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INTERNAL MEDICINE

ORIGINAL ARTICLE

The Significance of Enzyme Immunoassay for the Assessment of Hepatitis B Virus Core-Related Antigen following Liver Transplantation

Masumi Fujimoto[1], Tatsuki Ichikawa[1], Kazuhiko Nakao[1], Hisamitsu Miyaaki[1], Hidetaka Shibata[1], Susumu Eguchi[2], Mitsuhisa Takatsuki[2], Shinya Nagaoka[3], Hiroshi Yatsuhashi[3], Takashi Kanematsu[2] and Katsumi Eguchi[1]

Abstract

Purpose Recently, a new enzyme immunoassay for the detection of hepatitis B virus (HBV) core-related antigen (HBcrAg) has been reported. In this study, we proposed to account for feasibility of HBcrAg assay, and discuss the dynamics of HBV seen in patients following HBV-related living donor liver transplantation (LDLT).

Methods and results This study involved 12 patients; 11 patients had positive serum HBcrAg, and 6 patients had negative HBV-DNA. In the post-operation period, all cases were negative for HBV-DNA and HBsAg in sera under prophylaxis therapy. At post-operation, 5 of the 12 had positive serum HBcrAg, and at stable state, 6 had positive serum HBcrAg postoperatively. The mean levels of HBcrAg following LDLT were significantly lower than those seen in the preoperative-operation stage.

Conclusion This enzyme immunoassay is a readily utilizable marker of HBV replication in the post transplantation stage. Furthermore, the evaluation of HBV activity by HBcrAg assay must be studied to determine the appropriate prophylaxis for controlling replication of HBV following LDLT.

Key words: hepatitis B virus, liver transplantation, hepatitis B virus core-related antigen

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Introduction

Liver transplantation (LT) is a long established procedure for the treatment of end-stage liver disease. Patients with chronic or fulminant hepatitis B virus (HBV) infection are major candidates for LT. However, the recurrence of HBV following LT is implicated in severe and life-threatening graft failure (1). Therefore, the prevention of HBV recurrence following LT has been a serious concern. The advent of anti-HBsAg immune globulin (HBIG, Hebsbulin-IH, Mitsubishi Pharma Corporation, Tokyo, Japan), and HBV reverse transcriptase inhibitor, namely lamivudine (Lam, Zeffix, GlaxoSmithKline K.K., Tokyo, Japan) and adefovir dipivoxil (Adv, Hepsera, GlaxoSmithKline K.K., Tokyo, Japan), was a major breakthrough in controlling HBV recurrence in patients who received transplants for HBV-related liver disease. The ideal recurrence rate for HBV (<10%), has been observed in patients receiving HBIG and Lam combination prophylaxis versus just HBIG monotherapy (2, 3) or Lam monotherapy (4, 5). Lam monotherapy has been shown to be ineffective in controlling recurring HBV, and the long term administration of HBIG was necessary (6, 7). Therefore, presently, continuous combination therapy is the standard prophylaxis in the control of HBV recurrence following HBV-related LT.

[1]The First Department of Internal Medicine, Graduate School of Biomedical Sciences, Nagasaki University, Nagasaki, [2]Department of Transplantation and Digestive Surgery, Graduate School of Biomedical Sciences, Nagasaki University, Nagasaki and [3]Clinical Research Center, National Nagasaki Medical Center, Omura

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Correspondence to Dr. Tatsuki Ichikawa, ichikawa@net.nagasaki-u.ac.jp

Table 1. Clinical Characteristics

Case	Age at LDLT	Gender	Indication disease	HCC	HBV-DNA at LDLT (logcopy/mL)	Adefovir before LDLT	HBsAg (COI)	HBsAb (IU/mL)	HBeAg (IU/mL)	HbeAb (COI)	HBcrAg (logU/mL)
1	56	male	LC	+	<2.6	-	>2000	2.3	0.6	82.4	4.2
2	60	male	LC	+	<2.6	-	1789	0.1	0.2	97.6	5.8
3	59	male	LC	+	<2.6	-	>2000	0.3	0.1	>100	3.2
4	55	female	LC	-	<2.6	-	>2000	0.2	36.0	0	6.0
5	56	male	LC	+	<2.6	-	>2000	0.1	1.4	75.4	5.6
6	68	male	LC	+	<2.6	-	47.7	0.1	0.1	>100	<3.0
7	37	female	LC	+	2.6	+	>2000	0.1	0.2	81.5	5.5
8	57	male	LC	+	<2.6	+	188.5	0.5	0.8	54.0	5.1
9	48	male	LC	-	<2.6	+	562.5	0.1	1.1	57.7	5.0
10	53	male	LC	-	4.4	+	>2000	0.1	49.2	96.1	7.5
11	34	female	FHF	-	4.9	-	374	7.9	0.8	93.9	5.7
12	28	female	FHF	-	4.6	-	19.5	133.8	4.6	54.4	7.4

Previous reports showed only trace amounts of HBV replication in extra-hepatic sites following LT (8). If HBV was present in hepatocytes, Lam would have masked the appearance of HBV-DNA regardless of the presence of intrahepatic HBV covalently closed circular (ccc)DNA (9, 10). These factors make it difficult to understand HBV dynamics following LT. Recently, new enzyme immunoassays for detecting HBV core antigen (HBcAg) (11) and HBV core-related antigen (HBcrAg) (12, 13) have been reported. These antigens move parallel with HBV-DNA in the serum and have a wide detection range (14). In particular, the assay for HBcrAg is able to detect both HBcAg and HBeAg even in anti-HBc antibody and anti-HBe antibody-positive specimens. Additionally, it has shown a higher sensitivity than HBV-DNA transcription mediated amplification (TMA), and equivalent sensitivity to in-house real time detection PCR (15). Different from the assay for HBV genome, the HBcrAg assay detects translational products of HBV and is presumed to be a reflection of cccDNA (16, 17). The HBcrAg assay has never been used to assess transplant patients undergoing HBV prophylaxis, and the status of HBV replication markers has also never been discussed in the case of post-transplanted patients, negative for HBsAg and HBV-DNA, who were undergoing anti-HBV prophylaxis. Therefore, in this study, we proposed to account for availability of HBcrAg assay, and discuss HBV dynamics in patients following HBV-related LT.

Abbreviation: HBsAg: hepatitis B virus s antigen, HBeAg: hepatitis B virus e antigen, HBcAg: hepatitis B virus core antigen, HBcrAg: Hepatitis B virus core-related antigen, cccDNA: covalently closed circular DNA, Lam: Lamivudin, HBIG: anti-HBs antigen immune globulin

Materials and Methods

Patients and clinical samples

From 2001 to 2006, a total of 12 patients with HBV-related severe liver disease, were admitted to Nagasaki University Hospital, Nagasaki, Japan, and enrolled in this study (Table 1). There were 8 men and 4 women with a median age of 52.0 years (range 28-68 years). All 12 patients had received LDLT at this hospital. The graft survival rate was 100%, and not one showed evidence of graft hepatitis. Of the 12 patients, 10 had been diagnosed with liver cirrhosis (LC) (with 7 of those having hepatocellular carcinoma), and 2 patients had been diagnosed with fulminant hepatic failure (FHF). All patients had been receiving a daily dose of 100 mg Lam since the pre-operation period in order to prevent the recurrence of HBV infection [range 0.1-22 months, mean (standard deviation: SD); 7.81 (8.17) months] and following LT, 4 patients began receiving Adv therapy [range; 19-250 days, mean (SD) 102.3 (128.2) days] in addition to Lam due to Lam resistant HBV mutations present before and after LT. Donor status of HBV serological makers such as HBsAg, HBsAb and HBcAb were negative. Prophylactic infusion of HBIG was administered to all patients using a fixed dosing schedule: 10,000 units intravenously at the anhepatic period and on the day following LDLT. Afterwards, a dose of 2,000 units of HBIG was given routinely over the long term with the aim of keeping the serum titer greater than 100 units/L. After LDLT, serum HBsAg, HBeAg or HBV-DNA were not detected in any patient. Serum samples for HBV were collected from each patient at the following three specified intervals: 1) at the pre-operation period during Lam or Lam/Adv treatment (ranges; 7.9±8.7 days prior to LT). 2) at the immediate post-operation period during which patients received combined prophylaxis, and immunosuppression with steroid and calcineurin inhibitor (within 37.7±20.3 days after LT), and 3) at the stable state period when patients received combined prophylaxis, and immunosuppression without steroid (18.1±16.7 months after LT). Serum concentration of aspartate aminotransferase (AST), alanine aminotransferase (ALT), total bilirubin (T-Bil), prothrombin time (PT), and albumin (Alb) were obtained from patient's medical records. All patients underwent needle liver biopsy every year after transplantation.

Abbreviation: HBsAb: hepatitis B virus s antibody, HBcAb: hepatitis B virus core antibody

Serological markers for HBV

HBsAg, HBsAb, HBeAg, HBeAb, and HBcAb were assessed by the chemiluminescence enzyme immunoassay (CLEIA) method, using a commercially available enzyme immunoassay kit (Lumipulse, Fuji Rebio, Tokyo, Japan). Serum concentrations of HBV DNA were determined using a

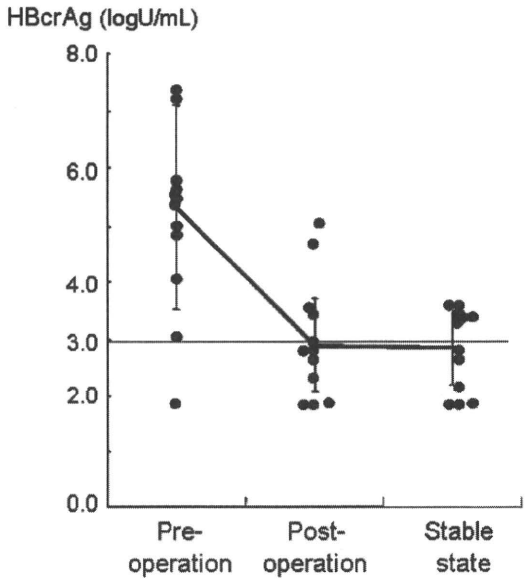

Figure 1. Serial changes of the HBcrAg levels. The HBcrAg levels are represented as mean values; the closed circles show the value of HBcrAg levels in all phases. Error bar is standard deviation. The mean value of HBcrAg levels in the post-operation period and in the stable state period were significantly lower than that in the pre-operation period (t-test, $p<0.05$). The detection range is above 3.0 logU/mL. To obtain the mean value, the value of 3.0 logU/mL or less and 2.0 logU/mL or more was added to the calculation.

polymerase chain reaction HBV monitoring kit (Roche, Tokyo, Japan), which had a quantitative range from 2.6 to 7.6 log copy/mL.

Serum concentrations of HBcrAg were measured using the CLEIA method reported previously (12, 18). In brief, 100 mL serum was mixed with 50mL of a pretreatment solution containing 15% sodium dodecylsulfate, and 2% Tween 60. After incubation at 70℃ for 30 minutes, 50mL of pretreated serum was added to test wells coated with monoclonal antibodies specific for denatured HBcAg and HBeAg (HB44, HB61, and HB114), and then filled with 100 mL assay buffer. The plate was incubated for 2 hours at room temperature and the wells were then washed with buffer. Alkaline phosphatase-labeled monoclonal antibodies specific for denatured HBcAg and HBeAg (HB91 and HB 110), were added to the wells, and the plate was again incubated at room temperature, this time for 1 hour. After washing, CDP-Star with Emerald II (Applied Biosystems, Bedford, MA) was added and the plate was incubated at room temperature one more time for 20 minutes. The relative chemiluminescent intensity was measured, and the HBcrAg concentration was determined by comparison with a standard curve generated using recombinant pro-HBeAg (amino acids, 10-183 of the precore/core gene product). The HBcrAg concentration was expressed as units/mL (U/mL) and the immunoreactivity of recombinant pro-HBeAg at 10 fg/ml corresponded to 1 U/mL. In this study, the cutoff value was tentatively set at 3.0 logU/mL (12).

Table 2. Comparison of the HBcrAg Levels between Lam Group and Combination Lam/Adv Group at Each Period

Group	Number	pre-operation	post-operation	stable
L: Lam	6	4.47 (1.62)	2.92 (1.19)	5.14 (0.72)
A: Lam+Adv	4	5.78 (1.17)	3.58 (0.78)	3.45 (0.17)

Abbreviation: HBeAb: hepatitis B virus e antibody

Statistical analyses

Statistical analyses were performed using the SPSS 11.0.1 J statistical software package (SPSS, Inc., Chicago, IL). The p-values of less than 0.05 were considered statistically significant.

Results

Serial changes in HBcrAg levels at indicated periods

Results of the HBcrAg assay showed differences in titers during the specific periods (Table 1 and Fig. 1); 11 cases had positive levels of HBcrAg, however 8 of them were negative for HBV-DNA. In the post-operation period, all cases were negative for HBV-DNA and HBsAg, however 5 of them (cases 4, 5, 7, 9 and 10) had positive levels of HBcrAg. In the stable state period, 6 of the cases (cases 2, 5, 7, 8, 9 and 10) had positive levels of HBcrAg. The 2 cases with FHF (cases11 and 12) had negative levels of HBcrAg in both post operation and the stable state periods. Of the 4 patients who received combined Lam/Adv treatment, 3 patients (cases 7, 9, and 10) also had positive HBcrAg levels in both post operation and the stable state periods. Two cases (cases 2 and 8) had negative levels of HBcrAg in the post operation period, but positive levels in the stable state period. The overall mean level of HBcrAg following LT [post-operation 3.05 (1.026) logU/mL and stable state periods 2.875 [(0.66) logU/mL] was significantly lower than that at pre-operation period [mean (SD); 5.25 (2.445) logU/mL] (Fig. 1). After LT, the levels of serum HBcrAg were decreased and steroid administration on early post-operation period did not seem to influence HBcrAg levels.

Comparison of the HBcrAg levels between combination Lam/Adv group and Lam group

A comparison of the mean values of HBcrAg levels between the group receiving only Lam treatment (6 patients with LC: Group L) and the group receiving combination Lam/Adv treatment (4 patients with LC: Group A) was made (Table 2). The mean value of HBcrAg in Group A was higher than that in Group L through all periods of the study [mean (SD) value (logU/mL) is as follows: pre-operation, Group A; 5.78 (1.17), Group L; 4.47 (1.62), post-operation, Group A; 3.58 (0.78), Group L; 2.92 (1.19), stable state, Group A 3.45 (0.17), Group L: 5.14 (0.72)]. No

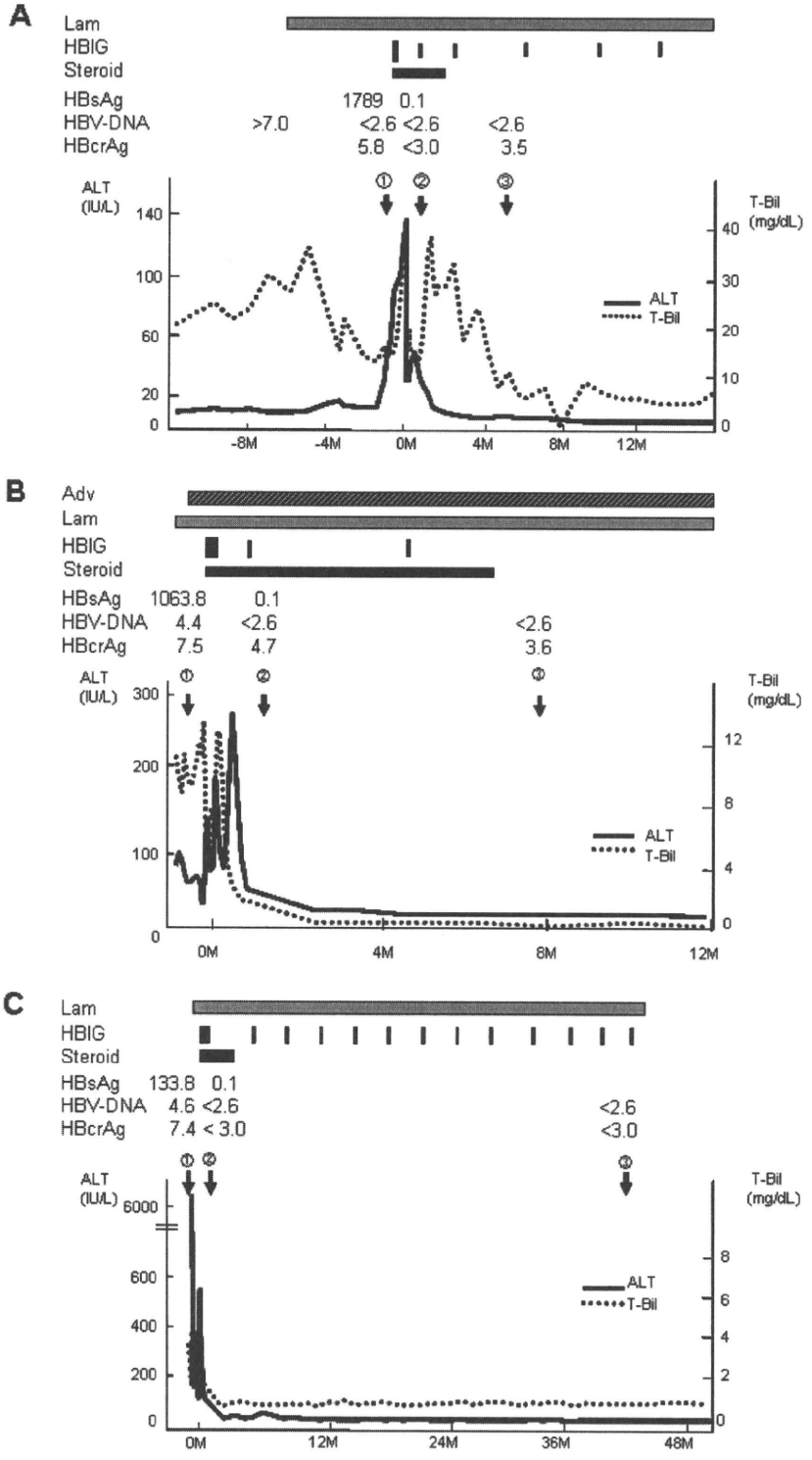

Figure 2. **Clinical course of representative cases. A: Case 2 had suffered from LC. The value of crAg was below sensitivity in the immediate post-LT phase, then returned to a positive value in the post-LT late phase. B: Case 7 suffered from LC with Lam resistance. Adv was added to the Lam treatment at the pre-LT period, and continued after LT. C: Case 9 suffered from fulminant hepatic failure.**

significant difference was noted between these groups in repeated statistical analysis of the data.

Patterns of serum HBcrAg levels compared with the clinical courses of selected cases

No correlation was made between the serum HBcrAg levels and AST, ALT, total bilirubin, prothrombin time, or albumin during any phase of this study (data not shown). Patterns of variation in serum HBcrAg levels were compared to the clinical courses of selected cases. A representative case (Fig. 3A, case 2) is a 60-year-old man with LC and hepatoma. He had been receiving Lam therapy for 5 months

prior to LT. His serum HBV-DNA became negative 1 month prior to LT. Liver function became worse and LT was performed for hepatic failure. His level of serum HBcrAg prior to LT was 5.8 logU/mL, and HBsAg was positive. Following LT, HBsAg became negative. His HBsAb titer was high due to HBIG. At post operation, his serum HBcrAg level fell below the cut-off level (2.8 logU/mL). However, levels of HBcrAg then rose to 3.5 logU/mL despite normal levels of ALT and total bilirubin. Negative levels of both HBsAg and HBV-DNA have continued to present. In case 8, whose HBcrAg level was negative in the post operation period but became positive in the stable state period, the levels of ALT and total bilirubin remained at normal levels in the observation period.

Case number 10 (Fig. 3B), a 53-year-old man with LC, began receiving Lam therapy 19 months prior to LT, however his ALT and T-Bil were in relapse due to Lam resistant HBV mutation. Therefore, the addition of Adv therapy was started 3 weeks prior to LT. Hepatic failure could not be prevented despite the addition of Adv, and LT was performed. At the time of LT, serum HBV-DNA and HBcrAg were 4.4 logU/mL and 7.5 logU/mL, respectively. Following LT, Lam and Adv therapy was continued and his levels of HBV-DNA have remained below the cut-off level (<2.6 logU/mL), but levels of HBcrAg have been positive, throughout both the post-operation period; 4.7 logU/mL, and the stable state period; 3.6 logU/mL. His liver function became stable following after LT.

Case number 12 (Fig. 3C), a 28-year-old woman with FHF, suffered from acute HBV infection. Since several courses of plasma aphaeresis, along with Lam therapy did not improve her condition, she underwent LT despite positive levels both of HBV-DNA (4.6 logU/mL) and HBcrAg (7.4 logU/mL). Following LT, serum HBV-DNA became negative, and serum HBcrAg levels have remained below the cut-off level (post-operating period; 2.0 logU/mL, stable state period; 2.3logU/mL). Her liver function became stable following LT.

Every case entered in this study underwent an annual liver biopsy in our hospital. All of the biopsy specimens in the stable state did not show any pathological features of chronic viral hepatitis despite the titer of HBcrAg in serum.

Discussion

This newly developed enzyme immunoassay for HBcrAg could be a useful measure of HBV activity in patients receiving anti-HBV prophylaxis following LT. Serum HBcrAg was detected prior to LT in all patients, and the levels varied in the early and late post operation period. Our use of HBcrAg assay shows that HBV replication is occurring in patients receiving combination prophylaxis following LT, and that LT itself decreased levels of HBcrAg. Since LT decreased the levels of serum HBcrAg, then the use of steroid did not have any influence on HBcrAg levels. The value of HBcrAg varies over time, but it has no relationship to hepatic function. However, further observation is necessary to evaluate the relationship between the detection of HBcrAg and the long-term prognosis of these patients.

It has been reported that serum HBcrAg levels can be thought of as a non-relapse marker at the time of Lam cessation (15), and a risk marker for HBV resistance at the 6 month point in Lam treatment (16). Lam blocked the reversed transcription of HBV-RNA to HBV-DNA, but did not inhibit translation or transcription. Cessation of Lam at the absence of serum HBV-DNA causing a flare up of HBV replication, due to the existence of HBV cccDNA, which is a template for the HBV pregenome RNA, may be a source of Lam resistant HBV strains in hepatocytes (8, 19). The levels of cccDNA in hepatocytes, as well as HBcrAg in serum, but not HBV-DNA in serum, are also a prediction marker of sustained anti-viral response in Lam treatment (20, 21). Production of HBcrAg in hepatocytes as a reflection of HBV replication activity, indicates the existence of cccDNA in hepatocytes. Therefore, the concentration of HBcrAg in the serum of a patient receiving Lam treatment may indicate an altered HBV replication status within the hepatocytes (22). We feel the HBcrAg assay is a reliable means for identifying HBV replication following HBV-related LT, and think that HBV replication continues following LT despite combination HBIG and Lam prophylaxis. The sensitivity of HBcrAg is not very high in HBsAg seroclearance patients (17). Since HBsAg and HBV-DNA had not been detected in post LT patients receiving combination prophylaxis, HBcrAg assay can be a predictive maker of HBV replication at this stage. Recently, it was reported that HBV cccDNA in hepatocytes (23), HBV-RNA (22, 24) and serum HBsAg quantitative (25) are HBV replication markers. In addition to the HBcrAg assay, we should evaluate these markers to fully understand HBV dynamics after LT.

Previous reports have suggested that Lam resistant, HBV-infection related-LT was as safe as wild type HBV-infection-related LT (26). These reports concluded that a combination of Adv and Lam therapy provides effective prophylaxis in patients with pre-LT Lam resistant HBV mutants (26, 27). However, positive HBV-DNA was observed in all of the patients in the present study, and Adv and Lam resistant HBV has recently been observed (28). In our study, in the stable period, the titer of HBcrAg in Lam group was relatively higher than Adv add-on group. Further study is needed to evaluate Adv add-on Lam combined prophylaxis.

The production site of HBcrAg was unclear in the post-LT period. In cases of HCV-related LT, non-hepatic virus sources, at the most, account for 4% of the total viral production, and post-LT viral clearance, after rapid initial decline, slows, possibly due to the filling of absorption sites in newly grafted liver (29). HBV re-infection may be caused by the over-production of HBV in extrahepatic sites or HBV circulating particles following LT (30). Escaped mutants from HBIG and Lam may also cause re-infection (31). According to a recent report (32), highly sensitive real-time PCR of cccDNA found that cccDNA in PBMCs is detected

only to a small degree. As such, PBMCs are unlikely to function as a reserve of HBV. In HCV-related LT, it has been reported that the virus immediately re-infects liver grafts (33, 34), but re-infection of the graft is not apparent in HBV. We can not disregard production of HBcrAg in hepatocytes following LT, but further studies are necessary to fully understand HBV replication sites following LT.

In addition to HBsAg and HBV-DNA, HBcrAg assessment could be a practical tool as a marker of HBV replication after LT. Because the levels of HBcrAg are a reflection of cccDNA, we think that the HBcrAg positive cases need continual prophylaxis following LT. In addition, the evaluation of HBV dynamics by HBcrAg assay must be studied to determine the appropriate prophylaxis against replication of HBV following LT.

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Interferon-α-induced mTOR activation is an anti-hepatitis C virus signal via the phosphatidylinositol 3-kinase-Akt-independent pathway

Azusa Matsumoto · Tatsuki Ichikawa · Kazuhiko Nakao · Hisamitsu Miyaaki · Kumi Hirano · Masumi Fujimito · Motohisa Akiyama · Satoshi Miuma · Eisuke Ozawa · Hidetaka Shibata · Shigeyuki Takeshita · Hironori Yamasaki · Masanori Ikeda · Nobuyuki Kato · Katsumi Eguchi

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Abstract
Object The interferon-induced Jak-STAT signal alone is not sufficient to explain all the biological effects of IFN. The PI3-K pathways have emerged as a critical additional component of IFN-induced signaling. This study attempted to clarify that relationship between IFN-induced PI3-K-Akt-mTOR activity and anti-viral action.
Result When the human normal hepatocyte derived cell line was treated with rapamycin (rapa) before accretion of IFN-α, tyrosine phosphorylation of STAT-1 was diminished. Pretreatment of rapa had an inhibitory effect on the IFN-α-induced expression of PKR and p48 in a dose dependent manner. Rapa inhibited the IFN-α inducible IFN-stimulated regulatory element luciferase activity in a dose-dependent manner. However, wortmannin, LY294002 and Akt inhibitor did not influence IFN-α inducible luciferase activity. To examine the effect of PI3-K-Akt-mTOR on the anti-HCV action of IFN-α, the full-length HCV replication system, OR6 cells were used. The pretreatment of rapa attenuated its anti-HCV replication effect in comparison to IFN-α alone, whereas the pretreatment with PI3-K inhibitors, wortmannin and LY294002 and Akt inhibitor did not influence IFN-induced anti-HCV replication.
Conclusion IFN-induced mTOR activity, independent of PI3K and Akt, is the critical factor for its anti-HCV activity. Jak independent mTOR activity involved STAT-1 phosphorylation and nuclear location, and then PKR is expressed in hepatocytes.

Keywords mTOR · STAT-1 · Interferon · HCV · PKR

Abbreviations

IFN	Interferon
HCV	Hepatitis C virus
STAT	Signal transducers and activators of transcription
ISGF-3	IFN-stimulated gene factor 3
ISRE	IFN-stimulated regulatory element
PKR	Double-stranded RNA-dependent protein kinase
Rapa	Rapamycin
PI3-K	Phosphatidylinositol 3-kinase
mTOR	Mammalian target of rapamycin
siRNA	Small interfering RNA

Introduction

Currently, a chronic hepatitis C virus (HCV) infection is the major cause of hepatocellular carcinoma worldwide [1]. Therefore, an anti-HCV strategy is important for prevention of carcinogenesis. Advancement in the treatment of HCV by a combination of pegylated interferon (IFN) and

A. Matsumoto · K. Hirano
Department of Clinical Pharmaceutics,
Graduate School of Biomedical Sciences,
Nagasaki University, Nagasaki, Japan

T. Ichikawa (✉) · K. Nakao · H. Miyaaki · M. Fujimito · M. Akiyama · S. Miuma · E. Ozawa · H. Shibata · S. Takeshita · K. Eguchi
The First Department of Internal Medicine,
Graduate School of Biomedical Sciences, Nagasaki University,
1-7-1 Sakamoto, Nagasaki 852-8501, Japan
e-mail: ichikawa@net.nagasaki-u.ac.jp

H. Yamasaki
Health Research Center, Graduate School of Biomedical Sciences, Nagasaki University, Nagasaki, Japan

M. Ikeda · N. Kato
Department of Molecular Biology, Graduate School of Medicine and Dentistry, Okayama University, Okayama, Japan

ribavirin is effective in 80% of HCV genotype 2 or 3 cases, but less than 50% of genotype 1 cases. To ameliorate the salvage rate of HCV infection, new anti-HCV agents have been developed to inhibit the life cycle of HCV and are combined with IFN-α [2]. Since IFN-α is the most basic agent for HCV treatment, it is necessary to improvement the salvage rate of HCV infection by clarifying the efficacy of IFN treatment.

The factors associated with a refractory response to IFN treatment are the HCV genotype, viral load, age, sex, fibrosis of the infected liver and metabolic factors such as insulin resistance and steatosis [3]. Increased hepatic expression of the suppressor of cytokine signaling (SOCS) family, known as the Jak-STAT signal inhibitors, especially SOCS-3, is associated with non-response to IFN treatment [4, 5]. It is thought that inflammatory cytokines, such as, interleukin 6, induced by HCV infection can induce SOCS-3 in hepatocyte [5]. SOCS-3 inhibits IFN-induced tyrosine phosphorylation of Jak, then intra-hepatocyte IFN signal transduction is inhibited. For HCV survival, Jak1, Tyk2 and STAT-1,-2 signaling, which is the essential pathway for type 1 IFN-induced anti-viral activity, becomes the attack targets from HCV. The relative lack of a viral response to IFN treatment is associated with blunted IFN signaling [6]. HCV coding proteins also inhibit STAT-1 tyrosine phosphorylation [7]. The cause of a refractory response to IFN treatment is thought to be HCV-induced Jak-STAT signal inhibition.

Type 1 IFN is a pleiotropic cytokine which activates various intra-cellular signal pathways other than the Jak-STAT signal [8]. Additional signaling pathways could either collaborate with STATs at the promoter level and contribute to the activation of the STATs plus transcription factor genes or function totally independent of any STAT factors, thus leading to the activation of transcription factor only genes [8]. The Jak-STAT signal alone is not sufficient to explain all the biological effects of type 1 IFN. The PI3-K and p38 kinase pathways have emerged as critical additional component of IFN-induced signaling [8–10]. p38, activated via IL-1β is enhanced STAT-1 tyrosine phosphorylation and express the anti-viral protein, PKR [9]. The IFN-induced PI3-K-Akt pathway has Jak independent activation, and it is the critical signal for cell survival and insulin action [10], but its relationship with the anti-viral action and PI3-K-Akt pathway is still unclear.

Recently, mTOR, a downstream kinase of PI3-K-Akt pathway, was shown to play a critical role in protein synthesis and anti-viral effects. Kaur and his colleagues [11] reported that the IFN activated mTOR pathway exhibits important regulatory effects in the generation of the IFN responses, including the anti-encephalo myocarditis virus effect. The IFN-induced mTOR is LY294002 sensitive and does not affect the IFN-stimulated regulatory element (ISRE) dependent promoter gene activity. Human cytomegalovirus is inhibited by 5′-AMP-activted protein kinase mediated inhibition of mTOR kinase [12]. In contrast, vesicular stomatitis virus is mTOR dependent [13]. A relationship has been reported between the replication of hepatitis virus and mTOR activity. p21-activated kinase 1 is activated through the mTOR/p70 S6 kinase pathway and regulates the replication of HCV [14]. mTOR activation is dependent upon the PI3-K-Akt and ERK pathways. Gao and colleagues reported that HCV-NS5A protein activates the PI3-K-Akt-mTOR pathway and could inhibit HBV RNA transcription and reduce HBV DNA replication in HepG2 cells [15]. The activation of the N-Ras-PI3-K-Akt-mTOR pathway by HCV is required for cell survival and HCV replication [16]. Therefore, PI3-K, Akt and mTOR activated by HCV are inhibitory signals of HCV replication and survival signals of HCV infected cells. Furthermore, the PI3-K-Akt-mTOR pathway, which is activated by HCV, is thought to be one mechanism for chronic HCV infection [14–16]. However, type 1 IFN-induced PI3-K, Akt and mTOR have not yet been fully evaluated regarding their influence on HCV replication.

This study investigated whether IFN-α induced the PI3-K-Akt-mTOR pathway, whether the Jak-STAT pathway has a relationship with the PI3-K-Akt-mTOR pathway, and, finally, whether IFN induced signal transduction, other than the Jak-STAT pathway, is associated with the anti-HCV activity.

Materials and methods

Reagents and cell culture

Recombinant human IFN-α2b was a generous gift from Schering-Plough KK (Tokyo, Japan). Wortmannin, LY 294002, Akt inhibitor and rapamycin were purchased from Calbiochem (La Jolla, CA, USA). Hc human hepatocyte cells (Applied Cell Biology Research Institute, Kirkland, WA, USA) and HuH-7 human hepatoma cells (American Type Culture Collection, Rockville, MD, USA) were maintained in a chemically defined medium, CS-C completed (Cell Systems, Kirkland, WA, USA) and RPMI (Invitrogen, Grand Island, NY, USA), respectively, supplemented with 5% fetal bovine serum. In the pretreatment of rapamycin and chemical inhibitors for 3 h, the cells were

cultured in 5% RPMI, and then exchanged the medium and treated the cells with IFN-α2b at the indicated time.

Cell viability assay

The cells were measured using the colorimetric cell viability assay method. Cell viability was determined by the colorimetric method using a Cell Counting kit (Wako Life Science, Osaka, Japan). The absorbance of each well was measured at 405 nm with a microtiter plate reader (Multiskan JX, Thermo BioAnalysis Co., Japan). After 2 days of 100 IU/mL IFN-α and 1000 nmol/L rapamycin treatment, Cell viability is expressed as a percentage of the viability in standard media without IFN-α and rapamycin. Data were expressed as the mean ± standard deviation (SD). Statistical significance was assessed using Student's *t* test. Statistical difference was defined as $P < 0.05$. All numerical results were reported as the mean of four independent experiments.

Western blotting and antibodies

Western blotting with anti-PKR, anti-STAT-1 (Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-tyrosine-701 phosphorylated STAT-1, anti-serine-727 phosphorylated STAT-1, anti-p48, anti-serine-437 phosphorylated Akt, anti-threonin-308 phosphorylated anti-Akt, anti-Akt, anti-serine-2448 phosphorylated mTOR, anti-serine-2481 phosphorylated mTOR, anti-mTOR, anti-JAK-1 or anti-tyrosine 1022/1023 JAK-1 (Cell Signaling, Beverly, MA, USA) was performed as described previously [9]. Briefly, Hc cells were lysed by the addition of a lysis buffer (50 mmol/L Tris–HCl, pH 7.4, 1% Np40, 0.25% sodium deoxycholate, 0.02% sodium azide, 0.1% SDS, 150 mmol/L NaCl, 1 mmol/L EDTA, 1 mmol/L PMSF, 1 μg/mL each of aprotinin, leupeptin and pepstatin, 1 mmol/L sodium o-vanadate and 1 mmol/L NaF). The samples were separated by electrophoresis on 8–12% SDS polyacrylamide gels and electrotransferred to nitrocellulose membranes, and then blotted with each antibody. The membranes were incubated with horseradish peroxidase-conjugated anti-rabbit IgG or anti-mouse IgG, and the immunoreactive bands were visualized using the ECL chemiluminescence system (Amersham Life Science, Buckinghamshire, England).

Fluorescence immunohistochemistry

The Hc cells were seeded onto 11-mm glass cover-slips in 24-well plates at 2.4×10^5 cells/well. The next day, the medium was replaced with serum-free medium, and the cells were pretreated with 10 or 100 nmol/L rapamycin, or vehicle, for 3 h and then stimulated with 100 IU/mL IFN-α for 10 min. Fluorescence immunohistochemistry was performed as described previously [17]. The cells were incubated with anti-tyrosine-701 phosphorylated STAT1 antibody for 1 h at room temperature, washed three times in PBS, incubated with rhodamine-conjugated donkey anti-rabbit IgG (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA, USA) for 1 h, washed in PBS, and mounted in Vectashield Mounting Medium (Vector Laboratories Inc., Burlingame, CA, USA). Nuclear staining was performed using Hoechst 33258 (Invitrogen Japan K.K., Tokyo, Japan). An immunofluorescence analysis was done using an Olympus BX50 microscope (Tokyo, Japan) and the image was captured by a Nikon DXM 1200 digital camera (Tokyo, Japan).

Reporter gene assay

A pISRE-Luc cis-reporter plasmid containing five copies of the ISRE sequence and the firefly luciferase gene and pRL-SV40 containing the SV40 early enhancer/promoter and the renilla luciferase gene were obtained from Clontech (San Diego, CA, USA) and Promega (Madison, WI, USA), respectively. The HuH-7 cells were grown in 24-well multiplates and transfected with 1 μg of pISRE-Luc and 10 ng of pRL-SV40 as a standard by the lipofection method. One day later, the cells were incubated in the absence or presence of varying concentrations of chemical blockers and IFN-α, and the luciferase activities in the cells were determined using a dual-luciferase reporter assay system and a TD-20/20 luminometer (Promega). The data were expressed as the relative ISRE-luciferase activity.

HCV replicon system

OR6 cells stably harboring the full-length genotype 1 replicon, ORN/C-5B/KE [18], were used to examine the influence of the anti-HCV effect of IFN. The cells were cultured in Dulbecco's modified Eagle's medium (Gibco-BRL, Invitrogen) supplemented with 10% fatal bovine serum, penicillin and streptomycin and maintained in the presence of G418 (300 mg/L; Geneticin, Invitrogen). This replicon was derived from the 1B-2 strain (strain HCV-o, genotype 1b), in which the *Renilla* luciferase gene is introduced as a fusion protein with neomycin to facilitate the monitoring of HCV replication. After the treatment, the cells were harvested with *Renilla* lysis reagent (Promega, Madison, WI, USA) and then were subjected to a luciferase assay according to the manufacturer's protocol. mTOR gene knock down is used siRNA (Cell Signaling). 100 nmol/L mTOR specific and non-targeted siRNA as a control was transfected to OR6 cells in accordance with the appended manual. One day later, the cells were incubated in either the absence or presence of 10 IU/mL IFN-α.

Results

IFN-α-induced activity of STAT-1 is inhibited by rapamycin pretreatment

To attempt to clearly identify the influence of mTOR to IFN-α-induced anti-viral protein expression rapamycin (rapa), the specific inhibitor of mTOR, was added prior to treatment with IFN-α. Hc cells have been used as normal hepatocytes in previous reports [19]. The Hc cells were incubated in the absence or presence of IFN-α with or without pretreatment with rapa for 2 h the cells were then harvested for the Western blot analysis (Fig. 1). IFN-α clearly induced tyrosine and serine phosphorylation of STAT-1 at 5 (Fig. 1a, lane 4) and 10 min (Fig. 1a, lane 6), respectively, in the absence of rapa. However, when the Hc cells were pretreated with rapa before IFN-α stimulation, the levels of tyrosine and serine phosphorylated STAT-1 were clearly and rapidly lower than those induced by IFN-α alone 5 min after treatment in tyrosine (Fig. 1a, lane 5). Jak-1, an upstream protein of STAT-1, was equally phosphorylated by IFN-α with (Fig. 1b, lane3) or without (Fig. 1b, lane2) pretreatment with rapa. The viability of the Hc cells was 1 in vehicle, 0.93±0.21 in IFN-α treatment and 0.88±0.34 in rapamycin treatment. No difference in the cell viability the among vehicle, IFN-α and rapamycin treatment was not recognized in our assay. The viability of the HuH-7 and OR6 cells also demonstrated no difference between the presence of IFN-α and rapamycin treatment and the absence thereof.

IFN inducible gene products are diminished by pretreatment of rapamycin

Since pretreatment with rapa inhibited the IFN-α induced STAT-1 activity, the phosphorylation of tyrosine and serine and nuclear translocation, the effect of pretreated with rapa on the IFN-α inducible gene product was examined. The protein levels of PKR, an anti-viral protein that acts as a mRNA translation inhibiter activated by double stranded RNA [20, 21], and p48, key component of ISGF-3 with activated STAT-1 and -2 [22], were induced by IFN-α treatment for 3 h in Hc cells (Fig. 1c, lanes 1, 2). However, pretreatment with rapa had an inhibitory effect on IFN-α-induced PKR and p48 in a dose dependent manner (Fig. 1c, lanes 2–4).

The serine 473 on Akt and serine 2448 on mTOR are phosphorylated by IFN-α

Because pretreatment with rapa affected the IFN-α signaling (Fig. 1), the ability of IFN-α to activate the Akt-mTOR pathway was investigated. The phosphorylation of serine-2448 residues of mTOR and serine-473 residue of

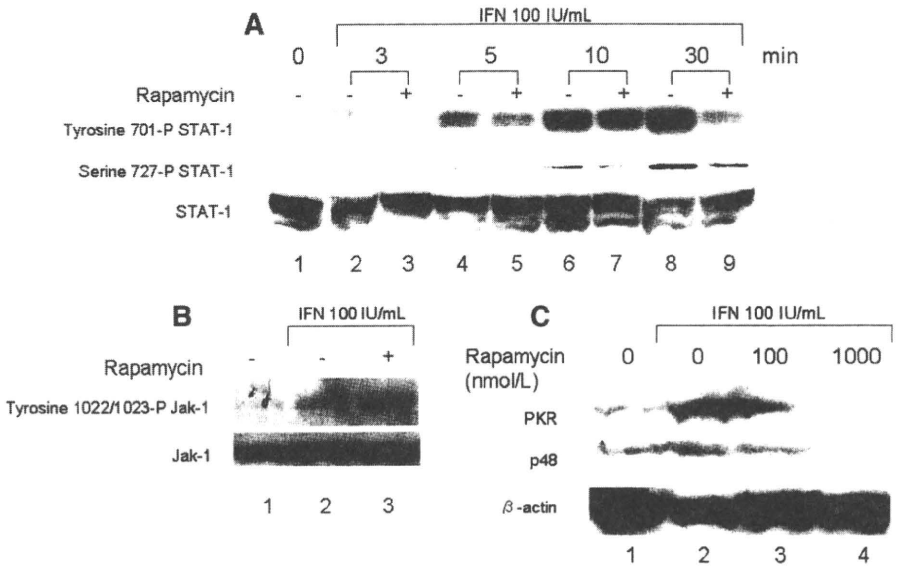

Fig. 1 Alteration in the distribution of IFN-α induced phosphorylated STAT-1 (**a**) and Jak-1 (**b**) by rapamycin and effect of rapamycin on IFN-α-induced PKR and p48 (**c**). Hc cells were pretreated without (*lanes 1, 2, 4, 6,* and *8*) or with 1 μmol/L rapa (*lanes 3, 5, 7,* and *9*). These Hc cells were stimulated by 100 IU/L IFN-α (*lane 2–9*) for 30 min. Phosphorylated STAT-1 at tyrosine-701 residue (*upper panel*) and at serine-727 residue (*lower panel*) were analyzed by Western blotting. **a** After pretreatment of 1000 nmol/L rapa (*lane 3*) for 3 h, Hc cells were untreated (*lane 1*) or treated with 100 IU/mL IFN-α (*lanes 2, 3*) for 3 min, then phosphorylated JAK-1 at tyrosine-1022/1023 residue (*first panel*), expression of JAK-1 (*second panel*) were analyzed by Western blotting (**b**). Hc cells were treated with 100 IU/mL of IFN-α in the absence (*lane 2*) or of the presence of pretreatment (*lane 3, 4*). *Lane 1* was not treated IFN-α and calcineurin inhibitors. One day latter, PKR and p48 was determined by Western blotting (**c**)

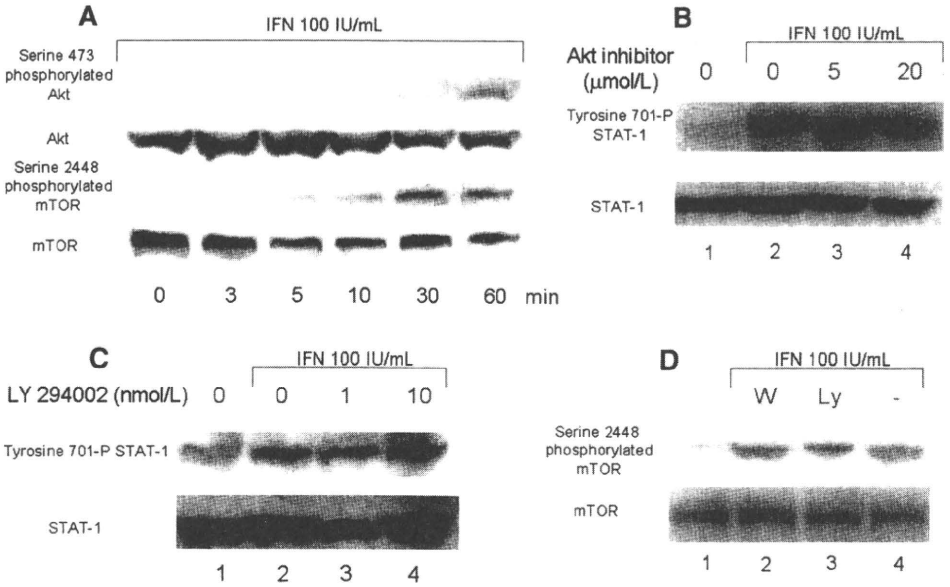

Fig. 2 Effect of IFN-α on Akt and mTOR (**a**) and effect of Akt inhibitor (**b**) and LY294002 (**c**) on IFN-α-induced tyrosine phosphorylated STAT-1 and Serine phosphorylated mTOR (**d**). Hc cells were stimulated by 100 IU/L IFN-α for 60 min. At the indicated time, the cells were harvested. Phosphorylated Akt at serine-473 residue (*first panel*), Akt (*second panel*), mTOR at serine-2448 residue (*third panel*) and mTOR (*fourth panel*) were analyzed by Western blotting. After pretreatment with 5 or 20 μmol/L Akt inhibitor (*lane 3*, and 4, respectively) (**b**) and 1 or 10 nmol/L LY294002 (*lane 3* and *4*, respectively) (**c**) for 3 h, Hc cells were untreated (*lane 1*) or treated with 100 IU/mL IFN-α (*lanes 2–4*) for 5 min and phosphorylated STAT-1 at tyrosine-701 residue (*first panel*), expression of STAT-1 (*second panel*) were analyzed by Western blotting. **d** After pretreatment with 100 nmol/L wortmannin (*lane 2*) and 1 nmol/L LY294002 (*lane 3*) for 3 h, the Hc cells were either untreated (*lane 1*) or treated with 100 IU/mL IFN-α (*lanes 2–4*) for 10 min and then were phosphorylated mTOR at Serine-2448 residue (*first panel*), the expression of mTOR (*second panel*) was analyzed by Western blotting

Akt by 100 IU/ml of IFN-α was detected at 5 min and at 60 min after IFN-α treatment, respectively (Fig. 2a). The band intensity of serine 2448 phosphorylated mTOR increased at 30 min and decreased at 60 min after IFN-α treatment. In contrast, a slight band intensity of serine phosphorylated 473 Akt was only detected at 60 min after IFN-α treatment. In addition, a Western blot analysis of phosphorylated serine 2481 of mTOR and threonine 308 Akt was conducted under the same conditions as Fig. 2a, but no bands were detected (data not shown). In Fig. 2d, IFN-α-induced Serine 2448 phosphorylated mTOR was not inhibited by PI3-K inhibitors (lanes 2, 3).

The IFN-α-induced nuclear translocation of tyrosine phosphorylated STAT-1 was inhibited by pretreatment with rapa

The location of tyrosine phosphorylated STAT-1 was evaluated by fluorescence immunohistochemistry of cultured Hc cells (Fig. 3). The IFN-α-induced nuclear translocation of tyrosine phosphorylated STAT-1 was observed (Fig. 3c), but its translocation was inhibited by pretreatment with rapa and the inhibition of the translocation of STAT-1 was more definitive at 1000 nmol/L rapa (Fig. 3e) than 100 nmol/L (Fig. 3g).

IFN-α-induced ISRE-contained promoter activity is inhibited by pretreatment of rapa, but not by wortmannin, LY294002 and Akt inhibitor

The influence of pretreatment of PI3-K-Akt-mTOR inhibitors on IFN-α inducible luciferase activity of the ISRE-containing promoter was examined. Since Hc cells were not sufficient for reporter gene transfection, HuH-7 cells were used in the transfection assay. HuH-7 cells were transfected with pISRE-Luc containing five repeats of the ISRE sequence and pRV-SV40 as a standard and then were treated with IFN-α after 3 h with or without pretreatment with rapa, wortmannin, LY294002 or Akt inhibitor. Rapa inhibited IFN-α inducible luciferase activity in a dose-dependent manner (Fig. 4, lane 2–4). However, wortmannin and LY294002, PI3-K inhibitor, and Akt inhibitor had no effect on IFN-α inducible luciferase activity (Fig. 4, lanes 2, 5–7).

The expression of IFN-α-induced tyrosine phosphorylated STAT-1 was determined after pretreatment with Akt inhibitor and LY294002 to evaluate the result of luciferase assay (Fig. 4). The Hc cells were incubated under the same conditions used in Fig. 4, but phosphorylated STAT-1 was not inhibited by the Akt inhibitor (Fig. 2b) and LY294002 (Fig. 2c).

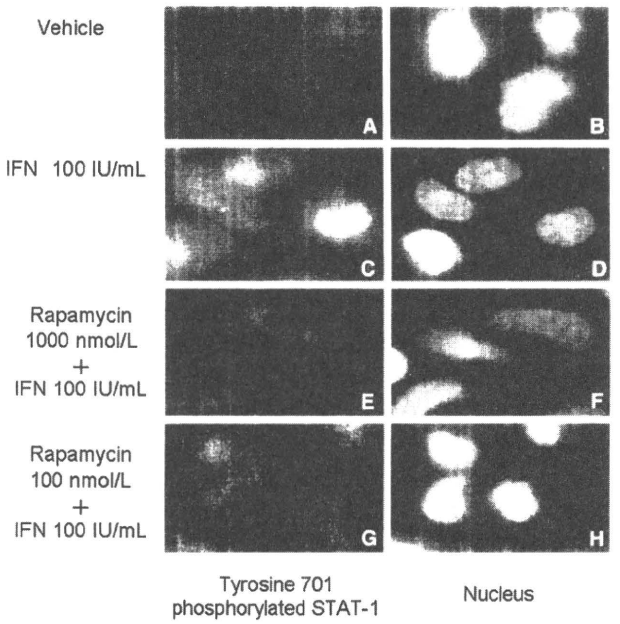

Fig. 3 Inhibition of IFN-α-induced nuclear translocation of phosphorylated STAT-1 by rapamycin. The Hc cells were pretreated without (**a–d**) or with 1000 nmol/L rapa (**e**, **f**) or 100 nmol/L rapa (**g**, **h**). After pretreatment, the Hc cells were stimulated by 100 IU/L IFN-α (**c–h**) for 30 min. Thereafter, the cells were fixed, permeabilized, processed for immunofluorescence (**a**, **c**, **e**, **g**) and Hoechst staining (**b**, **d**, **f**, **h**), and visualized by fluorescence microscopy. The results shown are from one representative experiment from a total of three performed

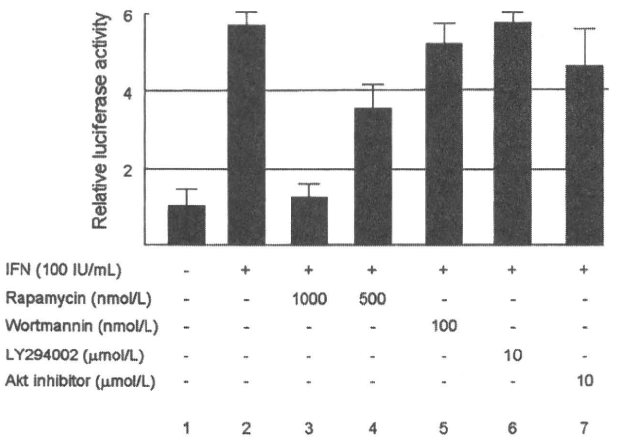

Fig. 4 Suppression effect of rapamycin, not PI3-k inhibitors and Akt inhibitor, on IFN-α-induced reporter gene assay. HuH-7 cells transfected with reporter gene (pISRE-Luc and pRL-SV40) were either untreated (*lane 1*) or pretreated with rapa (*lane 3*, *4*), wortmannin (*lane 5*), LY294002 (*lane 6*) or Akt inhibitor (*lane 7*) for 3 h, followed by IFN-α 100 IU/mL (*lanes 2–7*). Six hour later, the relative ISRE-luciferase activity ($n = 4$) was determined as described in the "Materials and methods". The data are expressed as the mean ± SD and are representative example of four similar experiments

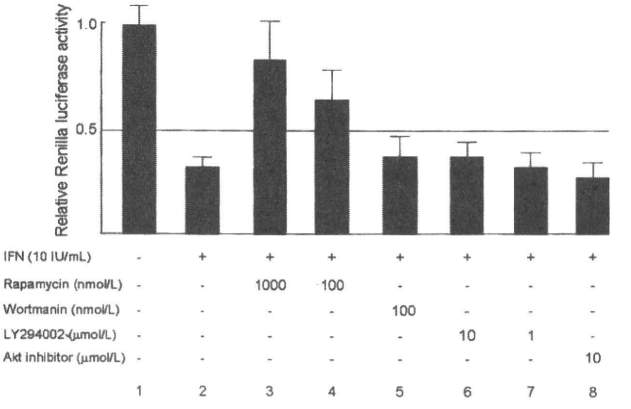

Fig. 5 Alternation of IFN-α suppressed HCV replication by rapamycin, but not PI3-K inhibitors and Akt inhibitor. OR6 cells, a full-length replicon system, were treated with 100 IU/mL of IFN-α in the absence (*lane 2*) or presence of pretreatment (*lanes 3–8*) for 3 h. *Lane 1* was not treated IFN-α alone. One day latter, *Renilla* luciferase activity was determined by luminometer ($n = 4$). The data are expressed as the mean ± SD and are representative example of four similar experiments

Rapamycin and mTOR specific siRNA, but not PI3-K inhibitor and Akt inhibitor can cancel the IFN-α-induced anti-HCV replicon activity

OR6 cells the full-length HCV replication system was used to examine the anti-viral effect of PI3-K, Akt and mTOR on IFN-α stimulation. The cells were treated with IFN-α after 3 h in the presence or absence of rapa, Akt inhibitor or PI3-K inhibitor (Fig. 5). Pretreatment with rapa attenuated its anti-HCV replication effect in comparison to IFN-α alone (Fig. 5, lanes 1–4), whereas pretreatment with PI3-K inhibitors and Akt inhibitor did not increase the *Renilla* luciferase activity (Fig. 5, lanes 1, 2, 5–8). We performed siRNA transfection for mTOR knock down (Fig. 6). Although transfection efficiency of siRNA is barely 10%, IFN-α-induced anti-HCV action was clearly inhibited in siRNA against mTOR transfected cells (lane 5) in comparison to the control cells (lane 6).

Discussion

Rapa inhibited the IFN-α-induced tyrosine and serine phosphorylation and nuclear translocation of STAT-1, the ISRE-promoter activity, the expression of PKR and the replication of HCV replicon. This suggests that the IFN-induced mTOR activity, through Jak independent STAT-1 phosphorylation, is a critical signal for IFN-induced anti-HCV action. Interestingly, mTOR activated by IFN was PI3-K-Akt independent in this study.

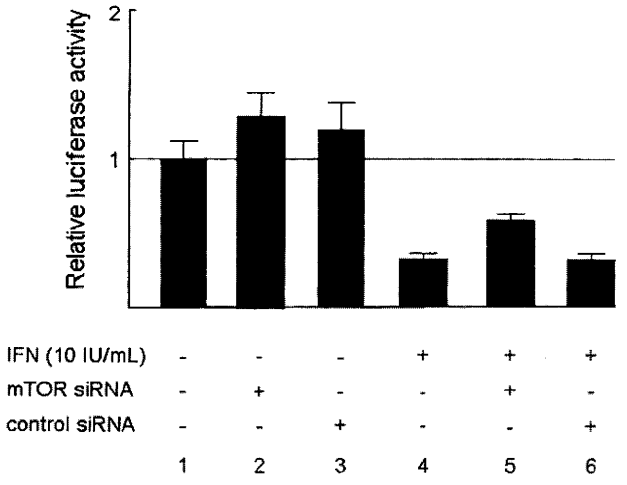

Fig. 6 Alternation of IFN-α suppressed HCV replication by siRNA against mTOR. The OR6 cells were transfected the siRNA against mTOR (*lanes 2, 5*) and the non-targeted siRNA (*lanes 3, 6*). One day later, the cells were IFN-α treatment (*lanes 4–6*). HCV replicon assay is same as Fig. 5. The data are expressed as the mean ± SD and are representative example of four similar experiments

mTOR activity may have an inhibitory action on HCV replication through STAT-1 phosphorylation, but not the translation initiation action of mTOR. This study assumed that IFN-induced PKR expression and ISRE-luciferase activity were inhibited by rapa as the result of a suppression effect on IFN inducible STAT-1 activation. IFN inducible PKR contributes the anti-HCV action [20], and anti-HCV action of ribavirin is also attributable to its ability to up-regulate PKR activity [21]. Previous reports revealed that the mTOR activity did not influence the HCV-IRES activity because the viral promoter has cap-independent translation [23]. Although mTOR is the mRNA translational regulator through phosphorylation of a downstream target such as 4E-BP and S6K [24], we think that the IFN-induced mTOR activity influences the phosphorylation of STAT-1 in our study (Fig. 1). In addition, it is thought that the alternation of STAT-1 phosphorylation by the mTOR activity influences the gene expression of anti-virus protein and IFN-induced anti-viral action.

In our study, serine-473 on Akt showed a delayed phosphorylation in comparison to that of serine-2448 on mTOR after IFN stimulation (Fig. 2a). Since serine-473 on Akt is phosphorylated by mTOR/Rictor/GβL [25, 26] and a PDK-1 independent pathway [25], IFN-induced serine-473 phosphorylated Akt may not involve the mTOR activity. Therefore, PI3-K inhibitor and Akt inhibitor had no effect on IFN inducible anti-HCV action. The pathway of mTOR activation is prismatic. PI-3Ks, upstream kinase of Akt and mTOR, are grouped into three classes (I–III), according to their substrate preference and sequence homology [27]. PI3-k inhibitor, wortmannin and LY294002, inhibit class I and III PI3-Ks, and to a lesser extent class II PI3-K, upstream kinase of Akt [27]. In our study, neither PI3-K nor Akt inhibitor inhibited IFN-induced ISRE luciferase activity and loss of HCV replication (Figs. 4, 5). These results indicate that the IFN-induced anti-HCV activity is mTOR dependent, but not PI3-K and Akt dependent. In the current report, the production of IL-1 receptor antagonist in IFN-stimulated monocytes depends on the PI3-K pathway, but not STAT-1 [28], and chronic myelogenous leukemia cells are differentially regulated by the IFN-induced PI3-K-Akt-mTOR pathway with no relation to STAT-1 phosphorylation [29]. Similar to the findings of those reports, the PI3-K-Akt pathway has been reported to be generally independent of the STAT activity [10]. Therefore, the difference in the cell type [8] may explain the discrepancy between these data and our data. We therefore speculate that in hepatocytes, unlike lymphoid cells, IFN-induced mTOR activity is not dependent on the PI3-K activity. In addition, the mTOR activity is not related to the STAT activity in lymphoid cells. However, in hepatocytes, the IFN-induced mTOR activity was closely linked to the IFN-induced STAT activity in our study.

mTOR is a serine and threonine kinase [10]. Phosphorylation of STATs by mTOR occurs also on a serine residue, but not tyrosine [10, 30]. The mTOR pathway is critical for IFN-γ-induced suppression of tyrosine phosphorylated STAT-3 in a prostate cancer cell line [31]. Although this is not consistent with the results of our study, this also showed mTOR to be associated with tyrosine phosphorylation without reference to SOCS and phosphatase. In addition, in a mouse embryo fibroblast cell line, IFN-γ-induced tyrosine and serine phosphorylation of STAT-1 is inhibited by rapa [32], while in the hepatoma cell line, HLF, IFN-β stimulated STAT-1 tyrosine phosphorylation partially decreases by LY294002, but the effect of rapa has not yet been studied [33]. In the current study [31–33], not only STAT-1 serine phosphorylation but also tyrosine was found to be downstream of the IFN induced mTOR activity; however, the mechanism controlling the tyrosine phosphorylation of STAT-1 and the mTOR activity, remains to be elucidated.

In conclusion, IFN-induced mTOR activity, independent of PI3-K and Akt, is the critical factor for anti-HCV action. The Jak independent mTOR activity is, therefore, involved in STAT-1 phosphorylation and nuclear location, thus resulting in the development of IFN-induced anti-HCV protein, especially the expression of PKR, in HCV-infected hepatocytes.

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Original Article

Predictive value of suppressor of cytokine signal 3 (*SOCS3*) in the outcome of interferon therapy in chronic hepatitis C

Hisamitsu Miyaaki, Tatsuki Ichikawa, Kazuhiko Nakao, Toshihisa Matsuzaki, Toru Muraoka, Takuya Honda, Shigeyuki Takeshita, Hidetaka Shibata, Eisuke Ozawa, Motohisa Akiyama, Satoshi Miuma and Katsumi Eguchi

The First Department of Internal Medicine, Nagasaki University School of Medicine, Sakamoto, Nagasaki, Japan

Aims: Suppressor of cytokine signaling 3 (*SOCS3*) can suppress Janus kinase (JAK)-signal transducers and activators of transcription (STAT) signaling by blocking an IFN-induced protein. In this study, the relationship between *SOCS3* and phosphorylation of STAT1 in the liver and outcome of interferon therapy were examined.

Methods: Prior to interferon treatment, we immunostained for *SOCS3* and phosphorylated-STAT1 (P-STAT1) in 59 liver specimens from chronic hepatitis C virus (CHC) patients and compared the expression of *SOCS3* and clinicopathological factors. Fifty-one patients were receiving peg-interferon alpha-2b and ribavirin therapy and also compared interferon therapy effect and the expression of *SOCS3*.

Results: Immunostaining for *SOCS3* was mainly seen in the periportal area. The concentration of P-STAT1 nuclei was significantly larger in specimens with < 30% area immunostaining to *SOCS3* than those in which this area was ≥ 30% (10.6 ± 8.8 vs. 4.6 ± 6.1, $P = 0.004$). *SOCS3* immunostaining score was significantly correlated with aspartate amino transferase ($r = 0.373$, $P = 0.003$), alanine amino transferase ($r = 0.337$, $P = 0.008$), platelets ($r = -0.273$, $P = 0.037$), and homeostatic model assessment ($r = 0.339$, $P = 0.008$). On univariate analysis and multivariate analysis, *SOCS3* immunostaining score (0 or 1) and age (<60 years old) were significant predictors of interferon response (odds ratio 10.888; $P = 0.010$; odds ratio 3.817, $P = 0.045$ respectively).

Conclusion: *SOCS3* expression in the liver prior to interferon therapy was correlated with increased insulin resistance and might be a useful predictor of HCV clearance by interferon therapy.

Key words: hepatic C virus, insulin resistance, interferon, signal transducers and activators of transcription, suppressor of cytokine signaling 3

INTRODUCTION

HEPATITIS C VIRUS (HCV) infects approximately 200 million people worldwide. In Japan, about 2 million people are chronically infected, and HCV is the leading cause of hepatocellular carcinoma. The current standard care for chronic hepatitis C virus (CHC) is peginterferon-α and ribavirin. This treatment is effective in approximately 50% of patients but has numerous adverse effects.[1-5]

The Janus kinase (JAK)-signal transducers and activators of transcription (STAT) pathway is critical in interferon's antiviral effect. This is because STATs are essential to trigger antiviral and cytoprotective mechanisms within the HCV-infected cell.[6-10] We previously reported that the phosphorylation level of STAT1, which is correlated with BMI and insulin resistance, impairs response to interferon treatment and might be a useful predictor of HCV clearance by interferon therapy.[11] The mechanisms by which phosphorylation of STAT-1 interferes with this IFN signaling have not been fully elucidated.

HCV infection leads to endogenous IFN production and also to increased expression of suppressor of cytokine signaling 3 (*SOCS3*), either directly by viral proteins or indirectly though IFN inhibitory factors.[12,13] *SOCS3* can suppress JAK-STAT signaling by blocking the IFN-induced formation of ISGF3.[14] Previous studies have reported that *SOCS3* is induced by various adipocytokines such as tumor necrosis factor (TNF-α) and that this is associated with a poorer treatment outcome.[15,16]

Correspondence: Dr Hisamitsu Miyaaki, The First Department of Internal Medicine, Nagasaki University School of Medicine, 1-7-1 Sakamoto, Nagasaki 852-8501, Japan. Email: miyaaki-hi@umin.ac.jp

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In this study, we used immunohistochemical techniques to examine the relationship between *SOCS3* and phosphorylation of STAT1 in the liver and outcome of interferon therapy.

PATIENTS AND METHODS

NEEDLE BIOPSIES OF the liver were obtained from 59 patients with positive hepatitis C virus (HCV) antibody prior to interferon treatment in Nagasaki University Hospital and associated hospitals. All patients underwent assessment of baseline clinical and biological parameters, including HCV load, which was determined by assaying HCV core protein (Eiken chemical, Tokyo, Japan). The degree of insulin resistance was determined by the homeostatic model assessment (HOMA) using the formula: insulin resistance = insulin (μU/mL) × glucose (mg/dL)/405. Clinical data are summarized in Table 1. Liver biopsy was performed by needle puncture for diagnostic purposes. The diagnosis of each case was independently confirmed histologically by liver pathologists according to the Japanese chronic hepatitis classification (New Inuyama classification). According to this classification, mild activity was defined as A0 or A1, severe activity as A2 or A3, mild fibrosis as F0 or F1, and severe fibrosis as F2, F3, or F4.

Fifty-one patients received peg-interferon alpha-2b (Schering-Plough, Tokyo, Japan) and ribavirin (Schering-Plough) therapy. Those with genotype 1 ($n = 39$) were treated for 48 weeks, and those with genotype 2 ($n = 12$) were treated for 24 weeks. The patients treated with the reduced dose of peg-interferon or ribavirin by more than 20% were excluded from the study. Peginterferon alpha-2b (1.5 μg/kg) was administered once per week, and ribavirin dose was titrated according to body weight. Sustained virological response (SVR) was defined as undetectable HCV RNA at 6 months after the end of interferon treatment.

Table 1 Clinical data from 59 HCV-positive patients who underwent liver biopsy

Age (years)	59.4 ± 9.9
Sex (male : female)	32:27
AST (IU/L)	71 ± 36
ALT (IU/L)	97 ± 55
Plt (×10⁴/uL)	16.7 ± 4.8
BMI (kg/m²)	22.4 ± 2.4
HOMA-IR	1.90 ± 0.87
LDL (mg/dL)	102 ± 21
HCV genotype (1:2)	46:13
HCV viral load core protein (fmol/L)	6004 (20–24200)
Interferon response ($n = 51$) (SVR: no SVR)	29:22

ALT, alanine amino transferase; AST, aspartate amino transferase; BMI, body mass index; HCV, hepatitis C virus; HOMA homeostatic model assessment; LDL, low-density lipoprotein; Plt, platelate; SVR, sustained virological response.

P-STAT1 and *SOCS3* immunohistochemistry

All tissue was fixed in 10% neutral buffered formalin and was then embedded in paraffin, and 4-μm-thick serial sections were cut from each paraffin block. In the immunohistochemical study, anti-phosphor STAT1 (Tyr701) antibody (dilution 1:100; Cell Signaling Technology, Beverly, MA, USA) was used for evaluation of P-STAT1 and anti-*SOCS3* (dilution 1:100, Affinity BioReagents, Rockford, IL, USA) was used for *SOCS3*. Immunohistochemistry was performed with the labeled streptavidin biotinylate antibody (LSAB) method and a commercially available kit (Histofine SAB-PO; Nichirei, Tokyo, Japan). The number of P-STAT1 positive nuclei was counted in liver specimens and the number of positive nuclei per 10 mm² was calculated. The area immunostaining for *SOCS3* was semiquantitatively scored according to the number of immunoreactive cells per unit area. Immunoreactive cases were further subclassified as follows: score 0, < 5% of the cells stained; score 1+, 5–30% of the cells stained; score 2+, 30–50% of the cells stained; and score 3+, > 50% or more of the cells stained.

Statistical analysis

The SPSS 9.0 for Windows (Microsoft, Redmond, WA, USA) statistical software program was used to assess correlations among multiple variables. When appropriate, clinical and laboratory data were compared with Student-*t* test or the Mann–Whitney *U*-test. A *P*-value of < 0.05 was considered to be statistically significant.

RESULTS

Comparison of p-STAT1 and *SOCS3* expression in the liver (Figs 1,2)

THE AVERAGE CONCENTRATION of P-STAT1 nuclei was 10.6 ± 9.9/10 mm². Immunostaining for *SOCS3* was mainly seen in the periportal area (Fig. 1). *SOCS3* immunostaining scores were as follows: 0, $n = 11$; 1, $n = 19$; 2, $n = 16$; and 3, $n = 13$. Concentration of P-STAT1 nuclei was significantly inversely correlated with *SOCS3* immunostaining score ($r = -0.372$, $P = 0.004$). Concentration of P-STAT1 nuclei was signifi-

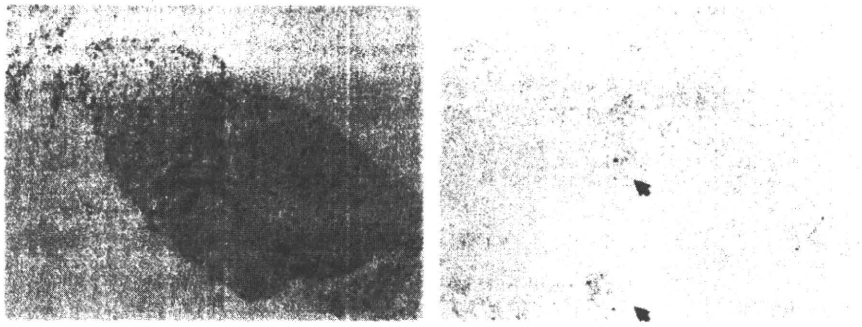

Figure 1 (a) Immunostaining for *SOCS3* (brown) was mainly seen in the periportal area. (b) Immunohistochemical detection of phosphorylated-signal transducer and activator of transcription (P-STAT) 1. The arrows indicate P-STAT-positive hepatocyte nuclei.

cantly greater in specimens showing *SOCS3* immunostaining score 0 or 1 than in those with scores of 2 or 3 (10.6 ± 8.8 vs. 4.6 ± 6.1; $P = 0.004$) (Fig. 2).

Correlation between *SOCS3* immunostaining and clinical factors (Tables 2,3)

SOCS3 immunostaining score did not differ significantly between either sex or HCV genotype. However, it did correlate significantly with AST ($r = 0.373$, $P = 0.003$), ALT ($r = 0.337$, $P = 0.008$), PLT ($r = -0.273$, $P = 0.037$), and HOMA ($r = 0.339$, $P = 0.008$). *SOCS3* immunostaining score was significantly higher in patients with insulin resistance (HOMA ≥ 2) than in patients without insulin resistance (HOMA < 2) (2.1 ± 0.9 vs. 1.1 ± 0.9; $P = 0.001$). No significant correlations were observed between *SOCS3* immunostaining score and other clinical factors (age, body mass index, low-density lipoprotein, or HCV viral load).

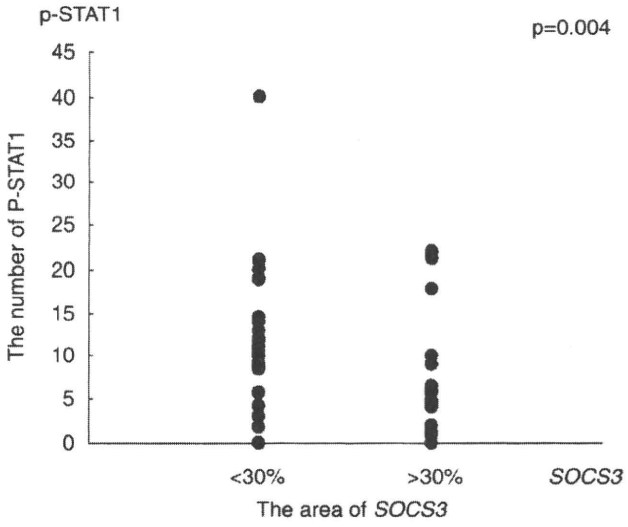

Figure 2 Correlation of P-STAT1 and *SOCS3* immunostaining score. Concentration of P-STAT1 nuclei was significantly higher in specimens with *SOCS3* immunostaining score 0 or 1 than in those with score 2 or 3 using Mann–Whitney *U*-test (10.6 ± 8.8 vs. 4.6 ± 6.1, $P = 0.004$).

Comparison of *SOCS3* expression and pathological factors (Table 3)

SOCS3 immunostaining score differed significantly according to both hepatitis activity level and fibrosis (mild grade vs. severe grade = 1.4 ± 0.5 vs. 1.9 ± 0.3; $P = 0.010$; mild stage vs. severe stage = 1.3 ± 0.5 vs. 1.8 ± 0.4, $P = 0.001$).

Table 2 Correlation of *SOCS3* immunostaining score with each parameter

		P-value†
Age	r = 0.200	0.094
AST	r = 0.373	0.003
ALT	r = 0.337	0.008
Plt	r = −0.273	0.037
BMI	r = 0.151	0.254
HOMA	r = 0.339	0.008
LDL	r = −0.051	0.708
Viral load	r = 0.510	0.702

†Correlations between the groups were determined by the Spearman analysis.
ALT, alanine amino transferase; AST, aspartate amino transferase; BMI, body mass index; HOMA homeostatic model assessment; LDL, low density lipoprotein; Plt, platelate.

Table 3 Imuunostaining score of *SOCS3* in each group

		P-value†
Sex (male : female)	1.7 ± 0.8 : 1.3 ± 1.2	0.071
Genotype (1:2)	1.5 ± 1.0 : 1.4 ± 1.1	0.723
Grading (mild : severe)	1.4 ± 0.5 : 1.9 ± 0.3	0.010
Staging (mild : severe)	1.3 ± 0.5 : 1.8 ± 0.4	0.001

†Differences between the groups were analyzed using Mann–Whitney U-test.

Table 4 On univariate analysis, factors associated with response to treatment (among 51 patients who underwent treatment)

	SVR ($n = 29$)	Non-SVR ($n = 22$)	*P*-value
Sex (female)	10	12	0.250
Age (<60 y)	19	8	0.033
AST (≥70 IU/L)	11	10	0.890
ALT (≥70 IU/L)	16	11	0.933
Plt ($>1.5 \times 10^3$)	18	9	0.133
BMI (≥25 kg/mm^2)	10	8	0.875
HOMA (≥2)	10	13	0.142
LDL (≥100 mg/dL)	21	12	0.186
Genotype (1b)	19	20	0.077
Viral load (≥300 fmol/L)	24	22	0.263
Grade (severe)	3	5	0.414
Stage (severe)	8	11	0.080
SOCS3 (<30%)	20	4	0.0009

ALT, alanine amino transferase; AST, aspartate amino transferase; BMI, body mass index; HOMA homeostatic model assessment; LDL, low density lipoprotein; Plt, platelate; SVR, sustained virological response.

Correlation between *SOCS3* expression and viral clearance (Tables 4–7)

Of the 51 patients receiving peg-interferon alpha-2b + ribavirn therapy, 26 achieved SVR. *SOCS3* immunostaining score in the SVR group was significantly lower than in those who did not achieve SVR (1.7 ± 1.1 vs. 2.5 ± 1.1; $P = 0.021$). On univariate analysis and multivariate analysis, *SOCS3* immunostaining score (0 or 1) and age (<60 years old) were significant predictors of SVR (*SOCS3* immunostaining score: odds ratio 10.888; $P = 0.001$; age: odds ratio 3.817; $P = 0.045$). Of the 39 cases with genotype 1, *SOCS3* immunostaining score (0 or 1) and age (<60 years old) were also significant predictors of SVR (*SOCS3* immunostaining score: odds ratio 13.740; $P = 0.003$; age: odds ratio 6.658, $P = 0.033$).

Table 5 On univariate analysis, factors associated with response to treatment of patients with genotype1

	SVR ($n = 19$)	Non-SVR ($n = 20$)	*P*-value
Sex (female)	8	10	0.621
Age (<60 years)	13	6	0.030
AST (≥70 IU/L)	8	10	0.862
ALT (≥70 IU/L)	11	10	0.862
Plt ($>1.5 \times 10^3$)	12	8	0.260
BMI (≥25 kg/mm^2)	7	8	0.899
HOMA (≥2)	6	11	0.249
LDL (≥100 mg/dL)	13	9	0.339
Viral load (≥300 fmol/L)	14	18	0.326
Grading (severe)	3	5	0.752
Staging (severe)	6	9	0.594
SOCS3 (<30%)	14	4	0.002

ALT, alanine amino transferase; AST, aspartate amino transferase; BMI, body mass index; HOMA homeostatic model assessment; LDL, low density lipoprotein; Plt, platelate; SVR, sustained virological response.

Table 6 On multivariate analysis, factors associated with responder to treatment

	Odds ratio	*P*-value
SOCS3 (<30%)	10.888	0.001
Age (<60 y)	3.817	0.045

SOCS3, suppressor of cytokine signal 3.

DISCUSSION

RECENT IMPROVEMENTS IN the efficiency of antiviral therapy have led to 50% of patients with HCV genotype 1 achieving sustained viral clearance.[1–5] However, some patients are refractory to interferon therapy. Previous studies have reported *SOCS3* as a factor associated with non-response to treatment.[15,16] Our results show that *SOCS3* expression in the liver could be a simple and useful predictor of response to interferon treatment.

Several recent reports have stated that HCV infection *per se* plays a role in *SOCS3* gene regulation and that HCV core protein directly affects *SOCS3* regulation.[12,13] In the present study, multivariable analysis identified age and *SOCS3* immunostaining score as independent factors associated with response to antiviral treatment. These findings suggest that the HCV virus itself induced *SOCS3* as a strategy to avoid clearance by the interferon system, prior to the commencement of interferon therapy. We previous reported that phosphorylation level of STAT1 might be a useful predictor of HCV clear-

Table 7 On multivariate analysis, factors associated with responder to treatment of patients with genotype1

	Odds ratio	*P*-value
SOCS3 (<30%)	13.740	0.003
Age (<60 years)	6.658	0.033

SOCS3, suppressor of cytokine signal 3.