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Model Incorporating the *ITPA* Genotype Identifies Patients at High Risk of Anemia and Treatment Failure With Pegylated-Interferon Plus Ribavirin Therapy for Chronic Hepatitis C

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This study aimed to develop a model for predicting anemia using the inosine triphosphatase (*ITPA*) genotype and to evaluate its relationship with treatment outcome. Patients with genotype 1b chronic hepatitis C (n = 446) treated with peg-interferon alpha and ribavirin (RBV) for 48 weeks were genotyped for the *ITPA* (rs1127354) and *IL28B* (rs8099917) genes. Data mining analysis generated a predictive model for anemia (hemoglobin (Hb) concentration <10 g/dl); the CC genotype of *ITPA*, baseline Hb <14.0 g/dl, and low creatinine clearance (CLcr) were predictors of anemia. The incidence of anemia was highest in patients with Hb <14.0 g/dl and CLcr <90 ml/min (76%), followed by Hb <14.0 g/dl and *ITPA* CC (57%). Patients with Hb ≥14.0 g/dl and *ITPA* AA/CA had the lowest incidence of anemia (17%). Patients with two predictors (high-risk) had a higher incidence of anemia than the others (64% vs. 28%, $P < 0.0001$). At baseline, the *IL28B* genotype was a predictor of a sustained virological response [adjusted odds ratio 9.88 (95% confidence interval 5.01–19.48), $P < 0.0001$]. In patients who achieved an early virological response, the *IL28B* genotype was not associated with a sustained virological response, while a high risk of anemia was a significant negative predictor of a sustained virological response [0.47 (0.24–0.91), $P = 0.026$]. For high-risk patients with an early virological response, giving >80% of the planned RBV dose increased sustained virological responses by 24%. In conclusion, a predictive model incorporating the *ITPA* genotype could identify patients with a high risk of anemia and reduced probability of sustained virological response. ***J. Med. Virol. 85:449–458, 2013.***

KEY WORDS: hemolytic anemia; ribavirin; creatinine clearance; antiviral therapy

INTRODUCTION

Hepatitis C virus (HCV) infection is a leading cause of cirrhosis and hepatocellular carcinoma worldwide [Kim, 2002]. The rate of eradication of HCV by pegylated interferon (PEG-IFN) plus ribavirin (RBV), defined as a sustained virological response, is around 50% in patients with HCV genotype 1 [Manns et al., 2001; Fried et al., 2002]. Failure of treatment is attributable to the lack of a virological response or relapse after completion of therapy. Genome-wide association studies and subsequent cohort studies

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have shown that single nucleotide polymorphisms (SNPs) located near the *IL28B* gene are the most important determinant of virological response to PEG-IFN/RBV therapy [Ge et al., 2009; Suppiah et al., 2009; Tanaka et al., 2009; Rauch et al., 2010]. On the other hand, among patients with a virological response, the probability of a sustained virological response decreases when the patients become intolerant to therapy because of RBV-induced hemolytic anemia and receive a reduced dose of RBV [McHutchison et al., 2002; Kurosaki et al., 2012]. Genome-wide association studies have shown that variants of the inosine triphosphatase (*ITPA*) gene protect against hemolytic anemia [Fellay et al., 2010; Tanaka et al., 2011]. These variants are associated with a reduced requirement for an anemia-related dose reduction of RBV [Sakamoto et al., 2010; Thompson et al., 2010a; Kurosaki et al., 2011d; Seto et al., 2011]. However, factors other than the *ITPA* gene also contribute to the risk of severe anemia or RBV dose reduction [Ochi et al., 2010; Kurosaki et al., 2011d] and the results of studies on the impact of the *ITPA* genotype on treatment outcome are inconsistent [Ochi et al., 2010; Sakamoto et al., 2010; Thompson et al., 2010a, 2011; Kurosaki et al., 2011d].

Data mining is a novel statistical method used to extract relevant factors from a plethora of factors and combine them to predict the incidence of the outcome of interest [Breiman et al., 1980]. Decision tree analysis, a primary component of data mining analysis, has found medical applications recently [Averbook et al., 2002; Miyaki et al., 2002; Baquerizo et al., 2003; Leiter et al., 2004; Garzotto et al., 2005; Zlobec et al., 2005; Valera et al., 2007] and has proven to be a useful tool for predicting therapeutic efficacy [Kurosaki et al., 2010, 2011a,b,c, 2012] and adverse events [Hiramatsu et al., 2011] in patients with chronic hepatitis C treated with PEG-IFN/RBV therapy. Because the results of data mining analysis are presented as a flowchart [LeBlanc and Crowley, 1995], they are easily understandable and usable by clinicians lacking a detailed knowledge of statistics.

For the general application of this genetic information in clinical practice, this study aimed to construct a predictive model of severe anemia using the *ITPA* genotype, together with other relevant factors. This study also aimed to analyze the impact of the risk of anemia on treatment outcome, after adjustment for the *IL28B* genotype. These analyses were carried out at baseline and during therapy, when the early virological response became evident.

MATERIALS AND METHODS

Patients

Data were collected from a total of 446 genotype 1b chronic hepatitis C patients who were treated with PEG-IFN alpha and RBV at five hospitals and universities throughout Japan. The inclusion criteria were: (1) infection by hepatitis C genotype 1b; (2) no co-infection with hepatitis B virus or human immunodeficiency virus; (3) no other causes of liver disease such as autoimmune hepatitis and primary biliary cirrhosis; and (4) availability of DNA for the analysis of the genetic polymorphisms of *IL28B* and *ITPA*. Patients received PEG-IFN alpha-2a (180 μg) and 2b (1.5 μg/kg) subcutaneously every week and a daily weight-adjusted dose of RBV (600 mg for patients weighing <60 kg, 800 mg for patients weighing 60–80 kg, and 1,000 mg for patients weighing >80 kg) for 48 weeks. Dose reduction or discontinuation of PEG-IFN and RBV was primarily based on the recommendations on the package inserts and the discretion of the physicians at each university and hospital. The standard duration of therapy was set at 48 weeks. No patient received erythropoietin or other growth factors for the treatment of anemia. Written informed consent was obtained from each patient, and the study protocol conformed to the ethical guidelines of the Declaration of Helsinki and was approved by the institutional ethics review committees.

Laboratory Tests

Blood samples obtained before therapy were analyzed for hematologic data, blood chemistry, and HCV RNA. Genetic polymorphisms in SNPs of the *ITPA* gene (rs1127354) and the *IL28B* gene (rs8099917) were determined using ABI TaqMan Probes (Applied Biosystems, Carlsbad, CA) and the DigiTag2 assay, respectively. Baseline creatinine clearance (CLcr) levels were calculated using the formula of Cockcroft and Gault [1976]: for males, CLcr = [(140 − age in years) × body weight in kg] ÷ (72 × serum creatinine in mg/dl) and for females, CLcr = 0.85 × [(140 − age in years) × body weight in kg] ÷ (72 × serum creatinine in mg/dl). The stage of liver fibrosis was scored according to the METAVIR scoring system: F0 (no fibrosis), F1 (mild fibrosis: portal fibrosis without septa), F2 (moderate fibrosis: few septa), F3 (severe fibrosis: numerous septa without cirrhosis), and F4 (cirrhosis). A rapid virological response was defined as undetectable HCV RNA by qualitative PCR with a lower detection limit of 50 IU/ml (Amplicor, Roche Diagnostic Systems, Pleasanton, CA) at week 4 of therapy and a complete early virological response was defined as undetectable HCV RNA at week 12. A sustained virological response was defined as undetectable HCV RNA at 24 weeks after completion of therapy. Severe anemia was defined as hemoglobin (Hb) <10 g/dl.

Statistical Analysis

Database for analysis included the following variables: age, sex, body mass index, serum aspartate aminotransferase (AST) levels, alanine aminotransferase (ALT) levels, gamma-glutamyltransferase (GGT) levels, creatinine levels, CLcr, Hb, platelet count, serum levels of HCV RNA, and the stage of liver fibrosis

TABLE I. Patients' Baseline Characteristics

Age (years)	58.6	(9.6)
Gender: male (n, %)	185	(42%)
Body mass index (kg/m^2)	23.1	(3.7)
AST (IU/L)	59.9	(53.8)
ALT (IU/L)	69.8	(53.8)
GGT (IU/L)	48.5	(41.6)
Creatinine (mg/dl)	0.7	(0.2)
Creatinine clearance (ml/min)	89.5	(23.0)
Hemoglobin (g/dl)	14	(1.4)
Platelet count (10^9/L)	154.5	(52.1)
HCV RNA > 600,000 IU/ml (n, %)	354	(79%)
Liver fibrosis: F3-4 (n, %)	108	(24%)
Initial ribavirin dose (n, %)		
600 mg/day	300	(67%)
800 mg/day	138	(31%)
1,000 mg/day	9	(2%)
Pegylated interferon (n, %)		
alpha2a 180 mcg	58	(13%)
alpha2b 1.5 mcg/kg	388	(87%)
ITPA rs1127354: CC (n, %)	317	(71%)
IL28B rs809917: TT (n, %)	311	(70%)

AST, aspartate aminotransferase; ALT, alanine aminotransferase; GGT, gamma-glutamyltransferase.
Data expressed as mean (standard deviation) unless otherwise mentioned.

(Table I). Based on these data set, a model for predicting the risk of developing severe anemia was constructed by data mining analysis using the IBM-SPSS Modeler 13 as described previously [Kurosaki et al., 2010, 2011a,b,c; Hiramatsu et al., 2011]. Briefly, the software was used to explore the database automatically to search for optimal predictors that discriminated most efficiently patients with severe anemia from those without. The software also determined the optimal cutoff values of each predictor. Patients were divided into two groups according to the predictor and each of the two groups was repeatedly divided in the same way until no significant factor remained or 20 or fewer patients were in a group.

The incidence of severe anemia, the total dose of RBV, and treatment outcome were compared between groups with high and low risks of anemia. On univariate analysis, Student's t-test was used for continuous variables, and Fisher's exact test was used for categorical data. Logistic regression was used for multivariate analysis. P values of <0.05 were considered significant. SPSS Statistics 18 was used for these analyses.

RESULTS

Predictive Model of Severe Anemia

The incidence of severe anemia in the whole cohort was 49% (Fig. 1). The best predictor of severe anemia was the baseline Hb concentration. Patients with a low baseline Hb concentration (<14 g/dl) were more likely to develop severe anemia (67%) than those with a higher Hb (>14 g/dl) (34%). The second best predictor for those patients with a baseline Hb <14.0 g/dl was CLcr. Patients with a CLcr below 90 ml/min had the highest incidence of severe anemia (76%). In those with a CLcr above >90 ml/min the incidence of severe anemia was 57% in patients with the CC allele of the *ITPA* gene while it was 37% in patients with the CA or AA allele. On the other hand, the second best predictor for those patients with a baseline Hb concentration above 14 g/dl was the *ITPA* genotype. Patients with the AA or AC allele had the lowest incidence of anemia (17%). For those with the *ITPA* CC allele, CLcr was the third best predictor; the optimal cutoff value was 85 ml/min for this group. The incidence of severe anemia was 49% in patients with a CLcr below 85 ml/min while it was 32% in those with a CLcr above 85 ml/min.

Following this analysis, the patients were divided into six groups, with the incidence of severe anemia ranging from 17% to 76%. Three groups with two predictors, having an incidence of anemia >40%, were defined as the high-risk group and the remainder were defined as the low-risk group. The incidence of severe anemia was higher in the high-risk group than the low-risk group (65% vs. 28%, $P = 0.029$) (Fig. 2). Comparison of the *ITPA* genotype and the predictive model showed that the sensitivity for the prediction of severe anemia was similar (75.9% vs. 76.4%) but the specificity of the predictive model was greater (33.6% vs. 59.3%).

The Risk of Anemia Impacts on Sustained Virological Responses by Patients Who Achieved an Early Virological Response

The impact of *IL28B* genotype, *ITPA* genotype, and risk group of anemia on the rate of sustained virological response was studied at baseline and week 12. At baseline, patients with the TT allele of the *IL28B* gene had a significantly higher rate of sustained virological response than those with the TG or GG allele (43% vs. 10%, $P < 0.0001$), the high-risk group for anemia had a significantly lower rate of sustained virological response than the low-risk group (28% vs. 40%, $P = 0.011$), and the *ITPA* genotype was not associated with a sustained virological response (Fig. 3A–C). At week 4, patients with rapid virological response had a high rate of sustained virological response, irrespective of the *IL28B* genotype (TT vs. TG/GG; 97% vs. 100%, $P = 1.000$), the *ITPA* genotype (CC vs. CA/AA; 95% vs. 100%, $P = 1.000$), and the risk of anemia (high vs. low; 95% vs. 100%, $P = 1.000$). Among the patients who did not achieve a rapid virological response, those with the *IL28B* TT allele had a significantly higher rate of sustained virological response than those with the TG or GG allele (38% vs. 8%, $P < 0.0001$), and the high-risk group for anemia had a significantly lower rate of sustained virological response than the low-risk group (24% vs. 35%, $P = 0.015$). At week 12, in patients who achieved a complete early virological response, the *IL28B* genotype was not associated with a sustained virological response, while the high-risk group for anemia had a

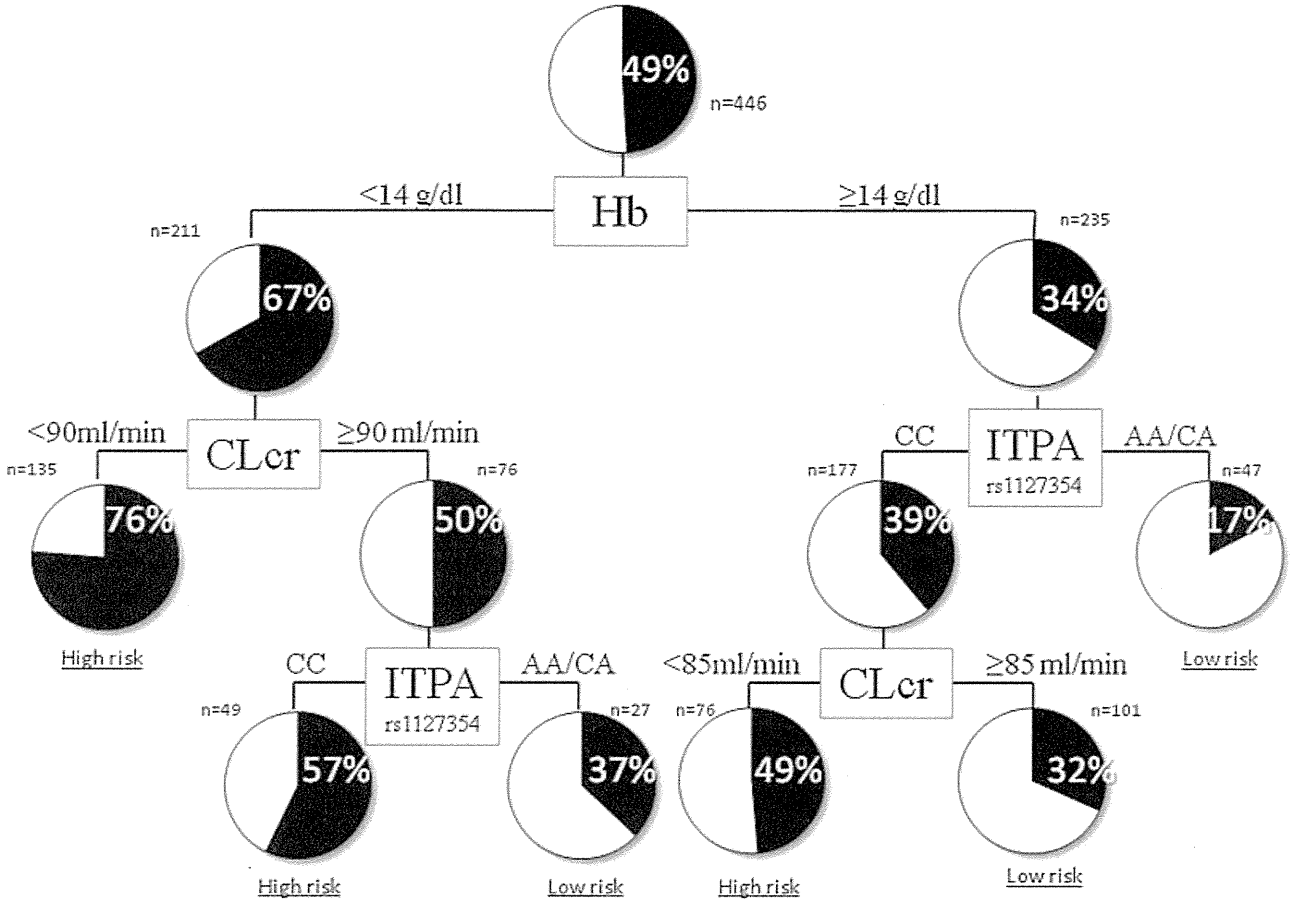

Fig. 1. The predictive model for severe anemia. The boxes indicate the factors used to differentiate patients and the cutoff values for the different groups. The pie charts indicate the rate of severe anemia (Hb <10.0 g/dl) for each group of patients, after differentiation. Terminal groups of patients differentiated by analysis are classified as at high risk if the rate is >40% and low risk if the rate is <40%. *ITPA*, inosine triphosphatase; CLcr, creatinine clearance; Hb, hemoglobin.

significantly lower rate of sustained virological response than the low-risk group (59% vs. 76%, $P = 0.013$) (Fig. 3D–F). In patients who did not achieve a complete early virological response, the *IL28B* genotype was a significant predictor of a sustained virological response (TT vs. TG/GG; 14% vs. 2%, $P < 0.0001$) but a high risk for anemia was not (high vs. low; 10% vs. 6%, $P = 0.361$).

From multivariate analysis (Table II), the *IL28B* genotype was the most important predictor of a sustained virological response at baseline [adjusted odds ratio 9.88 (95% confidence interval 5.01–19.48), $P < 0.0001$], along with female sex [0.42 (0.26–0.68), $P < 0.0001$], platelet count [1.09 (1.04–1.15), $P < 0.0001$], advanced fibrosis [0.49 (0.27–0.91), $P = 0.024$], and baseline HCV RNA load [4.14 (2.27–7.55), $P < 0.0001$]. At week 4, in patients without a rapid virological response, the *IL28B* genotype remained the most important predictor of a sustained virological response [7.16 (3.60–14.25), $P < 0.0001$], along with female sex and platelet count. At week 12, in patients with a complete early virological response, the risk of anemia was an independent and significant predictor of a sustained virological response [0.47 (0.24–0.91), $P = 0.026$], together with the platelet count and HCV RNA load, but the *IL28B* genotype was not associated with a sustained virological response. In patients without a complete early virological response, the *IL28B* genotype was a predictor of a sustained virological response [9.13 (2.02–41.3), $P = 0.004$] along with the platelet count. Thus, *IL28B* was a significant predictor of a sustained virological response at baseline and among virological non-responders at weeks 4 and 12. On the other hand, once a complete early virological response was achieved, the *IL28B* genotype was no longer associated with a sustained virological response but the risk of anemia was an independent predictor of a sustained virological response.

The Risk of Anemia, RBV Dose, and Treatment Outcome in Patients With a Complete Early Virological Response

Patients who achieved a complete early virological response were stratified according to adherence to

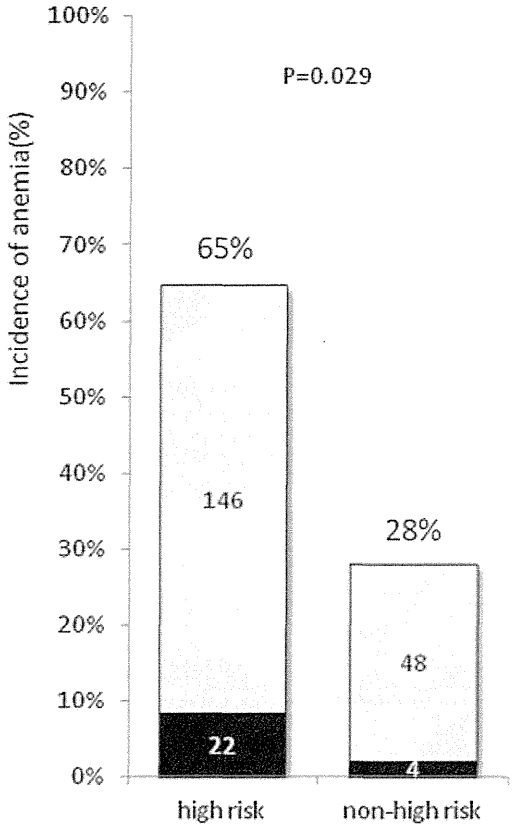

Fig. 2. The incidence of severe anemia stratified by risk of anemia. The incidence of anemia during therapy is shown for each group of patients at high and low risk of anemia. The black and white bars represent the percentages of patients with Hb concentrations below 8.5 g/dl and above 10 g/dl, respectively.

RBV (≤40%, 41–60%, 61–80%, and >80%), which showed that patients with a high risk of anemia were predominantly in subgroups with a lower adherence to RBV (≤40%, 41–60%, and 61–80%), whereas patients with a low risk of anemia were predominantly in subgroups with a higher adherence to RBV (>80%) (Fig. 4, upper panel). The percentage of patients who received >80% of the planned dose of RBV was significantly higher in the low-risk group for anemia than in the high-risk group (74% vs. 55%, $P < 0.0001$).

Within the groups with high and low risks of anemia, there was a stepwise increase in the rate of sustained virological response according to the increase in adherence to RBV (Fig. 4, lower panel). The rate of sustained virological response was higher in patients who received >80% of the planned dose of RBV than those who received less, for both high-risk patients (71% vs. 47%, $P = 0.016$) and low-risk patients (81% vs. 60%, $P = 0.072$). Within the same subgroup of RBV adherence, however, the rate of sustained virological response did not differ between patients with a high risk and a low risk of anemia. Taken together, these results suggest that patients with a high risk of anemia have a disadvantage because they are likely to be intolerant to RBV, leading to reduced adherence to RBV throughout the 48 weeks of therapy and a reduced rate of sustained virological response. However, if >80% adherence to RBV could be obtained, the rate of sustained virological response would increase by 24%.

DISCUSSION

This study confirmed previous reports that the *IL28B* genotype is the most significant predictor of a sustained virological response to PEG-IFN plus RBV therapy in chronic hepatitis C patients at baseline [Ge et al., 2009; Suppiah et al., 2009; Tanaka et al., 2009; Rauch et al., 2010; Kurosaki et al., 2011c] and at week 4 [Thompson et al., 2010b], but it had no impact on the rate of sustained virological response among those patients who achieved a complete early virological response [Thompson et al., 2010b; Kurosaki et al., 2011c]. In contrast, the risk of anemia, assessed by the combination of the *ITPA* genotype, baseline Hb concentration, and baseline CLcr, was found to be associated with a sustained virological response in patients who achieved a complete early virological response. Generally, a complete early virological response is the hallmark of a high probability of a sustained virological response, but the rate of sustained virological responses in patients who achieved a complete early virological response and had a high risk of anemia was as low as 59%. This reduced rate of sustained virological response in these patients was attributable to poor adherence to RBV throughout the 48 weeks of therapy. Because administration of >80% of the planned RBV dose increased the rate of sustained virological response by 24%, it may be postulated that personalizing the treatment schedule to achieve a sufficient dose of RBV, such as extension of treatment duration, may improve sustained virological response rates in these patients. Clearly, this postulate needs to be confirmed in future study. Thus, the findings presented here may have the potential to support selection of the optimum, personalized treatment strategy for an individual patient, based on the risk of anemia.

The degree of hemolytic anemia caused by RBV varies among individuals. A reduction of the Hb concentration early during therapy predicts the likely development of severe anemia [Hiramatsu et al., 2008, 2011] but there are no reliable predictors at baseline. A breakthrough came from the results of a genome-wide association study that revealed that variants of the *ITPA* gene are protective against hemolytic anemia [Fellay et al., 2010]. The *ITPA* genotype has been shown repeatedly to be associated with the degree of hemolytic anemia and dose reduction of RBV [Fellay et al., 2010; Sakamoto et al., 2010; Thompson et al., 2010a; Seto et al., 2011; Tanaka et al., 2011; Kurosaki et al., 2011d]. However, factors other than the *ITPA* gene, such as baseline Hb concentrations [Ochi et al., 2010; Kurosaki et al., 2011d], platelet counts [Ochi

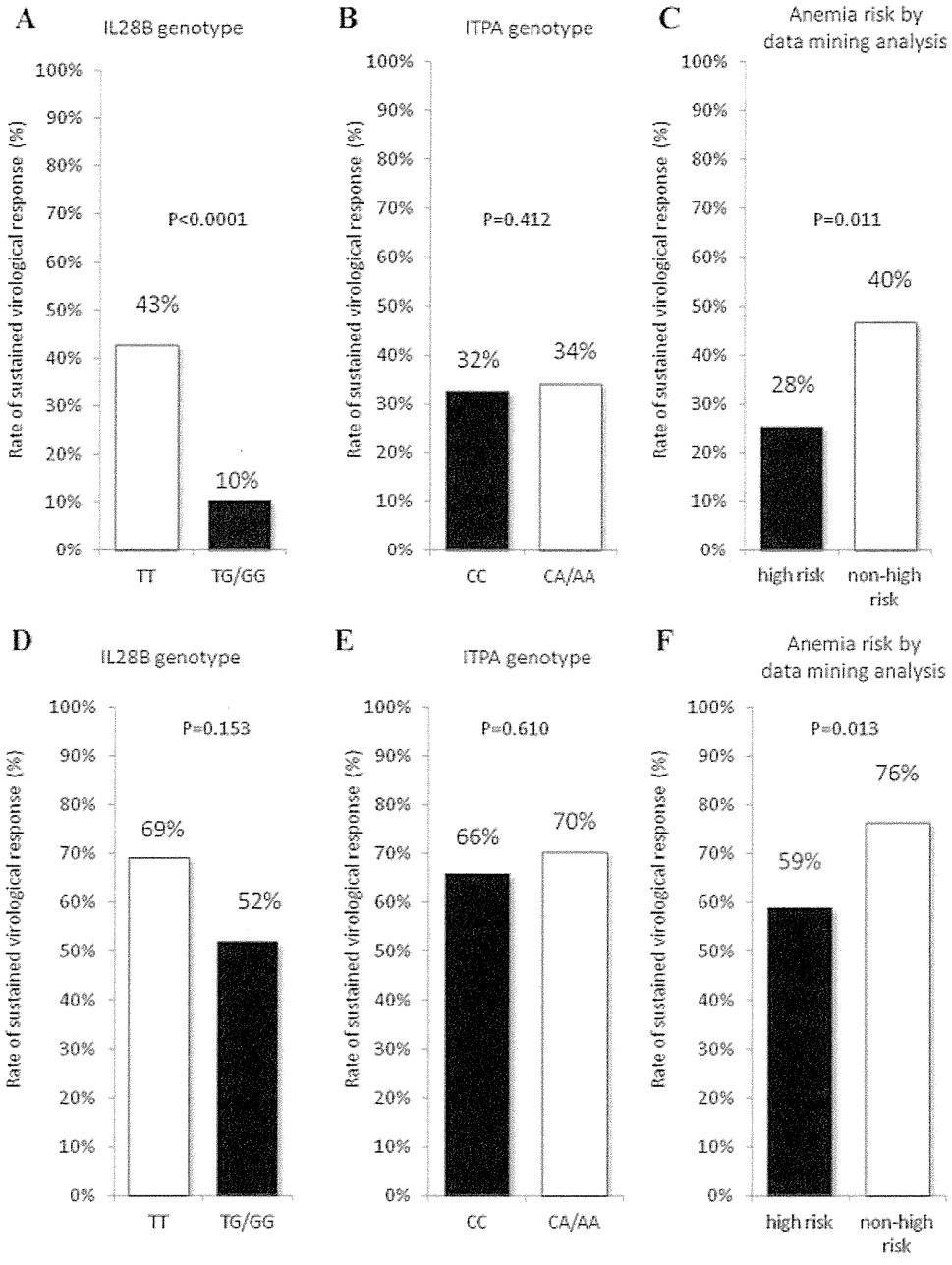

Fig. 3. Rates of sustained virological responses at baseline and among those with a virological response at week 12. The impacts of *IL28B* genotype, *ITPA* genotype, and risk group of anemia on the rate of sustained virological response were studied at baseline (**A–C**) and among those with complete early virological responses (defined as undetectable HCV RNA at week 12) (**D–F**). At baseline, those with the TT allele of the *IL28B* gene had a significantly higher rate of sustained virological response than those with the TG or GG allele and the group at high-risk of anemia had a significantly lower rate of sustained virological response than the low-risk group. Among patients with complete early virological responses, the *IL28B* genotype was not associated with a sustained virological response, while the group at high-risk of anemia had a significantly lower rate of sustained virological response than the low-risk group.

et al., 2010], and CLcr [Kurosaki et al., 2011d], also contribute to the risk of severe anemia or RBV dose reduction. In the present study, the predictive model of anemia based on the data mining analysis selected the *ITPA* genotype, baseline Hb concentration, and baseline CLcr as predictive factors and identified six subgroups of patients with a variable rate of severe anemia, ranging from 17% to 76%. The specificity of the prediction of severe anemia was improved by 25.7% in the predictive model, compared to *ITPA*

TABLE II. Logistic Regression Analysis for Factors Associated With Sustained Virological Response at Baseline, Week 4 and Week 12

	Multi-variable		
	Odds	95% CI	*P*-value
Pre-treatment			
Sex: female	0.42	0.26–0.68	<0.0001
Platelet (10^9/L)	1.09	1.04–1.15	<0.0001
Fibrosis: F3-4	0.49	0.27–0.91	0.024
HCV RNA: <600,000 IU/L	4.14	2.27–7.55	<0.0001
IL28B rs8099917: TT	9.88	5.01–19.48	<0.0001
At week 4			
Non-RVR patients			
Sex: female	0.45	0.28–0.72	0.001
Platelet (10^9/L)	1.10	1.05–1.16	0.000
IL28B rs8099917: TT	7.16	3.60–14.25	<0.0001
At week 12			
cEVR patients			
Platelet (10^9/L)	1.09	1.02–1.17	0.015
HCV RNA: <600,000 IU/L	3.21	1.39–7.55	0.007
High-risk of anemia[a]	0.47	0.24–0.91	0.026
At week 12			
Non-cEVR patients			
Platelet (10^9/L)	1.11	1.02–1.21	0.017
IL28B rs8099917: TT	9.13	2.02–41.3	0.004

RVR: rapid virological response, defined as undetectable HCV RNA at week 4.
cEVR: complete early virological response, defined as undetectable HCV RNA at week 12.
[a]High-risk of anemia defined by decision tree analysis includes the following groups: (1) baseline hemoglobin <14.0 g/dl and creatinine clearance <90 ml/min, (2) baseline hemoglobin <14.0 g/dl, creatinine clearance ≥90 ml/min and ITPA rs1127354 genotype CC, and (3) baseline hemoglobin ≥14.0 g/dl, ITPA rs1127354 genotype CC, and creatinine clearance <85 ml/min.

genotyping alone. Because hemolytic anemia induced by RBV is one of the major adverse events leading to premature termination of therapy [Fried et al., 2002], a method to predict the risk of severe anemia before treatment is important clinically. A predictive model of anemia may have the potential to support individualized treatment strategies; patients at high risk of anemia may be tested intensively for anemia or may be candidates for erythropoietin therapy, whereas those with a low risk of anemia may be treated with a higher dose of RBV. Prediction of anemia will remain important in the era of direct antiviral agents for chronic hepatitis C, because these newer therapies still require RBV and PEG-IFN in combination, and the degree of anemia complicating these therapies may be even greater than with the current combination therapy [McHutchison et al., 2009; Kwo et al., 2010].

Studies of the impact of the *ITPA* genotype on treatment outcome have produced conflicting results. Previous studies of American [Thompson et al., 2010a] and Italian [Thompson et al., 2011] cohorts did not find any association between the *ITPA* genotype and treatment outcome, whereas a marginal difference was observed in a report from Japan [Ochi et al., 2010]. Moreover, with a subgroup analysis of Japanese patients, the variant of the *ITPA* gene was associated with a sustained virological response in patients with the *IL28B* major genotype [Kurosaki et al., 2011d], in patients infected with HCV other than genotype 1[Sakamoto et al., 2010], and in patients with pre-treatment Hb concentrations between 13.5 and 15 g/dl [Azakami et al., 2011]. These inconsistent results may be because the impact of anemia may be greater on a cohort of aged patients, such as in Japan. Another reason may be that the *ITPA* genotype is not the sole determinant of anemia; the *ITPA* genotype alone was not associated with treatment outcome in the present study but a high-risk of anemia, defined by the combination of the *ITPA* genotype, baseline Hb concentration, and baseline CLcr, was associated with sustained virological responses by patients with complete early virological responses, even after adjustment for the *IL28B* genotype and other relevant factors. This is in contrast to the finding that the *IL28B* genotype is an independent and significant predictor at baseline of a sustained virological response by patients without a rapid virological response and those without a complete early virological response, but not those with a complete early virological response. These results indicate that the *IL28B* genotype could be used to predict a sustained virological response at baseline or during therapy in patients in whom HCV RNA has not yet become undetectable, but it has no predictive value in patients in whom HCV RNA has become undetectable. The risk of anemia may be used to predict sustained virological responses in a selected subgroup of patients who achieve a complete early virological response.

Patients who received more than 80% of the planned dose of PEG-IFN or RBV had a higher rate of sustained virological responses than those who received a lower cumulative dose [McHutchison et al., 2002; Davis et al., 2003]. Patients who achieve a complete early virological response usually have a good chance of a sustained virological response and the treatment duration is not extended beyond 48 weeks. However, reduced adherence to drugs in these patients was related to relapse after the completion of 48 weeks of therapy [Hiramatsu et al., 2009; Kurosaki et al., 2012]. In the present study, the rate of sustained virological response was 59% in patients who achieved a complete early virological response but had a high risk of anemia, 17% lower than in patients with a low risk of anemia. However, there was a stepwise increase in the rate of sustained virological response according to the increase in adherence to RBV, and the rate of sustained virological response was higher in high-risk patients who received >80% of the planned dose of RBV (71% vs. 47%). This 24% increase in sustained virological response was observed among the patients in the present study who received 48 weeks of treatment. These findings suggest that receiving a sufficient RBV dose is essential for patients with a complete early virological response to attain a sustained virological response and that the treatment strategy should be personalized for patients with a

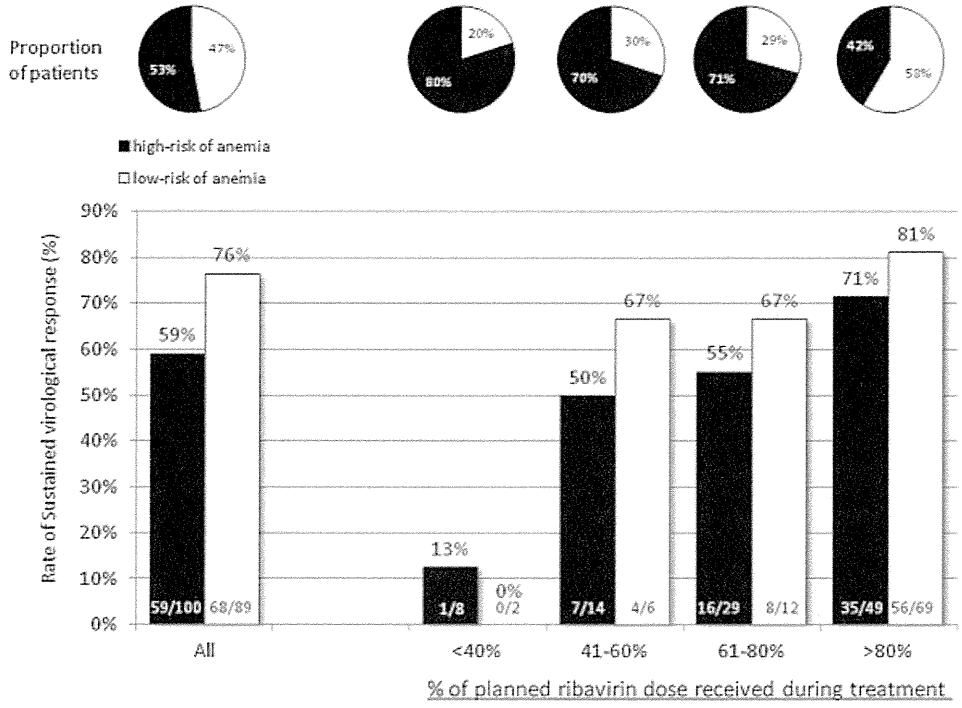

Fig. 4. The impact of risk of anemia and RBV dose on treatment outcome after a complete early virological response. Patients with complete early virological responses were divided into subgroups according to their adherence to RBV: ≤40%, 41–60%, 61–80%, and >80%. For each subgroup, the proportion of patients with a high risk and a low risk of anemia is shown in the upper panel by pie charts, and the rates of sustained virological responses, stratified by high risk and low risk of anemia, are shown in the lower panel by bar graphs. The black and white bars or charts represent patients with high and low risks of anemia, respectively.

high risk of anemia to extend the duration of treatment, even those patients with a complete early virological response, to obtain >80% adherence to RBV.

In conclusion, the combination of the *ITPA* genotype, baseline Hb concentration, and baseline CLcr could be used as a pre-treatment predictor of anemia. The risk of anemia thus identified is associated with adherence to RBV and impacts on the treatment outcome of patients who achieve a complete early virological response. This is in contrast to the major role of the *IL28B* genotype in the prediction of sustained virological responses at baseline and among non-responders at weeks 4 and 12. Patients who achieve a complete early virological response generally have a high probability of a sustained virological response but those who have a high risk of anemia have a high rate of relapse because of reduced adherence to RBV. To improve the rate of sustained virological responses in these patients, it may be postulated that the treatment schedule may be personalized to obtain >80% adherence to RBV. Clearly, this postulate needs to be confirmed in a future study.

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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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Running title: HCV NS4B suppresses IFN response

Footnotes

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Abbreviations used in this manuscript:

HCV - hepatitis C virus, CHC - Chronic hepatitis C, HCC - hepatocellular carcinoma, IFN - interferon, DAA(s) – direct antiviral agents, SVR - sustained viral response, TLR(s) - toll-like receptors, RLR(s) - RIG-I like receptors, RIG-I - retinoic-acid-inducible protein I, MDA5 - melanoma-differentiation-associated gene 5, LGP-2 - laboratory of genetics and physiology, dsRNA - double-stranded RNA, PAMP(s) - pathogen-related molecular patterns, CARD - caspase recruitment domain, TBK1 - TANK binding kinase 1, IKKε - IκB kinase ε, IRF - interferon-regulatory factor, pIRF3 - phospho-IRF3, STAT1 - signal transducer and activator of transcription protein-1, SOCS - suppressor of cytokine signaling, Jak - Janus kinase, E2 - envelope2, TM - transmembrane, ISRE - interferon-stimulated response element, STING - stimulator of interferon genes, Cardif - CARD adaptor inducing interferon-β, YFV - yellow fever virus, PDI - protein disulphide-isomerase, BiFC - Bimolecular Fluorescence Complementation, MAM - mitochondria-associated ER membrane, FACL4 - fatty acid-CoA ligase, long chain 4, TMEM173 - transmembrane protein 173

Keywords:

1. RIG-I-like receptors

2. Interferon-regulatory factor 3 (IRF-3)

3. Double-strandeded RNA

4. Bimolecular Fluorescence Complementation assay

5. NS3 protease

Summary

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 protein I (RIG-I) (Tasaka et al. J Gen Virol 2007); 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, while 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.

Introduction

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 (TLRs) and a family of RIG-I like receptors (RLRs), such as retinoic-acid-inducible protein I (RIG-I) and melanoma-differentiation-associated gene 5 (MDA-5), and then host antiviral responses are activated resulting in the production of cytokines such as type I and type III IFNs (5). RIG-I is activated by recognition of short double-strand RNA (dsRNA) or triphosphate at the 5'-end of dsRNA as pathogen-associated molecular patterns (PAMPs) (6, 7), forming a homo-oligomer that binds with the CARD of Cardif (also known as MAVS, VISA or IPS-1) (8-11). Cardif subsequently recruits TANK binding kinase 1(TBK1) and IκB kinase (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 IRF3 or IRF7, 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 STAT1 (13). The core protein also induces expression of suppressor of cytokine signaling-1 (SOCS1) and SOCS3, and blocks Janus kinase (Jak)-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 et al. 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 (11, 16, 17). On the other hand, Baril et al. reported that Cardif was still able to form a homo-oligomer and to activate downstream IFN production signaling despite delocalization from the mitochondria

(18). 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), which binds RIG-I, Cardif, TBK1, and IKKε. STING is thought to act as a scaffold for Cardif/TBK1/IRF3 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 have 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 RLR-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 Dr. Tschopp (11). Plasmids expressing STING were provided by Dr. Barber (20). Plasmids expressing HCV NS3/4A, NS4B and truncated NS4B have been described (25). Plasmid pIFNβ-Fluc was provided by Dr 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, HCV-JFH1 strain was used (29, 30).

Antibodies. Antibodies used were anti-IRF3 (FL-425, Santa Cruz Biotechnology), anti-HA (Invitrogen), anti-myc (Invitrogen), mouse anti-PDI (Abcam), rabbit anti-PDI (Enzo Life Science), anti-Flag (Sigma Aldrich), anti-Cardif (Enzo Life Science), anti-phospho-IRF3 (Ser396, Millipore), anti-monomeric Kusabira-Green C- or N-terminal fragment (MBL), and anti-FACL4 (Abgent).

Luciferase reporter assay. IFN-β reporter assays were carried out as previously described (19, 31). Plasmids pIFN-β-Fluc and pRL-CMV were cotransfected with NS3/4A or NS4B, and ΔRIG-I, Cardif, STING or poly(dA-dT)poly(dT-dA) (indicated as 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 done in triplicate and the results were expressed as means±SD.

Immunoblotting. Preparation of total cell lysates was carried out as described previously (19, 28). Protein was separated using NuPAGE 4-12% Bis/Tris gels (Invitrogen) and blotted onto an Immobilon PVDF 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-labelled secondary antibodies. Mitochondria were stained by MitoTracker (Invitrogen). Cells were visualized using a confocal laser microscope (Fluoview FV10, Olympus).

Bimolecular fluorescence complementation (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 PCR-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.

siRNA assay. Nucleotide sequences of STING-targeted siRNAs were as follows: #1: 5'-gcaacagcatctatgagcttctggagaac-3' #2: 5'- gtgcagtgagccagcggctgtatattctc;-3'#3:

5'-gctggcatggtcatattacatcggatatc-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's *t*-test. P values less than 0.05 were considered to be statistically significant.