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Assessment of Clinical Utility of Low and High Normal Alanine Aminotransferase Values in Patients with Chronic Hepatitis B Virus Infection in Bangladesh

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Key Words
Chronic hepatitis B · Alanine aminotransferase, low and high normal

Abstract
Background/Aims: There is a lack of consensus about the currently accepted range of normal values for serum alanine aminotransferase (ALT) levels because some investigators have claimed that the true values are significantly lower than those listed by kit manufacturers. ***Methods:*** A total of 255 chronic hepatitis-B-virus (HBV)-infected patients with traditional, normal levels of ALT (≤42 U/l) were divided into 2 groups: (1) low normal ALT (men: ≤30 U/l; women: ≤19 U/l) and (2) high normal ALT (men: 31–42 U/l; women: 20–42 U/l). The extent of hepatic inflammation and fibrosis was evaluated in these patients by examining liver biopsy specimens. ***Results:*** The levels of HBV DNA were >10,000 copies/ml in 58.4, 52.9 and 61.2% of the patients with traditional normal ALT, low normal ALT and high normal ALT values, respectively ($p > 0.05$). Also, the moderate degrees of hepatic necroinflammation [histological activity index (HAI)-NI score of ≥9] and severe hepatic fibrosis (HAI-F score of ≥3) were similar among the 3 groups of patients ($p > 0.05$). ***Conclusion:*** The newly defined low normal cutoff values for ALT did not exhibit any added clinical benefit for assessing the extent of liver damage in patients with chronic HBV infection in Bangladesh.

Introduction

An estimated 350 million people worldwide are chronically infected with hepatitis B virus (HBV), of which 15–40% are at risk of developing serious sequelae including liver cirrhosis, hepatic decompensation and hepatocellular carcinoma. About 500,000 chronic HBV-infected patients die every year because of complications of HBV-related liver diseases [1]. Even with the advent of an efficient hepatitis B vaccine, more than 50 million new cases of HBV infection occur annually [2]. The aim of chronic hepatitis B treatment is to prevent serious sequelae [3, 4]. All major liver organizations recommend that patients with chronic HBV infection should undertake periodic assessments of biochemical (serum levels of alanine aminotransferase, ALT), virological (levels of HBV DNA in the sera), immunological (hepatitis B e antigen, HBeAg,

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positivity or seroconversion), and histopathological parameters (extent of necroinflammation and fibrosis in liver biopsy specimens) [5–7].

Among these, serum ALT is the cheapest and most widely used laboratory parameter for the evaluation and follow-up of chronic HBV-infected subjects. In addition, ALT is the most convenient and reliable surrogate marker of liver injury. Current upper limits of normal (ULN) of ALT were set at 667 nkat/l (40 U/l; range: 500–833 nkat/l, 30–50 U/l) [8].

However, the currently accepted range of normal values for serum ALT levels has been challenged by different investigators who claim that the true values are significantly lower than those listed by kit manufacturers. An evaluation of the ULN of serum ALT (provided by kit manufacturers) may not properly reflect the magnitude of liver damage. This has been supported by large-scale population studies in normal control subjects [9–11]. Updated ULN (men: 30 U/l; women: 19 U/l) were lower than the current limits for men and women and have shown superior sensitivity in patients with hepatitis C virus viremia [8]. However, conflicting data have been reported about their clinical utility in patients with chronic HBV infection [12–14]. In addition, magnitudes of liver damage have not been compared in patients with chronic HBV infection according to traditional normal ALT values and low normal ULN of ALT.

In order to address these issues, we enrolled 255 consecutive patients with chronic HBV infection who were treatment naïve, HBeAg negative and had ALT values within ULN of traditional cutoff values in Bangladesh (≤42 U/l). These patients were divided into two more groups: one with low baseline ALT cutoff values (≤30 U/l for men and ≤19 U/l for women) and the other with high normal ALT cutoff values (31–42 U/l for men and 20–42 U/l for women). The extent of hepatic inflammation and fibrosis was evaluated in these patients by examining liver biopsy specimens to develop insights about the clinical utility of a low normal ALT cutoff value for assessing the extent of liver damage in patients with chronic HBV infection.

Patients and Methods

Patients

The study was conducted in patients with chronic HBV infection with normal levels of ALT at the Department of Hepatology, Bangabandhu Sheikh Mujib Medical University, Dhaka, Bangladesh. Inclusion criteria for the study were: (1) positive for hepatitis B surface antigen for >6 months; (2) serum ALT within ULN (≤42 U/l); (3) negative for serological markers of hepatitis C virus, IgM antibodies to hepatitis A virus and hepatitis E virus; (4) alcohol consumption of <20 g/day (most Bangladeshis consume almost no alcohol for religious reasons), and (5) no evidence of pregnancy. The patients had been under follow-up for >6 months prior to enrollment in the study and none of them had exhibited any flare of ALT above ULN. All patients were treatment naïve and none had received any antiviral or immune modulator drugs during the previous 6 months. The nature and purpose of the study were explained to all subjects. A total of 272 patients gave informed consent for a liver biopsy and also for the study protocol. The study was approved by the local ethics committee of the hospital. Patients were excluded from further analyses if adequate amounts of liver tissue were not available during liver biopsy, as recommended [15]. Finally, a total of 255 patients with chronic HBV infection were available for evaluation.

Biochemical and Serological Tests

Serum ALT levels and prothrombin time were assessed commercially. The cutoff value for abnormal ALT was 42 U/l. HBeAg was checked commercially by ELISA, using a commercial kit (Abbott Labs, Chicago, Ill., USA).

Quantification of Serum HBV DNA Levels

Serum HBV DNA was quantified by the polymerase chain reaction method, using a commercial kit (Amplicon HBV Monitor Assay, RT-PCR; Roche Molecular Systems, Pleasanton, Calif., USA). The lower limit of detection was 500 copies of HBV DNA/ml.

Liver Biopsy

A percutaneous liver biopsy was performed in all patients with prior, voluntary informed consent. In the case of minors, consent was obtained from a legal guardian. Biopsies were performed under local anesthesia, using a 16-gauge Tru-Cut biopsy needle (Cardinal Health, McGaw Park, Ill., USA). A biopsy specimen of >1.0 cm in length with 5–6 portal tracts was collected. The histology was graded according to the histological activity index (HAI) using to the criteria by Knodell et al. [16]. The total HAI score comprises necroinflammation (HAI-NI) and fibrosis (HAI-F) scores. The HAI-NI scale includes 3 components (0–10: piecemeal necrosis; 0–4: lobular necrosis and inflammation; 0–4: portal inflammation). The HAI-F is graded according to severity: 0 = absence of fibrosis; 1 = fibrous portal expansion; 3 = bridging fibrosis, and 4 = cirrhosis.

Statistical Analyses

Data are expressed as means ± SD. In some places, data are also shown as medians (ranges). The data were analyzed by the unpaired t test if the they were normally distributed, and by the Mann-Whitney rank sum test if they were skewed. Distribution was assessed by the F test. Differences were considered significant if $p < 0.05$.

Results

All patients had expressed hepatitis B surface antigen in the sera for >6 months. They were treatment naïve, HBeAg negative and had ALT values below the tradi-

Table 1. Age, sex and serum HBV DNA in relation to different levels of ALT in chronic HBV carriers

	Total patients (n = 255)	Patients with low normal ALT (n = 85; 33.33%)	Patients with high normal ALT (n = 170; 66.67%)
Age, years			
Mean ± SD	29.3 ± 9.5	29.0 ± 9.4	29.5 ± 9.6
Median (range)	28 (12–55)	28 (12–48)	27 (15–55)
Male	185 (72.5%)	72 (84.7%)	113 (66.5%)
Mean age ± SD, years	29.56 ± 9.6	29.00 ± 9.6	29.92 ± 9.6
Median age (range), years	28 (12–55)	28 (12–48)	28 (15–55)
Female	70 (27.5%)	13 (15.3%)	57 (33.5%)
Mean age ± SD, years	28.6 ± 9.5	29.0 ± 9.0	28.6 ± 9.7
Median age (range), years	25 (12–45)	27 (16–41)	25 (13–45)
HBV DNA in sera, copies/ml			
Mean ± SD	1,592,566 ± 7,666,612	1,366,005 ± 6,567,916	1,705,846 ± 8,176,762
Median (range)	14,000 (600–60,999,400)	11,000 (600–40,999,400)	14,825.5 (900–60,999,264)
HBV DNA ≤1 × 10^4	106 (41.6%)	40 (47.1%)	66 (38.8%)
HBV DNA >1 × 10^4	149 (58.4%)	45 (52.9%)	104 (61.2%)
HBV DNA ≤1 × 10^5	191 (74.9%)	62 (72.9%)	129 (75.9%)
HBV DNA >1 × 10^5	64 (25.1%)	23 (27.1%)	41 (24.1%)

tional baseline cutoff values (<42 U/l for both men and women).

Out of the total of 255 patients, 185 were men and 70 were women. Among the male patients, the levels of ALT were ≤30 U/l in 72 patients. In 113 male patients, the levels of ALT were 31–42 U/l. Among the females, 13 patients had ALT values of ≤19 U/l, and 57 had ALT values of 20–42 U/l. Taken together, out of the total of 255 patients, 85 patients had low normal levels of ALT and 170 patients had high normal baseline ALT values. The age of patients with low baseline ALT values (29.0 ± 9.4 years) and high ALT cutoff values (29.5 ± 9.6 years) did not differ significantly ($p > 0.05$) (table 1). Also, no significant difference could be detected between male and female patients in age distribution (table 1). The ratio of male patients was slightly but not significantly higher in the low normal ALT group compared to the high normal ALT group (table 1).

HBV DNA in Patients with Low Baseline ALT Values and High Baseline ALT Values

The levels of HBV DNA in the sera in patients with different ALT values are shown in table 1. Levels of HBV DNA of >10^4 copies/ml were seen in 58.4% of the patients with traditional ULN of ALT (≤42 U/l), in 52.9% of the patients with low normal ALT values (≤30 U/l for men and ≤19 U/l for women), and in 61.2% of the patients with high normal ALT values (31–42 U/l for men and 20–42 U/l for women; $p > 0.05$). Further analyses revealed that HBV DNA levels of >10^5 copies/ml were detected in 25.1, 27.1 and 24.1% of the patients with traditional ULN of ALT, low normal ALT values and high normal ALT values, respectively ($p > 0.05$).

Hepatic Necroinflammation in Inactive HBV Carriers from Bangladesh

The levels of necroinflammation and hepatic fibrosis in patients with traditional normal ALT, low normal ALT and high normal ALT values are summarized in table 2. The level of hepatic necroinflammation of HAI-NI scores of ≥9 was regarded as a moderate level of hepatic inflammation, which has been recommended for antiviral treatment [5]. In fact all patients with HAI-NI scores of ≥9 in this cohort had moderate levels in 2 of 3 parameters of the HAI-NI score (piecemeal necrosis, lobular necrosis and inflammation, and portal inflammation). Hepatic necroinflammation (HAI-NI scores ≥9) were detected in 33 of 255 patients (12.9%), 11 of 85 patients (12.9%) and 22 of 170 patients (12.9%) with traditional normal ALT values, low normal ALT values and high normal ALT values, respectively. Severe degrees of fibrosis (HAI-F scores ≥3) were detected in 35 of the 255 patients (13.7%) with traditional normal ALT values. Severe hepatic fibrosis (HAI-F scores ≥3) was detected in 11 of 85 (12.9%) of the patients with low normal ALT values and in 24 of the 170 patients (14.1%) with high normal ALT values (table 2).

Table 2. Histological findings from liver biopsy specimens in patients with chronic HBV infection in relation to ALT levels in sera

	Total patients (n = 255)	Patients with low normal ALT (n = 85)	Patients with high normal ALT (n = 170)
Extent of hepatitis, HAI-NI score			
Mean ± SD	4.8 ± 2.5	4.8 ± 2.6	4.8 ± 2.5
Median (range)	3 (1–11)	3 (1–11)	3 (1–11)
Extent of fibrosis, HAI-F score			
Mean ± SD	1.2 ± 0.9	1.2 ± 0.9	1.2 ± 0.9
Median (range)	1 (0–4)	1 (0–4)	1 (0–4)
Moderate hepatitis (HAI-NI ≥9)	33 (12.9%)	11 (12.9%)	22 (12.9%)
Severe fibrosis (HAI-F ≥3)	35 (13.7%)	11 (12.9%)	24 (14.1%)
Both hepatitis and fibrosis (HAI-NI ≥9 and HAI-F ≥3)	14 (5.5%)	5 (5.9%)	9 (5.3%)

Clinical Utility of Low ALT Values for Assessing Extent of Liver Damage in Patients with Chronic HBV Infection

As moderate levels of hepatic inflammation and severe degrees of hepatic fibrosis were detected in considerable numbers of patients in this study, we further assessed the clinical utility of low normal ALT values in these patients. A total of 14 of the total 255 patients (5.5%) with traditional normal ALT values had both moderate hepatic inflammation (HAI-NI scores ≥9) and severe hepatic fibrosis (HAI-F scores ≥3). Out of them, 5 of 85 patients (5.9%) belonged to the group of low normal ALT values, and 9 of 170 (5.3%) belonged to high normal ALT group (table 2).

Discussion

ALT is the most commonly used enzyme in the evaluation of liver diseases. It is probably the only parameter that is assessed in all medical facilities around the world. ALT also acts as a surrogate marker of liver injury. Compared to the assessments of HBV DNA, HBeAg and liver histology, the estimation of ALT is also the cheapest. Accordingly, ALT levels are used to assess the extent of liver damage, fix therapeutic recommendations and also follow up patients with chronic HBV infection around the world.

In addition, ALT is a sign of ongoing liver injury, and this marker is regarded as evidence for ongoing host immunity. This is important in the context of therapeutic recommendation because patients without elevated ALT levels are not recommended for antiviral therapy because in absence of adequate host immunity, antiviral drugs are not effective in patients with chronic HBV infection [4–7]. Thus, ALT has diagnostic, therapeutic and prognostic value. In addition, ALT represents the main parameter of the liver function test that is used to assess the condition of the liver during periodic health checkups in different countries of the world.

Recent studies conducted in apparently healthy normal individuals and in patients with chronic hepatitis C virus infection have shown that the ULN of ALT should be reevaluated for proper management of these patients [8–11]. However, there are controversies about the utility of low normal ALT values in chronic HBV infection. Assy et al. [14] reported that lower ALT values (< 30 U/l in men and <19 U/l in women) are important to differentiate HBeAg-negative chronic hepatitis B patients from inactive chronic HBV carriers if combined with lower baseline levels of HBV DNA. However, they did not evaluate the clinical importance of lower baseline values of ALT alone. In addition, they did not check liver histology in their patients. On the other hand, Tai et al. [12] showed that there is no difference in long-term outcomes between subjects with low normal maximum ALT (<0.5 × ULN) and those with high normal maximum ALT values (0.5–1.0 × ULN). Also, similar data about no impact of a low UNL of ALT were reported from Greece [13]. The data presented in this study support the results of the studies by Tai et al. [12] and Papatheodoridis et al. [13]. We too did not find any added clinical utility of low normal ALT values over traditional normal ALT values in patients with chronic HBV infection.

Chronic HBV infection represents a dynamic pathological process, and the patients show exacerbation and remission in their levels of HBV DNA, ALT and liver damage either spontaneously or due to treatment with

antiviral agents. Many of these patients require life-long follow-up. During the last decade, the cutoff values of HBV DNA in the sera for proper management of these patients have been changed several times due to availability of more sensitive assay methods [4–6]. The concept of a low baseline ALT cutoff value has shown clinical importance in some cases. However, the real implications of this marker should be fixed after conducting more controlled trials and long-term follow-ups.

This study, accomplished in Bangladesh, a country with 140 million people and about 8 million chronic HBV carriers, has significant public health importance. Usually, patients with chronic HBV infection are not properly followed up in Bangladesh and most other developing countries that harbor the bulk of global chronic HBV-infected subjects. Although these countries should develop their own treatment and management guidelines, mostly guidelines by the American Association for the Study of the Liver Diseases, the European Association for the Study of the Liver and the Asian Pacific Association for the Study of the Liver are adopted by hepatologists and physicians of these countries for the management of their patients. However, due to poorly developed health care delivery systems, a lack of medical insurance, poverty and other socioeconomic factors, most of the chronic HBV carriers are not regularly followed up in these countries. This study shows that the kinetics of ALT in Bangladesh is different from that in Israel [14], but may be similar to that in Taiwan or Greece [12, 13]. This study also shows important information. Many patients with normal levels of ALT had progressive liver damage. This study may inspire others to assess the utility of low baseline ALT cutoff values in other developing countries that share similar socioeconomic conditions with Bangladesh.

In conclusion, we found that a low baseline ALT cutoff value is of limited value for assessing the extent of liver damage in inactive HBV carriers of Bangladesh. However, a follow-up study should provide more information about this in the future.

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ORIGINAL ARTICLE

Histopathological Assessment of Liver Biopsy Specimen in the Context of HBV DNA and HBeAg in Patients with Chronic Hepatitis B

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ABSTRACT

Background: There is lack of consensus if hepatitis B virus (HBV) DNA can be regarded as a surrogate marker of liver damages in patients with chronic hepatitis B (CHB).

Methods: A total of 77 patients with CHB were enrolled in this study. The sera of the patients were tested for HBV DNA and hepatitis B e antigen (HBeAg). The extent of hepatitis and hepatic fibrosis was assessed by liver biopsy.

Results: Out of total of 77 patients with CHB, 29 were HBeAg-positive and 48 were HBeAg-negative. Twenty-seven of 29 HBeAg-positive patients and eight of 48 HBeAg-negative patients had high levels of HBV DNA (HBV DNA>10^5 copies/ml). The extent of hepatitis was minimal or mild in most HBeAg-positive CHB patients (27 of 29) irrespective of the levels of HBV DNA in the sera. Moderate levels of hepatitis were seen in two HBeAg-positive patients and five HBeAg-negative patients. Moderate levels of hepatic fibrosis were seen in four of eight HBeAg-negative patients with high HBV DNA.

Conclusion: This study shows that HBV DNA and HBeAg do not reflect the extent of hepatitis or hepatic fibrosis in CHB patients of Bangladesh. Combination of different HBV-related markers with liver biopsy is required for proper diagnosis and management of CHB in Bangladesh.

Abbreviations: HBV: Hepatitis B virus; HBsAg: Hepatitis B surface antigen; HBeAg: Hepatitis B e antigen; ALT: Alanine aminotransferase; CHB: Chronic hepatitis B; HAI: Histology activity index.

Keywords: HBV DNA, HBeAg, Hepatitis, Hepatic fibrosis, Bangladesh.

INTRODUCTION

HBV, a member of the family *Hepadnaviridae*, is a noncytopathic DNA virus. About 2 billion people have been infected globally at some point in their lives with HBV, and approximately 350 to 400 million people are chronically infected with this virus. Epidemiologic data indicate that HBV accounts for 0.5 to 1.2 million deaths annually, and approximately 15 to 25% of chronic hepatitis B patients will eventually die of HBV-related liver disease.[1,2]

Chronic HBV-infected subjects express different virological, biochemical, immunological and histological markers; some of which are detectable in the sera; whereas, others can be assessed in liver biopsy specimens. HBV DNA, HBsAg, HBeAg and ALT are detected in the sera for diagnosis of HBV infection and to assess extent of liver damages in patients with CHB. However, liver biopsy provides most critical evidences of liver injury. It is a matter of controversy whether HBV DNA may be used as surrogate marker of liver damages in CHB patients. Some investigators have shown that HBV DNA levels may be useful in this context.[3-8] However, data contrary to this conception also exist and it seems that serum HBV DNA may not reflect the extent of liver damages in CHB patients.[9-12]

Bangladesh, a South Asian country with 160 million people, exhibits an intermediate prevalence for HBV infection. The lifetime risk of acquiring this infection in Bangladesh is >40%. It has been shown that this virus is responsible for about 10 to 35% cases of acute viral hepatitis, about 35% cases of fulminant hepatic failure, about 33 to 40% cases of chronic hepatitis and 46% cases of hepatocellular carcinoma.[13]

The apparent discrepancies about usefulness of HBV DNA in determining extent of liver damages may be due to

differences of study population and other explored factors. However, this has tremendously comprised both proper diagnosis and effective management of CHB patients in most developing countries. This present study in which HBV DNA and HBeAg were compared with findings of liver biopsy in 77 consecutive patients with CHB in Bangladesh provided information about complexity of HBV management at Bangladesh.

MATERIALS AND METHODS

Patients with chronic HBV infection (HBsAg-positive for at least 6 months) attending Department of Hepatology, Bangabandhu Sheikh Mujib Medical University, Dhaka, Bangladesh between March 2008 and December 2008 were enrolled in this study. Written informed consent was obtained from each patient. The patients were positive for serum HBV DNA and negative for antihepatitis C virus antibody.

Serum ALT levels and prothrombin time were assessed commercially. The cutoff value for abnormal ALT was 42 U/L. HBeAg was checked commercially by ELISA using a commercial kit (Abbott Labs, Chicago, IL, USA). Serum HBV DNA was quantified in a polymerase chain reaction method using a commercial kit (Amplicon HBV Monitor Assay, RT-PCR, Roche Molecular Systems, CA, USA). The lower limit of detection was 500 copies of HBV DNA/ml.

A percutaneous liver biopsy was performed with written consent of the patients. Biopsies were performed under local anesthesia using a 16G Tru-cut biopsy needle (Cardinal Health, McGaw Park, IL, USA). A biopsy specimen of more than 1.0 cm in length with five to six portal tracts was accepted when examined microscopically. Histology was graded according to the histologic activity index (HAI) using the criteria of Knodell et al.[14] The total HAI score comprises necroinflammation (HAI-N1) and fibrosis (HAI-F) scores. The HAI-N1 scale includes three components (0–10, piecemeal necrosis; 0-4, lobular necrosis and inflammation; 0-4, portal inflammation). Minimal hepatitis, mild hepatitis, and moderate hepatitis were regarded when HAI-N1 score was 0-4, 5-8 and 9-12 respectively. HAI-F was graded according to severity: 0, absence of fibrosis; 1, fibrous portal expansion; 3, bridging fibrosis; 4, cirrhosis.

RESULTS

A total of 77 patients were enrolled in the study to assess the relationship of HBV DNA and HBeAg with extent of liver damages or hepatitis. HBV DNA and HBeAg were assessed in the sera and extent of hepatitis was evaluated by liver biopsy. On the basis of levels of HBV DNA in the sera, the patients were divided in two groups: (1) high HBV DNA; patients expressing HBV DNA of $>10^5$ copies/ml, and (2) low HBV DNA; patients with HBV DNA of $<10^5$ copies/ml in the sera.

Twenty-nine out of 77 patients were HBeAg-positive and the rest 48 were HBeAg-negative. Out of 29 HBeAg-positive patients, 27 (93%) had high levels of HBV DNA, whereas only 2 (7%) had low levels of HBV DNA in the sera. However, levels of HBV DNA showed a different picture in HBeAg-negative patients. Out of 48 HBeAg-negative patients, HBV DNA levels were $>10^5$ copies/ml in 8 patients, whereas the rest 40 patients of this group had low HBV DNA. Taken together, 35 patients had high HBV DNA, whereas 42 patients had low HBV DNA in the sera in this cohort.

The main purpose of this study was to assess if there is a relation between serum HBV DNA or HBeAg with extent of hepatitis or hepatic fibrosis in liver biopsy. As described in the Method section, the extent of hepatic inflammation and liver fibrosis was graded according to the criteria of Knodell et al. When the necrosis score was HAI-N1 0-4, the patients were regarded to have minimal hepatitis. On the contrary, if the levels of hepatic inflammation were HAI-N1 5-8 and HAI-N1 9-12, they were regarded as having mild and moderate hepatitis, respectively. A total of 36, 34 and seven patients had minimal, mild and moderate hepatitis (Table 1).

Next, we assessed if the levels of HBV DNA reflect the levels of liver injury in CHB patients. As shown in Table 2, out of 35 patients with high HBV DNA, only 5 had moderate levels of hepatitis. The levels of hepatitis were minimal or mild in rest 30 patients with high levels of HBV DNA. Moderate levels of hepatitis were detected in two out of 42 patients with low HBV DNA.

The relation between HBeAg expression and extent of hepatitis has been shown in Table 3. Mild hepatitis was equally distributed between HBeAg-positive and HBeAg-negative patients in this cohort. Moderate hepatitis was seen in higher ratio in HBeAg-negative patients (5 of 48, 10.4%) than in HBeAg-positive patients (2 of 29, 6.9%).

Table 1: Distribution of levels of hepatitis in patients with chronic hepatitis B

Extent of hepatitis	*Number of patients*	*Percentage*
Minimal hepatitis (HAI N1 0-4)	36	47%
Mild hepatitis (HAI N1 5-8)	34	44%
Moderate hepatitis (HAI N1 9-12)	7	9%
Total	77	100%

Note: All patients had a liver biopsy and the levels of inflammation were assessed, according to Knodell et al.

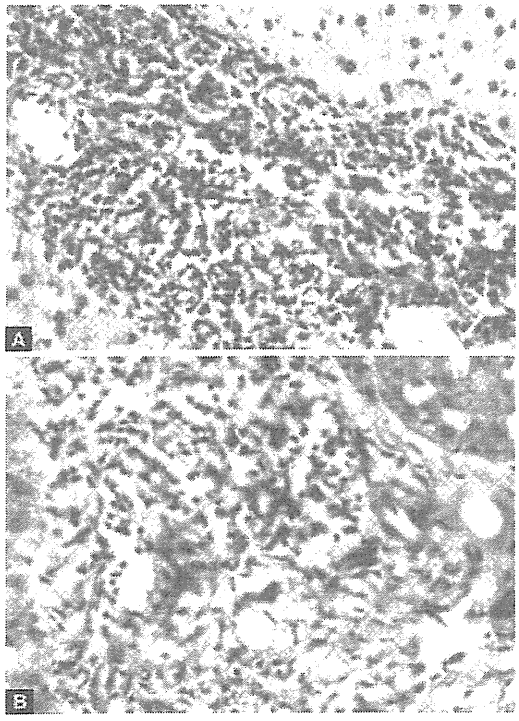
Table 2: Extent of hepatitis and levels of HBV DNA in patients with chronic hepatitis B

HBV DNA level (copies/ml)	*Minimal hepatitis*		*Mild hepatitis*		*Moderate hepatitis*	
	No.	*%*	*No.*	*%*	*No.*	*%*
High (>10^5)	11	31	19	56	5	71.42
Low (<10^5)	25	69	15	44	2	28.57
Total	36	100	34	100	7	100

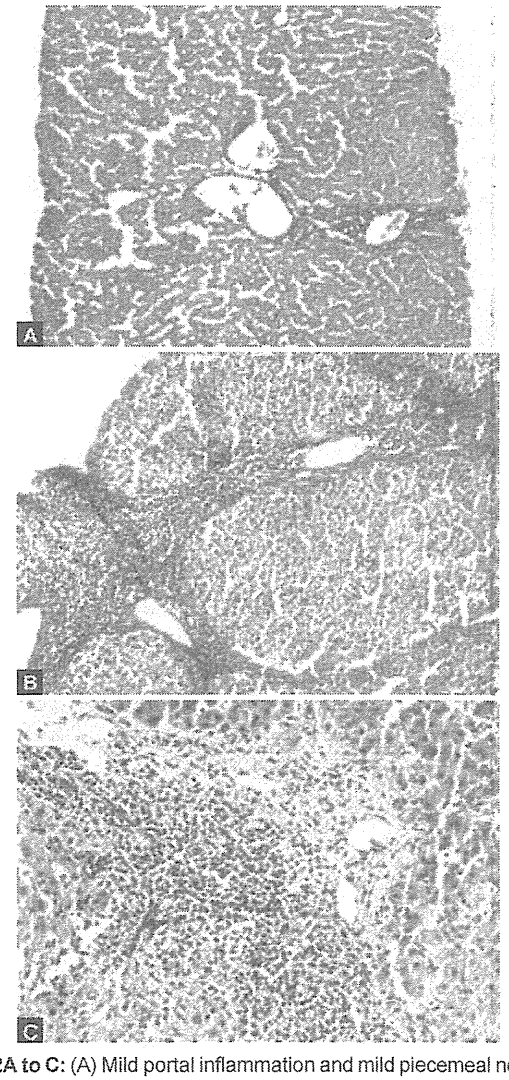
Table 3: Serum HBeAg and extent of hepatitis in patients with chronic hepatitis B

Serum HBeAg status	*Minimal hepatitis*		*Mild hepatitis*		*Moderate hepatitis*	
	No.	*%*	*No.*	*%*	*No.*	*%*
Positive	10	28	17	50	2	29
Negative	26	72	17	50	5	71
Total	36	100	34	100	7	100

The extents of hepatic fibrosis were mild in most HBeAg-positive patients with CHB irrespective of their HBV DNA levels. In 24 of 27 HBeAg-positive patients with high HBV DNA, had minimal to mild fibrosis (HAI-F 0-1). Also, two HBeAg-positive patients with low HBV DNA have minimal to mild fibrosis (HAI-F 0-1). However, in HBeAg-negative patients, four of eight patients with high HBV DNA had bridging fibrosis (HAI-F 3) and the rest four patients had mild hepatic fibrosis. In HBeAg-negative patients with low HBV



Figs 1A and B: (A) Moderate levels of hepatitis in liver biopsy specimen in HBeAg-positive patient with chronic hepatitis B. The patient was a 28-year-old female. The level of alanine aminotransferase (ALT) was 27 U/L. The HBV DNA was 4.7 × 10^{11} copies/ml. (B) Moderate level of hepatitis with interface hepatitis in HBeAg-positive patients with CHB. The patient was 19-year-old female with ALT of 28 U/L and HBV DNA was 4.8 × 10^5 copies/ml



Figs 2A to C: (A) Mild portal inflammation and mild piecemeal necrosis in HBeAg-negative CHB patient. The level of ALT was 30 U/L and HBV DNA was 1.1 × 10^4 copies/ml. (B) Moderate piecemeal necrosis with bridging necrosis and bridging fibrosis in HBeAg-negative CHB patient. The level of ALT was 36 U/L and HBV DNA was 1.88 × 10^5 copies/ml. (C) Moderate piecemeal necrosis with bridging necrosis and bridging fibrosis in a patient with HBeAg-negative CHB with ALT of 36 U/L and HBV DNA of 1.3 × 10^5 copies/ml

Table 4: Biochemical, serological and histological parameters of patients with chronic hepatitis B

Case no.	Age (in years)	Sex	Biochemical	Serological			Histology active index					
			S. ALT (U/L)	HBsAg	HBeAg	HBV DNA copies/ml	PN+BN	ID+FN	P1	Fibrosis	HAI/18	Knodell/22
1.	12	F	20	+ve	–ve	4.1×10^3	1	1	1	1	3	4
2.	39	M	80	+ve	–ve	2×10^3	1	1	3	1	5	6
3.	20	M	46	+ve	–ve	6.7×10^3	1	1	1	1	3	4
4.	28	M	65	+ve	–ve	1.1×10^3	1	1	1	1	3	4
5.	40	M	33	+ve	–ve	9.6×10^3	1	1	1	1	3	4
6.	28	M	84	+ve	–ve	9×10^3	3	1	3	3	7	10
7.	22	F	24	+ve	–ve	2.4×10^3	1	1	1	3	3	6
8.	20	M	32	+ve	–ve	7.7×10^3	1	1	1	1	3	4
9.	52	M	30	+ve	–ve	1.4×10^3	1	1	1	1	3	4
10.	34	M	60	+ve	–ve	1.6×10^3	3	1	3	1	7	8
11.	28	M	35	+ve	–ve	1.6×10^3	1	1	1	1	3	4
12.	26	M	54	+ve	–ve	6.2×10^3	1	1	1	1	3	4
13.	27	M	85	+ve	–ve	9.2×10^3	3	1	3	1	7	8
14.	36	M	38	+ve	–ve	2.9×10^3	1	1	1	1	3	4
15.	31	M	30	+ve	–ve	2.5×10^4	3	1	3	3	7	10
16.	19	M	54	+ve	+ve	5.5×10^{11}	3	1	3	3	7	10
17.	23	M	42	+ve	+ve	8.6×10^8	3	1	3	1	7	8
18.	24	M	49	+ve	+ve	6.9×10^{10}	1	1	1	1	3	4
19.	22	M	32	+ve	+ve	1×10^{12}	1	1	1	1	3	4
20.	26	M	31	+ve	–ve	2×10^4	6	1	3	3	10	13
21.	19	F	28	+ve	+ve	4.8×10^5	3	1	3	3	7	10
22.	31	M	26	+ve	–ve	2×10^4	1	1	1	1	3	4
23.	20	M	29	+ve	–ve	1.3×10^4	3	1	1	3	5	8
24.	20	M	59	+ve	+ve	6.1×10^6	1	1	0	1	2	3
25.	20	F	68	+ve	+ve	3.9×10^{11}	1	1	1	1	3	4
26.	23	M	36	+ve	–ve	1.74×10^5	1	1	1	1	3	4
27.	17	M	103	+ve	–ve	1.3×10^5	6	1	4	3	11	14
28.	28	F	27	+ve	+ve	4.7×10^{11}	1	1	3	1	5	6
29.	24	M	62	+ve	+ve	1.2×10^9	1	1	1	1	3	4
30.	24	M	47	+ve	+ve	7.7×10^9	3	1	3	1	7	8
31.	30	M	67	+ve	–ve	2.4×10^3	3	1	1	1	5	6
32.	28	M	25	+ve	–ve	5.1×10^3	1	1	1	1	3	4
33.	14	M	44	+ve	–ve	1.4×10^3	1	1	1	1	3	4
34.	38	F	33	+ve	–ve	1.3×10^3	1	1	1	1	3	4
35.	20	M	18	+ve	–ve	6.9×10^3	3	1	3	1	7	8
36.	24	M	47	+ve	–ve	1.2×10^3	3	1	3	1	7	8
37.	26	M	75	+ve	–ve	1.3×10^3	1	1	1	1	3	4
38.	30	M	41	+ve	–ve	6.5×10^3	3	1	3	3	7	10
39.	18	M	63	+ve	–ve	5.1×10^3	1	1	1	1	3	4
40.	24	M	28	+ve	+ve	4.9×10^8	1	1	3	1	5	6
41.	35	M	48	+ve	–ve	3×10^4	1	1	1	1	3	4
42.	27	F	33	+ve	–ve	2.8×10^3	1	0	1	1	2	3
43.	22	M	66	+ve	–ve	8.4×10^4	3	1	3	3	7	10
44.	20	M	71	+ve	+ve	3×10^8	3	1	3	1	7	8
45.	28	M	44	+ve	–ve	6.7×10^4	1	1	1	3	3	6
46.	15	M	46	+ve	+ve	1.4×10^9	1	1	3	3	5	8
47.	20	M	43	+ve	–ve	8.3×10^2	1	1	3	1	5	6
48.	26	M	41	+ve	–ve	2.7×10^3	3	1	1	1	5	6
49.	25	F	50	+ve	+ve	8.3×10^6	3	1	3	1	7	8
50.	23	F	30	+ve	–ve	3.7×10^3	1	1	1	1	3	4
51.	27	M	42	+ve	–ve	6.7×10^3	3	1	1	3	5	8
52.	30	M	22	+ve	–ve	1×10^3	1	1	1	1	3	4

Contd.

Contd.

Case no.	*Age (in years)*	*Sex*	*Biochemical*	*Serological*			*Histology active index*					
			S. ALT (U/L)	*HBsAg*	*HBeAg*	*HBV DNA copies/ml*	*PN+BN*	*ID+FN*	*PI*	*Fibrosis*	*HAI/18*	*Knodell/22*
53.	42	F	46	+ve	–ve	2.1×10^{3}	3	1	1	3	5	8
54.	55	M	30	+ve	+ve	1.9×10^{12}	3	1	3	3	7	10
55.	28	M	44	+ve	–ve	1.1×10^{7}	3	3	3	3	9	12
56.	25	M	44	+ve	+ve	9×10^{7}	1	1	1	1	3	4
57.	28	M	30	+ve	+ve	1.1×10^{4}	1	1	1	1	3	4
58.	20	M	35	+ve	+ve	1.4×10^{4}	1	1	1	1	3	4
59.	22	F	44	+ve	+ve	1.9×10^{5}	1	1	1	1	3	4
60.	36	M	91	+ve	–ve	1.8×10^{4}	3	3	4	3	10	13
61.	19	M	41	+ve	+ve	6.0×10^{11}	3	1	3	1	7	8
62.	28	M	58	+ve	–ve	1.5×10^{5}	1	1	1	0	3	3
63.	25	M	110	+ve	–ve	2.0×10^{3}	1	0	1	1	2	3
64.	42	M	70	+ve	–ve	5.25×10^{5}	3	1	1	1	5	86
65.	14	F	27	+ve	+ve	4.1×10^{9}	3	1	3	1	7	8
66.	22	M	23	+ve	+ve	2.0×10^{6}	3	1	3	1	7	8
67.	16	M	48	+ve	+ve	4.0×10^{9}	1	0	1	0	2	2
68.	18	F	28	+ve	+ve	4.5×10^{11}	3	1	3	1	7	8
69.	19	M	60	+ve	+ve	6.2×10^{5}	1	1	3	1	5	6
70.	56	M	48	+ve	+ve	1.1×10^{10}	3	4	3	1	10	11
71.	24	F	48	+ve	+ve	2.4×10^{12}	3	1	3	1	7	8
72.	21	M	50	+ve	+ve	1.15×10^{9}	5	3	1	1	9	10
73.	25	M	110	+ve	+ve	1.6×10^{6}	1	1	1	1	5	6
74.	25	M	35	+ve	–ve	1×10^{5}	1	3	1	1	3	4
75.	23	M	57	+ve	–ve	1.2×10^{3}	1	1	1	1	3	4
76.	35	M	36	+ve	–ve	1.3×10^{5}	3	1	3	3	7	10
77.	24	M	36	+ve	–ve	1.88×10^{5}	5	3	3	3	11	14

DNA, 13/15 had minimal to mild fibrosis (HAI-F 0-1) and 2/15 had bridging fibrosis (HAI-F 3) (Table 4).

The extents of hepatitis and hepatic fibrosis in two HBeAg-positive CHB patients and three HBeAg-negative patients were shown in Figures 1A and B and Figures 2A to C. It seems that liver histology was independent of the HBeAg expression or levels of ALT or HBV DNA concentrations.

DISCUSSION

The aim of this study was to assess whether HBV DNA may be used as a surrogate marker of liver damages in CHB patients of Bangladesh. Some investigators have reported about the utility of HBV DNA in assessing liver damages.[2-8] Also, a role of serum HBV DNA has been shown as a marker of prognosis of antiviral therapy in CHB patients.[15-19] As Bangladesh harbors about 8 to 10 million chronic HBV infected subjects, we studied if serum HBV DNA levels can be used to assess the extent of liver damages in CHB patients from Bangladesh. The present study provided mixed signals about the significance of serum HBV DNA in CHB patients. In HBeAg-positive patients, the high levels of serum HBV DNA were not related with increased levels of liver damages. This was seen in the context of both liver inflammation and hepatic fibrosis. However, in patients with HBeAg-negative CHB, some patients with high HBV DNA also had comparatively higher levels of liver damages. Taken together, it does not appear that HBV DNA or HBeAg reflect proper surrogate marker of liver damages in CHB patients of Bangladesh.

The study presented here contradicted what that have been reported by other investigators about the impact of serum HBV DNA for determining extent of liver damages in CHB patients.[2-8] But, this study has apparently supported several studies about little impact of HBV DNA from developing countries.[9-12]

In fact, studies from Bangladesh and India along with other countries have already shown that patients with very low levels of HBV DNA also exhibit considerable levels of liver damages. Al-Mahtab et al have shown that considerable numbers of HBeAg-negative patients with <10,000 copies/ml of HBV DNA, and normal ALT had severe liver inflammation and hepatic fibrosis.[20]

However, there are several limitations of this study and the outcomes should be cautiously analyzed to develop insights about HBV pathogenesis. We have enrolled few patients in this study, which may not be enough to draw a firm conclusion. The next, we took liver biopsies for once

only that may not be sufficient to assay the kinetics of hepatic damages of these patients. In addition, HBV DNA was measured once. In fact, this preliminary study indicates that a controlled study with considerable numbers of patients should be conducted in future.

In fact, management of CHB patients is a critical factor in developing countries including Bangladesh.[21-23] Lacks of proper surrogate markers of antiviral therapy make the management of CHB patients difficult. Taken together, it appears that pathogenesis of CHB is a complex issue and dependence of HBV markers should be cautiously planned for developing insights about HBV pathogenesis and management of CHB patients.

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BRIEF REPORT

Effects of Hepatitis B Virus Infection on the Interferon Response in Immunodeficient Human Hepatocyte Chimeric Mice

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Complementary DNA microarray analysis of human livers cannot exclude the influence of the immunological response. In this study, complementary DNA microarray analysis was performed under immunodeficient conditions with human hepatocyte chimeric mice, and gene expression profiles were analyzed by hepatitis B virus (HBV) infection and/or interferon treatment. The expression levels of 183 of 525 genes upregulated by interferon treatment were significantly suppressed in response to HBV infection. Suppressed genes were statistically significantly associated with the interferon signaling pathway and pattern recognition receptors in the bacteria/virus recognition pathway ($P = 1.0 \times 10^{-8}$ and $P = 1.2 \times 10^{-8}$, respectively). HBV infection attenuated virus recognition and interferon response in hepatocytes, which facilitated HBV escape from innate immunity.

Chronic hepatitis B virus (HBV) infection is associated with the development of virus-related liver diseases, including chronic hepatitis, liver cirrhosis, and hepatocellular carcinoma. Interferon α (IFN-α) has been used for the treatment of chronic hepatitis B, and many large clinical trials and meta-analyses have demonstrated the effectiveness of interferon [1–3]. However, the effect of IFN-α therapy is unsatisfactory, and the molecular basis for tolerance to IFN-α is not clearly defined.

DNA microarray technology has enabled genome-wide analysis of gene transcript levels with the use of clinical tissues and animal models, which has yielded insights into the molecular features of several liver diseases [4–6]. However, it has been difficult to determine whether the changes in gene expression were caused by viral interference or by the human immune response, because all of these studies that used clinical and experimental samples were analyzed under the influence of adaptive immune responses. Recently, Mercer and colleagues developed a human hepatocyte chimeric mouse model [7]. These mice were derived from severe combined immunodeficiency (SCID) mice, which are severely immunocompromised, and the mouse liver cells were extensively replaced with human hepatocytes [7, 8]. With the use of this chimeric mouse model, in which HBV can continuously infect human hepatocytes, the effect of drugs and the response of viral infection can be analyzed in human hepatocytes under immunodeficient conditions [9]. In this study, we performed microarray analysis with human hepatocyte chimeric mouse livers to assess the direct impacts of HBV infection and IFN treatments on gene expression profiles. We successfully demonstrated that HBV infection attenuated the expression of IFN-stimulating genes under immunodeficient conditions, which suggests that HBV proteins might afford escape mechanisms from cellular innate immunity.

METHODS

A serum sample was obtained from a HBV carrier after obtaining written informed consent for the donation and evaluation of the blood sample. The inoculum was positive for Hepatitis B surface and Hepatitis B e antigens, with slightly elevated levels of serum alanine aminotransferase and high-level viremia (HBV DNA load, 7.1 log copies/mL). The studied patient was infected with HBV genotype C. The experimental protocol conformed to the ethical guidelines of the Declaration of Helsinki and was approved by the Hiroshima University Hospital ethical committee (approval ID: D08-9).

The $uPA^{+/+}/SCID^{+/+}$ mice were prepared and the human hepatocytes were transplanted as described elsewhere [8]. The experiments were performed in accordance with the guidelines of the local committee for animal experiments at Hiroshima University.

Sixteen chimeric mice, in which >90% of the liver tissue was replaced with human hepatocytes, were divided into

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4 experimental groups. Group A contained 4 mice that were neither infected with HBV nor treated with IFN. Group B consisted of 3 mice that were treated with IFN-α for 6 h (7,000 IU per gram of body weight) just before being humanely killed but were not infected with HBV. Mice in groups C and D were inoculated via the mouse tail vein with human serum containing 6×10^6 copies of HBV. After inoculation, we collected mouse serum samples every 2 weeks and analyzed HBV DNA titers by real-time polymerase chain reaction (PCR) and human albumin levels by means of a human albumin enzyme-linked immunosorbent assay quantitation kit (Bethyl Laboratories), as described elsewhere [9]. Virus and human albumin titer levels are shown in Supplementary data 1. All 9 mice developed measurable viremia 4 weeks after inoculation. Eight weeks after inoculation, 4 of the 9 infected mice (group C) were humanely killed without IFN treatment and the remaining 5 mice (group D) were humanely killed after 6 h of IFN-α treatment (7,000 IU per gram of body weight). The mice were infected, had serum samples extracted, and were killed humanely under ether anesthesia, as described elsewhere [8].

All 16 chimeric mice were killed humanely, and human hepatocytes were finely dissected from the mouse livers and stored in liquid nitrogen after submerging in RNA later solution (Applied Biosystems). Total RNA was extracted with TRIzol reagent (Invitrogen) and labeled with cyanine 3 by use of a low RNA input linear amplification kit (Agilent Technologies) after amplification. Cyanine-3–labeled complimentary RNA was hybridized to a 44-K whole human genome oligo microarray (Agilent). Detailed protocols are described in Supplementary data 2.

Gene expression profiles were analyzed using GeneSpring GX software (version 10.0.2; Tomy Digital Biology). The detailed protocol is described in Supplementary data table 3. Complete linkage hierarchical clustering analysis was applied using Euclidean distance, and differentially expressed genes were annotated using information from the Gene Ontology (GO) Consortium. Global molecular networks and comparisons of canonical pathways were generated using Ingenuity Pathway Analysis (IPA) software (version 8.6; Ingenuity Systems).

Total RNA was extracted from the implanted human hepatocytes in the mouse livers by use of an RNeasy mini kit (Qiagen) and was reverse transcribed. The selected messenger RNA (mRNA) was quantified by real-time PCR using the 7300 real-time PCR system (Applied Biosystems), and the expression of glyceraldehyde-3-phosphate dehydrogenase served as a control. The amplification protocol and primer sequences are described in Supplementary data 4 and 5.

RESULTS

To analyze the direct effects of IFN in human hepatocytes, we compared the gene expression profiles between groups A (mice without IFN treatment) and B (mice with IFN treatment). Of the 1403 genes that remained after screening with the Welch *T* test, 685 genes showed a >3.0-fold change between groups. Of these 685 genes, 525 genes were up-regulated and the other 160 genes down-regulated by IFN. The top 20 IFN-regulated genes are listed in Supplementary data table 6. GO analysis revealed that 8 (40%) of the top 20 genes that were upregulated with IFN treatment were related to immune response.

To analyze the effect of HBV infection in human hepatocytes, we compared the gene expression profiles between groups A (mice without HBV infection) and C (mice with HBV infection). Among the 1,714 genes hat remained after screening, 373 genes showed a >3.0-fold change between groups. Of these 373 genes, 159 genes were up-regulated and the other 214 genes down-regulated by HBV. The top 20 HBV-regulated genes are listed in Supplementary data table 7. Several oncogenic genes such as growth differentiation factor 15 and glial cell derived neurotrophic factor were included in the top group. Most of the top 20 genes that were downregulated with HBV infection were associated with transcriptional regulation.

To examine whether HBV infection may alter the effect of IFN response in human hepatocytes, we compared gene expression profiles among all groups. As mentioned above, 525 genes were upregulated by >3.0-fold by IFN in the absence of HBV infection. A comparison of groups C (mice with HBV infection but no IFN treatment) and D (mice with both HBV infection and IFN treatment) revealed that 183 (34.9%) of the 525 genes showed statistically significantly reduced IFN response with HBV infection ($P < .01$) (Supplementary data 8A). The top 20 genes in which IFN response was significantly changed by HBV infection are shown in Table 1. The mRNA expression levels of 11 selected genes among the 183 genes with reduced IFN response were also analyzed by real-time PCR, and the reductions in IFN response by HBV infection were verified (Supplementary data 8B). Additionally, we used IPA software to analyze the influence of HBV infection on the IFN response of these 183 genes by means of a pathway-oriented approach. Pathway analysis revealed that several pathways were affected by HBV infection (Table 2). The IFN response was statistically significantly attenuated by HBV infection in the pathways related to IFN signaling and pattern recognition of bacteria and viruses ($P = 1.0 \times 10^{-8}$ and $P = 1.2 \times 10^{-8}$, respectively).

DISCUSSION

Elsewhere we have demonstrated a human hepatocyte chimeric mouse model that can be chronically infected with hepatitis B and C viruses [9–11]. This mouse model facilitates analysis of the effect of viral infection and the response to medication under immunodeficient conditions. In this study, we performed complementary DNA microarray analysis using the chimeric mouse model and obtained gene expression profiles to analyze

Table 1. Genes With Interferon Responsiveness Downregulated by Hepatitis B Virus (HBV) Infection

Gene symbol	GenBank accession no.	Function	Fold change in expression level		*P*
			Without HBV infection	With HBV infection	
ENST00000322831	None	Unknown	4.52	−1.45	4.15×10^{-7}
AA593970	AA593970	EST	9.70	1.61	5.58×10^{-7}
THC2533996	None	Unknown	3.74	−2.50	6.97×10^{-7}
LOC388532	None	Unknown	3.11	−2.48	1.61×10^{-6}
ZNF267	NM_003414	Transcription regulator	7.66	1.79	2.30×10^{-6}
ZNF217	NM_006526	Transcription regulator	3.69	1.03	3.62×10^{-6}
CRSP3	NM_015979	Transcription regulator	7.50	−1.02	4.06×10^{-6}
MGC39372	BC025340	Hypothetical protein	30.92	7.03	5.74×10^{-6}
BF972140	BF972140	EST	16.91	4.71	5.78×10^{-6}
LOC731599	XR_015536	Hypothetical protein	3.17	−4.18	8.58×10^{-6}
LOC645676	AK126559	Hypothetical protein	3.76	1.35	9.13×10^{-6}
THC2650457	None	Unknown	78.07	6.28	1.29×10^{-5}
ZNF24	NM_006965	Transcription regulator	3.69	1.36	1.64×10^{-5}
CCDC68	NM_025214	Unknown	5.88	−2.83	1.89×10^{-5}
SP110	NM_004510	Transcription regulator	5.00	10.77	2.00×10^{-5}
FLJ21272	AK024925	Hypothetical protein	14.70	2.49	3.18×10^{-5}
PLEKHF1	NM_024310	Unknown	6.65	1.84	4.70×10^{-5}
AK026418	AK026418	Unknown	9.50	2.58	5.02×10^{-5}
hCG_1790262	XM_001133847	Unknown	3.13	−2.94	6.25×10^{-5}
CEBPD	NM_005195	Transcription regulator	8.16	1.56	7.03×10^{-5}
FLJ20273	NM_019027	RNA binding	3.37	1.11	7.11×10^{-5}

NOTE. *P* values were analyzed by the Welch *T* test. *CEBPD,* CCAAT/enhancer binding protein (C/EBP) delta; *CCDC68,* coiled-coil domain containing 68; *CRSP3,* mediator complex subunit 23 (*MED23*); EST, expressed sequence tag; *FLJ20273,* RNA binding motif protein 47 (*RBM47*); *PLEKHF1,* pleckstrin homology domain containing, family F (with FYVE domain) member 1; *SP110,* SP110 nuclear body protein; *ZNF24,* zinc finger protein 24; *ZNF217,* zinc finger protein 217; *ZNF267,* zinc finger protein 267.

the direct influence of HBV infection and IFN-α treatment on human hepatocytes.

To avoid contamination with mouse tissue, human hepatocyte chimeric mice, in which liver tissue is largely (>90%) replaced by human hepatocytes, were used in the present study. However, a small amount of mouse-derived cells, such as interstitial cells, bile duct cells, and vascular cells, still remain in the chimeric mouse livers. Because of high homology between the human and mouse genomes, the signals from microarray analyses may be influenced by cross-hybridization with mouse mRNA. It is difficult to produce $uPA^{+/+}/SCID^{+/+}$ mice >10 weeks old without hepatocyte transplantation, and a previous study demonstrated that it is feasible to use microarray analysis in a functional genomics analysis of chimeric mice [12]. Therefore, to compensate for the contamination, the mice in group A, which were neither infected with HBV nor treated with IFN, were used as negative controls.

To analyze the effect of IFN treatment, we compared gene expression profiles between groups A (mice without IFN treatment) and B (mice with IFN treatment); 525 genes with >3.0-fold upregulation following IFN treatment were observed. Among them, chemokine (C-X-C motif) ligand 9, chemokine (C-X-C motif) ligand 10, and chemokine (C-X-C motif) ligand 11, which promote T cell adhesion, were remarkably highly induced with IFN treatment (Supplementary data table 6) [13]. These results suggest that the antiviral effects of IFN might involve not only direct activation of IFN-stimulated proteins such as myxovirus resistance protein A and double strand RNA-dependent protein kinase but also activation of immunity via chemokines.

Second, we compared the profiles between groups A (mice without HBV infection) and C (mice with HBV infection). As shown in Supplementary data table 7, more than half (12) of the top 20 genes upregulated by HBV infection localized to the cell membrane or the extracellular region, but 14 (70%) of the 20 downregulated genes localized to the nucleus. In addition, GO analysis demonstrated that genes related to cell cycle and DNA modification were affected by HBV infection. We speculate that HBV infection promotes cell growth and DNA damage in the hepatocyte nucleus and activates the immune response in the cytoplasm. From the clinical standpoint, some healthy HBV carriers develop hepatocellular carcinoma without chronic hepatitis or cirrhosis. The present results strongly support this observation, showing that most of the affected genes are known to be associated with carcinogenesis.

Clinically, HBV is known to develop tolerance to IFN treatment in patients with chronic hepatitis B, although the mechanism is not clear. We analyzed the IFN response with and

Table 2. Pathway Analysis of 183 Interferon-Induced Genes With Interferon Responsiveness Downregulated by Hepatitis B Virus Infection

Canonical pathways	*P*	Genes
Interferon signaling	1.00×10^{-8}	*IFIT3, SOCS1, IFIT1, MX1, IFNGR1, JAK2, STAT1, TAP1, IRF1*
Role of pattern recognition receptors in recognition of bacteria and viruses	1.20×10^{-8}	*IL12A, OAS2, OAS3*(includes EG:4940), *IFIH1, PIK3R3, TLR4, NOD2, TICAM1, DDX58, CASP1, NOD1, TLR3, RIPK2*
Type 1 diabetes mellitus signaling	2.00×10^{-4}	*SOCS1, IL12A, RIPK1, GAD1, SOCS6, SOCS2, IFNGR1, JAK2, STAT1, IRF1*
Prolactin signaling	2.70×10^{-4}	*PIK3R3, SOCS1, SOCS6, SOCS2, NMI, JAK2, STAT1, IRF1*
TREM1 signaling	3.50×10^{-4}	*TLR4, NOD2, ICAM1, CASP1, JAK2, TLR3, CASP5*
Production of nitric oxide and reactive oxygen species in macrophages	3.90×10^{-4}	*PIK3R3, TLR4, RND3, PPP2R2A, PPM1J, RHOU, IFNGR1, MAP3K8, IRF8, JAK2, STAT1, IRF1*
Pathogenesis of multiple sclerosis	1.10×10^{-3}	*CXCL10, CXCL9, CXCL11*
Activation of IRF by cytosolic pattern recognition receptors	2.60×10^{-3}	*IFIH1, RIPK1, DDX58, STAT1, IFIT2, ISG15*
Dendritic cell maturation	2.60×10^{-3}	*B2M, PIK3R3, TLR4, ICAM1, IL12A, IL1RN, IRF8, JAK2, TLR3, STAT1*
Interleukin 12 signaling and production in macrophages	3.60×10^{-3}	*PIK3R3, TLR4, IL12A, IFNGR1, MAP3K8, IRF8, STAT1, IRF1*
Sphingosine-1-phosphate signaling	3.60×10^{-3}	*PIK3R3, S1PR2, RND3, CASP1, RHOU, CASP4, CASP7, CASP5*
JAK-STAT signaling	4.00×10^{-3}	*PIK3R3, SOCS1, SOCS6, SOCS2, JAK2, STAT1*
Growth hormone signaling	4.70×10^{-3}	*PIK3R3, SOCS1, SOCS6, SOCS2, JAK2, STAT1*
Retinoic acid mediated apoptosis signaling	8.50×10^{-3}	*TNFRSF10B, PARP8, TNFSF10, TIPARP, IRF1*

NOTE. *B2M,* beta-2-microglobulin; *CASP1,* caspase 1, apoptosis-related cysteine peptidase (interleukin 1, beta, convertase); *CASP4,* caspase 4, apoptosis-related cysteine peptidase; *CASP5,* caspase 5, apoptosis-related cysteine peptidase; *CASP7,* caspase 7, apoptosis-related cysteine peptidase; *CXCL9,* chemokine (C-X-C motif) ligand 9; *CXCL10,* chemokine (C-X-C motif) ligand 10; *CXCL11,* chemokine (C-X-C motif) ligand 11; *DDX58,* DEAD (Asp-Glu-Ala-Asp) box polypeptide 58; *GAD1,* glutamate decarboxylase 1 (brain, 67kDa); *ICAM1,* intercellular adhesion molecule 1; *IFIH1,* interferon induced with helicase C domain 1; *IFIT1,* interferon-induced protein with tetratricopeptide repeats 1; *IFIT2,* interferon-induced protein with tetratricopeptide repeats 2; *IFIT3,* interferon-induced protein with tetratricopeptide repeats 3; *IFNGR1,* interferon gamma receptor 1; *IL1RN,* interleukin 1 receptor antagonist; *IL12A,* interleukin 12A (natural killer cell stimulatory factor 1, cytotoxic lymphocyte maturation factor 1, p35); IRF, interferon regulatory factor; *IRF1,* interferon regulatory factor 1; *IRF8,* interferon regulatory factor 8; *ISG15,* ISG15 ubiquitin-like modifier; *JAK2,* Janus kinase 2; *MAP3K8,* mitogen-activated protein kinase kinase kinase 8; *MX1,* myxovirus (influenza virus) resistance 1, interferon-inducible protein p78 (mouse); *NMI,* N-myc (and STAT) interactor; *NOD1,* nucleotide-binding oligomerization domain containing 1; *NOD2,* nucleotide-binding oligomerization domain containing 2; *OAS2,* 2′-5′-oligoadenylate synthetase 2, 69/71kDa; *OAS3,* 2′-5′-oligoadenylate synthetase 3, 100kDa; *PARP8,* poly (ADP-ribose) polymerase family, member 8; *PIK3R3,* phosphoinositide-3-kinase, regulatory subunit 3 (gamma); *PPM1J,* protein phosphatase, Mg2+/Mn2+ dependent, 1J; *PPP2R2A,* protein phosphatase 2, regulatory subunit B, alpha; *RHOU,* ras homolog gene family, member U; *RIPK1,* receptor (TNFRSF)-interacting serine-threonine kinase 1; *RIPK2,* receptor-interacting serine-threonine kinase 2; *RND3,* Rho family GTPase 3; *S1PR2,* sphingosine-1-phosphate receptor 2; *SOCS1,* suppressor of cytokine signaling 1; *SOCS2,* suppressor of cytokine signaling 2; *SOCS6,* suppressor of cytokine signaling 6; *STAT1,* signal transducer and activator of transcription 1, 91kDa; *TAP1,* transporter 1, ATP-binding cassette, sub-family B (MDR/TAP); *TICAM1,* Toll-like receptor adaptor molecule 1; *TIPARP,* TCDD-inducible poly(ADP-ribose) polymerase; *TLR3,* Toll-like receptor 3; *TLR4,* Toll-like receptor 4; *TNFRSF10B,* tumor necrosis factor receptor superfamily, member 10b; *TNFSF10,* tumor necrosis factor (ligand) superfamily, member 10; *TREM1,* triggering receptor expressed on myeloid cells 1.

without HBV infection, focusing on the 525 upregulated genes with IFN treatment and using all obtained gene expression profiles. Interestingly, 61.3% of the extracted genes maintained an IFN response, but in 34.9% of those genes, IFN responses were attenuated by HBV infection (Supplementary data 8A). Genes corresponding to interferon signaling, including suppressor of cytokine signaling 1 (*SOCS1*) and interferon regulatory factor 1, and those corresponding to pattern recognition of bacteria and viruses, including nucleotide-binding oligomerization domain containing 1 (*NOD1*) and receptor-interacting serine-threonine kinase 2 (*RIPK2*), were statistically significantly associated with HBV-mediated attenuation to IFN response ($P = 1.0 \times 10^{-8}$ and $P = 1.2 \times 10^{-8}$, respectively). According to these results, HBV infection significantly up-regulated *SOCS1* expression and reduced the IFN responsiveness of *SOCS1*. Thus, *SOCS1* might support chronic infection of HBV in escaping the effects of innate immunity or IFN therapy. On the other hand, genes involved in recognition of viral infection were also inhibited following HBV infection. Both *NOD1* and *RIPK2* are related to innate and adaptive immune responses [14, 15]. We speculated that inhibition of *NOD1* or *RIPK2* expression facilitates HBV survival. Although further study is needed, these results may have important implications for the mechanisms of viral escape from innate immunity.

In conclusion, we performed complementary DNA microarray analysis using human hepatocyte chimeric mice. With this system, we could analyze the direct effects of IFN treatment and HBV infection without the confounding effects of the lymphocyte immunological response and obtained evidence that HBV infection attenuated the virus recognition and IFN response in

hepatocytes, by which means HBV could evade innate immune detection and response.

Supplementary Data

Supplementary data are available at *The Journal of Infectious Diseases* online.

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Hepatitis C Virus Infection Suppresses the Interferon Response in the Liver of the Human Hepatocyte Chimeric Mouse

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Abstract

Background and Aims: Recent studies indicate that hepatitis C virus (HCV) can modulate the expression of various genes including those involved in interferon signaling, and up-regulation of interferon-stimulated genes by HCV was reported to be strongly associated with treatment outcome. To expand our understanding of the molecular mechanism underlying treatment resistance, we analyzed the direct effects of interferon and/or HCV infection under immunodeficient conditions using cDNA microarray analysis of human hepatocyte chimeric mice.

Methods: Human serum containing HCV genotype 1b was injected into human hepatocyte chimeric mice. IFN-α was administered 8 weeks after inoculation, and 6 hours later human hepatocytes in the mouse livers were collected for microarray analysis.

Results: HCV infection induced a more than 3-fold change in the expression of 181 genes, especially genes related to Organismal Injury and Abnormalities, such as fibrosis or injury of the liver (P = 5.90E-16 ~ 3.66E-03). IFN administration induced more than 3-fold up-regulation in the expression of 152 genes. Marked induction was observed in the anti-fibrotic chemokines such as *CXCL9*, suggesting that IFN treatment might lead not only to HCV eradication but also prevention and repair of liver fibrosis. HCV infection appeared to suppress interferon signaling via significant reduction in interferon-induced gene expression in several genes of the IFN signaling pathway, including *Mx1*, *STAT1*, and several members of the *CXCL* and *IFI* families (P = 6.0E-12). Genes associated with Antimicrobial Response and Inflammatory Response were also significantly repressed ($P = 5.22 \times 10^{-10} \sim 1.95 \times 10^{-2}$).

Conclusions: These results provide molecular insights into possible mechanisms used by HCV to evade innate immune responses, as well as novel therapeutic targets and a potential new indication for interferon therapy.

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Introduction

Chronic hepatitis C virus (HCV) infection is one of the most serious global health threats, affecting more than 170 million people worldwide [1–3]. Interferon is administered to chronic hepatitis C patients to attempt to eradicate the virus and to prevent the development of advanced liver diseases such as chronic hepatitis, cirrhosis, and hepatocellular carcinoma (HCC), with limited success. While the overall eradication rate of HCV has improved since the introduction of pegylated-interferon (PEG-IFN) and ribavirin (RBV) combination therapy, the sustained viral response (SVR) rate of genotype 1b with high viral load still remains only 40–50% [4–6]. Viral and host factors, such as HCV RNA titer, viral substitutions in HCV core or NS5A region, age, gender, liver fibrosis, and SNPs in *IL-28B locus*, are significantly associated with the effects of PEG-IFN and RBV combination therapy [7–15], but the precise molecular mechanisms remained unclear.

Recently, some HCV-related structural as well as non-structural proteins have been reported to be associated with host proteins and affect innate immunity or lipid metabolism. RIG-I (retinoic acid inducible gene I) and Mda5 (melanoma differentiation-

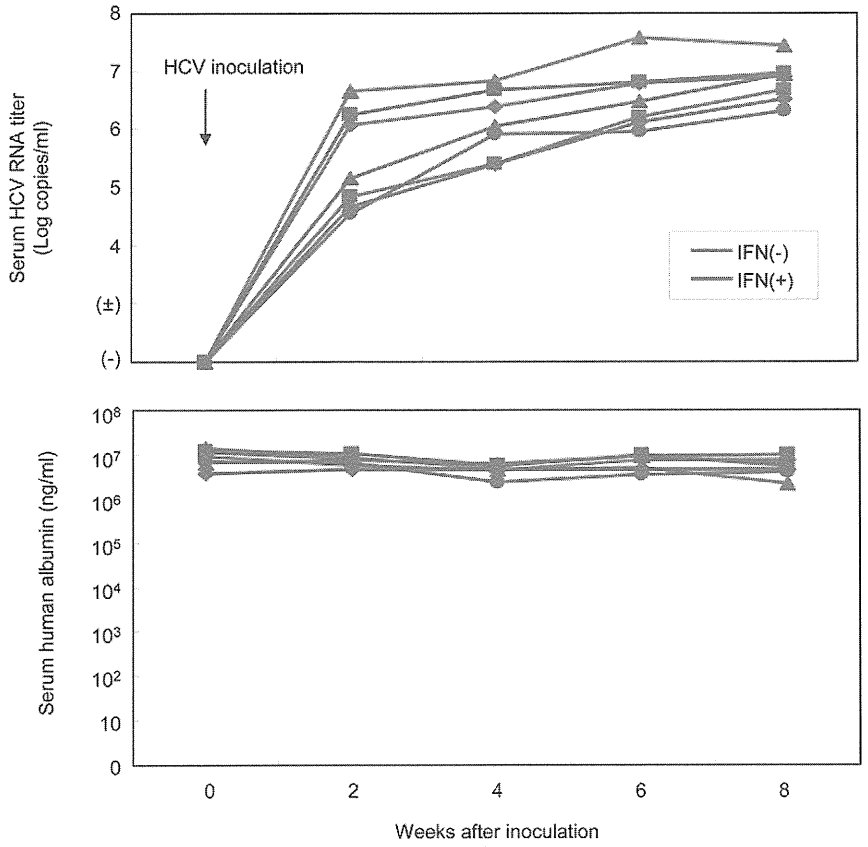

Figure 1. Change in HCV titers and human albumin levels in mouse serum. HCV RNA titers (upper panel) and human albumin levels (lower panel) in chimeric mouse sera after inoculation are shown. The horizontal axis indicates weeks after inoculation. Mouse sera were collected every two weeks after inoculation, and serum HCV RNA and human albumin levels were measured. Results were similar for all mice.
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associated gene 5) are known to activate the type I interferon signaling pathway by interacting with adaptor protein IPS-1/MAVS/VISA/Cardif [16–18]. In the presence of HCV infection, the viral non-structural protein NS3/4A, which has serine protease activity, can cleave and inactivate IPS-1 [19]. TLR (Toll like receptor) is a sensor of RNA or DNA and is known to play various roles in viral infection. Abe et al. demonstrated that HCV non-structural protein NS5A inhibits the recruitment of interleukin-1 receptor-associated kinase 1 by interacting with MyD88 and impairs cytokine production in response to TLR ligands [20]. HCV core protein is also known to interact with host proteins. The core protein promotes hepatic steatosis, insulin resistance and hepatocarcinogenesis through activation of host proteins such as PPARα and MAPK [21–26]. However, these reports were based on *in vitro* analysis of cell lines or used human liver tissues in which results were complicated by adaptive immune responses, and it has been difficult to evaluate the direct impact of HCV infection and interferon administration on human hepatocytes.

Mercer and colleagues developed a human hepatocyte chimeric mouse [27] derived from the severely immunocompromised SCID mouse, in which mouse liver cells were extensively replaced with human hepatocytes [27,28]. This mouse model facilitates continuous HCV infection and makes it possible to analyze the effects of drugs and viral infection on human hepatocytes under immunodeficient conditions [29,30]. To analyze the putative effects of HCV infection or IFN administration without the adaptive immune response, we constructed an HCV carrier mouse model using the human hepatocyte chimeric mouse and performed cDNA microarray analysis using human hepatocytes dissected from the mouse livers. The results are intended to reflect the direct impacts of HCV infection and IFN administration on human hepatocytes and may help in elucidating HCV immune evasion mechanisms.

Materials and Methods

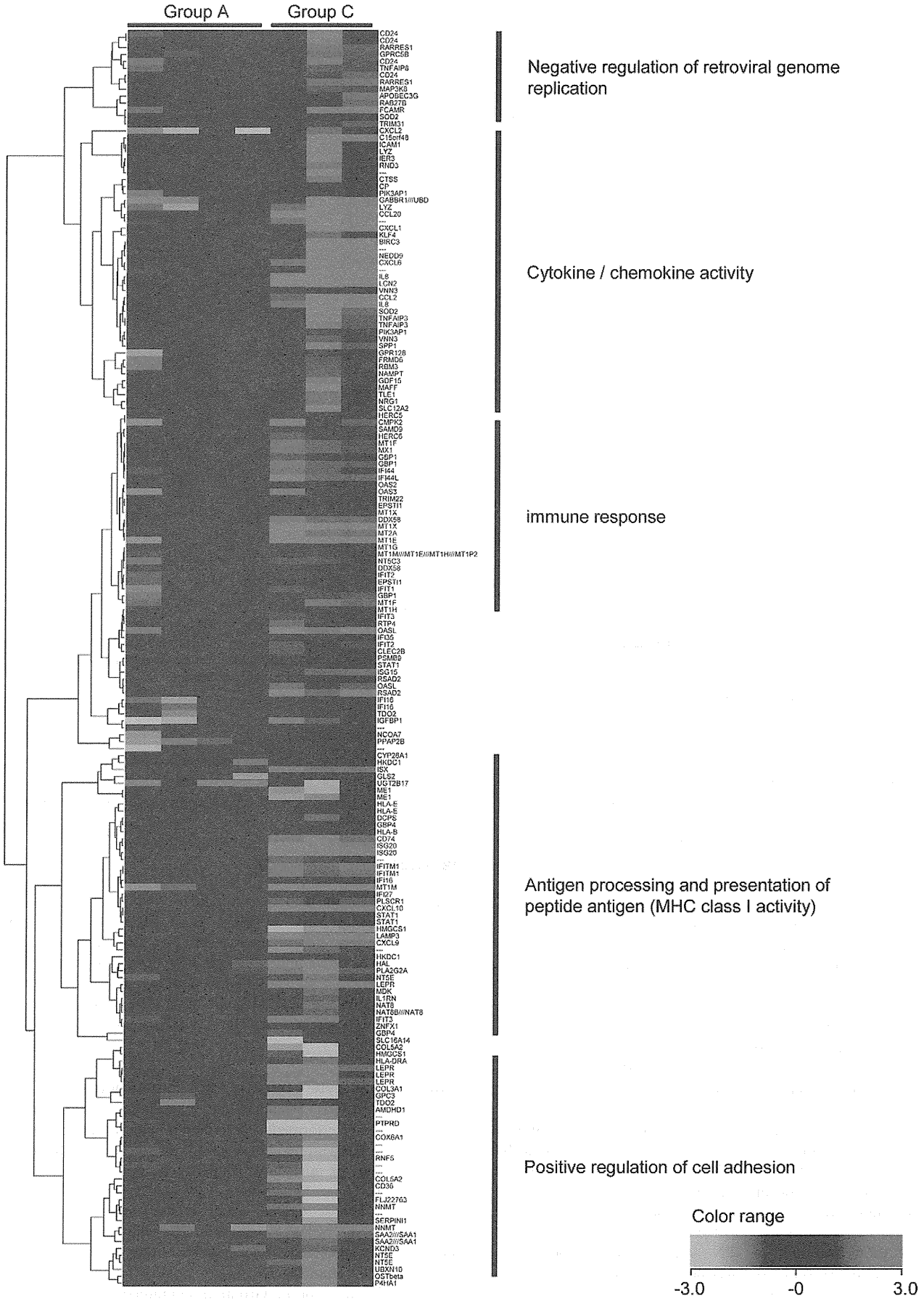
Human Serum Samples

Serum samples were obtained from HCV carriers after obtaining written informed consent for the donation and evaluation of blood samples. Inocula contained high viral loads of genotype 1b HCV RNA (6.9 log copies/ml). The experimental protocol met the ethical guidelines of the 1975 Declaration of Helsinki and was approved by the Hiroshima University Ethical Committee.

Human Hepatocyte Chimeric Mice Experiments

The $uPA^{+/+}/SCID^{+/+}$ mice and transplantation of human hepatocytes were performed as described previously [28]. All mice were transplanted with hepatocytes from the same donor. Human hepatocyte chimeric mice, in which liver cells were largely (>90%) replaced with human hepatocytes, were used to reduce potential influence by mouse-derived mRNA. The experiments were performed in accordance with the guidelines of the local committee for animal experiments at Hiroshima University.

A total of 15 chimeric mice were prepared and assigned to four experimental groups. Group A contained four mice that


Group A
Group C
Negative regulation of retroviral genome replication
Cytokine / chemokine activity
immune response
Antigen processing and presentation of peptide antigen (MHC class I activity)
Positive regulation of cell adhesion
Color range
-3.0
-0
3.0