclei were stained with DAPI. Cells were observed by confocal microscopy. Scale bars = 5 μm.

NS4B-N, and C-Cardif and N-NS4B (Fig. 4A,B). The percentage of cells positive for BiFC signal increased with the combination of N-Cardif and NS4B-C, and C-Cardif and NS4B-N (Fig. 4C). Fluorescence microscopy indicated that mKG-Cardif, but not Cardif-mKG, was partially colocalized with mitochondria, possibly due to disruption of mitochondria anchor domain by C-terminal fusion with mKG (Fig. 4D). These results indicate the lack of significant molecular interactions between NS4B and Cardif.

Binding of NS4B to STING Blocks Molecular Interaction Between Cardif and STING. It has been reported that STING binds Cardif directly.[20,22] Thus, we hypothesized that NS4B, through a competitive