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CLINICAL STUDIES

Study of liver-targeted regulatory T cells in hepatitis B and C virus in chronically infected patients

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Keywords
Foxp3 – hepatitis B virus – hepatitis C virus – regulatory T cell

Abbreviations
HBV, hepatitis B virus; HCV, hepatitis C virus; CHB, chronic hepatitis B; CHC, chronic hepatitis C; GITR, glucocorticoid-induced TNF receptor-related protein; Foxp3, forkhead box P3

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Abstract

Introduction: Regulatory T cells (Tregs) play a critical role in chronic viral infections. The role of Tregs in chronic hepatitis B (CHB) and chronic hepatitis C (CHC) is unknown. This study examined the distribution and frequency of forkhead box $p3^+$ ($Foxp3^+$) Tregs in the liver tissue and compared the clinicopathological characteristics of CHB and CHC patients. *Methods:* Liver needle biopsies were obtained from 26 patients who were hepatitis B surface antigen positive and 27 patients who were hepatitis C virus antibody positive. *Results:* The ratio of $Foxp3^+$ Tregs in $CD3^+$ T cells was similar in HBV and in HCV cases. In HBV cases, the variables that were positively associated with the ratio of $Foxp3^+$ Tregs in $CD3^+$ T cells included the serum alanine aminotransferase level ($R=0.402$, $P=0.025$) and the ratio of $CD8^+$ T cell plus $CD56^+$ NK cell against $CD4^+$ T cell ($R=0.53$, $P=0.005$). The ratio of $Foxp3^+$ Tregs in $CD3^+$ T cells increased more in the severe activity group than in the mild activity group ($P=0.04$). In HCV cases, the ratio of $Foxp3^+$ Tregs in $CD3^+$ T cells increased significantly in terms of the genotype2 ($P=0.0002$) and male gender ($P=0.04$). In addition, the ratio of $Foxp3^+$ Tregs in $CD3^+$ T cells showed a negative correlation with the ratio of $CD8^+$ T cell plus $CD56^+$ NK cell against $CD4^+$ T cell ($R=-0.508$, $P=0.005$) and HCV viral load ($R=-0.482$, $P=0.001$). *Conclusions:* Liver-targeted regulatory T cells present similarly in CHB and CHC, but their relationship with the effector cell population, the inflammation grade or the viral load is different between CHB and CHC.

Hepatitis B virus (HBV) and hepatitis C virus (HCV) are global health problems and both cause chronic hepatitis, cirrhosis and hepatocellular carcinoma (1, 2). In some cases, these viruses lead to a chronic infection in the liver, but the mechanisms by which these hepatitis viruses are able to evade the immuno system are still only poorly understood.

Recent studies have focused on regulatory T cells (Tregs). Tregs are engaged in the maintenance of self-tolerance by suppressing the activation and expansion of self-reactive lymphocytes (3–5). This suppressing function may lead to chronic inflammation and/or autoimmunity (6–12). Most of these Treg markers, including CD25, CTLA-4 and glucocorticoid-induced TNF receptor-related protein (GITR), do not accurately represent CD4+ T cells with regulatory activity, and they overlap with activated T cells, which do not necessarily possess a regulatory activity (13, 14). Currently, the best indicator of the Tregs function is thought to be the intracellular expression of forkhead box P3 (Foxp3), which is also crucial for Treg development (15). Previous reports have indicated that Tregs play a role in viral persistence by suppressing the virus-specific T cell responses in a cell-to-cell contact manner (16, 17). A more detailed analysis of this cell reaction is necessary not only in the peripheral blood but also in the target organ tissue. The liver is the target organ of both HBV and HCV. This study examined the distribution and frequency of Tregs in the liver and compared the clinicopathlogical characteristics of chronic hepatitis B (CHB) and chronic hepatitis C (CHC) patients.

Patients and methods

Liver needle biopsies were obtained from 26 patients who were hepatitis B surface antigen positive (mean age: 33.3 ± 10.4, male: female = 23:3) and 27 patients who were hepatitis C positive virus antibody positive (mean age: 57.8 ± 10.6, male: female = 15:12) at Nagasaki Universities and associated hospitals.The clinical and biological parameters, including HBV viral load, of all the patients were assessed using the HBV transcription-mediated amplification method or HCV virus load by the quantitative RT-PCR method. None of the HBV cases had been administered any antiviral treatment, while 10 of 27 HCV cases received antiviral treatment.

The clinical data of the patients are summarized in Table 1. Liver biopsy tissue specimens were taken by a needle puncture for diagnostic purposes. The diagnosis of each case was

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Table 1. Clinical data of the patients

	HBV 26 cases	HCV 27 cases
Mean age (years old)	33.6 ± 10.4	57.8 ± 10.6
Male:female	23:3	15:12
ALT (IU/L)	310 ± 472	91 ± 54
AST (IU/L)	208 ± 333	67 ± 36
Plt (× 10^4/μl)	16.6 ± 5.6	16.9 ± 4.7
Inflammation grade (mild:severe)	19:7	22:5
Fibrosis stage (mild:severe)	15:11	14:13
HBV viral load LEG/ml (TMA)	6.8 ± 1.8	
HCV genotype (1:2)		21:6
Antiviral treatment (naïve:former treatment)		17:10
HCV viral load KIU/ml (RT-PCR)		1637 (5–5000)

ALT, alanine aminotransferase; AST, aspartate aminotransferase; HBV, hepatitis B virus; HCV, hepatitis C virus; RT-PCR, reverse transcriptase polymerase chain reaction; TMA, transcription-mediated amplification.

independently confirmed histologically by liver pathologists according to the Japanese chronic hepatitis classification (New Inuyama classification). Mild activity was defined as A0 or A1, and severe activity was defined as A2 or A3 by the Inuyama classification. Mild fibrosis was defined as F0 or F1, and severe fibrosis was defined as F2, F3 or F4 by the Inuyama classification. All tissues were fixed in 10% neutral-buffered formalin and were then embedded in paraffin, and 4-μm-thick serial sections were cut from each paraffin block.

T cells were examined immunohistochemically with the anti-CD3 antibody (Novocastra, Newcastle, UK), regulatory T cells were examined with the anti-Foxp3 antibody (eBioscience, San Diego, CA, USA), $CD4^+$ T cells were examined with the anti-CD4 antibody (Novocastra), $CD8^+$ T cells were examined with the anti-CD8 antibody (Novocastra) and NK cells were examined with the anti-CD56 antibody (Novocastra). The number of $Foxp3^+$, $CD4^+$, $CD8^+$, $CD56^+$ and $CD3^+$ cells contained within three portal tracts selected in each specimen were counted at a magnification of × 200. To correct for differences in the size of the portal tracts, the average proportion of $FoxP3^+$ Tregs among the total number of $CD3^+$ T cells and the average ratios of $CD8^+$ T cell plus $CD56^+$ NK cells against $CD4^+$ T cells were determined.

Statistical analysis

The SPSS statistical software program (SPSS, Chicago, IL, USA) was used to assess any correlations among multiple variables. Differences between the groups were analysed using the Mann–Whitney *U* test. Correlations between the groups were determined by the Spearman analysis. A *P* value of < 0.05 was considered to be statistically significant.

Results

The population of $Foxp3^+$ regulatory T cells in the liver

In HBV and HCV cases, both $CD3^+$ and $Foxp3^+$ lymphocytes were mainly seen in the portal areas (Fig. 1). In HBV cases, the average ratio of $Foxp3^+$ Tregs in $CD3^+$ T cells in the portal tract was 0.09 ± 0.04, and the average ratio of $CD8^+$ T cell plus $CD56^+$ NK cell against $CD4^+$ T cell was 0.93 ± 0.63, while in HCV cases, the average ratio of $Foxp3^+$ Tregs in $CD3^+$ T cells was 0.09 ± 0.05, and the average ratio of $CD8^+$ T cell plus $CD56^+$ NK cell against $CD4^+$ T cell was 0.95 ± 0.51.

Association between the laboratory data and the frequency of $Foxp3^+$ regulatory T cells in the liver

In HBV and HCV cases, there was no significant correlation between the ratio of $Foxp3^+$ Tregs in $CD3^+$ T cells and age. In HBV cases, the ratio of $Foxp3^+$ Tregs in $CD3^+$ T cells showed no significant difference in either gender (Table 2). On the other hand, in HCV cases, the ratio of $Foxp3^+$ Tregs in $CD3^+$ T cells was significantly increased in males in comparison with females (male:female = 0.11 ± 0.05:0.07 ± 0.03, $P = 0.04$; Table 3). In HBV cases, there was a significant correlation between the ratio of $Foxp3^+$ Tregs in $CD3^+$ T cells and the serum ALT level ($R = 0.481$, $P = 0.026$; Fig. 2, Table 2). Similarly, there was a significant correlation between the ratio of $Foxp3^+$ Tregs in $CD3^+$ T cell and AST level ($R = 0.402$, $P = 0.025$; Table 2). On the other hand, in HCV cases, there was no significant correlation between the ratio of $Foxp3^+$ Tregs in $CD3^+$ T cells and either serum AST or ALT level ($R = -0.177$, $P = 0.38$, $R = -0.127$, $P = 0.53$; Fig. 2, Table 3).

Both in HBV and in HCV cases, there was no significant correlation between the ratio of $Foxp3^+$ Tregs in $CD3^+$ T cells and the platelet count ($R = -0.10$, $P = 0.91$, $R = -0.18$, $P = 0.37$, respectively; Tables 2 and 3).

In HBV cases, there was no significant correlation between the ratio of $Foxp3^+$ Tregs in $CD3^+$ T cells and HBV DNA viral load ($R = -0.314$, $P = 0.19$; Table 2). In HCV cases, there was a significant inverse correlation between the ratio of $Foxp3^+$ Tregs in $CD3^+$ T cells and HCV viral load ($R = -0.487$, $P = 0.01$; Fig. 3a, Table 3). In addition, the ratio of $Foxp3^+$ Tregs in $CD3^+$ T cells was significantly increased in the genotype2 group in comparison with the genotype1 group (genotype1:genotype2 = 0.07 ± 0.03:0.11 ± 0.05, $P = 0.0002$; Fig. 3b, Table 3). In HCV cases, the ratio of $Foxp3^+$ Tregs in $CD3^+$ T cells showed no significant difference between the naïve group and the former antiviral treatment group (0.08 ± 0.05:0.10 ± 0.03, $P = 0.40$; Table 3).

Association between the histological findings and the frequency of $Foxp3^+$ regulatory T cells in the liver

In HBV cases, the ratio of $Foxp3^+$ Tregs in $CD3^+$ T cells was significantly increased in the severe activity group in comparison with the mild activity group (mild:severe = 0.08 ± 0.03:0.11 ± 0.04, $P = 0.04$; Fig. 4, Table 2). In HCV cases, there was no significant difference in either activity group (mild:severe = 0.100.05:0.06 ± 0.03, $P = 0.09$; Fig. 4, Table 3). There were no significant differences among the fibrosis groups in the HBV and HCV cases (mild:severe = 0.09 ± 0.04:0.10 ± 0.03, $P = 0.58$, mild: severe = 0.09 ± 0.05:0.10 ± 0.05, $P = 0.65$, respectively; Tables 2 and 3).

Association between the ratio of $Foxp3^+$ regulatory T cells in $CD3^+$ T cells and the ratio of $CD8^+$ T cells plus $CD56^+$ NK cells against $CD4^+$ T cells in the liver

In HBV cases, a positive correlation was observed between the ratio of $Foxp3^+$ Tregs in $CD3^+$ T cells and the ratio of $CD8^+$ T cells plus $CD56^+$ NK cells in $CD4^+$ T cells ($R = 0.53$, $P = 0.005$; Table 2). In contrast, in the HCV cases, a negative correlation was observed between them ($R = -0.508$, $P = 0.005$; Table 3).

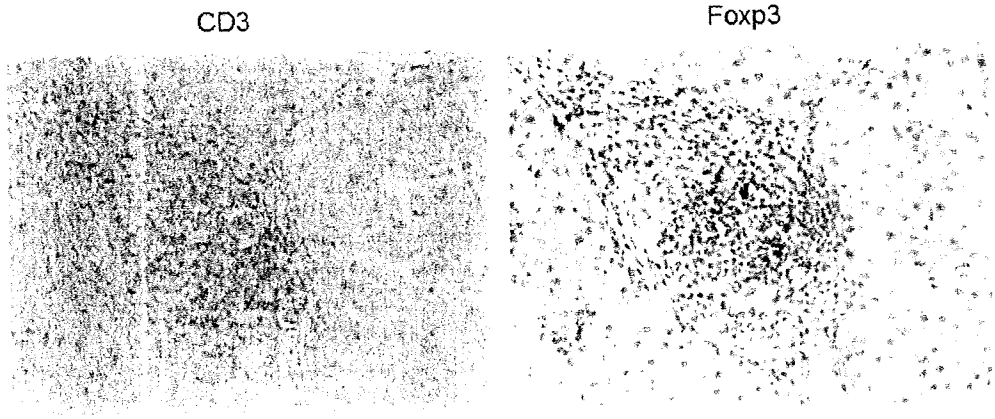

Fig. 1. Immunostaining of $CD3^+$ lymphocytes and $Foxp3^+$ Tregs in representive liver sections of patients with CHB and CHC.

Table 2. Comparison between Foxp3/CD3 and each parameter in Chronic hepatitis B

		P value*
Sex (male:female)	0.09 ± 0.04:0.09 ± 0.03	0.97
Age	$R = -0.118$	0.57
AST	$R = 0.402$	0.025
ALT	$R = 0.481$	0.026
Plt	$R = -0.10$	0.90
Grading (mild:severe)	0.08 ± 0.03:0.11 ± 0.04	0.04
Staging (mild:severe)	0.09 ± 0.04:0.10 ± 0.03	0.58
Viral load	$R = 0.314$	0.19
(CD8+CD56)/CD4†	$R = 0.53$	0.005

*Differences between the groups were analysed using the Mann–Whitney *U* test. Correlations between the groups were determined by the Spearman analysis.
†The ratio of $CD8^+$ T cell plus $CD56^+$ NK cell against $CD4^+$ T cell.
ALT, alanine aminotransferase; AST, aspartate aminotransferase; NK, natural killer.

Table 3. Comparison between Foxp3/CD3 and each parameter in Chronic hepatitis C

		P value*
Sex (male:female)	0.11 ± 0.05:0.07 ± 0.03	0.04
Age	$R = 0.255$	0.20
AST	$R = -0.177$	0.38
ALT	$R = -0.127$	0.53
Plt	$R = -0.18$	0.37
Grading (mild:severe)	0.10 ± 0.05:0.06 ± 0.03	0.09
Staging (mild:severe)	0.09 ± 0.05:0.10 ± 0.05	0.65
Genotype (1:2)	0.07 ± 0.03:0.11 ± 0.05	0.0002
Antiviral treatment (naïve:former treatment)	0.08 ± 0.05:0.10 ± 0.03	0.40
Viral load	$R = -0.482$	0.01
(CD8+CD56)/CD4†	$R = -0.508$	0.005

*Differences between the groups were analysed using the Mann–Whitney *U* test. Correlations between the groups were determined by the Spearman analysis.
†The ratio of $CD8^+$ T cell plus $CD56^+$ NK cell against $CD4^+$ T cell.
ALT, alanine aminotransferase; AST, aspartate aminotransferase.

Discussion

Both HBV and HCV infect the liver and cause acute and chronic liver disease (1, 2). However, the mechanism of persistent infection of these viruses is not clear. Some studies have reported that $CD4^+$ $CD25^{high}$ regulatory T cells (Tregs) play a critical role in persistent virus infection (18–20). Recently, forkhead box P3 (Foxp3) was detected as a specific marker of Tregs (12). The present study examined the distribution of $Foxp3^+$ Tregs in the liver and clarified the role of Tregs in chronic viral hepatitis.

A previous study showed that the normal human liver does not display a significant population of Tregs. This may mean that the tolerance of the liver is not primarily maintained by Tregs. In this study, $Foxp3^+$ regulatory T lymphocyte populations in the liver constitute 9% of $CD3^+$ T cell in both HBV and HCV cases. Other studies have shown that Tregs accumulate and expand locally at the site of infection where they exert their suppressive activity (21–23). This suggests that Tregs may be a key factor in the pathogenesis of chronic viral hepatitis.

In HBV cases, the variables that were significantly associated with the ratio of $Foxp3^+$ Tregs in $CD3^+$ T cells included the transaminase level (AST and ALT) and pathological grading. These factors are characteristics of the degree of inflammation in the liver. Indeed, the ratio of CD8/CD3 correlated significantly with the transaminase level (AST and ALT) in HBV cases ($r = 0.428$, $P = 0.029$, $r = 0.495$, $P = 0.010$; data not shown). These results indicated that, when severe hepatitis occurred in CHB, the number of $Foxp3^+$ Treg increases. The ratio of $CD8^+$ T cell+$CD56^+$ NK cell against $CD4^+$ cells significantly correlated with the ratio of $Foxp3^+$ Tregs in $CD3^+$ T cells. These observations suggest that Tregs may migrate into the liver during the necroinflammatory reactions induced by the virus-specific effector T cells. If Treg acts too late, the unlimited effector T cells may lead to excessive destruction and the death of hepatocyte. Therefore, it is possible that Tregs suppress the excessive activity of effector T cells in severe hepatitis by HBV.

In contrast, in HCV cases, no correlation was found between alanine aminotransferase or pathological grading and the ratio of $Foxp3^+$ Tregs in $CD3^+$ T cells. Moreover, the ratio of $CD8^+$ T cell+$CD56^+$ NK cell against $CD4^+$ cells showed a significant inverse correlation with the ratio of $Foxp3^+$ Tregs in $CD3^+$ T cells. In daily medical examination, the patients rarely experienced acute exacerbation of chronic hepatitis in HCV cases.

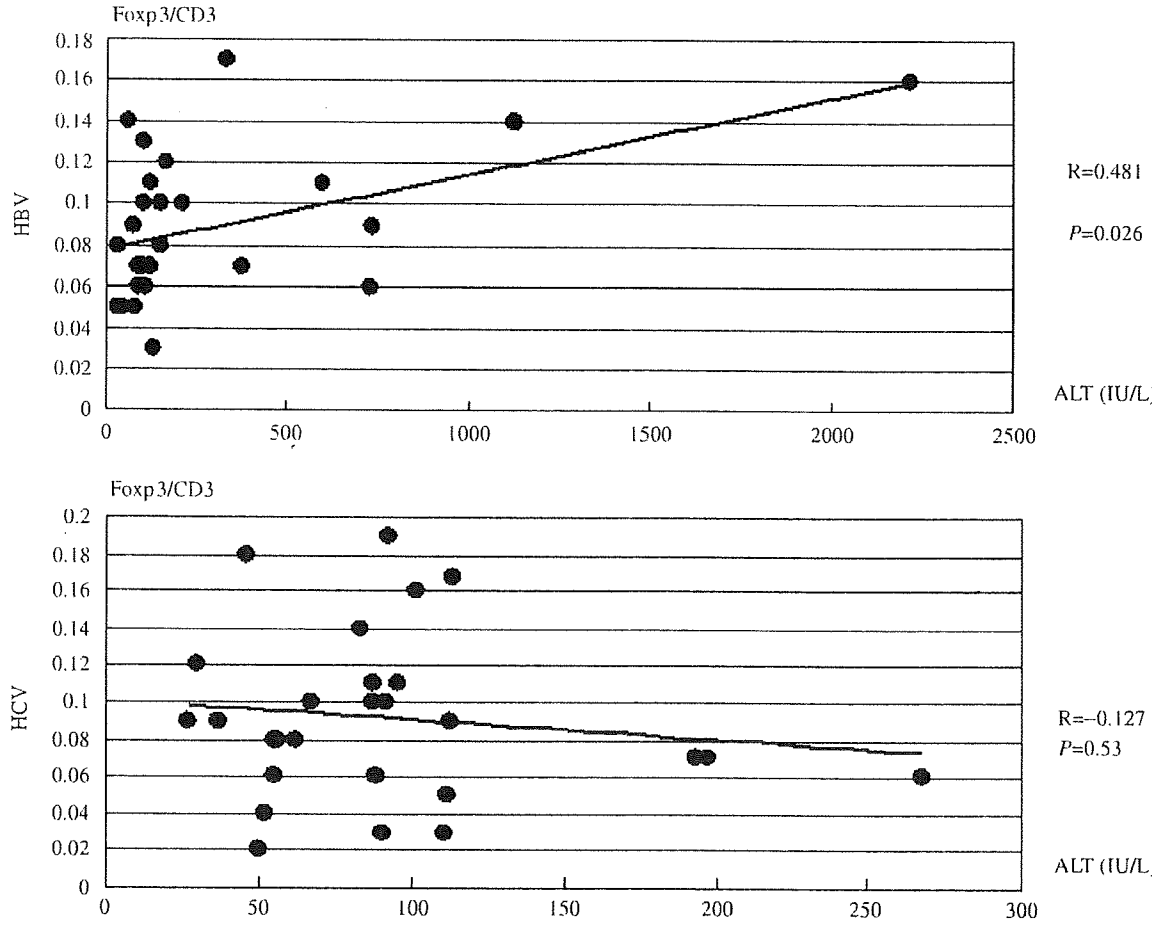

Fig. 2. Comparison between the ratio of Foxp3$^+$ Tregs in CD3$^+$ T cell and serum alanine aminotransferase level in chronic hepatitis B (CHB) and in chronic hepatitis C. A significantly positive correlation was observed between the frequency of Foxp3$^+$ Treg and the serum alanine aminotransferase level in chronic hepatitis B by the Spearman analysis ($R = 0.402$, $P = 0.025$).

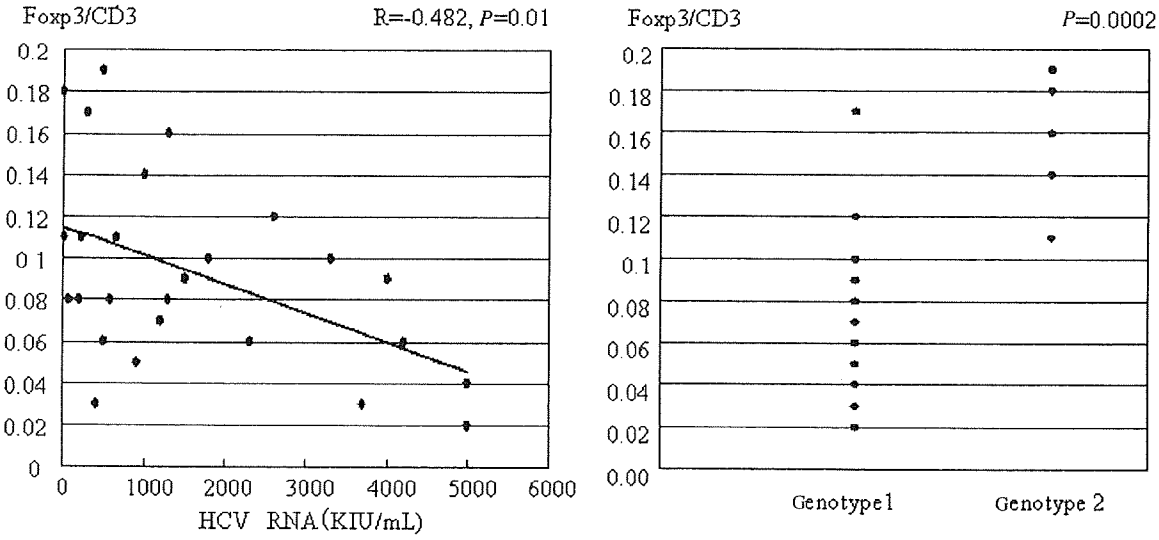

Fig. 3. (a) Comparison between the ratio of Foxp3$^+$ Tregs in CD3$^+$ T cells and HCV RNA (KIU/ml). A significantly negative correlation was found between the frequency of Foxp3$^+$ Tregs and HCV core protein by the Spearman analysis ($R = -0.482$, $P = 0.01$), (b) Comparison between the ratio of Foxp3$^+$ Tregs in CD3$^+$ T cells and HCV genotype. A significant difference was observed in the genotype by the Mann–Whitney *U* test ($P = 0.0002$).

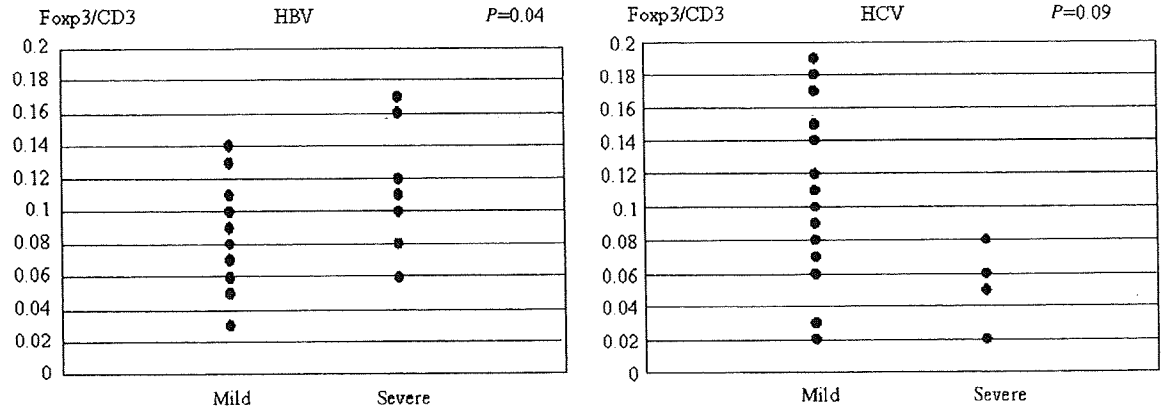

Fig. 4. Comparison between the ratio of Foxp3$^+$ Tregs in CD3$^+$ T cells and the histological fibrosis grade in chronic hepatitis B and in chronic hepatitis C. A significant difference was observed in chronic hepatitis B using the Mann–Whitney *U* test ($P = 0.04$).

In this study, the transaminase level was > 1000 IU/ml in some of the HBV cases, but in all HCV cases, it was < 300 IU/ml. These results suggest that Treg could constantly suppress the flare of inflammation induced by HCV.

The prevalence of regulatory T cells in the peripheral blood of patients with autoimmune encephalitis or systemic lupus erythematosus (24) is higher in males than in females. Similarly, in the present study, the ratio of Foxp3$^+$ Tregs in CD3$^+$ T cells in HCV cases in the liver was significantly higher in females than in males. Sex hormones may act directly on Tregs to alter their functions because immune cells express oestrogen and androgen receptors (25, 26). In the HCV cases, most of the female patients were over 50 years old (76%) and in a postmenopausal state. These findings suggested that oestrogen deficiency is therefore associated with the Tregs in the liver.

Viral genotype, viral load and gender have been shown to be key factors associated with HCV clearance by interferon therapy (27, 28, 29). The mechanism responsible for this difference in interferon treatment outcome is unknown. In this study, in HCV cases, the variables that were significantly associated with the ratio of Foxp3$^+$ Tregs in CD3$^+$ T cell were genotype2 and male gender. These results suggested that regulatory T cells in the liver may be related to the clearance of HCV by interferon therapy and may be a useful indicator for interferon therapy. The ratio of Foxp3$^+$ Tregs in CD3$^+$ T cells showed a significant inverse correlation with the HCV viral load. Tregs were induced by antigen-presenting cells such as dendritic cells (30). Therefore, a decreasing number of Tregs may indicate an impairment of antigen-presenting cells; conversely, the presence of Tregs may represent a normal function of antigen-presenting cells. Taken together, it is possible that, in CHB, regulatory T cells suppress the excessive activity of effector T cells during acute exacerbation, and that, in CHC, the presence of regulatory T cells may be a marker of the normal response of the host immunosystem against HCV.

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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

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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.

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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

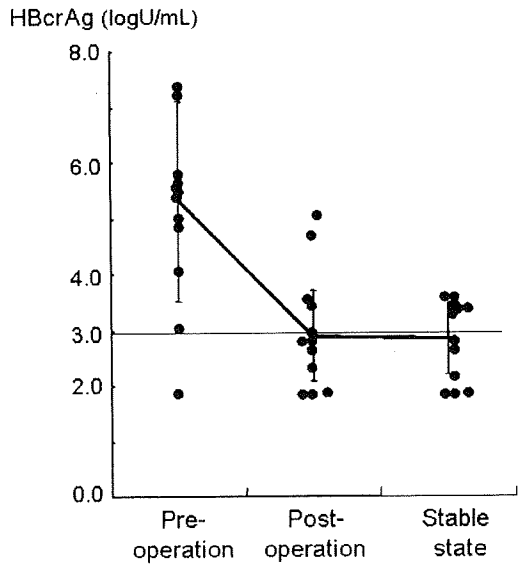

Figure 1. Serial changes of the HBcrAg levels. The HBcrAg levels are represented as mean values; the closed circles show the value of HBcrAg levels in all phases. Error bar is standard deviation. The mean value of HBcrAg levels in the post-operation period and in the stable state period were significantly 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

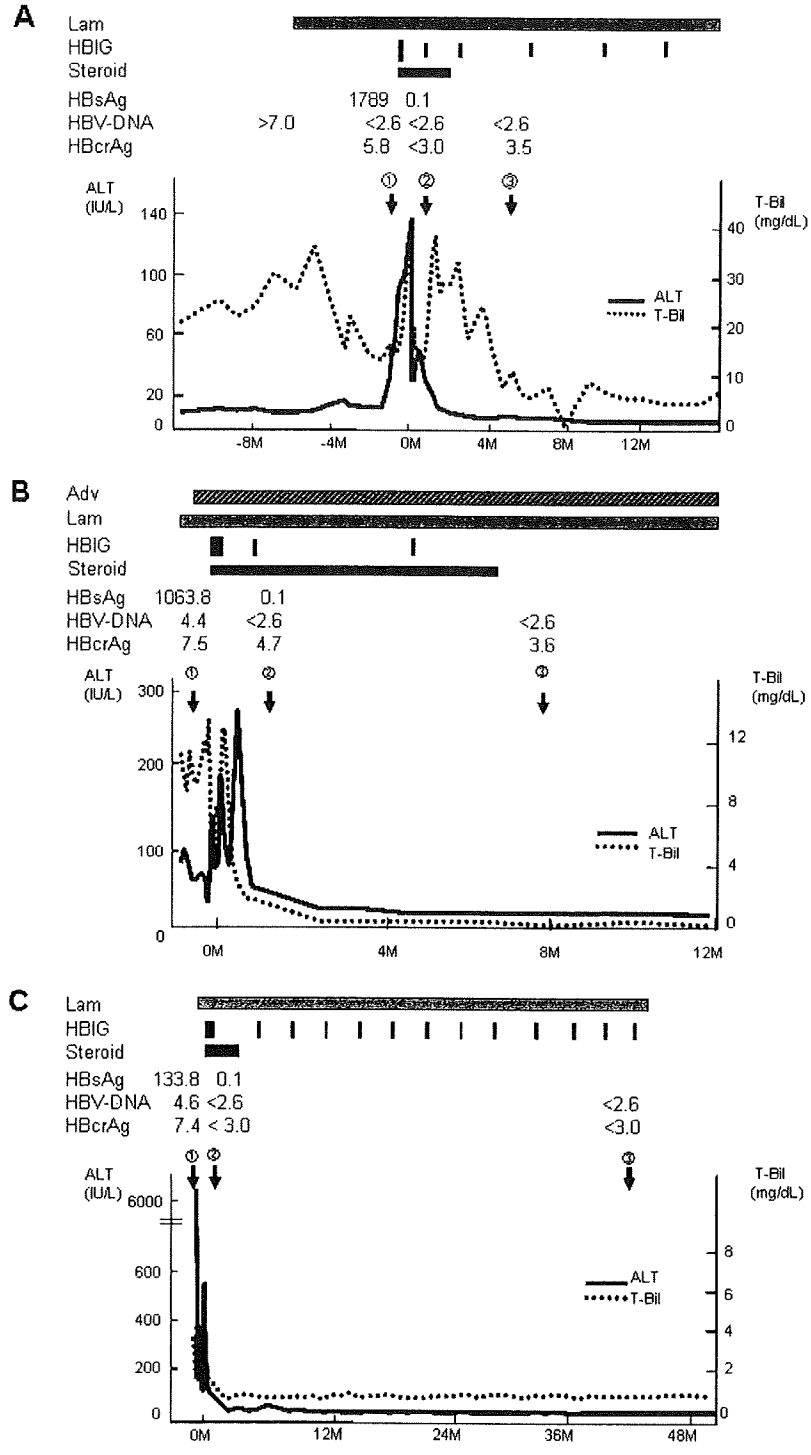

Figure 2. Clinical course of representative cases. A: Case 2 had suffered from LC. The value of crAg was below sensitivity in the immediate post-LT phase, then returned to a positive value in the post-LT late phase. B: Case 7 suffered from LC with Lam resistance. Adv was added to the Lam treatment at 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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Original Article

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

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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]

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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-

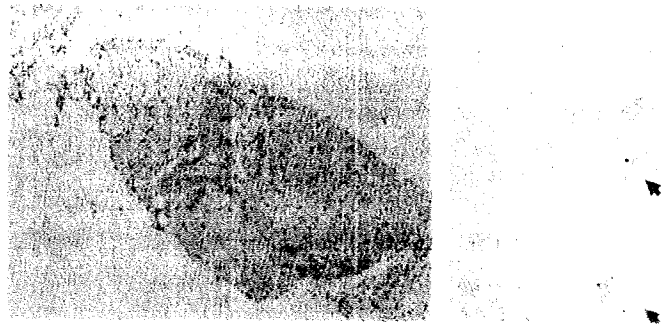

Figure 1 (a) Immunostaining for *SOCS3* (brown) was mainly seen in the periportal area. (b) Immunohistochemical detection of phosphorylated-signal transducer and activator of transcription (P-STAT) 1. The arrows indicate P-STAT-positive hepatocyte nuclei.

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).

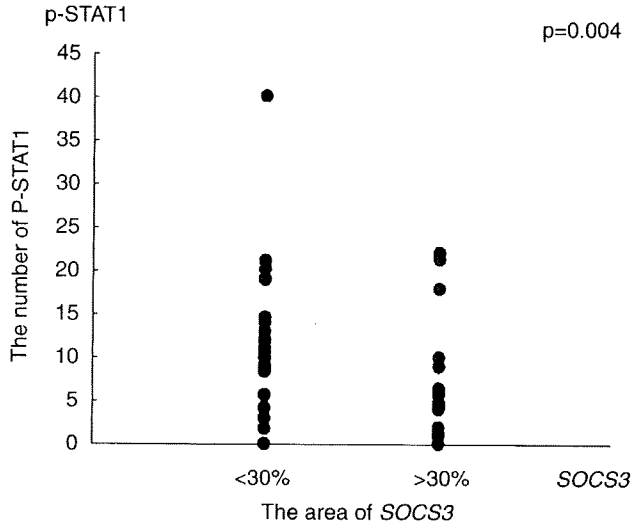

Figure 2 Correlation of P-STAT1 and *SOCS3* immunostaining score. Concentration of P-STAT1 nuclei was significantly higher in specimens with *SOCS3* immunostaining score 0 or 1 than in those with score 2 or 3 using Mann–Whitney *U*-test (10.6 ± 8.8 vs. 4.6 ± 6.1, $P = 0.004$).

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.

ance by interferon therapy.[11] The present *in vivo* findings also showed that increased *SOCS3* expression in the liver prior to interferon treatment impaired the phosphorylation of STAT1 and that the area immunostaining to *SOCS3* was an important factor for predicting HCV clearance by interferon therapy.

In this study, area immunostaining to *SOCS3* was significantly correlated with HOMA-IR. Because the liver is the primary site of insulin resistance, insulin resistance caused by increased hepatic expression of *SOCS3* protein may lead to persistent hyperinsulinemia that further exacerbates insulin resistance.[17,18] Recent data indicate that the incidence of altered baseline glucose level and the frequency of diabetes type 2 were greater in non-responder cases than in SVR cases.[19] Experimental data obtained using the replicon model also indicate that when an insulin level similar to that seen clinically in hyperinsulinemia was added to interferon, the ability to block HCV replication disappeared.[20] Taken together, these findings show that insulin signaling is associated with interferon signaling. Our data suggest that one factor important in the association between insulin signaling and interferon signaling is *SOCS3*.

SOCS3 immunostaining was mainly seen in the periportal area. *SOCS3* is induced by proinflammatory stimuli such as TNF-α and LPS,[12,21] indicating that these proinflammatory stimuli may enhance *SOCS3* expression via portal vein flow. Adipocytokines such as TNF-α induced by visceral fat have been shown to cause insulin resistance by increasing serine phosphorylation of insulin receptor substrate (IRS)-1.[22,23] On basis of these data, these adipocytokines are likely to enhance *SOCS3* expression and induce both insulin resistance and interferon resistance.

SOCS3 immunostaining score was observed to significantly correlate with AST and ALT and was significantly higher in patients with severe inflammation than in those with mild inflammation. This finding was consistent with previous reports that *SOCS3* expression is influenced by inflammatory state.[24,25]

Moreover, *SOCS3* immunostaining score was negatively correlated with platelet count and was also significantly higher in patients with severe fibrosis than in those with mild fibrosis. *SOCS3* is known to act as a negative regulator in hepatocyte proliferation, and the progression of fibrosis is associated with reduced cellular proliferation.[26,27] Our results showed that *SOCS3* expression increased and liver regeneration was impaired during the progression of liver fibrosis.

In this study, 14 patients were younger than 60 and had *SOCS3* immunostaining of < 30% of specimen area. These 14 patients all demonstrated SVR. Specificity of the combination of *SOCS3* and age was 100%. Therefore, prior to interferon therapy, consideration of age and quantification of the area immunostaining to *SOCS3* on hepatic biopsy can be considered simple and useful for predicting a positive response to interferon. Some previous study also reported that *SOCS3* expression was a factor associated with response to treatment.[15,16] Most of previous study was subjected to Caucasoid, few study subjected to Asian patients. Our study showed that immunostaing of *SOCS3* in the liver also significant predictor of HCV clearance by interferon therapy in Asian patients. Recently, Persico *et al.* reported that the *SOCS3* -4874 AA genotype was strongly associated with failure of antiviral therapy, and that the AA genotype carriers had significantly higher *SOCS3* mRNA and protein levels,[28] suggesting that the high expression of *SOCS3* in the liver tissue, which we observed in non-SVR patients, may be related to *SOCS3* -4874 AA genotype. Further study is needed to confirm this.

In conclusion, response to antiviral therapy may be conditioned by *SOCS3* expression. Moreover, expression of *SOCS3* was influenced by insulin resistance. Taken together, a good response to interferon therapy is needed for improvement in insulin resistance.

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.

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