Liver International ISSN 1478-3223

CLINICAL STUDIES

Association of interferon regulatory factor-7 gene polymorphism with liver cirrhosis in chronic hepatitis C patients

Radsamee Sermasathanasawadi, Naoya Kato, Ryosuke Muroyama, Narayan Dharel, Run-Xuan Shao, Jin-Hai Chang, Chang-Zheng Li, Takao Kawabe and Masao Omata

Department of Gastroenterology, Graduate School of Medicine, University of Tokyo, Tokyo, Japan

Keywords
chronic hepatitis C – interferon regulatory factor 7 – liver cirrhosis – polymorphism

Abbreviations
AFP, α-fetoprotein; ALT, alanine aminotransferase; CI, confidence interval; HCC, hepatocellular carcinoma; HCV, hepatitis C virus; IFN, interferon; IRF, interferon regulatory factor; ISRE, interferon-stimulated response element; IU, international unit; OR, odds ratio; PCR, polymerase chain reaction; PT, prothrombin time; SNP, single nucleotide polymorphism.

Correspondence
Naoya Kato, MD, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-8655, Japan
Tel: 81 3 3815 5411 ext. 37198
Fax: 81 3 3814 0021
e-mail: kato-2im@h.u-tokyo.ac.jp

Received 27 October 2007
Accepted 1 February 2008

DOI:10.1111/j.1478-3231.2008.01725.x

Abstract

Background and aims: Interferon (IFN) regulatory factor 7 (IRF-7) has been shown to play an essential role in the transcriptional activation of virus-inducible cellular genes, especially IFN genes. Polymorphisms of the IRF-7 gene may probably affect both the quality and the quantity of IRF-7. We investigated the role of IRF-7 polymorphisms in Japanese patients with chronic hepatitis C virus (HCV) infection. *Methods:* We studied a total of nine polymorphisms of the IRF-7 gene including SNP1047A/G (Lys/Glu) and SNP2157A/G (Gln/Arg) using the Taqman allelic discrimination and sequencing techniques in 406 Japanese patients with chronic HCV infection. We further performed functional analysis of SNP1047 and SNP2157 by transcriptional activation of the IFNA promoter. *Results:* We found that SNP1047AG and SNP2157AG genotypes were in complete linkage disequilibrium and were present in a significantly higher proportion in HCV-infected patients with cirrhosis (5.6%) than in those without cirrhosis (1.7%) ($P = 0.03$). Multivariate analysis also revealed that SNP1047 and SNP2157 were independently associated with cirrhosis at an odds ratio of 2.5. Functional analysis revealed that SNP1047G and SNP2157G alleles increased IFNA expression. *Conclusion:* SNP1047AG and SNP2157AG genotypes were strongly associated with cirrhosis. SNP1047G and SNP2157G alleles might be used as markers of host factors associated with a higher risk of cirrhosis in Japanese patients with chronic HCV infection.

It has been estimated that hepatitis C virus (HCV) infects at least 170 million people worldwide (1), which often leads to the dreadful sequels of liver cirrhosis, end-stage liver disease and hepatocellular carcinoma (HCC) (1–4). The risk of HCC development increases with the severity of inflammation and liver fibrosis (5–8). Several factors, such as alcohol intake, older age at the time of infection, male gender and co-infection with the hepatitis B virus or human immunodeficiency virus, are known to accelerate disease progression in HCV infection (6, 8, 9). In addition, host genetic factors have been reported to affect the outcome of HCV infection (10–17). Actually, we have reported previously that genetic polymorphisms including single nucleotide polymorphisms (SNPs) in interleukin-1β, UDP glucuronosyltransferase 1A7, SCYB14, GFRA1, CRHR2 and MDM2 genes were associated with the development of HCC in Japanese patients with chronic HCV infection (14–17).

The endogenous production of interferons (IFNs) serves as an initial defence mechanism against viral infection. IFN regulatory factor 7 (IRF-7), one of the IFN-stimulated genes synthesized, regulates the transcription of IFNA genes. IFN-β produced in response to a viral challenge (18) induces transcription of IRF-7, and IRF-7 then binds and induces the promoter of IFNA genes (19). The transcription effecter action of IRF-7 promotes IFNA subtype expression and diversification of the IFN-stimulated gene response, establishing a positive-feedback loop that amplifies IFN production and antiviral action (20). The importance of murine IRF-7 in the Type I IFN gene expression was demonstrated by Taniguchi's group, who showed that virus-activated transcription of Type I IFN genes

depends on the synthesis of IRF-7 (21). There are many reports showing that induction of the human IFNA subtypes by viral infection requires IRF-7 (22–26). A recent study using IRF-7 knockout mice has demonstrated that transcription of both IFN-α and IFN-β is dependent on IRF-7, indicating that IRF-7 is a master regulator of type I IFNs (20). Polymorphisms of the IRF-7 gene may probably affect both the quality and the quantity of IRF-7.

In this study, we investigated the role of IRF-7 SNPs in Japanese patients with chronic HCV infection.

Methods

Patients

We studied 406 consecutive Japanese patients with chronic HCV infection who consulted at the outpatient clinic of the University of Tokyo Hospital between August 2001 and June 2003 (227 men and 179 women; ages 22–84 years; median age 62 years; 178 with cirrhosis). The genomic DNA of these patients was made available after obtaining written informed consent for genotyping. Approval was obtained from the institutional ethics committee (No. 400), and all the procedures followed institutional guidelines (27). Patients selected for this study were those who tested positive for the HCV antibody by the second-generation enzyme immunoassay (Ortho Diagnostics, Tokyo, Japan), and HCV RNA was measured using the Amplicor HCV assay version 1 (Roche, Tokyo, Japan). All patients were negative for the hepatitis B surface antigen (Abbott Laboratories, North Chicago, IL, USA). HCV genotypes were determined using a genotyping assay (SRL Laboratory Co., Tokyo, Japan). Patients with an ethanol intake of $\geq$80 g/day for $>$ 10 years were considered to have a positive history of alcohol abuse. The following clinical variables were obtained for each patient at the time of whole-blood collection: age, gender, serum albumin level, serum total bilirubin level, serum alanine aminotransferase (ALT) level, serum α-fetoprotein (AFP) level, prothrombin time (PT), platelet count and serum viral load. The diagnosis of cirrhosis was made based on liver histology according to the criteria of Desmet (28) and Scheuer *et al.* (29). In patients without biopsy specimens, the diagnosis of cirrhosis was based on the presence of clinical manifestations of portal hypertension (e.g. varices, encephalopathy or ascites), biochemical abnormalities (elevated serum bilirubin, decreased serum albumin or prolonged PT) and obvious morphological change of the liver detected by hepatic imaging (e.g. ultrasonography, computed tomography, arteriography or magnetic resonance imaging).

Polymorphism genotyping

Genomic DNA was extracted from 100 μl whole blood as described previously (16). We studied two polymorphisms, 2157A/G (Gln/Arg) and 1047A/G (Lys/Glu), that are nonsynonymous polymorphisms. To determine the 2157A/G polymorphism, we could identify other three SNPs that located very close: 2068C/A, 2173A/G and 2200C/G. Furthermore, we selected four polymorphisms, −667C/T, −486A/G, −206C/G and −424–425, located close to the IFN-stimulated response element (ISRE) in the promoter region. Finally, we studied total nine polymorphisms of the IRF-7 gene (Table 1).

Genetic polymorphisms in the promoter region of IRF-7 were determined by direct sequencing of amplified gene fragments. We used two sets of primers: the outer primer, forward primer 5′-ccctccactcctccctact c-3′ and backward primer 5′-gtgtcacaggtgtccacagg-3′ for first polymerase chain reaction (PCR) and the inner primers, forward primer 5′-tcctccctactccgcgc tgg-3′ and backward primer 5′-gctgcctcggtatggatct c-3′ for nested PCR to amplify the specific IRF-7

Table 1. Interferon regulatory factor 7 gene polymorphisms studied

dbSNP rs#	Gene position	Alleles	Role	Amino acid	Method
rs10902179	−667	C/T	Promoter	–	Sequencing
rs2277270	−486	A/G	Intron	–	Sequencing
rs3832720	−424–425	−/GCCTCC	Intron	–	Sequencing
rs11544076	−206	C/G	Intron	–	Sequencing
rs1061502	1047	A/G	Nonsynonymous	Lys/Glu	Real time PCR
rs1061505	2068	C/A	Synonymous	–	Sequencing
rs3178010	2157	A/G	Nonsynonymous	Gln/Arg	Sequencing
rs12422022	2173	A/G	Intron	–	Sequencing
rs1051390	2200	C/G	Intron	–	Sequencing

SNP, single nucleotide polymorphism.

promoter fragment that covered all four polymorphisms. For the first PCR, a 617 bp fragment of the IRF-7 promoter was amplified by PCR using 20 ng of extracted genomic DNA template. PCR was performed with 5 × Green GoTaq Reaction Buffer and GoTaq DNA Polymerase (Promega, Madison, WI, USA). The thermocycling conditions were as follows: 95 °C for 2 min, followed by 35 cycles of 95 °C for 30 s, 65 °C for 30 s and 72 °C for 1 min then 72 °C for 5 min. To verify the size of the PCR product, amplified products were visualized on a 2% agarose gel with the appropriate size marker. Nested PCR was performed only for samples that did not give clear bands using 4 μl of the first PCR product. The thermocycling conditions of nested PCR were as follows: 95 °C for 2 min followed by 35 cycles of 95 °C for 30 s, 67 °C for 30 s and 72 °C for 1 min then 72 °C for 5 min. The 561 bp fragment of the nested PCR product was verified on 2% agarose gel with the appropriate size marker. For SNP determination, direct sequencing was performed bidirectionally using 10 ng of QIAquick Spin (Qiagen, Hilden, Germany)-purified PCR product, either the forward or backward PCR primer, and the Big Dye Terminator Cycle Sequencing Ready Reaction (PE Applied Biosystems, Foster City, CA, USA) followed by detection on an ABI 310 automated sequencer (PE Applied Biosystems).

We studied the SNP2157A/G genotype using direct sequencing of amplified gene fragments. We used the following primers: forward primer 5′-gctacacggaggaa ctgctg-3′ and backward primer 5′-ggctctggtctcacctt cac-3′ to amplify the specific IRF-7 fragment that covered other three SNPs: 2068C/A, 2173A/G and 2200C/G. A 428 bp fragment of IRF-7 was amplified by PCR using 20 ng of extracted genomic DNA template. PCR was performed with the same protocol as mentioned above. The thermocycling conditions were as follows: 95 °C for 2 min followed by 35 cycles of 95 °C for 30 s, 60 °C for 30 s and 72 °C for 40 s then 72 °C for 5 min. To verify the size of the PCR product, amplified products were visualized on a 2% agarose gel with the appropriate size marker. For SNP determination, sequencing was performed in the same way as mentioned above.

We performed SNP1047A/G genotype using the Taqman SNP genotyping assays and ABI 7000 sequence detection system, which were previously described in Dharel *et al.* (16).

Plasmids and site mutagenesis

pIFNA-pGL3 (pIFNA2 and pIFNA4) having Firefly luciferase gene downstream of the IFNA promoter and pFLAG-CMV-2IRF-7A expressing IRF-7 were generous gifts of Prof. John Hiscott (McGill University, Montreal, Canada). The plasmids were transformed in competent *Escherichia coli* and purified using a High Pure Plasmid Isolation Kit (Roche Diagnostics, Mannheim, Germany). Mutation was introduced into the IRF-7 gene of pFLAG-CMV-2IRF-7A using a QuickChange site-directed mutagenesis kit (Stratagene, La Jolla, CA, USA). Introduction of mutation was verified by dye-terminator cycle sequencing with an automated 310 DNA sequencer (Applied Biosystems).

Cell cultures, transient transfection and luciferase assay

Transfections for luciferase assays were carried out with human embryonic kidney 293 cells grown in Dulbecco's modified Eagle medium (GIBCO BRL, Gaithersburg, MD, USA) supplement with 10% foetal bovine serum and antibiotics. Subconfluent cells were transfected with 20 ng of pRL-TK expressing Renilla luciferase for internal control to adjust transfection efficiency, 600 ng of the pIFNA2 or pIFNA4, and 1200 ng of pFLAG-CMV-2IRF-7A using FuGene6 transfection reagent (Roche Diagnostics) in a six-well plate. At 48 h after transfection, the reporter gene activities were measured by a dual-luciferase reporter assay according to the manufacturer's instructions (Promega). All experiments were independently repeated for at least three times. Relative luciferase activities were calculated for each transfectant and compared with that of a blank pFLAG-CMV-2 expression vector (Sigma, St Louis, MO, USA).

Statistical analysis

The association between the clinical variables and the presence of cirrhosis was evaluated using the two-tailed *t*-test, the Wilcoxon test and the χ^2 test where appropriate. The association between different SNP genotypes and the presence of cirrhosis was evaluated using the χ^2 test. Possible confounding effects among the variables were adjusted using a multivariate logistic regression model (30), and odds ratios (ORs) and 95% confidence intervals (95% CI) were calculated. All data analyses were done using JMP software (version 5.1.2, SAS Institute Inc., Cary, NC, USA). The Hardy–Weinberg equilibrium of alleles at individual loci was evaluated using HWE. For all tests, $P < 0.05$ was considered significant.

Liver International (2008)

Results

Patient characteristics

The characteristics of the total 406 patients with chronic HCV infection involved in this study are shown in Table 2.

One hundred seventy-eight patients (43.8%) had cirrhosis. There was no significant difference in the proportion of gender, HCV genotype, serum viral load, alcohol drinking > 50 g/day, and serum ALT level between patients with and without cirrhosis. In patients with cirrhosis, age, presence of HCC, serum total bilirubin level and serum AFP level were higher than in patients without cirrhosis; however, serum albumin level, PT and platelet count were lower than in patients without cirrhosis.

Interferon regulatory factor-7 gene polymorphisms in hepatitis C virus-infected Japanese patients

Table 3 shows the distributions of the of IRF-7 gene polymorphisms. Since IRF-7 SNP1047, SNP2068,

Table 2. Patient demographics

Variable*	Total ($n = 406$)	Without cirrhosis ($n = 228$)	With cirrhosis ($n = 178$)	*P*-value
Sex (male)	227 (55.91%)	127 (55.70%)	100 (56.18%)	0.92†
HCC	177 (43.60%)	51 (22.37%)	126 (70.79%)	< 0.0001†
Age (year)	62 (22–84)	60 (22–83)	65 (37–84)	< 0.0001‡
Platelet count ($\times 10^4/\mu l$)	12.7 (1.7–38.67)	16 (4.1–38.67)	9.2 (1.7–27.1)	< 0.0001‡
Albumin (g/dl)	3.9 (2.3–5)	4.1 (2.8–5)	3.6 (2.3–4.8)	< 0.0001‡
ALT (U/L)	65 (9–429)	63.5 (9–341)	66 (13–429)	0.09‡
Total bilirubin (mg/dl)	0.8 (0.2–6.7)	0.7 (0.2–6.7)	0.9 (0.3–3.5)	< 0.0001‡
AFP (μg/L)	12 (1–21 700)	6 (1–3222)	25 (2–21 700)	< 0.0001‡
PT (%)	77.8 (7.3–100)	85 (12.6–100)	71.3 (7.3–100)	< 0.0001‡
HCV load (KIU/ml)	429 (0.5–1910)	453 (7.1–1910)	409.5 (0.5–1407)	0.3‡
Alcohol > 50 g/day	50 (12.92%)	24 (11.32%)	26 (14.86%)	0.36†
HCV genotype 1	259 (75.73%)	143 (75.26%)	116 (76.32%)	0.8†

*Age, albumin, total bilirubin, alanine aminotransferase, AFP, prothrombin time, platelet count and HCV load are shown as median (range). Male, cirrhosis, alcohol and HCV serotype 1 are shown as frequency (%).

†Calculated using the χ^2 test.

‡Calculated using the Wilcoxon test.

AFP, α-fetoprotein; ALT, alanine aminotransferase; HCC, hepatocellular carcinoma; HCV, hepatitis C virus; PT, prothrombin time.

KIU/ml (= 1000 HCV/ml).

Table 3. Interferon regulatory factor 7A single nucleotide polymorphism genotype frequencies in hepatitis C virus-infected patients

SNP	Gene position	Function	Homozygote	Heterozygote	Homozygote
rs10902179	−667C/T	Promoter	355 (87.44%) CC	50 (12.31%) CT	1 (0.25%) TT
rs3832720	−424–425 −/GCCTCC	Intron	224 (55%) (4 Repeat)	153 (37.6%)	30 (7.4%) (3 Repeat)
rs2277270	−486A/G	Intron	371 (91.38%) AA	35 (8.62%) AG	0 (0%) GG
rs11544076	−206C/G	Intron	406 (100%) CC	0 (0%) CG	0 (0%) GG
rs1061502	1047A/G	Nonsynonymous (Lys/Glu)	392 (96.55%) AA	14 (3.45%) AG	0 (0%) GG
rs1061505	2068A/C	Synonymous	392 (96.55%) AA	14 (3.45%) AC	0 (0%) CC
rs3178010	2157A/G	Nonsynonymous (Gln/Arg)	392 (96.55%) AA	14 (3.45%) AG	0 (0%) GG
rs12422022	2173A/G	Intron	392 (96.55%) AA	14 (3.45%) AG	0 (0%) GG
rs1051390	2200C/G	Intron	392 (96.55%) CC	14 (3.45%) CG	0 (0%) GG

SNP, single nucleotide polymorphism.

SNP2157, SNP2173 and SNP2200 were in complete linkage disequilibrium, only SNP1047 was described in this haplotype analysis. Concerning SNP 1047, the proportion of patients with heterozygosity (AG) was only 3.45% among Japanese HCV-infected patients. In addition, we could not find homozygote of rare alleles (GG) in our patients. When the HCV-infected patients were segregated into two groups based on the presence or absence of cirrhosis, the genotype frequency and allelic frequency of SNP1047 were significantly different between patients with and without cirrhosis. The proportion of the SNP1047AG genotype was significantly different between the groups of patients with and without cirrhosis (5.62 vs. 1.75%; $P=0.034$) (Table 4). In other words, a higher number of patients with cirrhosis had AG genotype compared with the patients without cirrhosis. Having a G allele increased the proportion of patients with cirrhosis with an OR of 3.27 (95% CI: 1.02–10.5, $P=0.036$).

We examined the four polymorphisms in the promoter region and first intron using the direct sequencing technique in all 406 patients. We found only three polymorphisms and the distribution of −424–425 polymorphism was common (Table 3). SNP-206 was absent in all of the 406 patients. Nevertheless, there was no association between polymorphisms in this region and cirrhosis.

Table 4. Association of SNP1047 with liver cirrhosis

	Patient with HCV			
SNP1047	Without cirrhosis ($n=228$)	With cirrhosis ($n=178$)	OR (95% CI)	*P*-value
Genotype				
AA	224 (98.25%)	168 (94.4%)	1	0.034
AG	4 (1.75%)	10 (5.6%)	3.34 (1.03–10.8)	
GG	0 (0%)	0 (0%)		
Allele				
A	456 (99%)	350 (96%)	1	0.036
G	4 (1%)	14 (4%)	3.27 (1.02–10.5)	

CI, confidence interval; HCV, hepatitis C virus; OR, odds ratio; SNP, single nucleotide polymorphism.

Factors associated with the presence of cirrhosis in hepatitis C virus-infected patients

The following factors significantly associated with the presence of cirrhosis according to univariate analysis (Table 5) including SNP1047 ($P=0.03$, OR = 3.34), age > 60 years ($P < 0.0001$), serum albumin < 3.3 g/dl ($P < 0.0001$), serum total bilirubin > 0.7 mg/dl ($P < 0.0001$), serum AFP > 20 μg/L ($P < 0.0001$) and platelet count $< 12.5 \times 10^4$/μl ($P < 0.0001$). To evaluate the effects of SNP1047 polymorphism on the presence of cirrhosis, a multivariate logistic regression analysis was carried out with six variables. Only five variables (SNP1047 genotypes, age > 60 years, serum AFP > 20 μg/L, serum total bilirubin > 0.7 mg/dl and platelet count $< 12.5 \times 10^4$/μl were included in the final model with OR (95% CI) of 2.5 (1.2–5.6; AA vs. AG), 1.5 (1.2–1.9), 1.7 (1.3–2.2), 2.0 (1.5–2.6) and 2.7 (2.1–3.5) respectively.

Evaluation of transcriptional activity of SNP1047 and SNP2157 of interferon regulatory factor-7

To evaluate the functional significance of SNP1047 and SNP2157 of IRF-7, transcriptional activity was examined by an IFNA promoter luciferase reporter assay. A complete linkage was observed in IRF-7 SNP1047, SNP2068, SNP2157, SNP2173 and SNP2200; nonetheless, only SNP1047 and SNP2157 were nonsynonymous SNPs that had amino acid change. Hence, we made mutation at these two points to compare the effect against IFNA promoter activity *in vitro* between SNP1047A/SNP2157A and SNP1047G/SNP2157G. We performed mutagenesis at

Table 5. Factors associated with liver cirrhosis in univariate and multivariate analyses

		Univariate analysis			Multivariate analysis		
Factor	Category	OR	95% CI	*P*-value	OR	95% CI	*P*-value
Age	> 60 years	2.6	1.7–3.9	< 0.0001	1.5	1.2–1.9	0.003
Albumin	< 3.3 g/dl	5.9	2.8–12.7	< 0.0001	–	–	–
AFP	> 20 μg/L	4.8	3.1–7.5	< 0.0001	1.7	1.3–2.2	< 0.0001
Platelet count	$< 12.5 \times 10^4$/μl	10.7	6.7–17.1	< 0.0001	2.7	2.1–3.5	< 0.0001
Total bilirubin	> 0.7 mg/dl	4.8	3.0–7.8	< 0.0001	2.0	1.5–2.6	< 0.0001
SNP1047	A/A	1			1		
	A/G	3.34	1.03–10.8	0.03	2.5	1.2–5.6	0.02

AFP, α-fetoprotein; CI, confidence interval; HCC, hepatocellular carcinoma; OR, odds ratio; SNP, single nucleotide polymorphism.

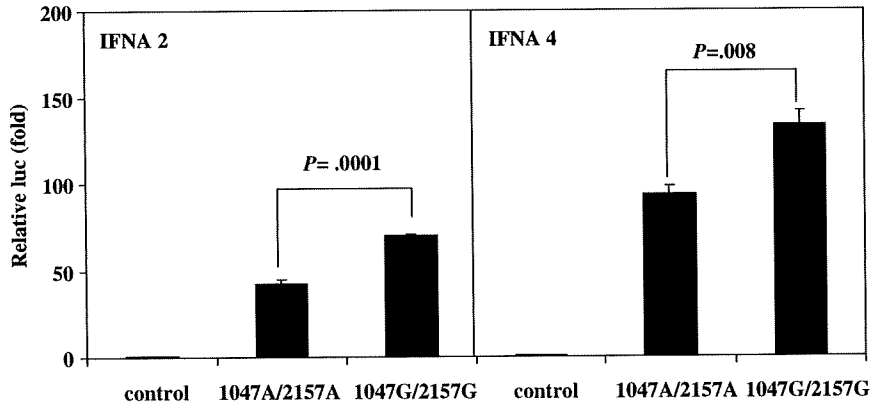

Fig. 1. Activation of luciferase reporter gene having IFNA2 or IFNA4 promoter by IRF-7 1047G/2157G and 1047A/2157A. Human embryonic kidney 293 cells were transfected with the pRL-TK (a control plasmid for transfection efficiency), pIFNA2 or pIFNA4 (reporter constructs containing luciferase gene) and IRF-7 expression plasmids, and luciferase activity was analysed at 48 h post-transfection. Relative luciferase activity was measured as fold activation (relative to the basal level for the reporter gene in the presence of the pFlag-CMV-2 plasmid after normalization to cotransfected relative light unit activity); the values represent the average of at least triplicate experiments. Standard error bars are shown. IFN, interferon; IRF, interferon regulatory factor.

both sites, and then we cotransfected SNP1047A/SNP2157A or SNP1047G/SNP2157G with pIFNA2 or pIFNA4 reporter plasmid. In addition, we used pRL-TK for the internal control of transfection efficiency. The relative luciferase activity was significantly higher in SNP1047G/SNP2157G than in SNP1047A/SNP2157A: the relative luciferase activity increased from 40 to 70 and from 90 to 130 when using pIFNA2 and pIFNA4 reporter plasmids respectively ($P = 0.0001$ and 0.008 respectively) (Fig. 1). IFNA4 promoter was significantly more highly activated than IFNA2 promoter by both SNP1047G/SNP2157G and SNP1047A/SNP2157A. We also performed Western blot analysis to confirm almost equal amounts of IRF-7 expression between SNP1047G/SNP2157G and SNP1047A/SNP2157A (data not shown).

Discussion

In this study, we identified a potential genetic marker for susceptibility to cirrhosis in chronic hepatitis C. We evaluated the relationships between the IRF-7 SNPs and the outcome of chronic HCV infection. Our results showed an effect of the nonsynonymous IRF-7 SNP1047 and SNP2157 on the presence of cirrhosis after controlling for other confounding clinical variables. The proportion of patients with cirrhosis was greater in SNP1047 and SNP2157 AG genotype than in AA genotype. The multivariate model confirmed an independent association between the SNP1047 and SNP2157 AG genotype and the presence of cirrhosis.

We studied the SNP1047 and SNP2157 because they are only two nonsynonymous SNPs that had been validated by multiple, independent submissions to the refSNP cluster and by frequency or genotype data: minor alleles were observed in at least two chromosomes. We studied the function of these SNPs by making two points of mutation because they were in complete linkage disequilibrium. We found that 1047G/2157G induced significantly higher expression of IFNA than 1047A/2157A did. As a result, A-to-G substitution at both SNPs increased their function of transcriptional activation. The structure of IRF-7 gene shows that SNP1047 is located in the constitutive activation domain and SNP2157 is located in the inhibitory domain (23, 31–33). The activation domain of IRF-7 contained acidic region, so changing amino acid from glutamine to lysine at SNP1047 might modify the transcription activity of IRF-7.

SNP1047G and SNP2157G that conferred higher activity to induce IFNA expression associated with cirrhosis in HCV-infected patients. This circumstance was possibly delineated by two postulates. Firstly, the role of IRF-7 was also implied in virus-mediated induction of IFNB and RANTES (regulated on activation normal T cell expressed and secreted; CCL5) genes (24, 34, 35), and it was a strong transactivator of RANTES chemokine gene expression (35). RANTES serves as a key ligand for CCR5 and plays an important role in attracting T cells to the portal area of the liver infected with HCV, and its expression is significantly elevated, especially in periportal and lobular areas that have the most lymphocytic infiltration (36, 37). RANTES has been suggested to be involved in the progression of chronic hepatitis C to advanced forms of liver disease (38–40). The deletion

mutation of inhibitory domain of IRF-7 activated the IFN and RANTES promoter more than the wild type of IRF-7 (40). Hence, overexpression of IFNA from SNP1047G and SNP2157G may also increase the expression of RANTES, and it might be involved in the progression of chronic hepatitis C to advanced forms of liver disease. Nonetheless, we could not see the difference of transaminase level between 1047G/2157G and 1047A/2157A. To attest these arguments, we should compare the transcriptional activity of RANTES promoter among SNP1047G/SNP2157G and SNP1047A/SNP2157A. Secondly, besides type I IFNs, IRF-7 also displayed an increment of the expression of genes encoding tumor necrosis factor-related apoptosis-inducing ligand, interleukin-12, interleukin-15 and CD80 (41). In addition to the role in innate immunity, IRF-7 modulated the expression of a large number of cellular genes. Most of them were upregulated gene especially the categories of signal transduction, transcription factors and apoptosis (42). Furthermore, IRF-7 was found to be a macrophage differentiation factor (43). These postulates might account for the increasing rate of cirrhosis in IRF-7 gene 1047G/2157G.

In our study, we also studied the gene function of IRF-7 in both IFNA2 and IFNA4. IFN type I genes can be subdivided into two groups: (i) immediate-early genes (IFNB and murine IFNA4, which is equivalent to human IFNA1); and (ii) delayed-type genes (which include the other IFNA subtypes) (26, 32). IRF-7 was used to induce the IFNA promoter; at least five IFNA promoters (1, 2, 4, 7, 14), and they were strongly transactivated by wild-type IRF-7 (50-fold induction of IFNA7 to 100-fold induction of IFNA4) (40). So we used IFNA4 because it was most strongly transactivated by IRF-7 among IFNA subtypes, and we used IFNA2 because IFNA2 was the treatment of choice in HCV infection. The result confirmed that IRF-7 could induce both IFNA2 and IFNA4 promoter. In addition, IFNA4 were significantly more highly transactivated than IFNA2. In particular, 1047A/2157A (wild-type) IRF-7 transactivated about 90-fold induction of IFNA4, and it was nearly the same as the previous report (40).

Interferon regulatory factor-7 is essential for the induction of type I IFN via virus-mediated (20). IFN activation of the ISGF3 complex results in the transcriptional upregulation of IRF-7 by binding to the ISRE within promoter region (44). So we tried to identify the role of promoter SNPs of IRF-7 in HCV-infected patients. However, we could not identify any association between promoter polymorphisms and the outcome of chronic HCV infection.

Our analyses showed a prominent effect of the IRF-7 nonsynonymous SNPs on the risk of developing cirrhosis. Although SNP1047 and SNP2157 G alleles were found in a minority of Japanese patients with chronic HCV infection, these polymorphisms might be used as markers of host factors associated with higher risk of cirrhosis in patients with chronic HCV infection.

Acknowledgements

Grant support: Supported in part by grants-in-aid for scientific research from the Ministry of Education, Culture, Sports, Science and Technology of Japan and by Health and Labor Sciences Research Grants for Research on Hepatitis from the Ministry of Health, Labour and Welfare, Japan. The authors thank Prof. John Hiscott (McGill University, Montreal, Canada) for providing us with plasmids. We also thank M. Tsubouchi for her technical assistance.

References

1. Lauer GM, Walker BD. Hepatitis C virus infection. *N Engl J Med* 2001; **345**: 41–52.
2. Omata M, Yoshida H, Shiratori Y, Shiina S. Progression from chronic hepatitis to hepatocellular carcinoma: natural course and treatments. *J Gastroenterol Hepatol* 2002; **17**(Suppl. 3): S434–6.
3. Shiratori Y, Shiina S, Imamura M, *et al.* Characteristic difference of hepatocellular carcinoma between hepatitis B- and C-viral infection in Japan. *Hepatology* 1995; **22**(Part 1): 1027–33.
4. Poynard T, Yuen MF, Ratziu V, Lai CL. Viral hepatitis C. *Lancet* 2003; **362**: 2095–100.
5. Yoshida H, Shiratori Y, Moriyama M, *et al.* Interferon therapy reduces the risk for hepatocellular carcinoma: national surveillance program of cirrhotic and noncirrhotic patients with chronic hepatitis C in Japan. IHIT Study Group. Inhibition of Hepatocarcinogenesis by Interferon Therapy. *Ann Intern Med* 1999; **131**: 174–81.
6. Liang TJ, Rehermann B, Seeff LB, Hoofnagle JH. Pathogenesis, natural history, treatment, and prevention of hepatitis C. *Ann Intern Med* 2000; **132**: 296–305.
7. Hoofnagle JH. Course and outcome of hepatitis C. *Hepatology* 2002; **36**(Suppl. 1): S21–9.
8. Seeff LB. Natural history of chronic hepatitis C. *Hepatology* 2002; **36**(Suppl. 1): S35–46.
9. Cerny A, Chisari FV. Pathogenesis of chronic hepatitis C: immunological features of hepatic injury and viral persistence. *Hepatology* 1999; **30**: 595–601.
10. Laurent-Puig P, Legoix P, Bluteau O, *et al.* Genetic alterations associated with hepatocellular carcinomas define distinct pathways of hepatocarcinogenesis. *Gastroenterology* 2001; **120**: 1763–73.

11. Mcilroy D, Theodorou I, Ratziu V, *et al.* FAS promoter polymorphisms correlate with activity grade in hepatitis C patients. *Eur J Gastroenterol Hepatol* 2005; **17**: 1081–8.
12. Rossi L, Leveri M, Gritti C, *et al.* Genetic polymorphisms of steroid hormone metabolizing enzymes and risk of liver cancer in hepatitis C-infected patients. *J Hepatol* 2003; **39**: 564–70.
13. Silvestri L, Sonzogni L, de Silvestri A, *et al.* CYP enzyme polymorphisms and susceptibility to HCV-related chronic liver disease and liver cancer. *Int J Cancer* 2003; **104**: 310–7.
14. Wang Y, Kato N, Hoshida Y, *et al.* UDP-glucuronosyltransferase 1A7 genetic polymorphisms are associated with hepatocellular carcinoma in Japanese patients with hepatitis C virus infection. *Clin Cancer Res* 2004; **10**: 2441–6.
15. Wang Y, Kato N, Hoshida Y, *et al.* Interleukin-1beta gene polymorphisms associated with hepatocellular carcinoma in hepatitis C virus infection. *Hepatology* 2003; **37**: 65–71.
16. Dharel N, Kato N, Muroyama R, *et al.* MDM2 promoter SNP309 is associated with the risk of hepatocellular carcinoma in patients with chronic hepatitis C. *Clin Cancer Res* 2006; **12**: 4867–71.
17. Kato N, Ji G, Wang Y, *et al.* Large-scale search of single nucleotide polymorphisms for hepatocellular carcinoma susceptibility genes in patients with hepatitis C. *Hepatology* 2005; **42**: 846–53.
18. Moriyama MKN, Otsuka M, Shao R-X, Taniguchi H, Kawabe T, Omata M. Interferon-beta is activated by hepatitis C virus NS5B and inhibited by NS4A, NS4B, and NS5A. *Hepatol Int* 2007; **1**: 302–10.
19. Seth RB, Sun L, Chen ZJ. Antiviral innate immunity pathways. *Cell Res* 2006; **16**: 141–7.
20. Honda K, Yanai H, Negishi H, *et al.* IRF-7 is the master regulator of type-I interferon-dependent immune responses. *Nature* 2005; **434**: 772–7.
21. Sato M, Hata N, Asagiri M, Nakaya T, Taniguchi T, Tanaka N. Positive feedback regulation of type I IFN genes by the IFN-inducible transcription factor IRF-7. *FEBS Lett* 1998; **441**: 106–10.
22. Au WC, Yeow WS, Pitha PM. Analysis of functional domains of interferon regulatory factor 7 and its association with IRF-3. *Virology* 2001; **280**: 273–82.
23. Au WC, Moore PA, Lafleur DW, Tombal B, Pitha PM. Characterization of the interferon regulatory factor-7 and its potential role in the transcription activation of interferon A genes. *J Biol Chem* 1998; **273**: 29210–7.
24. Lu R, Au WC, Yeow WS, Hageman N, Pitha PM. Regulation of the promoter activity of interferon regulatory factor-7 gene. Activation by interferon and silencing by hypermethylation. *J Biol Chem* 2000; **275**: 31805–12.
25. Yeow WS, Au WC, Juang YT, *et al.* Reconstitution of virus-mediated expression of interferon alpha genes in human fibroblast cells by ectopic interferon regulatory factor-7. *J Biol Chem* 2000; **275**: 6313–20.
26. Marie I, Durbin JE, Levy DE. Differential viral induction of distinct interferon-alpha genes by positive feedback through interferon regulatory factor-7. *Embo J* 1998; **17**: 6660–9.
27. Hara K, Ohe K, Kadowaki T, *et al.* Establishment of a method of anonymization of DNA samples in genetic research. *J Hum Genet* 2003; **48**: 327–30.
28. Desmet V. *Histopathology of Chronic Hepatitis.* Amsterdam: Elsevier, 1986.
29. Scheuer PJ, Ashrafzadeh P, Sherlock S, Brown D, Dusheiko GM. The pathology of hepatitis C. *Hepatology* 1992; **15**: 567–71.
30. Collett D. *Modelling Binary Data*, 2nd edn. FL, USA: Chapman and Hall/CRC, 2003.
31. Marie I, Smith E, Prakash A, Levy DE. Phosphorylation-induced dimerization of interferon regulatory factor 7 unmasks DNA binding and a bipartite transactivation domain. *Mol Cell Biol* 2000; **20**: 8803–14.
32. Lin R, Mamane Y, Hiscott J. Multiple regulatory domains control IRF-7 activity in response to virus infection. *J Biol Chem* 2000; **275**: 34320–7.
33. Lin R, Genin P, Mamane Y, Hiscott J. Selective DNA binding and association with the CREB binding protein coactivator contribute to differential activation of alpha/beta interferon genes by interferon regulatory factors 3 and 7. *Mol Cell Biol* 2000; **20**: 6342–53.
34. Wathelet MG, Lin CH, Parekh BS, Ronco LV, Howley PM, Maniatis T. Virus infection induces the assembly of coordinately activated transcription factors on the IFN-beta enhancer in vivo. *Mol Cell* 1998; **1**: 507–18.
35. Genin P, Algarte M, Roof P, Lin R, Hiscott J. Regulation of RANTES chemokine gene expression requires cooperativity between NF-kappa B and IFN-regulatory factor transcription factors. *J Immunol* 2000; **164**: 5352–61.
36. Hellier S, Frodsham AJ, Hennig BJ, *et al.* Association of genetic variants of the chemokine receptor CCR5 and its ligands, RANTES and MCP-2, with outcome of HCV infection. *Hepatology* 2003; **38**: 1468–76.
37. Nischalke HD, Nattermann J, Fischer HP, Sauerbruch T, Spengler U, Dumoulin FL. Semiquantitative analysis of intrahepatic CC-chemokine mRNas in chronic hepatitis C. *Mediators Inflamm* 2004; **13**: 357–9.
38. Apolinario A, Majano PL, Alvarez-Perez E, *et al.* Increased expression of T cell chemokines and their receptors in chronic hepatitis C: relationship with the histological activity of liver disease. *Am J Gastroenterol* 2002; **97**: 2861–70.
39. Leroy V, Vigan I, Mosnier JF, *et al.* Phenotypic and functional characterization of intrahepatic T lymphocytes during chronic hepatitis C. *Hepatology* 2003; **38**: 829–41.
40. Bone-Larson CL, Simpson KJ, Colletti LM, *et al.* The role of chemokines in the immunopathology of the liver. *Immunol Rev* 2000; **177**: 8–20.
41. Romieu-Mourez R, Solis M, Nardin A, *et al.* Distinct roles for IFN regulatory factor (IRF)-3 and IRF-7 in the activation of antitumor properties of human macrophages. *Cancer Res* 2006; **66**: 10576–85.

42. Barnes BJ, Richards J, Mancl M, Hanash S, Beretta L, Pitha PM. Global and distinct targets of IRF-5 and IRF-7 during innate response to viral infection. *J Biol Chem* 2004; **279**: 45194–207.
43. Hijikata M, Mishiro S, Miyamoto C, Furuichi Y, Hashimoto M, Ohta Y. Genetic polymorphism of the MxA gene promoter and interferon responsiveness of hepatitis C patients: revisited by analyzing two SNP sites (− 123 and − 88) in vivo and in vitro. *Intervirology* 2001; **44**: 379–82.
44. Gale M Jr, Foy EM. Evasion of intracellular host defence by hepatitis C virus. *Nature* 2005; **436**: 939–45.

Cancer Science
The official journal of the Japanese Cancer Association

Report

Characteristic mutations in hepatitis C virus core gene related to the occurrence of hepatocellular carcinoma

Zhongjie Hu,[1,2,3] Ryosuke Muroyama,[1] Norie Kowatari,[1] Jinhai Chang,[2] Masao Omata[2] and Naoya Kato[1,4]

[1]Unit of Disease Control Genome Medicine, The Institute of Medical Science, The University of Tokyo, Tokyo; [2]Department of Gastroenterology, Graduate School of Medicine, The University of Tokyo, Tokyo, Japan; [3]Department of Gastroenterology and Hepatology, Beijing You'an Hospital, Capital Medical University, Beijing, China

(Received May 06, 2009/Revised August 13, 2009/Accepted August 20, 2009)

Chronic hepatitis C virus (HCV) infection often results in hepatocellular carcinoma (HCC). Previous studies have shown that there might be some characteristic mutations in the core region of HCV related to HCC. Thus, we downloaded and analyzed HCV genotype 1b core gene sequences from HCV databases online to identify them. Based on the information of the sequences, 63 from patients with HCC and 188 from non-HCC were enrolled into our analysis. Then, the nucleotides at each position were compared by χ^2-test between the two groups, and 24 polymorphisms were found to be associated with HCC. Further analysis of these 24 polymorphisms by logistic regression indicated that eight were significantly related to the increased HCC risk: A028C, G209A, C219U/A, U264C, A271C/U, C378U/A, G435A/C, and G481A. Moreover, U303C/A was associated with the decreased HCC risk. These mutations could bring about four amino acid substitutions: K10Q, R70Q, M91L, and G161S. In conclusion, eight characteristic mutations in the *HCV-1b* core gene related to the occurrence of HCC were identified. The structural and functional alterations of core protein due to these mutations and the relationship with the occurrence of HCC need to be further studied. (*Cancer Sci* 2009; 100: 2465–2468)

Hepatitis C virus (HCV) is a major cause of chronic hepatitis worldwide and a major risk factor for hepatocellular carcinoma (HCC). More than 70% of patients with HCC in Japan are infected with HCV, and HCC has become the major cause of death in patients chronically infected with HCV.[1–3] In Asia, especially in Japan, HCV-1b is the most prevalent genotype and is more likely to develop into HCC than other genotypes.[4,5] Although there are many published data about the correlation between HCV infection and HCC development, details of hepatocarcinogenesis by HCV remain unclear. HCV core protein is implicated in hepatocarcinogenesis for its ability to modulate cellular gene transcription and protein expression, intracellular signal transduction, cell proliferation and apoptosis.[6–9]

Being an RNA virus, the HCV genome exhibits a considerable degree of sequence variation. Studies have proved that certain kinds of mutations may lead to functional changes of the virus, such as the resistance to antiviral treatments and the association with HCC.[10,11] Furthermore, there are more mutations in the core region in patients with HCC than that in those without HCC. Moreover, the rate of nucleotide substitutions in the core gene is significantly greater for isolates from HCC patients compared to those from individuals with chronic hepatitis.[12] It was also found that there was a significantly higher variability within the core region of tumor tissue isolates than that of isolates from non-tumor tissue. Mutant sequence diversity ranged from silent mutations, as well as amino acid substitutions, to appearance of in-frame stop codons and deletions leading to frame-shifts. In contrast, the variability of the NS5 region sequences between isolates from tumor and non-tumor tissue was not significantly different.[13] Recently, a report indicated that amino acid substitutions in the HCV core region are important predictor of hepatocarcinogenesis.[11] Even in patients without HCC, the substitution of amino acid 70 in the hepatitis C virus core region of genotype 1b is an important predictor of elevated alpha-fetoprotein.[14]

Based on the data above, we suppose that there might be some characteristic mutations in the *HCV-1b* core gene related to the occurrence of HCC. Because we can find thousands of HCV sequences from databases online, we have tried to identify these mutations by analyzing such sequences in this study.

Materials and Methods

Collection of HCV core gene sequences. HCV core gene sequences were downloaded from the following databases: European HCV database (euHCVdb), HCV Databases from Los Alamos National Laboratory, HCV Database (HCVdb) from the Viral Bioinformatics Resource Center (VBRC), and National Center for Biotechnology Information (NCBI).

Inclusion criteria. All the sequences enrolled into our analysis needed to comply with the following inclusion criteria: (i) the diagnosis of patients could be confirmed; (ii) the genotype of HCV could be confirmed and samples were isolated from sera of patients infected with HCV-1b; and (iii) the core gene sequence was of full length.

Exclusion criteria. Sequences were excluded if: (i) the diagnosis of patients or the genotype of HCV was unknown; (ii) samples came from liver, other tissues, ascites, or pooled sera; (iii) patients were co-infected with hepatitis B virus or human immunodeficiency virus; (iv) patients had ever been treated with interferon (IFN); (v) patients had ever undergone orthotopic liver transplantation (OLT); (vi) samples came from cell culture, animal experiments, chimeric DNA, or fusion protein; (vii) sequences came from repeated samples; and (viii) existence of deletion mutations or insertion mutations.

Statistical analysis. According to the diagnosis of the cases, sequences enrolled were divided into two groups: group HCC and group non-HCC. The first base of the start codon (AUG) was designated position 1. All the 573 nucleotides of the core region were compared respectively between group HCC and non-HCC. All the 573 nucleotides of the core region were compared respectively between group HCC and non-HCC by Pearson χ^2-test to find out significantly different mutations (when

[4]To whom correspondence should be addressed.
E-mail: kato-2im@ims.u-tokyo.ac.jp

doi: 10.1111/j.1349-7006.2009.01338.x

there were cells that the expected count was less than 5 or 1, we chose different χ^2-test methods – Continuity correction or Fisher's exact test). Then, the significant bases were further analyzed by logistic regression to identify the mutations significantly related to the occurrence of HCC. A P-value of <0.05 was considered statistically significant.

Results and Discussion

Enrollment of sequences. We downloaded sequences of HCV core region from HCV databases online, mainly from euHCVdb. A total of 2841 sequences of HCV-RNA with full-length core region were downloaded. Then, we checked the information provided by the databases and the published papers so that we could get the details about these sequences such as the diagnoses of patients, genotypes of HCV, the origin of samples, and so on. Finally, we picked out the sequences that complied with the inclusion and exclusion criteria and enrolled them into our analysis. As a result, from 2841 sequences, we found 1336 sequences of genotype 1b. Of these, 547 sequences were from identified samples and the others lacked information that identified the types of the samples. Among the 547 sequences with definite origin, 292 were from sera and the others (228 from livers, 20 from cultured cells, seven from plasmids) were ruled out. Of the 292 sequences derived from sera, seven were from animal experiments, 24 were from repeated cases at different time points, and four and six were from cases that had undergone OLT and IFN treatments, respectively. Finally, 251 sequences complied with the inclusion and exclusion criteria and were enrolled into our analysis. Sixty-three were from patients with HCC and 188 were from HCV carriers or patients with acute or chronic hepatitis without HCC. For the 251 sequences, 73.7% (185/251) were directly sequenced and 26.3% (66/251) represented one clone of available clones. Thus, it is possible that about a quarter of our data could contain mutations due to PCR error. However, the sequences were collected from 42 different studies and the statistical analysis may have overcome this problem.

Identification of the wild-type nucleotides. For the reason that there are frequent mutations of the HCV sequence, it is impossible to know the real wild-type nucleotides. It might be reasonable to identify the wild-type nucleotide based on the consensus of a large number of sequences. In our analysis, we defined the consensus of the 1336 HCV-1b sequences as the wild type, except the position of nucleotide (nt) 209. Because 209G was treated as wild type in almost all the published papers and it was also the consensus type of 2841 all genotypes sequences, we still defined "G" as the wild type even if "A" was the consensus.

Significantly different mutations between group HCC and non-HCC. By performing the χ^2-test or Fisher's exact test, we compared all the 573 nucleotides of the core gene between group HCC and non-HCC. A total of 24 nucleotide mutations were found to be associated with occurrence of HCC. Of the 24 nucleotide mutations, 21 increased the risk of HCC while three decreased the risk of HCC (Table 1).

Gene polymorphisms and amino acid substitutions associated with HCC. The 24 significant nucleotide mutations found by χ^2-tests were further analyzed by logistic regression. Eight polymorphisms were significantly related to the increased HCC risk: A028C, G209A, C219U/A, U264C, A271C/U, C378U/A, G435A/C, and G481A; and U303C/A was significantly associated with decreased HCC risk (Table 2). Furthermore, four polymorphisms brought about amino acid substitutions which might change the structure and functions of core protein and lead to HCC. For the other five synonymous mutations, it is possible that they might lead to amino acid substitutions of ARFP (alternative ribosomal frame shift protein) which has been reported to be able to affect cell proliferation and apoptosis[15] (Table 3). However, HCC is thought to be a consequence of long-term infection of HCV and chronic hepatitis. Although the gene polymorphisms and amino acid substitutions in the HCV core region identified in this analysis were proven to be significantly associated with HCC, whether they are "causes" or "effects" of HCC remains to be determined.

Recently, a similar study[16] determined four amino acid substitutions associated with increased HCC risk, and three of them were the same as our results (G209A, A271C/U, and G481A). Moreover, up to now, the G209A polymorphism has been thought to be related with both IFN/Ribavirin (RBV) treatment resistance and HCC.[10,11] The relationship between them is an interesting question. We guess that apoptosis might make an important function in this process. Apoptosis is considered to be a common pathway of virus clearance. HCV core protein could suppress apoptosis and escape this clearance mechanism.[9] On the other hand, inhibition of apoptosis might be able to make the

Table 1. Significant nucleotide mutations found by χ^2-tests

Gene position	Types of Nucleotide			Percentage of mutant			Amino acid			P-values
	Wild		Mutant	Non-HCC		HCC	Wild type		Mutant	
nt 009	G	→	A/U	20.2	→	68.3	Thr		Thr	<0.001*
nt 028	A	→	C	1.1	→	7.9	Lys	→	Gln	0.015†
nt 039	U	→	G/A	0	→	4.8	Arg		Arg	0.015‡
nt 048	C	→	G/U	0	→	4.8	Asn	→	Lys/Asn	0.015‡
nt 066	C	→	U	14.4	→	30.2	Val		Val	0.005*
nt 078	C	→	U	28.7	→	3.2 ↓	Gly		Gly	<0.001*
nt 116	G	→	C	0	→	7.9	Arg	→	Pro	0.001†
nt 209	G	→	A	28.7	→	52.4	Arg	→	Gln	0.001*
nt 219	C	→	U/A	7.4	→	23.8	Gly		Gly	<0.001*
nt 264	U	→	C	12.2	→	36.5	Asn		Asn	<0.001*
nt 270	C	→	U	8	→	33.3	Gly		Gly	<0.001*
nt 271	A	→	C/U	21.8	→	65.1	Met	→	Leu	<0.001*
nt 303	U	→	C/A	64.9	→	47.6 ↓	Arg		Arg	0.015*
nt 309	U	→	C	22.3	→	39.7	Ser		Ser	0.007*
nt 378	C	→	U/A	16.5	→	34.9	Leu		Leu	0.002*
nt 417	C	→	U	5.9	→	20.6	Leu		Leu	0.001*
nt 435	G	→	A/C	10.1	→	34.9	Gly		Gly	<0.001*
nt 446	G	→	A	0.5	→	7.9	Arg	→	Lys	0.004†
nt 456	G	→	A	24.5	→	47.6	Ala		Ala	0.001*
nt 462	C	→	U	25	→	49.2	Gly		Gly	<0.001*
nt 471	U	→	C/G	38.3	→	20.6 ↓	Val		Val	0.01*
nt 472	C	→	G/U	2.1	→	9.5	Leu	→	Val/Leu	0.026†
nt 481	G	→	A	2.7	→	20.6	Gly	→	Ser	<0.001†
nt 549	C	→	U	14.4	→	30.2	Ser		Ser	0.005*

*Pearson χ^2-tests; †continuity correction; ‡Fisher's exact test.

Table 2. Significant nucleotide mutations identified in logistic regression

Gene position	Mutation types	Odds ratio	95% CI		P-values
			Lower	Upper	
nt 028	A → C	14.65	1.31	163.51	0.029
nt 209	G → A	3.30	1.40	7.78	0.006
nt 219	C → U/A	4.37	1.49	12.77	0.007
nt 264	U → C	4.13	1.62	10.52	0.003
nt 271	A → C/U	5.50	2.26	13.37	<0.001
nt 303	U → C/A	0.42	0.18	0.98	0.045
nt 378	C → U/A	2.69	1.08	6.73	0.034
nt 435	G → A/C	4.16	1.61	10.72	0.003
nt 481	G → A	10.484	2.77	39.62	0.001

Nagelkerke R^2 = 0.572.

doi: 10.1111/j.1349-7006.2009.01338.x

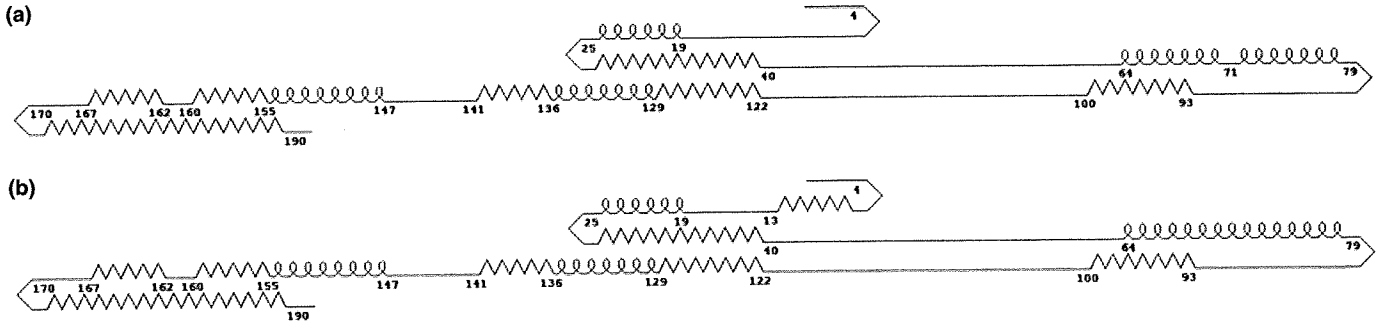

Fig. 1. Secondary structure of wild-type (a) and mutant (b) hepatitis C virus (HCV)-1b core protein predicted by Chou–Fasman analysis with Genetyx. The straight, bent, looped, and zigzag lines represent coil, β-sheet, α-helix, and turn structures, respectively. The numbers indicate the amino acid positions. A028C might turn the coil stucture of AA8-12 into β-sheet and G209A might turn the coil stucture of AA71 into α-helix. (The secondary structure of the wild type was predicted based on the following consensus amino acid sequence of 1336 HCV-1b core, except 70R: MSTNPKPQRKTKRNTNRRPQDVKFPGGGQIVGGVYLLPRRGPRLGVRATRKTSERSQPRGRRQPIPKARRPEGRAWAQPGYPWPLYGNEGMGWAGWLLSPR GSRPSWGPTDPRRRSRNLGKVIDTLTCGFADLMGYIPLVGAPLGGAARALAHGVRVLEDGVNYATGNLPGCSFSIFLLALLSCLTIPASA.)

genetically damaged hepatocytes survive and lead to neoplastic transformation. Whether the amino acid substitution of R70Q could enhance these effects is worth studying.

Alteration of the RNA and protein secondary structures of the *HCV-1b* core gene. Gene mutations of A271C/U, G435A/C, and G481A, were found to be able to cause the changes of the RNA secondary structure of *HCV-1b* core gene using RNAdraw software[17] (Karolinska Institute, Stockholm, Sweden). Furthermore, Chou–Fasman protein secondary prediction with the gene analysis software Genetyx (Genetyx, Tokyo, Japan) showed that A028C might turn the coil structure of AA8-12 into β-sheet and G209A might turn the coil stucture of AA71 into α-helix (Fig. 1). The results indicated that the point-mutations of the core gene might change the secondary structure of not only RNA but also protein. As a result, the functions of both RNA and protein of the core region, such as interaction with other DNA/RNA or proteins (lymphotoxin B receptor, heterogeneous-nuclear ribonucleoprotein, tumor necrosis factor receptor 1, etc.),[18–20] might change and lead to HCC.

In conclusion, eight characteristic mutations in the *HCV-1b* core gene related to the occurrence of HCC were identified: A028C, G209A, C219U/A, U264C, A271C/U, C378U/A, G435A/C, and G481A. Meanwhile, U303C/A was identified to be significantly associated with the decreased HCC risk. These mutations could bring about four amino acid substitutions: K10Q, R70Q, M91L, and G161S. The structural and functional alterations of core protein due to these mutations and the relationship with the occurrence of HCC need to be further studied.

Table 3. Amino acid substitutions of core protein and ARFP due to nucleotide mutations

Codon	Gene position	Amino acid position	Nucleotide			Amino acid		
			Wild type		Mutant	Wild type		Mutant
Normal	nt 028	aa 010	AAA	→	CAA	Lys	→	Gln
	nt 209	aa 070	CGG	→	CAG	Arg	→	Gln
	nt 219	aa 073	GGC	→	GGU/A	Gly		Gly
	nt 264	aa 088	AAU	→	AAC	Asn		Asn
	nt 271	aa 091	AUG	→	C/UUG	Met	→	Leu
	nt 303	aa 101	CGU	→	CGC/A	Arg		Arg
	nt 378	aa 126	CUC	→	CUU/A	Leu		Leu
	nt 435	aa 145	GGG	→	GGA/C	Gly		Gly
	nt 481	aa 161	GGC	→	AGC	Gly	→	Ser
+1 Codon	nt 028	aa 010	GAA	→	GAC	Glu	→	Asp
	nt 209	aa 070	GGC	→	AGC	Gly	→	Ser
	nt 219	aa 073	GCA	→	GU/AA	Ala	→	Val/Glu
	nt 264	aa 088	AUG	→	ACG	Met	→	Thr
	nt 271	aa 091	GCA	→	GCC/U	Ala		Ala
	nt 303	aa 101	GUG	→	GC/AG	Val	→	Ala/Glu
	nt 378	aa 126	UCA	→	UU/AA	Ser	→	Leu/Stop
	nt 435	aa 145	GGG	→	GA/CG	Gly	→	Glu/Ala
	nt 481	aa 161	ACG	→	ACA	Thr		Thr
−1 Codon	nt 028	aa 010	AAA	→	ACA	Lys	→	Thr
	nt 209	aa 070	CCG	→	CCA	Pro		Pro
	nt 219	aa 073	CAG	→	T/AAG	Gln	→	Stop/Lys
	nt 264	aa 088	UGA	→	CGA	Stop	→	Arg
	nt 271	aa 091	CAU	→	CC/UU	His	→	Pro/Leu
	nt 303	aa 101	UGG	→	C/AGG	Trp	→	Arg
	nt 378	aa 126	CAC	→	U/AAC	His	→	Tyr/Asn
	nt 435	aa 145	GGG	→	A/CGG	Gly	→	Arg
	nt 481	aa 161	CGG	→	CAG	Arg	→	Gln

ARFP, alternative ribosomal frame shift protein.

Acknowledgments

The study was supported by the Global COE Program "Center of Education and Research for Advanced Genome-Based Medicine: For personalized medicine and the control of worldwide infectious diseases", the Ministry of Education, Culture, Sports, Science and Technology of Japan; by Grants-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology of Japan; by Health and Labor Sciences Research Grants for Research on Hepatitis from the Ministry of Health, Labor and Welfare, Japan; and by a Japan–China Sasakawa Medical Fellowship.

References

1 Saito I, Miyamura T, Ohbayashi A *et al.* Hepatitis C virus infection is associated with the development of hepatocellular carcinoma. *Proc Natl Acad Sci U S A* 1990; **87**: 6547–9.
2 Shiratori Y, Shiina S, Imamura M *et al.* Characteristic difference of hepatocellular carcinoma between hepatitis B- and C- viral infection in Japan. *Hepatology* 1995; **22**: 1027–33.
3 Kato Y, Hamasaki K, Aritomi T *et al.* Most of the patients with cirrhosis in Japan die from hepatocellular carcinoma. *Oncol Rep* 1999; **6**: 1273–6.
4 Takada A, Tsutsumi M, Zhang SC *et al.* Relationship between hepatocellular carcinoma and subtypes of hepatitis C virus: a nationwide analysis. *J Gastroenterol Hepatol* 1996; **11**: 166–9.
5 Lee CM, Hung CH, Lu SN *et al.* Viral etiology of hepatocellular carcinoma and HCV genotypes in Taiwan. *Intervirology* 2006; **49**: 76–81.
6 Kato N, Yoshida H, Ono-Nita SK *et al.* Activation of intracellular signaling by hepatitis B and C viruses: C-viral core is the most potent signal inducer. *Hepatology* 2000; **32**: 405–12.

7 Otsuka M, Kato N, Lan K-H *et al.* Hepatitis C virus core protein enhances P53 function through augmentation of DNA-binding affinity and transcriptional ability. *J Biol Chem* 2000; **275**: 34122–30.
8 Yoshida H, Kato N, Shiratori Y *et al.* Hepatitis C virus core protein activates NF-κB-dependent signaling through tumor necrosis factor receptor-associated factor. *J Biol Chem* 2001; **276**: 16399–405.
9 Otsuka M, Kato N, Taniguchi H *et al.* Hepatitis C virus core protein inhibits apoptosis via enhanced Bcl-x_L expression. *Virol* 2002; **296**: 84–93.
10 Akuta N, Suzuki F, Sezaki H *et al.* Association of amino acid substitution pattern in core protein of hepatitis C virus genotype 1b high viral load and non-virological response to interferon-ribavirin combination therapy. *Intervirology* 2005; **48**: 372–80.
11 Akuta N, Suzuki F, Kawamura Y *et al.* Amino acid substitutions in the hepatitis C virus core region are the important predictor of hepato-carcinogenesis. *Hepatology* 2007; **46**: 1357–64.
12 Shimizu I, Yao DF, Horie C *et al.* Mutations in a hydrophilic part of the core gene of hepatitis C virus in patients with hepatocellular carcinoma in China. *J Gastroenterol* 1997; **32**: 47–55.
13 Rüster B, Zeuzem S, Krump-Konvalinkova V *et al.* Comparative sequence analysis of the core- and NS5-region of hepatitis C virus from tumor and adjacent non-tumor tissue. *J Med Virol* 2001; **63**: 128–34.
14 Akuta N, Suzuki F, Kawamura Y *et al.* Substitution of amino acid 70 in the hepatitis C virus core region of genotype 1b is an important predictor of elevated alpha-fetoprotein in patients without hepatocellular carcinoma. *J Med Virol* 2008; **80**: 1354–62.
15 Shao SW, Wu WB, Bian ZQ *et al.* Hepatitis C virus F protein inhibits cell apoptosis by activation of intracellular NF-kappaB pathway. *Hepatol Res* 2009; **39**: 282–9.
16 Fishman SL, Factor SH, Balestrieri C *et al.* Mutations in the hepatitis C virus core gene are associated with advanced liver disease and hepatocellular carcinoma. *Clin Cancer Res* 2009; **15**: 3205–13.
17 Matzura O, Wennborg A. RNAdraw: an integrated program for RNA secondary structure calculation and analysis under 32-bit Microsoft Windows. *Comput Appl Biosci* 1996; **12**: 247–9.
18 Matsumoto M, Hsieh TY, Zhu N *et al.* Hepatitis C virus core protein interacts with cytoplasmic tail of lymphotoxin-beta recepor. *J Virol* 1997; **71**: 1301–9.
19 Hsieh TY, Matsumoto M, Chou HC *et al.* Hepatitis C virus core protein interacts with heterogeneous nuclear ribonucleoprotein. *J Biol Chem* 1998; **273**: 17651–9.
20 Zhu N, Khosnan A, Schneider R *et al.* Hepatitis C virus core protein binds to the cytoplasmic domain of tumor necrosis factor (TNF) receptor 1 and enhances TNF-induced apoptosis. *J Virol* 1998; **72**: 3691–7.

doi: 10.1111/j.1349-7006.2009.01338.x

Liver INTERNATIONAL
OFFICIAL JOURNAL OF THE INTERNATIONAL ASSOCIATION FOR THE STUDY OF THE LIVER

Liver International ISSN 1478-3223

BASIC STUDIES

Double-stranded RNA-activated protein kinase inhibits hepatitis C virus replication but may be not essential in interferon treatment

Jin-Hai Chang[1], Naoya Kato[1,2], Ryosuke Muroyama[1,2], Hiroyoshi Taniguchi[1], Bayasi Guleng[1], Narayan Dharel[1], Run-Xuan Shao[1], Keisuke Tateishi[1], Amarsanaa Jazag[1], Takao Kawabe[1] and Masao Omata[1]

1 Department of Gastroenterology, Graduate School of Medicine, University of Tokyo, Tokyo, Japan
2 Unit of Disease Control Genome Medicine, Institute of Medical Science, University of Tokyo, Tokyo, Japan

Keywords
double-stranded RNA-activated protein kinase – hepatitis C virus – interferon – interferon-stimulated gene – RNA interference

Abbreviations
DMEM, Dulbecco's modified Eagle's medium; dsRNA, double-stranded RNA; eIF, eukaryotic translation initiation factor; FBS, fetal bovine serum; HCC, hepatocellular carcinoma; HCV, hepatitis C virus; IRES, internal ribosomal entry site; ISG(s), interferon-stimulated gene(s); MEF(s), mouse embryonic fibroblast(s); MTT, 3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide; PBS, phosphate-buffered saline; PKR, double-stranded RNA-activated protein kinase; RLU, relative light units; SD, standard deviation; SDS-PAGE, sodium dodecyl sulphate polyacrylamide gel electrophoresis; shRNA, short hairpin RNA; siRNA, short interfering RNA; UTR, untranslated region.

Correspondence
Dr Naoya Kato, Unit of Disease Control Genome Medicine, Institute of Medical Science, University of Tokyo, 4-6-1 Shirokanedai, Minato-ku, Tokyo 108-8639, Japan
Tel: +81 3 6409 2335
Fax: +81 3 6409 2336
e-mail: kato-2im@ims.u-tokyo.ac.jp

Received 21 May 2008
Accepted 4 September 2009

DOI:10.1111/j.1478-3231.2009.02144.x

Abstract

Background: Double-stranded RNA-activated protein kinase (PKR), an interferon (IFN)-stimulated gene, is activated by binding with double-stranded RNA, a putative replicative intermediate of the hepatitis C virus (HCV). Activated PKR phosphorylates the α subunit of eukaryotic initiation factor-2 to inhibit the translation of viral protein. *Aims/methods:* We established stable PKR knockdown Huh7 cells using RNA interference and investigated the effect of PKR against HCV replication using a subgenomic replicon that expressed luciferase reporter protein and the JFH1 full-length HCV genome. *Results:* In stable PKR knockdown cells that harboured a subgenomic replicon, luciferase activity was approximately three times higher than that of control cells, indicating that the subgenomic replicon replicated with a higher efficiency in stable PKR knockdown cells than that in control cells. Furthermore, stable PKR knockdown cells secreted significantly more HCV particles than did control cells after transfection with the full-length HCV genome. The replication of the subgenomic replicon was suppressed by the addition of IFN-α in both cells. Although the extent of suppression was significantly lower in stable PKR knockdown than control cells using a low concentration (2.5–5 U/ml) of IFN-α, even 10 U/ml IFN-α suppressed the replication of subgenomic replicon by > 98% in both cells. *Conclusions:* Double-stranded RNA-activated protein kinase plays an important role in suppressing HCV replication in an innate state, but may not be essential in IFN therapy.

The hepatitis C virus (HCV) is a positive-stranded RNA virus that causes chronic hepatitis, cirrhosis and hepatocellular carcinoma (HCC) worldwide (1–3). Type-1-interferon (IFN)-induced antiviral signals are regarded as the major defence against HCV infection, and anti-HCV therapy is primarily based on IFNs (4, 5). IFNs allow cells to become innately primed for defence against an eventual virus attack by inducing the transcription of IFN-stimulated genes (ISGs) that exert antiviral, anti-tumour and immunomodulatory actions by producing a complex set of proteins. Some ISGs have an important role in controlling viral replication.

Double-stranded RNA-activated protein kinase (PKR), one of the best-known ISGs, is a serine–threonine-type phosphorylation enzyme that combines with double-stranded RNA (dsRNA) and is then activated.

Given that the replication of the HCV genome is catalysed by its RNA-dependent RNA polymerase NS5B, dsRNA may be formed during its life cycle (6, 7). These HCV-replicative intermediates may be targeted by the PKR-based antiviral response (8). Activated PKR inhibits protein synthesis by phosphorylating the eukaryotic translation initiation factor (eIF)2α regulatory site, Ser51 (9). This triggers the general shutdown of protein synthesis and inhibition of viral propagation. In fact, the HCV internal ribosome entry site forms a binary complex with the 40S ribosomal subunit, recruits initiation factor eIF3 and the ternary eIF2/GTP/Met-tRNA(i)(Met) complex and joins 60S subunits to assemble translation-competent 80S ribosomes (10–12).

However, the exact role of PKR in the regulation of HCV replication in an innate state of liver cells has not been well documented. Furthermore, it remains unknown whether PKR plays an important role in IFN treatment against HCV infection. We evaluated whether PKR has anti-HCV activity using an HCV subgenomic replicon (13) and recently developed full-length HCV genome JFH1 (14) in human hepatoma cells.

Materials and methods

Cell lines

Human hepatoma cells (Huh7) that allow high replication of the HCV subgenomic replicon were a gift from Dr N. Sakamoto (Tokyo Medical and Dental University, Japan) [Tanabe *et al.* (15)]. Human cervical carcinoma cells (HeLa) were obtained from the Riken cell bank (Tsukuba, Japan). These cells were maintained in Dulbecco's modified Eagle's medium (DMEM; Sigma, St Louis, MO, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS).

Establishment of the Huh7 cell line with a stable knockdown expression of double-stranded RNA-activated protein kinase

A sequence that targets the PKR gene was selected, and sense and antisense oligonucleotides (5′-CACCGCGGGAAATTAGATAAAGTACGTGTGCTGTCCGTACT TTGTCTAGTTTCTCGCTTTTT-3′ and 5′-GCATAAAAAGCGAGAAACTAGACAAAGTACGGACAGCACACGTACTTTATCTAATTTCCCGC-3′ respectively) were designed to generate a short hairpin RNA (shRNA). The short interference RNA (siRNA) expression vector for PKR, pcPUR+U6-PKRi, was designed and constructed according to the manufacturer's instructions using the pcPUR+U6i cassette vector (iGENE Therapeutics, Tsukuba, Japan) (16, 17). These vectors were introduced into Huh7 cells to establish stable PKR knockdown cells and control cells. Briefly, the targeting (pcPUR+U6PKRi) or control (pcPUR+U6i) vector was transfected into Huh7 cells using FuGene6 (Roche, Basel, Switzerland), and the puromycin (Sigma)-resistant clones were selected as stable transfectants. The knockdown of PKR was confirmed by Western blotting.

Western blotting

To determine the expression level of PKR, immunoblotting was performed. Cell extracts were adjusted to the same protein concentration using a Micro BCA protein assay reagent kit (Pierce, Rockford, IL, USA), resolved by sodium dodecyl sulphate-polyacrylamide gel electrophoresis, and transferred to polyvinylidene difluoride membranes (Hybond-P; Amersham, Buckinghamshire, UK). Rabbit anti-PKR antibody (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) and rabbit polyclonal anti-PKR[pT^{451}] antibody (Invitrogen, Carlsbad, CA, USA) were used as a primary antibody. The HeLa cell lysate was used as a positive control for PKR and p-PKR bands, according to the manufacturer's instructions. β-actin protein was detected using the anti-β-actin antibody (Sigma) as a primary antibody at a dilution of 1:2500. Horseradish peroxidase-conjugated secondary antibody was purchased from Amersham. Bound antigens were detected using the ECL-Plus system (Amersham).

In vitro transcription and transfection of the hepatitis C virus subgenomic replicon and the full-length genome

The pRep-Feo, an HCV subgenomic replicon plasmid that expresses a chimeric protein that consists of neomycin phosphotransferase and firefly luciferase, was kindly provided by N. Sakamoto (13, 15). The pJFH1 that contained the full-length HCV JFH1 cDNA downstream of the T7 RNA promoter was a gift from T. Wakita (National Institute of Infectious Diseases, Japan) (14). *In-vitro*-transcribed HCV subgenomic RNA and full-length JFH1 RNA were prepared as described previously (13, 14). Briefly, after linearizing pRep-Feo or pJFH1 with *Xba*I, RNA transcripts were synthesized from 2 μg of the linearized plasmid using the MEGAscript T7 system (Ambion, Austin, TX, USA), according to the manufacturer's instructions.

To examine the effect of PKR knockdown on the luciferase activity generated by the subgenomic replicon, high replication permissive Huh7 cells grown under optimum conditions were trypsinized and collected by centrifugation (100*g*, 5 min). The cells were washed three times in ice-cold RNAse-free phosphate-buffered saline (PBS) and resuspended at 1×10^7 cells/ml in PBS. A transfection mixture that contained 10 μg of RNA transcripts made from pRep-Feo was prepared. We mixed 0.42 ml of washed Huh7 cells with the transfection mixture, added this mixture electroporation cuvettes (Bio-Rad Laboratories, Hercules, CA, USA), and immediately pulsed (0.270 kV, pulse-length 30 μs, one pulse) samples using a Bio-Rad electroporation system. After pulsed cells were allowed to recover for 10 min at room temperature, they were plated onto five 10 cm culture

dishes. At 24 h after transfection, we replaced the medium with DMEM that contained 10% FBS and 250 μg/ml G418. Approximately 3 weeks later, surviving colonies were picked up, and a luciferase assay was performed. The luciferase activity of subgenomic replicon-harbouring stable PKR knockdown cells and control cells was measured and compared. In addition, the effect of IFN-α 2b (Schering-Plough K.K., Osaka, Japan) against the replication of the subgenomic replicon was investigated in these subgenomic replicon-harbouring cells. Similarly, RNA transcripts made from pJFH1 were transfected into Huh7 cells to examine the effect of PKR knockdown on the amount of core protein secreted from the replicating JFH1 genome.

Cell growth assay

To examine the growth of control and stable PKR knockdown cells, 3×10^4 cells/well were seeded onto 24-well plates. After 24 or 48 h, the number of viable cells was determined using the 3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay (Sigma) (18). The data were reported as the mean ± standard deviation determined from triplicate wells.

Transfection of plasmid DNA that expresses double-stranded RNA-activated protein kinase

Three days after transfection, JFH1 RNA-transfected Huh7 cells, which were grown in six-well culture plates to ~60% confluency, were transfected with 0.4 μg of plasmid DNA using the Effectene Transfection Reagent (Qiagen, Hilden, Germany). Expression plasmid pRc-PKR, which contained the PKR gene, was provided by B. R. G. Williams (The Cleveland Clinic, Cleveland, OH, USA). The pRc-CMV (empty vector) plasmid was used as a control.

Luciferase assay and quantification of the hepatitis C virus core protein

Subgenomic replicon-harbouring cells were harvested after G418 selection, and the luciferase assay was performed using the PicaGene Dual SeaPansy system (Toyo Ink, Tokyo, Japan) with a luminometer (Lumat LB9507; EG&G Berthold, Bad Wildbad, Germany) to measure firefly luciferase activity as relative light units. Firefly luciferase activity was normalized for cell quantity based on the MTT assay. The luciferase assay was performed at least three times. The HCV core protein in the culture supernatant of JFH1 RNA-transfected cells was quantified using an immunoassay as described previously (19).

Statistical analyses

Statistical analyses were performed using the *t*-test (StatView J; Abacus Concepts, Berkeley, CA, USA), and $P < 0.05$ was deemed statistically significant.

Results

Establishing stable knockdown Huh7 cells

The expression of PKR and activated PKR (p-PKR) proteins in Huh7 cells was detected by Western blotting. Although PKR and p-PKR were expressed weakly in control cells without IFN, their expression was weaker in stable knockdown cells than that in control cells (Fig. 1A). The expression of PKR and p-PKR in control cells increased after adding 10 U/ml IFN-α 2b, whereas the expression in stable knockdown cells was much weaker, even after the addition of IFN-α 2b, compared with control cells (Fig. 1A). This indicated successful knockdown of PKR mRNA expression. Cell proliferation was analysed using the MTT assay, and no difference was observed between stable knockdown cells and control cells (Fig. 1B).

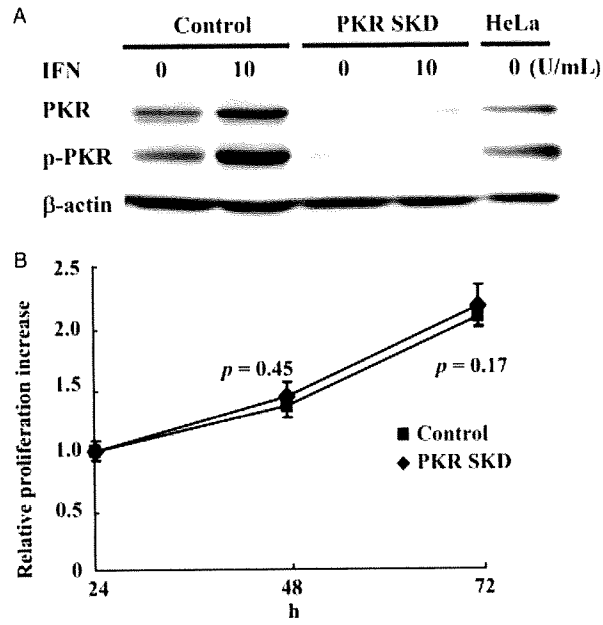

Fig. 1. Establishing SKD Huh7 cells. Huh7 cells were transfected with pcPUR+U6i or pcPUR+U6i-PKRi and incubated with puromycin to select stably transfected cells. The total cell lysates of single clones were subjected to Western blotting to monitor PKR and β-actin protein expression. (A) The endogenous expression of PKR and p-PKR in control and stable PKR knockdown cells and 24 h after the addition of IFN-α 2b. HeLa cell lysates were used as a positive control. (B) The proliferation of SKD and control cells. Stable PKR knockdown and control cells were seeded in 24-well plates, and the number of viable cells was determined using the MTT assay at 24, 48 and 72 h after passage. The error bars indicate the standard deviation of triplicate samples. IFN, interferon; MTT, 3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide; PKR, double-stranded RNA-activated protein kinase; SKD, stable knockdown.

Double-stranded RNA-activated protein kinase knockdown increases hepatitis C virus subgenomic replicon replication in Huh7 cells

To evaluate whether PKR affects HCV replication, HCV subgenomic replicon RNA was transfected into stable knockdown Huh7 cells and control Huh7 cells. High levels of luciferase activity were detected from G418-resistant clones after a few weeks of G418 selection. Relative luciferase activity was significantly higher in lysates from stable knockdown cells compared with lysates from control cells, indicating that the subgenomic replicon replicated better in stable knockdown cells (Fig. 2). Although Figure 2 shows the relative luciferase activities obtained from monoclonal G418-resistant cells, similar results were obtained for polyclonal G418-resistant cells (data not shown). The expression of PKR and p-PKR protein in cells with or without the HCV subgenomic replicon was detected by Western blotting, which revealed much weaker PKR and p-PKR expression in stable PKR knockdown cells compared with control cells (Fig. 3A). No difference in proliferation was observed between stable knockdown cells and control cells harbouring the subgenomic replicon (Fig. 3B), suggesting that cell growth did not contribute to the difference in the relative luciferase activity between stable knockdown cells and control cells harbouring the subgenomic replicon.

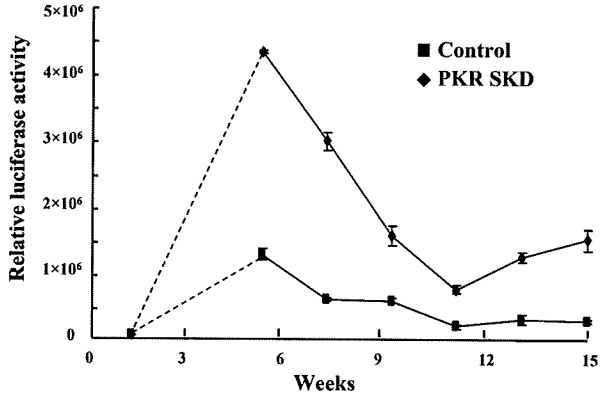

Fig. 2. Subgenomic replicon-related luciferase activities in SKD and control cells. A subgenomic replicon expressing a chimeric protein that consisted of neomycin phosphotransferase and firefly luciferase was transfected into SKD and control cells. The subgenomic replicon-related luciferase activity in G418-resistant cells was measured for 15 weeks. The MTT assay was performed at the same time, and firefly luciferase activity was normalized against cell quantity based on the MTT assay. The results are expressed as the mean of three wells. The error bars indicate the standard deviation of triplicate samples. MTT, 3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide; PKR, double-stranded RNA-activated protein kinase; SKD, stable knockdown.

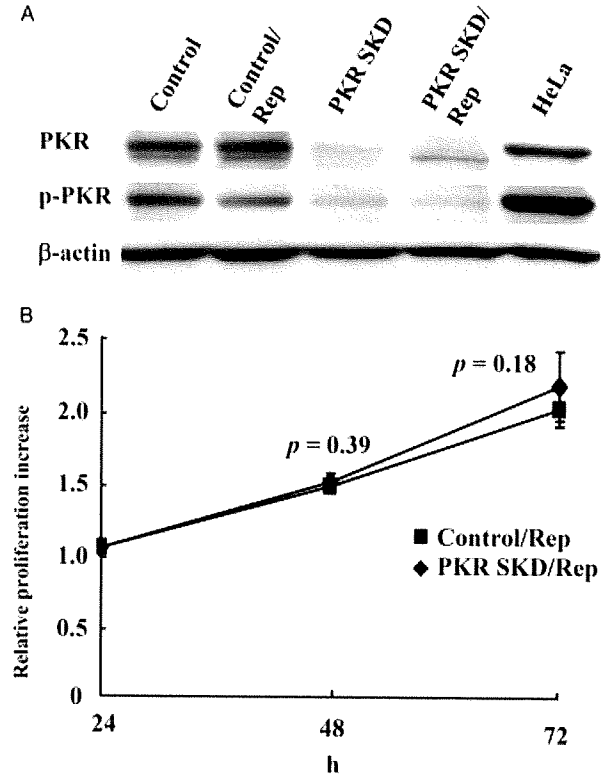

Fig. 3. (A) Expression of endogenous PKR and p-PKR in control cells and SKD cells with or without the HCV subgenomic replicon. HeLa cell lysates were used as a positive control. (B) The proliferation of control and SKD cells with the HCV subgenomic replicon. The number of viable cells was determined using the MTT assay at 24, 48 and 72 h after passage. The error bars indicate the standard deviation of triplicate samples. HCV, hepatitis C virus; MTT, 3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide; PKR, double-stranded RNA-activated protein kinase; Rep, pRep-Feo HCV subgenomic replicon; SKD, stable knockdown.

Double-stranded RNA-activated protein kinase limits the secretion of hepatitis C virus particles from the JFH1 full-length hepatitis C virus genome

To determine whether PKR is involved in regulating HCV replication, the amount of core protein secreted into the culture medium was measured for up to 16 days after transfection with JFH1 full-length HCV RNA. Significantly more core protein was secreted from JFH1 replicating within stable knockdown cells than within control cells (Fig. 4). In addition, the amount of core protein secreted into the culture medium was measured 48 h after the transfection of a PKR-expressing or control plasmid. Significantly less core protein was secreted from PKR-overexpressing cells harbouring JFH1 compared with control cells (Fig. 5). Because PKR knockdown significantly upregulated the replication of not only the HCV subgenomic replicon but also the full-length HCV JFH1 genome, these results confirmed that PKR is one of the factors limiting HCV replication.

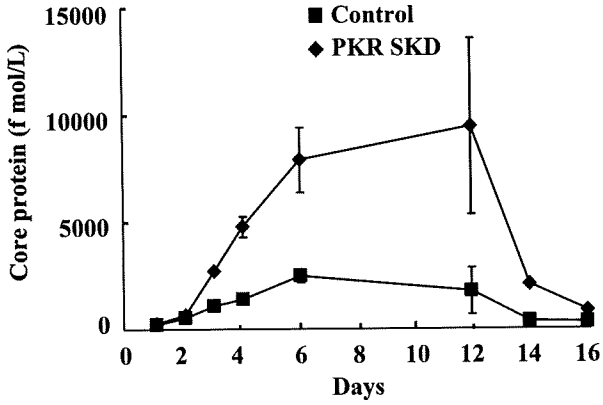

Fig. 4. Amount of core protein secreted from SKD and control cells harbouring JFH1 HCV. The JFH1 full-length HCV genome was transfected into SKD and control cells, and secreted core protein in the culture supernatant was quantified using an immunoassay. Core protein was measured up to 16 days after transfection. The error bars indicate the standard deviation of triplicate samples. HCV, hepatitis C virus; PKR, double-stranded RNA-activated protein kinase; SKD, stable knockdown.

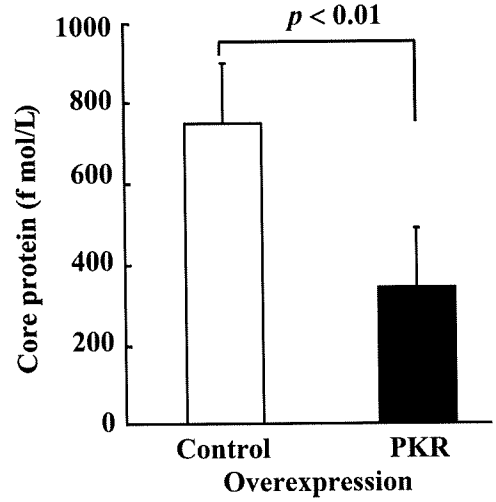

Fig. 5. The amount of core protein secreted from Huh7 cells harbouring the JFH1 full-length hepatitis C virus genome, with or without PKR overexpression. The core protein was measured 48 h post-transfection with pRc-PKR or pRc-CMV (control). The error bars indicate the standard deviation of triplicate samples. PKR, double-stranded RNA-activated protein kinase.

Interferon is fully effective against the hepatitis C virus subgenomic replicon in stable knockdown cells

To evaluate whether PKR knockdown had any effect on the HCV response to IFN treatment, control and stable knockdown cells harbouring the subgenomic replicon were treated with IFN-α 2b at a concentration of 2.5, 5 or 10 U/ml. Replication of the subgenomic replicon was suppressed and luciferase activity was reduced in both control and stable knockdown cells after adding IFN-α 2b (Fig. 6A). The extent of suppression was significantly lower in stable knockdown cells than that in control cells in the presence of a relatively lower concentration of IFN-α 2b ($P < 0.01$; Fig. 6B), whereas 10 U/ml IFN-α 2b suppressed replication of the subgenomic replicon by > 98% in both stable knockdown cells and control cells. This confirmed that the expression of PKR and p-PKR protein was not induced in stable knockdown cells harbouring the subgenomic replicon (Fig. 6C). Because patients with hepatitis C receiving IFN therapy show a maximum serum concentration of 50 U/ml, PKR may not be essential in IFN therapy for HCV.

Discussion

Hepatitis C virus infects approximately 170 million individuals worldwide and is a major aetiological agent of chronic liver disease and HCC (20). Although knowledge of hepatitis C has increased since HCV was identified in 1989 (21), the fundamental aspects of HCV biology still remain unclear. The study of HCV replication and virus–host interaction during HCV infection had been hampered by the lack of efficient cell culture systems for HCV replication. However, the HCV subgenomic replicon system has been developed (13, 22), and several groups have successfully developed reliable and robust cell culture systems for infectious HCV production and propagation in human hepatoma cells using cDNA derived from genotype 2a HCV, JFH1 (13, 14, 23–25). The development of these HCV replication systems has allowed various molecular studies of HCV replication, host–cell interactions and antiviral strategies.

Here, we provided a detailed description of how PKR suppresses HCV replication, using recently developed HCV replication systems for human liver cells, especially a system for the production of infectious HCV particles, and the RNA interference technique to make stable PKR knockdown liver cells. Both PKR and p-PKR were induced by addition of IFN in control cells but not in stable PKR knockdown cells, indicating successful knockdown of PKR. There was no difference in the levels of PKR and p-PKR between replicon-naïve and replicon-transfected control cells, and almost no PKR and p-PKR were detected in stable PKR knockdown cells regardless of the existence of a replicon as was expected. This indicates that replicon (HCV-derived) RNA hardly induces PKR and p-PKR in these cells. We provided evidence that a PKR-based antiviral response does control HCV replication. It was previously shown that an HCV subgenomic replicon replicates more efficiently in PKR knockout mouse embryonic fibroblasts (MEFs) than in wild-type MEFs; however, data from human liver cells and the full-length HCV genome are lacking (26). Recently, it was shown that the overexpression of PKR transiently suppresses the replication of the HCV subgenomic replicon in liver cells; however, data from the full-length HCV genome and PKR knockdown are lacking (27). Moreover, it was shown in liver cells transfected with genotype 1a full-length HCV cDNA that the

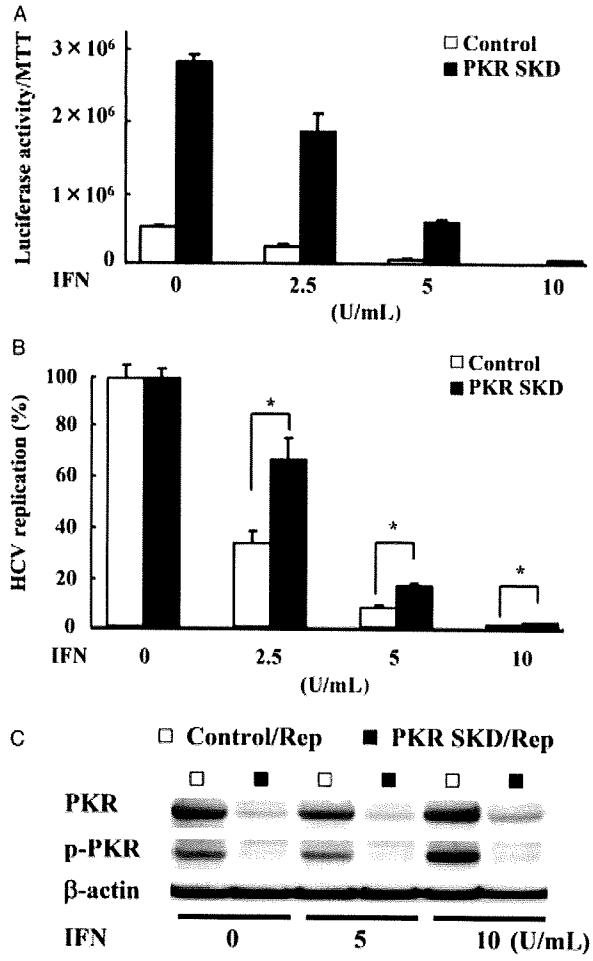

Fig. 6. (A) Subgenomic replicon-related luciferase activities in SKD and control cells that were treated with IFN-α 2b for 48 h. The luciferase activities were normalized against cell quantity based on the MTT assay. (B) Subgenomic replicon-related luciferase activities in SKD and control cells treated with IFN-α 2b. Stable PKR knockdown and control cells were treated with IFN-α 2b for 48 h, and luciferase activity was expressed as a percentage of that without IFN. The error bars indicate the standard deviation of triplicate samples. Asterisks indicate $P < 0.01$. (C) The expression of PKR and p-PKR during IFN treatment. At 24 h after IFN treatment, extracts of cells with the HCV subgenomic replicon were subjected to Western blotting. PKR and p-PKR were detected. HCV, hepatitis C virus; IFN, interferon; MTT, 3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide; PKR, double-stranded RNA-activated protein kinase; Rep, pRep-Feo HCV subgenomic replicon; SKD, stable knockdown.

transient overexpression of PKR results in decreased HCV core protein levels. Transient downregulation of PKR expression by siRNA results in increased HCV core protein levels; however, data for stable HCV replication and stable PKR knockdown are lacking (28). In contrast, our results from the stable knockdown and transient overexpression of PKR show that PKR suppresses the replication of the HCV subgenomic replicon and the full-length genome in human liver cells.

Therapies against HCV are based on IFN, which primarily acts by enhancing host innate immunity. However, sustained eradication of the virus is achieved only in a fraction of patients, even with the best-available therapy of combined pegylated-IFN and ribavirin (29). Understanding the factors that contribute to the suppression of HCV replication is critical to the development of better therapeutic measures. IFN stimulates the expression of a number of ISGs with antiviral activity, including PKR (27). However, it has been demonstrated previously that IFN-α/β inhibits HCV RNA replication in PKR(− / −) MEFs as efficiently as in PKR(+/+) MEFs (26). Our detailed experiments indicate that PKR plays an important role in suppressing HCV replication by low-concentration IFN-α 2b; however, IFN-α 2b is not essential at high concentrations because even 10 U/ml IFN-α 2b efficiently (> 98%) suppressed HCV replication. It is possible that the level of PKR expression induced by IFN at a dose of 10 U/ml is sufficient for suppressing HCV replication fully, even if the level was lower than that in control cells. For this reason, the expressions of PKR and p-PKR were analysed during IFN treatment. Both PKR and p-PKR were induced by addition of IFN in control cells but not in stable PKR knockdown cells, indicating successful knockdown of PKR. In stable knockdown cells harbouring the HCV subgenomic replicon, treatment with 10 U/ml IFN (1.8% residual HCV replicon) resulted in a much weaker expression of PKR and p-PKR than that in control cells treated with 5 U/ml IFN (7.5% residual HCV replicon). Furthermore, in stable knockdown cells harbouring the HCV subgenomic replicon, on treatment with 10 U/ml IFN, lower luciferase activities were found than that in control cells harbouring the HCV subgenomic replicon, without IFN or with 5 U/ml of IFN treatment. Moreover, suppression of replication of the HCV subgenomic replicon seems to be independent of activated p-PKR, total amount of PKR and their combination in stable PKR knockdown cells. These details clearly show that the suppression of HCV replication by IFN is not mainly PKR and p-PKR dependent. In fact, the maximum IFN concentration in the serum of patients receiving 3 MIU IFN-α thrice weekly is approximately 30–54.9 U/ml (30). Considering this information, PKR plays an important role in suppressing HCV replication in innate liver cells, but may not be essential in IFN treatment.

It was reported previously that U6 shRNA constructs induce an IFN response in mammalian cells (31). Therefore, it is possible that this IFN response influences the replication of the HCV subgenomic replicon and the full-length genome. Even under such conditions, the replication of HCV was upregulated by stable PKR knockdown, strengthening the conclusion that PKR plays an important role in suppressing HCV replication in innate cells.

Interestingly, a recent study has suggested that the anti-HCV action of ribavirin is partly attributable to its ability to upregulate PKR activity (32). PKR may play a more important role in IFN and ribavirin combination treatment than in IFN monotherapy.

Hepatitis C virus is thought to be able to counteract the host response in various ways. This ability of HCV to counter the host defences may contribute to the establishment of persistent HCV infection. HCV envelope protein E2 has been shown to inhibit PKR (33–35). E2 contains a sequence identical to phosphorylation sites of PKR, inhibits the kinase activity of PKR and blocks its inhibitory effect on protein synthesis and cell growth (35). HCV NS5A binds PKR and inhibits its catalytic activity by the disruption of PKR-dependent translational control and signalling actions (36, 37). Although these interactions of HCV E2 and NS5A with PKR have been assumed to be one mechanism by which HCV circumvents the antiviral effect of IFN, their inhibition of PKR seems to be incomplete. This is because the knockdown of PKR still increased the replication of the HCV subgenomic replicon that contained NS5A and the full-length genome that contained E2 and NS5A. Moreover, the knockdown of PKR decreased the efficacy of IFN treatment against HCV replication, which suggests that E2 and NS5A do not fully suppress PKR function.

Interestingly, HCV core proteins encoded by sequences derived from HCC tumour tissues, but not those derived from their non-tumour counterparts in the same liver, have been reported to activate PKR by direct interaction (8). This suggests that PKR plays an important role not only in the antiviral activity against HCV but also in hepatocarcinogenesis.

In summary, we demonstrated that PKR is an important regulator of intracellular HCV replication, especially in innate liver cells. PKR is an important molecule in the host innate defence against HCV infection, and any functional and physical deficiency of PKR can lead to an intracellular environment highly conducive to efficient HCV replication. However, PKR may not be an important determinant of IFN treatment against hepatitis C. This virus–cell interaction may play an important role in the pathogenesis of hepatitis C.

Acknowledgements

This study was supported by Global COE Program 'Center of Education and Research for Advanced Genome-Based Medicine: For personalized medicine and the control of worldwide infectious diseases'; the Ministry of Education, Culture, Sports, Science and Technology of Japan; Research Fellowships of the Japan Society for the Promotion of Science for Young Scientist; grant-in-aid for scientific research from the Ministry of Education, Culture, Sports, Science and Technology of Japan and Health and Labor Sciences Research Grants for Research on Hepatitis from the Ministry of Health, Labor and Welfare, Japan. We thank Dr N. Sakamoto, Dr B. R. G. Williams and Dr T. Wakita for providing us with plasmids and cells. We also thank Dr F. Kanai for advice on the experiments and M. Tsubouchi for technical assistance.

References

1. Lauer GM, Walker BD. Hepatitis C virus infection. *N Engl J Med* 2001; **345**: 41–52.
2. Saito I, Miyamura T, Ohbayashi A, *et al.* Hepatitis C virus infection is associated with the development of hepatocellular carcinoma. *Proc Natl Acad Sci USA* 1990; **87**: 6547–9.
3. Shiratori Y, Shiina S, Imamura M, *et al.* Characteristic difference of hepatocellular carcinoma between hepatitis B- and C-viral infection in Japan. *Hepatology* 1995; **22**: 1027–33.
4. Omata M, Yokosuka O, Takano S, *et al.* Resolution of acute hepatitis C after therapy with natural beta interferon. *Lancet* 1991; **338**: 914–5.
5. Fried MW, Shiffman ML, Reddy KR, *et al.* Peginterferon alfa-2a plus ribavirin for chronic hepatitis C virus infection. *N Engl J Med* 2002; **347**: 975–82.
6. Penin F, Dubuisson J, Rey FA, Moradpour D, Pawlotsky JM. Structural biology of hepatitis C virus. *Hepatology* 2004; **39**: 5–19.
7. Butcher SJ, Grimes JM, Makeyev EV, Bamford DH, Stuart DI. A mechanism for initiating RNA-dependent RNA polymerization. *Nature* 2001; **410**: 235–40.
8. Delhem N, Sabile A, Gajardo R, *et al.* Activation of the interferon-inducible protein kinase PKR by hepatocellular carcinoma derived-hepatitis C virus core protein. *Oncogene* 2001; **20**: 5836–45.
9. Dey M, Cao C, Dar AC, *et al.* Mechanistic link between PKR dimerization, autophosphorylation, and eIF2alpha substrate recognition. *Cell* 2005; **122**: 901–13.
10. Fukushi S, Okada M, Kageyama T, Hoshino FB, Katayama K. Specific interaction of a 25-kilodalton cellular protein, a 40S ribosomal subunit protein, with the internal ribosome entry site of hepatitis C virus genome. *Virus Genes* 1999; **19**: 153–61.
11. Otto GA, Puglisi JD. The pathway of HCV IRES-mediated translation initiation. *Cell* 2004; **119**: 369–80.
12. Siridechadilok B, Fraser CS, Hall RJ, Doudna JA, Nogales E. Structural roles for human translation factor eIF3 in initiation of protein synthesis. *Science* 2005; **310**: 1513–5.
13. Yokota T, Sakamoto N, Enomoto N, *et al.* Inhibition of intracellular hepatitis C virus replication by synthetic and vector-derived small interfering RNAs. *EMBO Rep* 2003; **4**: 602–8.
14. Wakita T, Pietschmann T, Kato T, *et al.* Production of infectious hepatitis C virus in tissue culture from a cloned viral genome. *Nat Med* 2005; **11**: 791–6.
15. Tanabe Y, Sakamoto N, Enomoto N, *et al.* Synergistic inhibition of intracellular hepatitis C virus replication by combination of ribavirin and interferon-alpha. *J Infect Dis* 2004; **189**: 1129–39.
16. Miyagishi M, Taira K. U6 promoter-driven siRNAs with four uridine 3′ overhangs efficiently suppress targeted gene expression in mammalian cells. *Nat Biotechnol* 2002; **20**: 497–500.
17. Jazag A, Ijichi H, Kanai F, *et al.* Smad4 silencing in pancreatic cancer cell lines using stable RNA interference and gene expression profiles induced by transforming growth factor-beta. *Oncogene* 2005; **24**: 662–71.