Table 4 Comparison of clinical and virological characteristics between patients with rapid and non-rapid decrease of HBsAg

Characteristic	Rapid decrease ($n = 31$)	Non-rapid decrease ($n = 67$)	P
At baseline			
Age (years)[a]	52 (15 to 65)	49 (19 to 83)	0.338
Male[b]	17 (55%)	38 (57%)	1.000
With cirrhosis[b]	6 (19%)	13 (19%)	1.000
ALT (IU/L)[a]	27 (10 to 108)	36 (13 to 447)	0.230
HBV genotype (A:B:C:UD)	1:4:26:0	2:4:59:2	0.617
HBeAg-positive[b]	7 (23%)	31 (46%)	0.028
HBsAg (log IU/ml)[a]	2.8 (−1.0 to 5.0)	3.3 (0.8 to 5.3)	0.001
HBcrAg (log U/ml)[a]	<3.0 (<3.0 to >6.8)	5.1 (<3.0 to >6.8)	<0.001
HBV DNA (log copies/ml)[a]	3.7 (<1.7 to >9.5)	5.9 (neg. to >9.5)	0.002
During follow-up			
Followed period (years)[a]	3 (1 to 9)	6 (1 to 10)	<0.001
Change in HBeAg status			0.012
Persistent positive[b]	0 (0%)	15 (22%)	
Became negative[b]	7 (23%)	16 (24%)	
Persistent negative[b]	24 (77%)	36 (54%)	
Clearance of HBsAg[b]	18 (58%)	0 (0%)	<0.001
Complication of HCC[b]	2 (7%)	12 (18%)	0.214
Introduction of NAs[b]	4 (13%)	19 (28%)	0.125

UD undetermined

[a] Data are expressed as median (range)

[b] Data are expressed as positive number (%)

were more uniformly distributed with age in both HBeAg-positive and -negative patients. Therefore, it can be inferred that HBsAg level is affected by age in the natural course of HBV, even when the factor of viral activity is excluded. The precise mechanism of this trend is at present unclear, but may be attributed to the character of HBsAg itself, and not to that of HBV antigens, because HBcrAg levels showed a similar trend as HBV DNA levels. Chan et al. [10] reported that a stronger correlation between HBV DNA and HBsAg was found in the HBeAg-positive phase than in the HBeAg-negative phase. This observation was clearly confirmed by our results in that the distribution pattern analyzed by age was similar between HBsAg and HBV DNA levels in HBeAg-positive patients but differed in HBeAg-negative ones.

The rate of change of HBsAg in the present study suggested the existence of two groups centered around a value of −0.4 log IU/year. A necessary decline in HBV replication was evident in the rapid decrease group, whose median HBV DNA level was lower than the 4.0 log copy/ml usually seen in inactive carriers of HBV. Since no patient with persistently positive HBeAg was classified into the rapid increase group, we presume that a loss of HBeAg is essential for a rapid decrease in HBsAg. In patients with persistently negative HBeAg, HBV DNA levels were significantly lower in the rapid decrease group than in the non-rapid decrease group. Therefore, not only a loss of HBeAg, but also a decline in HBV replication, appears to be fundamental factors necessary for a rapid decrease in HBsAg. Chan et al. [10] concluded that HBs antigen level remained

stable in HBe antigen-positive patients and reduced slowly in HBe antigen-negative patients. Our results are similar, but further imply that a decline in HBV replication is also required. The rate of HBsAg level decrease was similar before and after starting NA treatment in the present study. However, additional studies in larger cohorts will be required to determine this particular relationship.

We analyzed HBcrAg in addition to HBsAg as an HBV-related antigen in the present study to further clarify the characteristics of HBsAg. The HBcrAg assay measures serum levels of HBcAg, HBeAg, and the 22 kDa precore protein [12] simultaneously using monoclonal antibodies that recognize the common epitopes of these denatured antigens. Since the assay measures all antigens transcribed from the pre-core/core gene, it is regarded as core-related [14]. It is possible that levels of HBsAg and HBcrAg have different properties because transcriptions of these two antigens are regulated by alternative enhancer-promoter systems in the HBV genome [15]. Recent studies have shown that HBsAg quantification may represent a surrogate marker of cccDNA concentration in the liver and a potential tool to monitor virologic response to interferon treatment [4, 5, 16]. On the other hand, serum HBcrAg has been reported to accurately reflect intracellular levels of HBV cccDNA even during nucleos(t)ide treatment [11, 17, 18], and was found to be useful for identifying patients who were likely to show relapse of hepatitis after the discontinuation of NAs [19, 20] or who had a higher possibility to develop hepatocellular carcinoma even under NA treatment [17]. Our results here suggest that there exists a

difference in natural course changes between HBsAg and HBcrAg levels. We recently reported that the combined use of these two antigens was useful for predicting the occurrence of hepatitis relapse after cessation of NAs [21]. Such results also indicated that levels of HBsAg and HBcrAg had different clinical significance despite the fact that both antigen levels are generally considered to reflect the amount of HBV cccDNA in hepatocytes.

Complicating HCC occurred during the first 6 years of follow-up in our study at an annual occurrence rate of 2.3% per year for that period. This complication was seen at similar frequencies in patients with high and low baseline HBsAg levels as well as in patients who showed rapid and non-rapid decreases in HBsAg. Patients with lower HBsAg levels and those with rapid decreases in HBsAg have been shown to have lower levels of HBV replication, which would indicate a lower risk of complicating HCC. However, such patients also tend to be older and presumably more predisposed to HCC. The similar occurrence of HCC irrespective of HBsAg status may be attributed to the existence of these two contrary factors. Yuen et al. [7] reported that the risk of HCC in patients with HBsAg seroclearance was higher in those older than 50 years of age; indeed, the single patient who developed HCC after HBsAg seroclearance in the present study was a 90 year-old woman.

In conclusion, lower HBsAg levels were significantly associated with older age and lower viral activity, but not with gender or genotype. Both a loss of HBeAg positivity and a decline in HBV replication are suggested to be fundamental factors necessary for a rapid decrease in HBsAg. Furthermore, the clinical significance of HBsAg may be different from that of HBcrAg with regard to age. Future studies are required to clarify the difference between the two antigens.

Conflict of interest The authors declare that they have no conflict of interest.

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HEPATOLOGY

Long-term follow-up of patients with hepatitis B e antigen negative chronic hepatitis B

Dan Bekku,[*][1] Makoto Arai,[*][1] Fumio Imazeki,[*] Yutaka Yonemitsu,[*] Tatsuo Kanda,[*] Keiichi Fujiwara,[*] Kenichi Fukai,[*] Kenichi Sato,[†] Sakae Itoga,[†] Fumio Nomura[†] and Osamu Yokosuka[*]

Departments of *Medicine and Clinical Oncology and †Department of Molecular Diagnosis, Graduate School of Medicine, Chiba University, Chiba, Japan

Abstract

Background and Aim: After hepatitis B virus (HBV) e antigen (HBeAg) seroconversion, HBV-DNA continues to replicate, and HBeAg-negative patients still face the risk of liver disease progression. We investigated the predictive factors for alanine aminotransferase (ALT) elevation, antiviral drug use, and hepatocellular carcinoma (HCC) occurrence in HBeAg-negative patients.

Methods: Age, sex, ALT, platelet counts, HBV-DNA levels, genotype, antidiabetic drug use, body mass index, smoking, and alcohol consumption were analyzed for a total of 244 HBV carriers who were HBeAg-negative.

Results: Of 244 HBeAg-negative patients, 158 (64.8%) showed normal ALT levels at baseline. Multivariate Cox hazard regression analysis identified high HBV-DNA levels and high ALT at baseline as independent risk factors for ALT elevation in the patients with normal ALT at baseline. The threshold ALT and HBV-DNA levels were determined to be 31 IU/L and 5.3 logcopies/mL, respectively. Seventeen (7.0%) patients used antiviral drugs. Multivariate Cox hazard regression analysis identified high HBV-DNA levels (threshold, 5.7 log copies/mL), the use of antidiabetic drugs, and daily alcohol consumption at baseline as an independent risk factor for the use of antiviral drugs in HBeAg-negative patients. In 10 patients (4.1%), HCC was detected, and a low platelet count (threshold, $10.0 \times 10^4/mm^3$) was associated with the occurrence of HCC.

Conclusion: This study identified predictors of future active liver disease in HBeAg-negative patients, i.e. ALT elevation, unavoidable use of antiviral drugs, and occurrence of HCC.

Introduction

Chronic hepatitis caused by hepatitis B virus (HBV) often follows a fluctuating course characterized by periods of active hepatitis interspersed with quiescence. Therefore, close follow-up is necessary to understand the natural history of HBV patients. On the other hand, patients in which HBV is truly inactive have persistently quiescent disease with an excellent prognosis. Determining an accurate prognosis for HBV carriers based on clinical presentation is important for clinical management of the disease. Various studies have been performed to distinguish the positive and negative prognostic factors among HBV carriers.[1–3]

Hepatitis e antigen (HBeAg) seroconversion is an important event in the natural history of HBV infection. HBV-infected patients usually have a very good prognosis after HBeAg seroconversion.[4] Therefore, HBeAg seroconversion has become an important treatment goal during follow-up of HBV carriers.[5] However, it has also been shown that HBV-DNA replication and hepatic inflammation in seroconverted patients continue despite the

persistent loss of HBeAg; thus, HBeAg-negative patients are likely to develop liver cirrhosis or hepatocelluar carcinoma.[6] In this study, we focused on the natural history of patients with HBeAg-negative chronic hepatitis B, particularly with respect to alanine aminotransferase (ALT) elevation, antiviral drugs, and hepatocellular carcinoma (HCC).

Recently, prognostic factors for HBeAg-negative patients have been investigated in Taiwan and Canada.[7,8] We expect to identify a unique constellation of prognostic factors for HBeAg-negative chronic hepatitis B in the Japanese population, due to differences in race and HBV genotype.

Methods

Patients

Between January 1985 and April 2007, all patients visiting the Chiba University Hospital with HBV infection were approached for participation in the study. This study was carried out only at

one institute, Chiba University Hospital and was approved by ethical the committee of Chiba University. Written informed consent was obtained from all of the patients in accordance with the Declaration of Helsinki. New patients since 1985 and those who were already being followed-up in 1985 were eligible for inclusion in the study. A total of 881 patients were enrolled; of which, 862 were HBsAg positive at enrollment, and 319 were hepatitis B e antibody (HBeAb) positive. Patients who were positive for hepatitis C virus antibody or hepatitis D virus antibody or who had other potential cause of chronic liver diseases (autoimmune hepatitis, primary biliary cirrhosis) were excluded. Patients followed for less than 12 months were also excluded from the analysis. In total, 244 patients were included in the analysis. Serum samples from patients were stored at −20°C and the oldest sample for each patient was used for defining the level of HBV-DNA. The date of evaluation of HBV DNA level by PCR was defined as the baseline. Patient consent was obtained for storage and analysis of serum samples.

Laboratory methods

Serum ALT level was measured using a routine automated method. HBeAg and HBeAb were measured by standard enzyme-linked immunosorbent assays. Patients were screened for hepatitis C virus, hepatitis delta virus, and human immunodeficiency virus antibodies by a third-generation enzyme-linked immunosorbent assay.

HBV-DNA quantitative assay and genotyping

To investigate the level of HBV-DNA in serum, we chose polymerase chain reaction (PCR) assay with an accurate range of 500–200 000 copies/mL (Amplicor HBV monitor test, Roche Diagnostic Systems, Basel, Switzerland). The six major genotypes of HBV (A–F) were determined by enzyme-linked immunosorbent assay (ELISA) (HBV Genotype EIA, Institute of Immunology, Co., Ltd, Tokyo, Japan).

Statistical analysis

ALT elevation was defined as a change from normal ALT (< 42 IU/L) to elevated ALT (≥ 42 IU/L), and normalization was defined as a change from elevated ALT to normal from one visit to the next. Baseline data are presented as mean ± standard deviation (SD). Differences in clinical parameters between groups were analyzed by unpaired *t*-test, Welch *t*-test, and χ^2 tests. The Cox proportional hazards model was used to identify predictive factors for future ALT elevation/normalization, use of antviral drugs, and HCC occurrence using SPSS version 16.1 software (SPSS Inc., Chicago, IL, USA).

Results

Patient characteristics

To investigate the natural course of HBV carriers with HBeAb, 244 carriers (HBeAg-negative and HBeAb-positive) were enrolled in the study. Follow-up was terminated when the use of

antiviral drugs was started or the occurrence of HCC. The baseline clinical and virological characteristics of the 244 HBeAg-negative carriers are shown in Table 1. Because liver biopsy was performed only in 44 (18.0%) out of 244 patients, liver biopsy results could not be analyzed further. Age, sex, ALT, platelet count (PLT), HBV-DNA level, genotype, antidiabetic drug use, body mass index, smoking, and alcohol consumption were analyzed. The average (± SD) period of follow-up was 103.6 ± 74.8 months. Seventeen (7.0%) patients used antiviral drugs (lamivudine in eight and entecavir in nine) and HCC was detected in 10 (4.1%) patients. Two (0.82%) patients died of HCC. In addition, one died of intrahepatic cholangiocarcinoma, one of liver failure due to gastrointestinal bleeding, and one of tongue cancer during the follow-up period. In Japan, the majority of HBV cases are genotype C and B and these genotypes do not cause HBV carrier by way of horizontal infection in adults; therefore, the HBV infection in our HBV carriers mainly occurred by vertical infection or infection during childhood.[9] Thus, the period of HBV infection roughly coincided with the age of HBV carriers in Japan.

ALT and HBV-DNA levels

One hundred and fifty-eight of 244 (64.8%) HBeAg-negative patients had normal ALT levels at baseline. Of these 158 subjects, 85 (53.8%) continued to have normal ALT levels during follow-up, whereas 73 (46.2%) showed fluctuation of ALT levels with intermittently elevated ALT (Fig. 1). A total of 34 (21.5%) patients had ALT ≥ 84 IU/L (more than double the normal limit). Of the 86 patients who had elevated ALT levels at baseline, ALT elevation persisted in 10 (11.6%) and 76 (88.4%) showed ALT fluctuations with intermittently elevated ALT. Although HBV-DNA levels were associated with higher ALT levels in general, correlation was weak ($r^2 = 0.13$).

Platelet count

Patients were sub classified based on PLT as follows: (I) $< 100\,000$ (II) $100\,000$–$149\,000$ (III) $150\,000$–$199\,000$ (IV) $200\,000$–$249\,000$ (V) $> 250\,000$ and more (/mm^3). The numbers of patients in groups I, II, III, IV, and V were 17, 28, 73, 68, and 58, respectively. A total of 84 (34.4%) patients reached a lower platelet count at the end of follow-up.

Risk factors for future ALT elevation in patients with normal ALT levels

Although 158 (64.8%) out of 244 HBeAb-positive patients had normal ALT levels at baseline, 73 patients showed fluctuation of ALT levels with intermittently elevated ALT. We investigated the risk factors for future ALT elevation in these patients. The predictive factors of ALT elevation (ALT > 42 IU/L) in patients with normal ALT levels were HBV-DNA and ALT levels at baseline (Table 2). We carried out an additional univariate analysis changing the threshold of HBV DNA from 3.5 to 7.0 log copies/mL in 0.1 log increments and that of ALT from 15 to 41 IU/L in 1.0 increments. We determined the threshold when the value of probability was smallest; the thresholds for ALT and HBV-DNA levels were 31 IU/L and 5.3 logcopies/mL, respectively. The time

Table 1 Baseline characteristics of hepatitis B virus (HBV) e antigen (HBeAg)-negative patients

	Total	Normal ALT	Elevated ALT	*P*
Number	244	158	86	
Age(years) : (mean ± SD)	44.1 ± 12.5	44.1 ± 13.1	44.0 ± 11.4	NS*
<30	35 (14.3%)	24 (15.2%)	11 (12.8%)	
30–39	52 (21.3%)	32 (20.3%)	20 (23.2%)	
40–49	66 (27.0%)	44 (27.8%)	22 (25.6%)	
50–	91 (37.3%)	58 (36.7%)	33 (38.4%)	
Sex				<0.001**
Male	141 (57.8%)	76 (48.1%)	66 (75.9%)	
Female	103 (42.2%)	82 (51.9%)	21 (24.1%)	
Alanine aminotransferase (ALT) (IU/L) (mean ± SD)	58.9 ± 108.1	20.9 ± 8.7	127.9 ± 160	<0.001*
<20	84			
21–30	47			
31–40	27			
42–84	47			
85–	39			
Platelet count (×10⁴/mm³) (mean ± SD)	205.5 ± 69.6	211.4 ± 60	193.3 ± 81.8	NS*
HBV-DNA (log copies/mL) (mean ± SD)	4.3 ± 1.5	3.8 ± 1.1	5.1 ± 1.7	<0.001*
<4.0	116 (47.5%)	91 (57.6%)	25 (29.1%)	
4.0–4.9	54 (22.1%)	38 (24.1%)	16 (18.6%)	
5.0–5.9	27 (11.1%)	18 (11.4%)	9 (10.5%)	
6.0–6.9	26 (10.7%)	5 (3.2%)	21 (24.4%)	
7.0–	16 (6.6%)	3 (1.9%)	13 (15.1%)	
Genotype				NS**
A	3 (1.2%)	2 (1.3%)	1 (1.2%)	
B	30 (12.3%)	16 (10.1%)	14 (16.3%)	
C	87 (35.7%)	49 (31%)	38 (44.2%)	
Not detected	124 (50.8%)	91 (57.6%)	33 (38.4%)	
Liver Histology (*n* = 44)				
Fibrosis 4/3/2/1	7/8/9/20	0/1/4/13	7/7/5/7	NS**
Activity 3/2/1	7/16/21	1/4/13	6/12/8	NS**
Use of anti-Diabetes drug	20 (8.2%)	3 (1.9%)	6 (7.0%)	NS**
Body mass index (kg/m²) (mean ± SD)	23.3 ± 3.3	23.1 ± 3.2	24.0 ± 3.5	NS**
Smoker/ ever smoker/ non-smoker	32/15/89	16/5/56	16/10/33	NS**
Daily alcohol consumption	46 (27.1%)	24 (23.1%)	22 (33.3%)	NS**
Follow-up (months) (mean ± SD)	103.6 ± 74.8	109.5 ± 76.1	101.8 ± 74.6	NS*

*Unpaired *t*-test and **χ^2 test. NS, not significant difference.

interval from a visit with a normal ALT to a visit with an elevated ALT was used for Kaplan–Meier and Cox regression analysis. Kaplan–Meier curves were constructed for ALT and HBV-DNA levels (Fig. 2).

Risk factors for future use of antiviral drugs for HBV in HBeAg-negative patients

Seventeen (7.0%) patients used an antiviral drug (lamivudine in 8 and entecavir in 9). We investigated the risk factors for future use of antiviral drugs for HBV. The time interval from baseline to the use of an antiviral drug for HBV was used for Cox regression analysis. HBV-DNA levels, use of antidiabetic drugs, and daily alcohol consumption were predictive of future antiviral drug use for HBV, according to the results of multivariate Cox hazard regression analysis. Hazard ratios for HBV-DNA levels, antidiabetic drug use, and daily alcohol consumption were 1.519 (1.130–2.042, 95% confidence interval [CI]), 3.769 (1.203–11.81), and 3.011 (1.086–8.348), respectively. We repeated the univariate

analysis, changing the threshold for HBV DNA from 3.5 to 7.0 log copies/mL in 0.1 log increments. We determined the threshold when the probability value was lowest; the HBV-DNA threshold level was 5.7 log copies/mL. Kaplan–Meier curves were constructed for HBV-DNA levels, antidiabetic drug use, and daily alcohol consumption (Fig. 3).

Risk factors for hepatocellular carcinoma in HBeAg-negative patients

In 10 patients (4.1%), HCC was detected. We investigated the risk factors for HCC in HBeAg-negative patients. The time interval from baseline to occurrence of HCC was used for Cox regression analysis. According to the results of multivariate Cox regression analysis, PLT was predictive of the development of HCC. The hazard ratio for PLT was 0.807 (0.724–0.899, 95% CI). We performed univariate analyses, changing the PLT threshold from 8.0 to 30.0 × 10⁴/mm³ in 1.0 × 10⁴/mm³ increments. We determined the threshold when the value of probability was smallest; the

(a)

(b)
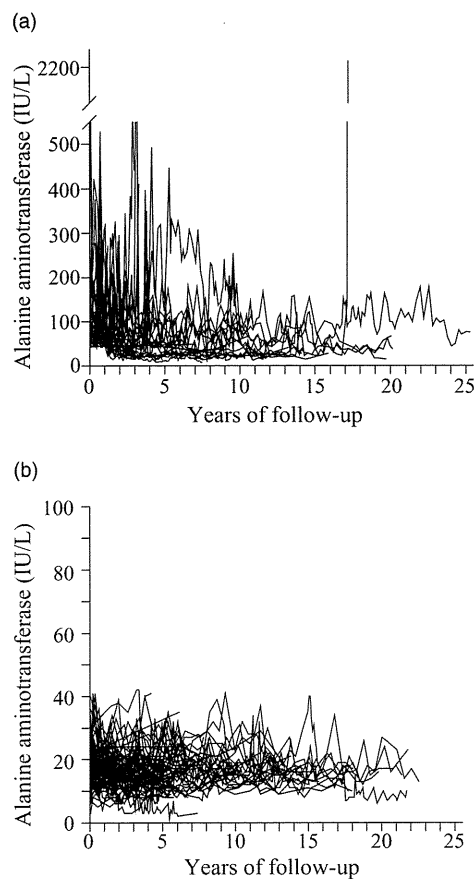
Figure 1 Level of alanine aminotransferase (ALT) in (a) patients with normal ALT at baseline and intermittently elevated ALT during follow-up ($n = 73$) and (b) patients with normal ALT at baseline and during follow-up ($n = 85$).

(a)

(b)
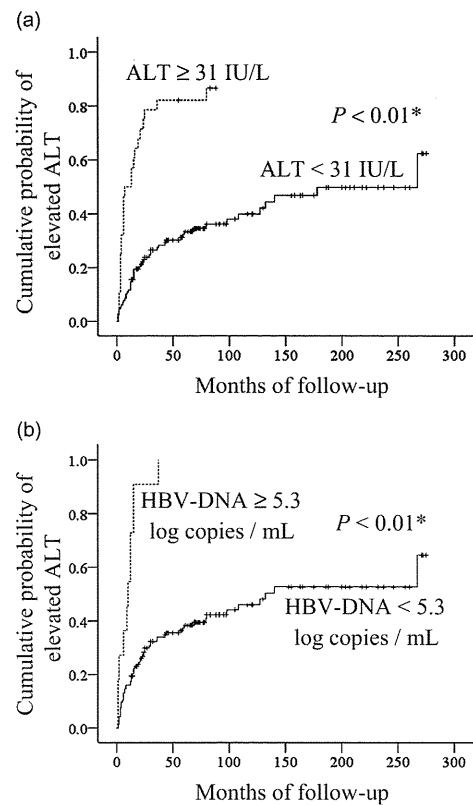
Figure 2 Cumulative occurrence of abnormal alanine aminotransferase (ALT) in HBeAg-negative patients with normal ALT based on (a) ALT and (b) HBV-DNA levels. We determined the threshold for ALT and HBV-DNA levels when the probability value was lowest in the univariate analysis. Kaplan–Meier curves show the time to ALT elevation. Solid lines indicated the control group. *A significant difference was determined by log-rank test.

Table 2 Univariate and multivariate analysis of factors associated with alanine aminotransferase (ALT) elevation in hepatitis B virus (HBV) e antigen (HBeAg)-negative patients with normal ALT levels

	Univariate analysis				Multivariate analysis			
	Standard error	Wald statistic	P-value	Hazard ratio (95% confidence interval)	Standard error	Wald statistic	P-value	Hazard ratio (95% confidence interval)
Sex (Male)	0.263	0.203	0.652	1.126 (0.673–1.885)				
Age (years)	0.011	5.704	0.017	1.027 (1.005–1.049)	0.252	0.068	0.794	1.015 (0.572–1.534)
HBV-DNA	0.109	17.773	<0.001	1.587 (1.280–1.966)	0.111	10.602	0.001	1.437 (1.155–1.788)
Genotype								
B	0.459	0.22	0.639	0.806 (0.328–1.982)				
C	0.435	0.055	0.815	1.107 (0.472–2.600)				
Alanine aminotransferase	0.014	42.440	<0.001	1.097 (1.067–1.128)	0.015	29.496	<0.001	1.086 (1.054–1.119)
Platelet count	0.019	5.928	0.015	0.955 (0.920–0.991)	0.021	0.754	0.385	0.982 (0.942–1.023)
Use of anti-diabetes drug	0.427	0.470	0.493	1.340 (0.581–3.091)				
Body mass index (kg/m²)	0.042	0.033	0.855	0.992 (0.913–1.078)				
Smoker and ever smoker	0.374	0.111	0.739	1.133 (0.544–2.359)				
Daily alcohol consumption	0.333	0.512	0.474	1.269 (0.661–2.435)				

(a)

(b)

(c)

Figure 3 Cumulative occurrence of antiviral drug use for hepatitis B virus (HBV) in HBeAg-negative patients based on (a) HBV-DNA levels (b) use of antidiabetic drug, and (c) daily alcohol consumption. We determined the threshold for HBV-DNA levels when the probability value was lowest in the univariate analysis. Kaplan–Meier curves show the time to use of antiviral drugs for HBV. Solid lines indicated the control group. *A significant difference was determined by log-rank test.
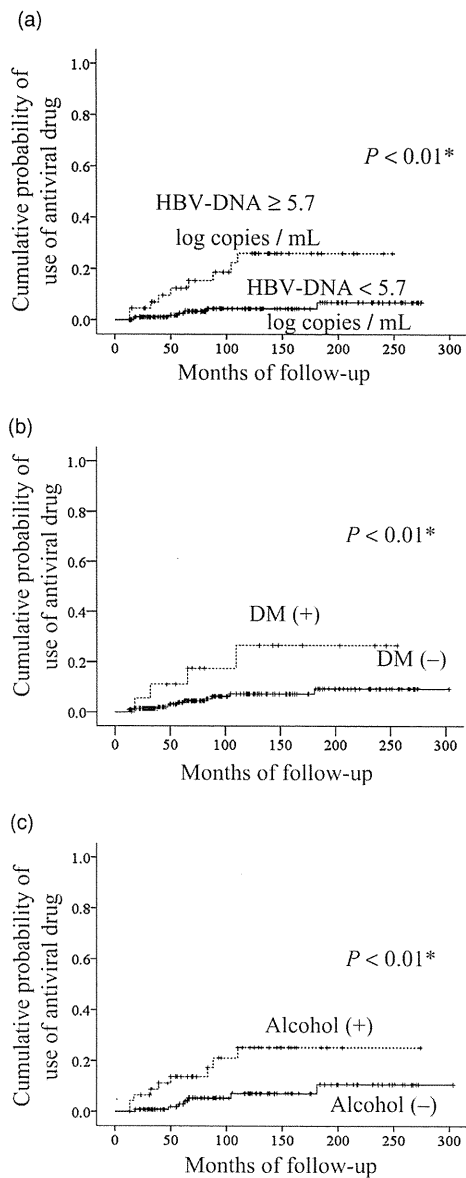
PLT threshold was $10.0 \times 10^4/mm^3$. Kaplan–Meier curves were constructed for PLT (Fig. 4).

Stratification analyses of risk factors for clinical outcomes in HBeAg-negative patients by age, sex, and HBV genotype

The stratification analyses by age, sex, and HBV-genotype were performed to evaluate the risk factors for future ALT elevation in
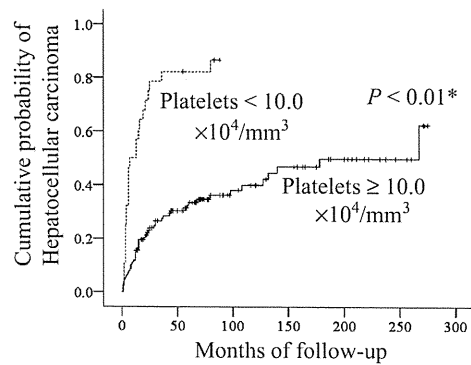
Figure 4 Cumulative occurrence of hepatocellular carcinoma (HCC) based on the platelet counts. We determined the threshold for HBV-DNA levels when the probability value was lowest in the univariate analysis. Kaplan–Meier curves show the time to HCC. Solid lines indicated the control group. *A significant difference was observed by log-rank test.

patients with normal ALT levels, future use of antiviral drugs for HBV, and HCC in HBeAg-negative patients (Table 3). The age threshold was 45 years, which was the average age of all the patients. We did not perform stratification analysis for patients infected with HBV genotype B because the number of such cases was very small.

Discussion

Most patients who have undergone HBeAg seroconversion have normal serum ALT levels, which is indicative of a good clinical outcome.[10] Therefore, various therapies for early seroconversion have been used.[5] Recently, HBeAg-negative viral mutants have been shown to be responsible for continuous HBV-DNA replication.[7] That is, there exists the possibility that liver disease will get worse after HBeAg seroconversion. In fact, previous reports revealed that HBeAg status is not a predictive factor for HCC,[11,12] and fulminant hepatitis can occur by the infection of HBV with HBeAg-negative.[13] HBeAg-negative patients should be monitored closely, even though most of these patients show normal ALT levels and no progressive liver disease.[14] Therefore, predictive factors for active liver disease in HBeAg-negative patients need to be identified in order to facilitate optimal disease management. This study provides data regarding the prediction of future active liver disease, i.e. ALT elevation, unavoidable use of antiviral drugs, and occurrence of HCC.

Many previous reports have attempted to define a threshold HBV-DNA level that corresponds to the presence of active liver disease.[15] A National Institute of Health workshop demonstrated that an HBV-DNA level of 10^5 copies/mL could be used to distinguish active HBV infection from inactive HBV infection.[16] Other studies also suggested that the threshold HBV-DNA level lies somewhere between 10^4 and 10^6 copies/mL.[8] In this study, in order to clarify the natural course of HBeAg-negative patients with normal ALT levels, we used a HBV-DNA threshold of $10^{5.3}$ copies/mL. By log rank analysis, the ALT levels in patients with $>10^{5.3}$ copies/mL HBV-DNA level were significantly higher than in patients with HBV-DNA below this level. In HCV patients, ALT is

Table 3 Stratification analysis multivariate analysis of factors associated with alanine aminotransferase (ALT) elevation in hepatitis B virus (HBV) e antigen (HBeAg)-negative patients with normal ALT levels, future use of antiviral drugs for HBV, and occurrence of hepatocellular carcinoma

	Age (years) ≥45 years n=126			Age (years) <45 years n=118			Sex Male n=141			Sex Female n=103			Genotype C n=87		
	Factors	Hazard ratio (95% CI)	P-value	Factors	Hazard ratio (95% CI)	P-value	Factors	Hazard ratio (95% CI)	P-value	Factors	Hazard ratio (95% CI)	P-value	Factors	Hazard ratio (95% CI)	P-value
Future ALT elevation in the patients with normal ALT level	HBV-DNA	1.535 (1.146–2.057)	0.004	ALT	1.106 (1.059–1.156)	<0.001	ALT	1.194 (1.075–1.325)	<0.001	ALT	1.060 (1.015–1.108)	0.008	ALT	1.149 (1.075–1.228)	<0.001
	ALT	1.077 (1.035–1.122)	<0.001												
Future use of anti-viral drugs for HBV	Alcohol	4.744 (1.362–16.52)	0.014	HBV-DNA	2.238 (1.107–4.526)	0.025	HBV-DNA	1.486 (1.053–2.098)	0.024	HBV-DNA	1.739 (1.213–2.492)	0.003	HBV-DNA	1.902 (1.223–2.956)	0.004
										DM†	86.14 (1.842–4027.3)	0.023	Alcohol	5.617 (1.431–22.05)	0.013
										BMI†	0.408 (0.187–0.890)	0.024			
Occurrence of hepatocellular carcinoma	PLT	0.772 (0.659–0.905)	0.001	(–)†			PLT	0.832 (0.731–0.948)	0.006	PLT†	0.775 (0.635–0.947)	0.013	PLT	0.833 (0.732–0.948)	0.006

†Three patients were used in subgroup for HBV antiviral drugs and HCC occurrence.
Alcohol, Daily alcohol consumption; BMI, Body mass index; CI, confidence interval; DM, use of antidiabetic medication; PLT, platelet count.

a poor surrogate marker for inflammation and fibrosis.[17] Therefore, even if the patient's ALT level was within normal limits, they should still be monitored closely, and HCV eradication therapy is recommended under certain circumstances. Similarly, even if the ALT levels are within normal limits in HBV-infected patients who are HBeAg-negative, the higher their ALT levels were, the more frequently their ALT levels would be high in the future, which might cause progressive liver disease.[18]

Some of the patients with progressive liver disease caused by HBV infection were treated with the antiviral drugs lamivudine and entecavir. The use of lamivudine or entecavir might result in mutant HBV resistance to antiviral drugs[19,20] and the associated costs are not trivial. The baseline levels of HBeAg, ALT, and HBV-DNA, and the presence of either chronic hepatitis or cirrhosis have been established as determinants for eligibility for antiviral treatment.[21] According to treatment guidelines in the Unites States (National Guideline Clearinghouse, http://www.guideline.gov), patients with HBeAg-negative chronic hepatitis B should be considered for antiviral treatment based on their HBV-DNA and ALT levels (serum HBV-DNA >20 000 IU/mL and elevated ALT >2 times normal). In this study, only four out of 17 patients treated with an antiviral drug showed normal ALT levels at baseline, and all four patients showed elevated ALT levels in 8–57 months later. Therefore, this study revealed that patients with high HBV-DNA levels tended to have high ALT levels at baseline or in the future; as a result, such patients have a tendency for future treatment with and antiviral drug.

Hepatocellular carcinoma occurrence was noted in only 10 cases (4.1%). The only predictive factor for HCC occurrence was PLT, which meant that patients with advanced liver disease tended to develop HCC later, because the decrease in PLT corresponded to the extent of liver fibrosis. Four patients (1.6%) died of liver-related diseases and one (0.4%) died of cancer in another organ. The number of deaths was too small to determine the predictive factors for death of HBeAg-negative HBV carriers. Further analysis is needed to properly address this factor.

Stratification analyses of risk factors for clinical outcomes by age, sex, and HBV-genotype were performed. Because the numbers of female patients with future use of antiviral drugs for HBV (n = 3), HCC occurrence (n = 3), or who were under 45 years old with HCC occurrence (n = 3) were very small, it was not possible to properly evaluate these subgroups. The risk factors among subgroups for future ALT elevation in patients with normal ALT levels, and for HCC were almost equal to those of the entire patient population. However, daily alcohol consumption, not HBV-DNA level, was predictive of future use of antiviral drugs for HBV in patients ≥45 years old or in patients infected with HBV genotype C. In these subgroups, alcohol consumption was an important factor for predicting the clinical course of HBV carriers; i.e. advising patients to abstain from drinking might reduce the need for antiviral drugs in the future.

Coffee or caffeine consumption is reported to be strongly related to ALT levels and HCC occurrence.[22–24] In our study, we did not survey caffeine consumption; therefore, further analysis is needed to determine the importance of coffee or caffeine consumption as a predictive factor of the clinical course in HBeAg-negative HBV carriers.

In conclusion, we established that low HBV-DNA levels and ALT levels at baseline were good predictors for future ALT eleva-

tion in HBeAg-negative HBV carriers with normal ALT levels. In addition, this study provides data on the prediction of unavoidable antiviral drug use and HCC occurrence.

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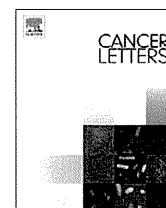
Nuclear receptor mRNA expression by HBV in human hepatoblastoma cell lines

Shuang Wu [a], Tatsuo Kanda [a,*], Fumio Imazeki [a], Shingo Nakamoto [a,b], Hiroshi Shirasawa [b], Osamu Yokosuka [a]

[a] Department of Medicine and Clinical Oncology, Chiba University, Graduate School of Medicine, 1-8-1 Inohana, Chuo-ku, Chiba 260-8677, Japan
[b] Department of Molecular Virology, Chiba University, Graduate School of Medicine, 1-8-1 Inohana, Chuo-ku, Chiba 260-8677, Japan

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ABSTRACT

Recent studies have implicated nuclear receptors (NRs) in the development of hepatocarcinogenesis. We assumed that hepatitis B virus (HBV) alters the expression of NRs and coregulators, and compared the gene expression profiling for 84 NRs and related genes between HpeG2.2.15, which secretes complete HBV virion, and HepG2 by real-time RT-PCR with SyBr green. Forty (47.6%) genes were upregulated 2-fold or greater, and only 5 (5.9%) were downregulated 2-fold or more, in HepG2.2.15 compared to HepG2. These results suggest that HBV affects NRs and their related signal transduction, and that they play important roles in viral replication and HBV-related hepatocarcinogenesis.

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1. Introduction

Hepatocellular carcinoma (HCC) is a common cancer worldwide, accounting for ~5.6% of all cancers [1]. It is the fifth most common cancer in the world and the third most common cause of cancer death [2]. Nearly 450,000 liver cancer deaths occur in Asia each year. Hepatitis B virus (HBV) infection is considered one of the major etiological factors associated with HCC. HBV is a small hepatotropic and highly species-specific enveloped DNA virus member of the Hepadnaviridae family [3,4]. HBV represents the prototype virus of the Orthohepadnavirus genus. More than 2 billion people have been exposed to HBV, and 350 million remain infected worldwide. HBV causes acute and chronic hepatitis, cirrhosis, and HCC [5,6].

Differential control of gene expression has become a central theme in cancer biology. Lipophilic hormones, because they both originate diffusely from a source and permeate to a target, are ideal candidates to serve as regulators of this process. These hormones, including steroids, retinoids, thyroid hormones, and vitamin D3, are potent regulators of development, cell differentiation, and organ physiology [7]. Many of these are associated with known human diseases. These hormones as ligands interact with nuclear receptors (NRs), leading to a suggested link between transcriptional control and physiology. NRs are characterized by a central DNA-binding domain (DBD), which targets the receptor to specific DNA sequences known as hormone response elements. The C-terminal half of the receptor encompasses the ligand-binding domain (LBD), which possesses the essential property of hormone recognition and ensures both specificity and selectivity of the physical response. In its simplest terms, LBD can be thought of as a molecular switch that, upon binding with ligand, shifts the receptor to a transcriptionally active state [7,8]. The effects of NRs on transcription are mediated through recruitment of coregulators. A subset of receptors also binds with corepressor factors, and they actively repress target gene expression in the absence of ligand. The characterization of corepressor and coactivator complexes, in concert with the identification of the specific interaction motifs in the receptors, has

demonstrated the existence of a general molecular mechanism by which different receptors elicit their transcriptional responses in target genes.

NRs such as androgen receptor (AR), estrogen receptor (ER), hepatocyte nuclear factor 4 (HNF4), and retinoid acid receptor (RXR) alpha are implicated in the development of HCC in association with or without HBV [9–12]. Human alpha-fetoprotein (AFP) contains potential heterodimerization motifs capable of interacting with NRs and transcription/growth factors [13]. Moreover, it is also possible that multiple NRs and their signals might regulate HBV replication [13,14].

Corepressors are found within multicomponent complexes that contain histone deacetylase (HDAC) activity. Deacetylation leads to chromatin compaction and transcriptional repression. Upon ligand binding, the receptors undergo a conformational change that allows the recruitment of multiple coactivator complexes. Some of these proteins are chromatin remodeling factors or possess histone acetylase activity, whereas others may interact directly with the basic transcriptional machinery. Recruitment of coactivator complexes to the target promoter causes chromatin decompaction and transcriptional activation.

HepG2.2.15 cells assemble and secrete complete virions that cause hepatitis in chimpanzees [15–17]. Quantitative real-time reverse transcription-polymerase chain reaction (RT-PCR) is the most sensitive technique for mRNA detection and quantification [18]. In the present study, we investigated NRs and their related molecule expression levels in the human hepatoma cell lines HepG2.2.15 and HepG2 using real-time RT-PCR. We observed that multiple NRs expressions in HepG2.2.15 cells are different from those in their parental HepG2 cells. These observations suggest that HBV affects NRs expression in hepatocytes, and it is considered that changes in NR expression possibly play an important role in HBV replication and lead to hepatocarcinogenesis.

2. Materials and methods

2.1. Cell culture

Human hepatoblastoma cell lines HepG2 and HepG2.2.15 were cultured in Roswell Park Memorial Institute medium (RPMI-1640) (Sigma–Aldrich, St. Louis, MO, USA) supplemented with 10% fetal bovine serum (FBS) under 5% CO_2 at 37 °C. HepG2.2.15 cells are derived from HepG2 and are characterized by stable 1.3-fold HBV (serotype adw) genome expression and replication in the culture system [15–17]. Trichostatin A (TSA), 1α, 25-dihydroxy vitamin D3 (Calcitriol) and 5α-androgen-17β-ol-3-one (DHT) were purchased from Wako Pure Chemical Industries Ltd. (Osaka, Japan), Cayman Chemical Company (Ann Arbor, MI, USA) and Sigma–Aldrich, respectively.

2.2. RNA extraction

Cells were seeded into 6-well plates, and total cellular RNA was extracted 48 h later using the RNeasy Mini kit (Qiagen, Tokyo, Japan) according to the manufacturer's instructions. RNA samples were then stored at −80 °C until

use. RNA quality was examined using the A_{280}/A_{260} ratio (Pharmacia Biotech, Bedford, MA, USA).

2.3. cDNA synthesis and real-time PCR

cDNA synthesis was performed using RT^2 First Strand Kit (SABiosciences, Frederick, MD, USA). Each 1 μg of RNA was subjected to one reaction. cDNA synthesis reaction was as follows: incubation at 42 °C for 15 min and then reaction stoppage by heating at 95 °C for 15 min. RNA quantitation was conducted by real-time PCR with SyBr Green I, as we described previously [18]. Gene quantification was determined using the ABI Prism 7300 from Applied Biosystems (Foster City, CA, USA). Thermal cycling conditions were 95 °C for 10 min followed by 40 cycles at 95 °C, 15 s for denaturation, and 1 min at 60 °C for annealing and extension. All primers for examining the gene expression of human NRs and coregulators were purchased from SABiosciences. Gene expression was normalized to beta-actin to determine the fold change in gene expression between test sample (HepG2.2.15) and control sample (HepG2) by 2^{-ddCT} (comparative cycle threshold) method [18]. Genes were annotated by Entrez Gene (NCBI, Bethesda, MD, USA). Data were analyzed with RT^2 prolifer PCR array data analysis software (http://www.superarry.com/pcrarraydataanalysis.php).

2.4. Quantitative PCR (Q-PCR) assay for HBV DNA

Total cellular DNA was extracted using a specific isolation kit (Sanko-junyaku, Tokyo, Japan). Reactions were determined with the SYBR Green I detection chemistry system using an ABI Step One real-time PCR system (Applied Biosystems). To detect HBV genome sequences, PCR was performed using the sense (5′-GTGTCTGCGGCGTTTTAT-CA-3′) and antisense (5′-GACAAACGGGCAACATACCTT-3′) primers. GAPDH was used as a control gene for normalization, and data were analyzed by the comparative threshold cycle method.

2.5. Cell viability assay

To evaluate cell growth and cell viability, dimethylthiazol carboxymethoxyphenyl sulfophenyl tetrazolium (MTS) assay was performed using CellTiter 96 Aqueous One-Solution Cell Proliferation Assay Kit (Promega, Madison, WI, USA) [18].

2.6. siRNA

The siRNA si-AR was named on the basis of its target gene AR. The si-C was negative control siRNA [19]. They were ON-TARGETplus siRNAs purchased from Thermo Fisher Scientific Inc. (Waltham, MA, USA). siRNAs were transfected into HepG2 and HepG2.2.15 cell by electroporation. Briefly, subconfluent cells were detached by trypsin treatment, collected by centrifugation (500g; 10 min), and washed two times in RNase-free phosphate-buffered saline. siRNAs were mixed at 0.4 mL of 2.0×10^6 cells in a 4-mm-gap-width cuvette (Bio-Rad, Hercules, CA, USA) and pulsed using a Bio-Rad GenePulser X cell (electroporation conditions, 270 V and 950 μF). The pulsed cells were

left to recover for 10 min at room temperature and then seeded into two 35-mm-diameter culture dishes. MTS assays were harvested at 48 h posttransfection.

2.7. Statistical analysis

All experiments were performed at least in triplicate. Results were expressed as mean ± SD. Student's t test was used to determine statistical significance.

3. Results

3.1. Intracellular NR gene expression in HepG2.2.15 cell lines

Hepatocytes are a major site of HBV replication. We examined NR-related gene expression profiles using real-time PCR-based focused microarrays. A comparison of NR-related genes between HepG2.2.15 and HepG2 cells is shown in Fig. 1A. Out of 84 cytokine-related genes examined, 40 (47.6%) were upregulated by 2-fold or greater in HepG2.2.15 than in HepG2. Eleven genes [NR subfamily 1, group H, member 4 (NR1H4), NR5A1, NR0B2, NR1I2, NR6A1, RAR-related orphan receptor A (RORA), HNF4A, HDAC2, thyroid hormone receptor beta (THRB), NR1I3 and thyroid hormone receptor associated protein 5 (THRAP5: MED16)] were upregulated 5-fold or more in HepG2.2.15 cells. The results of the genes upregulated 2-fold or more are summarized in Fig. 1B. On the other hand, out of 84 genes, 5 (5.9%) genes [NR1D2, notch homolog 2 (NOTCH2), NR co-repressor 2 (NCOR2), retinoic acid receptor gamma (RARG) and estrogen-related receptor gamma (ESRRG)] were downregulated 2-fold or more in HepG2.2.15 than in HepG2, with two of them (RARG, ESRRG) downregulated 5.0-fold or more.

3.2. NR genes were differentially expressed in HepG2.2.15 and HepG2

Further analysis revealed that among ARs and related molecules, five genes were upregulated 2-fold or greater, and NR1I3 and THRAP5 were upregulated 5-fold or greater in HepG2.2.15 cells (Fig. 2A). Among ERs and related molecules, two genes [peroxisome proliferator-activated receptor gamma, coactivator 1 beta (PPARGC1B) and NCOA6] were upregulated 2-fold or greater in HepG2.2.15 cells (Fig. 2B). NCOA6 also belongs to glucocorticoid receptors and related molecules (Fig. 2C), to RXRs and related molecules (Fig. 2D) and to thyroid hormone receptors and related molecules (Fig. 2E). Among RXRs and related molecules, RXR alpha (RXRA) was also upregulated 2-fold or greater in HepG2.2.15 cells, while RXR gamma (RARG) was downregulated 2-fold or more in HepG2.2.15 cells (Fig. 2D). It was reported that RXRA can support HBV transcription and replication [20]. Among thyroid hormone receptors and related molecules, nine genes were upregulated 2-fold or greater, and THRB, NR1I3 and THRAP5 were upregulated 5-fold or greater in HepG2.2.15 cells (Fig. 2E). THRAP5 also belongs to vitamin D receptors and related molecules (Fig. 2F). Among steroid hormone receptors and other NRs, 17 genes were up-regulated 2-fold or greater and NR1H4, NR5A1, NR0B2, NR1I2, NR6A1, RORA, HNF4A and THRB were upregulated 5-fold or greater while NR1D2, RARG and ESRRG were downregulated 2-fold or more in HepG2.2.15 cells (Fig. 2G and H). NR5A1, also known as human HBV enhancer II B1 binding factor (hB1F), plays important roles in HBV replication and infection [21]. Bioinformatics analysis revealed RORA to be one of the potential cellular anti-HBV targets [22]. HNF4A is also known to affect the HBV life cycle [23].
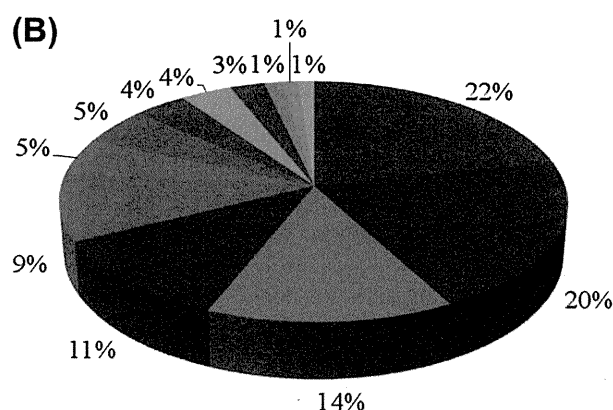
3.3. Transcription factors and regulators associated with NRs were differentially expressed in HepG2.2.15 and HepG2

Next, we analyzed transcription factors and regulators associated with NRs and revealed that among ligand-dependent NR transcription coactivators, seven genes were upregulated 2-fold or greater, and THRAP5 was upregulated 5-fold or greater in HepG2.2.15 cells (Fig. 3A). Among positive regulators of transcription factors, 10 genes were upregulated 2-fold or greater, and NR5A1 and NR1I3 were upregulated 5-fold or greater in HepG2.2.15 cells (Fig. 3B). Among negative regulators of transcription factors, four genes were upregulated 2-fold or greater and NR0B2 and THRB were upregulated 5-fold or greater while NCOR2 was downregulated 2-fold or more in HepG2.2.15 cells (Fig. 3C). Among other

(A) Real-time PCR for 84 nuclear receptor and coregulator gene expressions

HepG2.2.15 cells
vs.
HepG2 cells

Upregulated genes: 40 (≥ 2-fold)
Downregulated genes: 5 (≥ 2-fold)

(B)

■ Steroid hormone receptors
■ Other transcription factors and regulators
■ Positive regulators of transcription
■ Thyroid hormone receptors and related molecules
■ Ligand-dependent nuclear receptor transcription coactivators
■ Androgen receptors and related molecules
■ Vitamin D receptors and related molecules
■ Estrogen receptors and related molecules
■ Negative regulators of transcription
■ Retinoic acid receptors and related molecules
■ Glucocorticoid receptors and related molecules
■ Histone acetylation
■ Other nuclear receptors

Fig. 1. Differential expression of NR and coregulator genes between HepG2.2.15 and HepG2 cells. (A) RNAs were extracted from HepG2.2.15 and HepG2 cells and used in focused microarray analysis for 84 genes. All gene expression levels were corrected by comparison with the levels of actin. Three sets of real-time PCR arrays were performed, and the results were analyzed using RT^2 prolifer PCR array data analysis software (SABiosciences, Frederick, MD, USA). (B) The details of genes upregulated 2-fold or greater in HepG2.2.15 than HepG2 cells are shown.

transcription factors, 15 genes were upregulated 2-fold or greater, and NR1H4, NR6A1, RORA, HNF4A and HDAC2 were upregulated 5-fold or greater while NR1D2, NOTCH2, RARG and ESRRG were downregulated 2-fold or more in HepG2.2.15 cells (Fig. 3D). It was reported that HDAC2 was highly expressed in HBV-associated HCC [24].

3.4. Chromatin modification molecules associated with NRs and other NR co-regulators were differentially expressed in HepG2.2.15 and HepG2

We also examined chromatin molecules. Among histone acetylation molecules, NCOA1 was upregulated 2-fold or greater (Fig. 4A). Among histone deacetylation molecules, five genes were upregulated 2-fold or greater, and HDAC2 was upregulated 5-fold or greater in HepG2.2.15 cells (Fig. 4B). Among other chromatin modification molecules, two genes
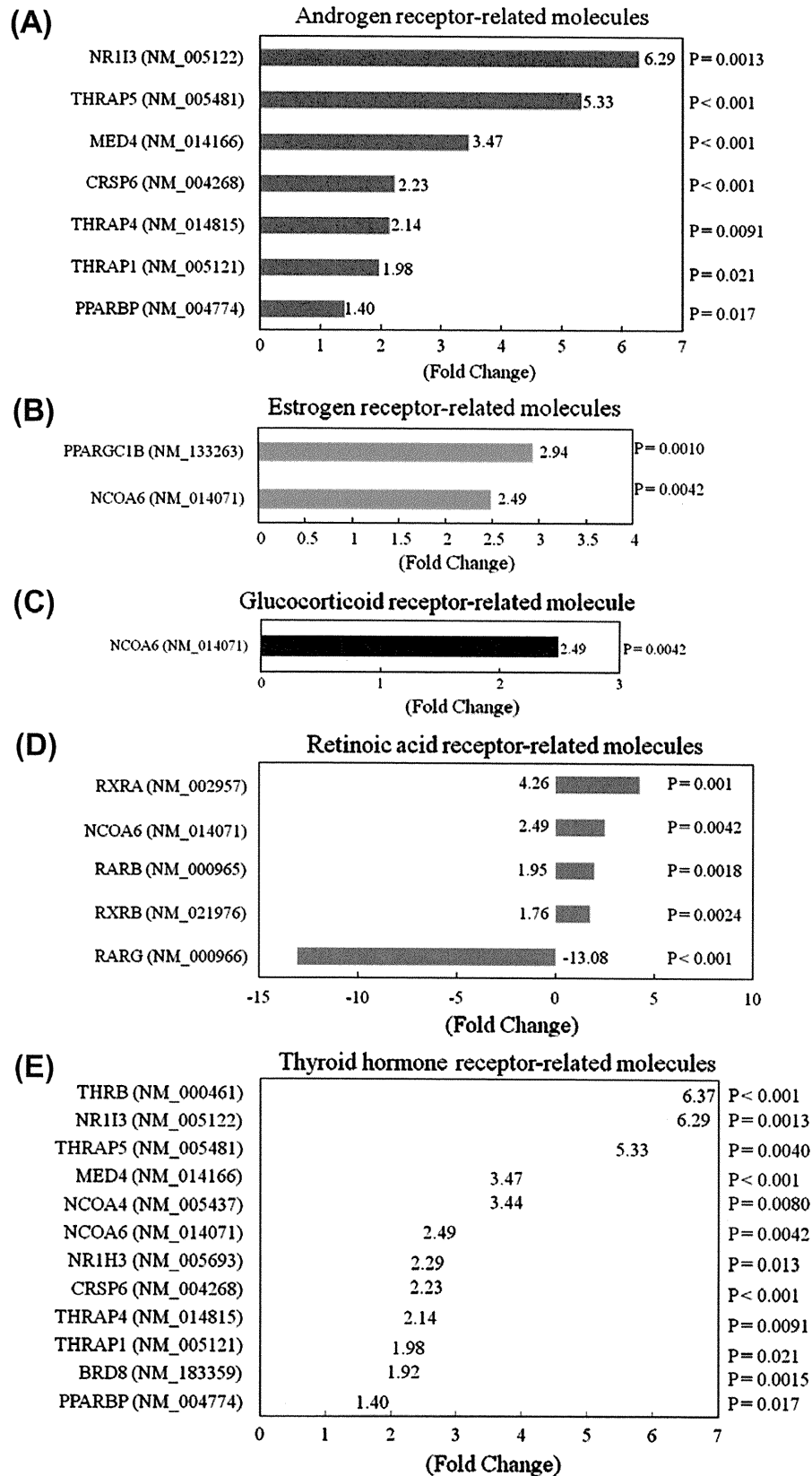
Fig. 2. NR genes differentially expressed ($P < 0.05$) in HepG2.2.15 were classified into eight categories according to their biological functions. (A) Androgen receptors and related molecules, (B) estrogen receptors and related molecules, (C) glucocorticoid receptor and related molecule, (D) retinoic acid receptors and related molecules, (E) thyroid hormone receptors and related molecules, (F) vitamin D receptors and related molecules, (G) steroid hormone receptors and related molecules and (H) other NRs. The bar lengths were proportional to the ratios of gene expression levels between HepG2.2.15 and HepG2 indicated on the left of each bar: gene name and accession number. Positive and negative numbers indicated that the genes were upregulated or downregulated in HepG2.2.15, respectively.
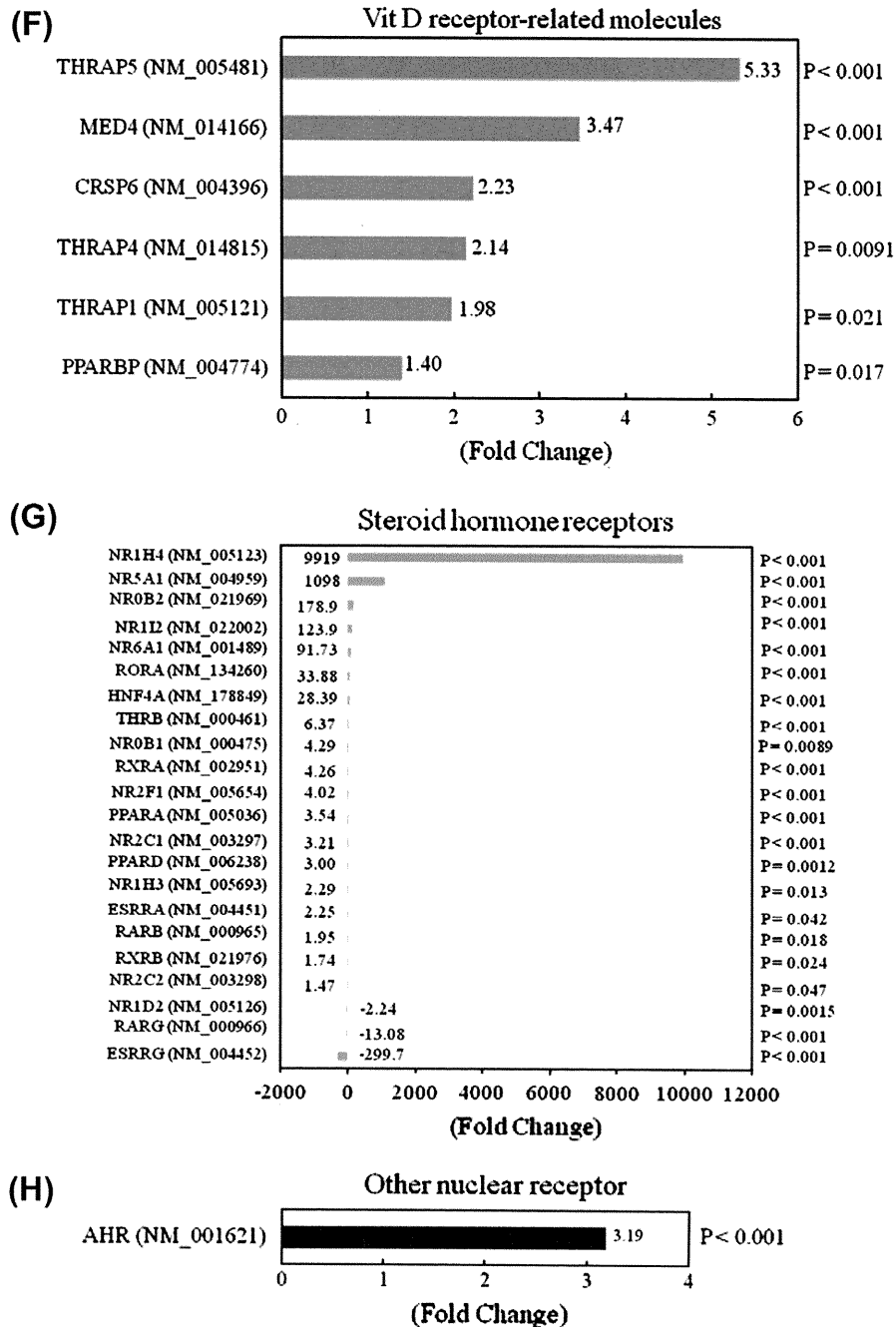
(F)

Vit D receptor-related molecules

Gene	Fold Change	P-value
THRAP5 (NM_005481)	5.33	P < 0.001
MED4 (NM_014166)	3.47	P < 0.001
CRSP6 (NM_004396)	2.23	P < 0.001
THRAP4 (NM_014815)	2.14	P = 0.0091
THRAP1 (NM_005121)	1.98	P = 0.021
PPARBP (NM_004774)	1.40	P = 0.017

(Fold Change)

(G)

Steroid hormone receptors

Gene	Fold Change	P-value
NR1H4 (NM_005123)	9919	P < 0.001
NR5A1 (NM_004959)	1098	P < 0.001
NR0B2 (NM_021969)	178.9	P < 0.001
NR1I2 (NM_022002)	123.9	P < 0.001
NR6A1 (NM_001489)	91.73	P < 0.001
RORA (NM_134260)	33.88	P < 0.001
HNF4A (NM_178849)	28.39	P < 0.001
THRB (NM_000461)	6.37	P < 0.001
NR0B1 (NM_000475)	4.29	P = 0.0089
RXRA (NM_002951)	4.26	P < 0.001
NR2F1 (NM_005654)	4.02	P < 0.001
PPARA (NM_005036)	3.54	P < 0.001
NR2C1 (NM_003297)	3.21	P < 0.001
PPARD (NM_006238)	3.00	P = 0.0012
NR1H3 (NM_005693)	2.29	P = 0.013
ESRRA (NM_004451)	2.25	P = 0.042
RARB (NM_000965)	1.95	P = 0.018
RXRB (NM_021976)	1.74	P = 0.024
NR2C2 (NM_003298)	1.47	P = 0.047
NR1D2 (NM_005126)	-2.24	P = 0.0015
RARG (NM_000966)	-13.08	P < 0.001
ESRRG (NM_004452)	-299.7	P < 0.001

(Fold Change)

(H)

Other nuclear receptor

Gene	Fold Change	P-value
AHR (NM_001621)	3.19	P < 0.001

(Fold Change)

Fig. 2 (*continued*)

were upregulated 2-fold or greater in HepG2.2.15 cells (Fig. 4C). Among other NR co-regulators, no gene was upregulated 2-fold or greater in HepG2.2.15 cells (Fig. 4D).

3.5. DHT, a synthetic androgen, enhanced HBV replication, but TSA, an HDAC inhibitor, inhibited HBV replication

These results showed up-regulations of several NRs and related gene expressions in the presence of HBV, which may ultimately possess the control of HBV replication. To address this issue, replication assays were performed. HCC, especially HBV-related HCC, is one of the male-dominant diseases [9,10]. Epigenetic silencing mechanisms are increasingly thought to play a major role in the development of human cancers, including HCC. Promoter CpG island hypermethylation and histone hypoacetylation, catalyzed by DNA methyltransferase (DNMT) and HDAC, respectively, are associated with transcriptional repression in a number of cancers [25].

In the present study, we focused on AR and HDACs among NRs. After adding 10 nM DHT (Fig. 5A) or 10 ng/mL TSA (Fig. 5B) into cell culture media, the cells were harvested 48 h later and HBV DNA was isolated and quantified by real-time PCR. The results revealed that 10 nM DHT potently enhanced HBV replication (Fig. 5A) and that 10 ng/mL TSA inhibited HBV replication at a level similar to 1000 IU/mL interferon alpha (Fig. 5B), indicating AR and HDAC were both involved in HBV replication.

3.6. Knockdown of AR inhibited cell viability in HepG2.2.15 but not in HepG2 cells, and this effect was enhanced by TSA

We performed a cell viability assay to examine whether there is any difference in cell growth or cell viability between HepG2.2.15 and its parental HepG2 cell line (Fig. 5C). Without any treatment, 48 h after cells were seeded, cell growth of HepG2.2.15 was significantly better than that of HepG2 (1.14 ± 0.022 vs. 1.00 ± 0.035; $n = 6$, $P < 0.001$).
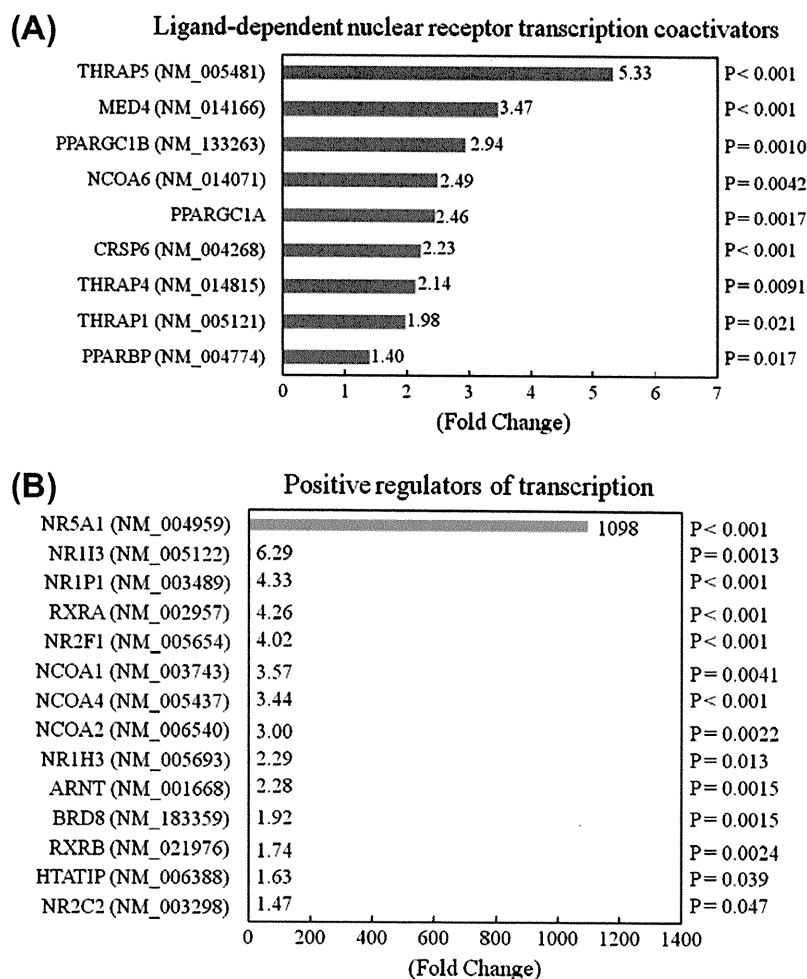
(A)   **Ligand-dependent nuclear receptor transcription coactivators**

Gene	Fold Change	P-value
THRAP5 (NM_005481)	5.33	P< 0.001
MED4 (NM_014166)	3.47	P< 0.001
PPARGC1B (NM_133263)	2.94	P= 0.0010
NCOA6 (NM_014071)	2.49	P= 0.0042
PPARGC1A	2.46	P= 0.0017
CRSP6 (NM_004268)	2.23	P< 0.001
THRAP4 (NM_014815)	2.14	P= 0.0091
THRAP1 (NM_005121)	1.98	P= 0.021
PPARBP (NM_004774)	1.40	P= 0.017

(Fold Change)

(B)   **Positive regulators of transcription**

Gene	Fold Change	P-value
NR5A1 (NM_004959)	1098	P< 0.001
NR1I3 (NM_005122)	6.29	P= 0.0013
NR1P1 (NM_003489)	4.33	P< 0.001
RXRA (NM_002957)	4.26	P< 0.001
NR2F1 (NM_005654)	4.02	P< 0.001
NCOA1 (NM_003743)	3.57	P= 0.0041
NCOA4 (NM_005437)	3.44	P< 0.001
NCOA2 (NM_006540)	3.00	P= 0.0022
NR1H3 (NM_005693)	2.29	P= 0.013
ARNT (NM_001668)	2.28	P= 0.0015
BRD8 (NM_183359)	1.92	P= 0.0015
RXRB (NM_021976)	1.74	P= 0.0024
HTATIP (NM_006388)	1.63	P= 0.039
NR2C2 (NM_003298)	1.47	P= 0.047

(Fold Change)

Fig. 3. Transcription factors and regulators differentially expressed ($P < 0.05$) in HepG2.2.15 were classified into four categories according to their biological functions. (A) Ligand-dependent NR transcription coactivators, (B) positive regulators of transcription, (C) negative regulators of transcription, and (D) other transcription factors. The bar lengths were proportional to the ratios of gene expression levels between HepG2.2.15 and HepG2 indicated on the left of each bar: gene name and accession number. Positive and negative numbers indicated that the genes were upregulated or downregulated in HepG2.2.15, respectively.

Next, we investigated the different effects of TSA and knockdown of AR between HepG2 and HepG2.2.15 (Fig. 5C). With 50 ng/mL TSA treatment, cell viabilities of HepG2 and HepG2.2.15 were 73 ± 1.5 (%) and 76 ± 5.2 (%), respectively. At this concentration of TSA, there was little difference between the two cell lines (Fig. 5C).

However, we observed a reduction in cell viability of HepG2.2.15, compared to that of HepG2, with AR knockdown using specific siRNA against AR. Knockdown of AR protein expression after transfection of si-C or si-AR used in the present study were previously reported [19]. The added use of 50 ng/mL of TSA enhanced the effects on cell viability by si-AR especially in HepG2.2.15 cells (Fig. 5C).

4. Discussion

In this study, we demonstrated that several genes encoding NRs and their coregulators, were differentially expressed in paired cell lines with or without HBV (Fig 1). Glucocorticoid receptor [26], PPARalpha (PPARA) [27], HNF4 [28], and RXRA [28,29] were reported to be involved in HBV gene expression and viral replication. Yu and Mertz reported that interactions between NRs and NR response elements (NRREs) present in the HBV genome may have

critical roles in regulating its transcription and replication during HBV infection of hepatocytes [30]. Our results also showed that some NRs, such as AR and HDAC seem to control HBV replication (Fig. 5A and B). Recently, Wang et al. [31] reported that HBV enhancer I contains a DNA element responsive to transcriptional regulation by androgen-stimulated AR for higher transcriptional activities of HBV mRNAs, leading to higher viral titers. Furthermore, we observed that knockdown of AR leads to a reduction in cell viability of HepG2.2.15 but not of HepG2 (Fig. 5C). AR may also promote HBV-induced hepatocarcinogenesis through modulation of HBV RNA transcription and replication [32].

Estrogen was shown to suppress HBV replication in male athymic mice transplanted with HBV-transfected HepG2 cells [33]. Estrogen exerts its function through its two NRs, ER alpha and beta. It has been reported that HBx protein, ER alpha and HDAC1 form a ternary complex and that TSA, a specific inhibitor, can restore the transcriptional activity of ER alpha inhibited by HBx [34]. Our study showed that TSA without estrogen suppressed HBV replication.
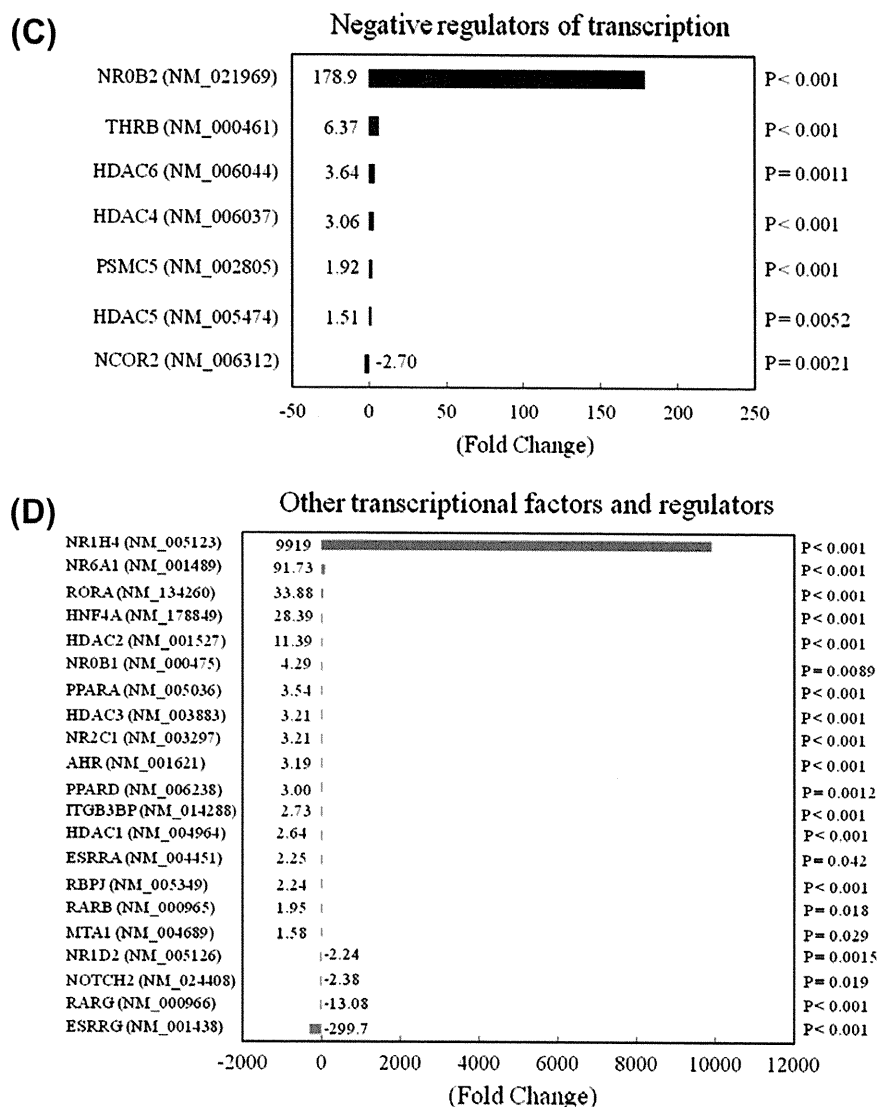
(C)

Negative regulators of transcription

Gene	Fold Change	P-value
NROB2 (NM_021969)	178.9	P< 0.001
THRB (NM_000461)	6.37	P< 0.001
HDAC6 (NM_006044)	3.64	P= 0.0011
HDAC4 (NM_006037)	3.06	P< 0.001
PSMC5 (NM_002805)	1.92	P< 0.001
HDAC5 (NM_005474)	1.51	P= 0.0052
NCOR2 (NM_006312)	-2.70	P= 0.0021

-50   0   50   100   150   200   250

(Fold Change)

(D)

Other transcriptional factors and regulators

Gene	Fold Change	P-value
NR1H4 (NM_005123)	9919	P< 0.001
NR6A1 (NM_001489)	91.73	P< 0.001
RORA (NM_134260)	33.88	P< 0.001
HNF4A (NM_178849)	28.39	P< 0.001
HDAC2 (NM_001527)	11.39	P< 0.001
NR0B1 (NM_000475)	4.29	P= 0.0089
PPARA (NM_005036)	3.54	P< 0.001
HDAC3 (NM_003883)	3.21	P< 0.001
NR2C1 (NM_003297)	3.21	P< 0.001
AHR (NM_001621)	3.19	P< 0.001
PPARD (NM_006238)	3.00	P= 0.0012
ITGB3BP (NM_014288)	2.73	P< 0.001
HDAC1 (NM_004964)	2.64	P< 0.001
ESRRA (NM_004451)	2.25	P= 0.042
RBPJ (NM_005349)	2.24	P< 0.001
RARB (NM_000965)	1.95	P= 0.018
MTA1 (NM_004689)	1.58	P= 0.029
NR1D2 (NM_005126)	-2.24	P= 0.0015
NOTCH2 (NM_024408)	-2.38	P= 0.019
RARG (NM_000966)	-13.08	P< 0.001
ESRRG (NM_001438)	-299.7	P< 0.001

-2000   0   2000   4000   6000   8000   10000   12000

(Fold Change)

Fig. 3 (continued)

HBV covalently closed circular DNA (cccDNA) basal core promoter-bound histones are crucial for regulating the transcription, expression, and replication of HBV chromatin. Chung and Tsai [35] reported that the promyelocytic leukemia (PML)-associated HDAC activity affects the acetylation status on the HBV cccDNA basal core promoter and determines the activation or repression of HBV transcription, expression, and replication. The reversal of HDAC-mediated repression on HBV cccDNA basal core promoter resulted in the amplification of HBV-core and pregenomic expression. Sorin et al. [36] reported that siRNA knockdown of HDAC1 in human immunodeficiency virus type-1 (HIV-1) results in decreased infectivity of HIV-1 in target cells. HDAC plays a critical role in the regulation of many viral replications such as varicella-zoster virus [37], herpes simplex virus type-1 [38], human papillomavirus [39], and SV40 [40]. We observed high expression of HDACs 1-6 in HepG2.2.15 cells (Fig 4B). Wu et al. [24] reported that class I HDAC isoforms (HDAC1, HDAC2 and HDAC3) are highly expressed in HCC, and that patients with a high expression

level of HDAC2 or HDAC3 were prone to earlier recurrence of HCC. In the present study, we observed a reduction of cell viability in both cell lines, but we did not see any differences in cell viabilities between HepG2 and HepG2.2.15 after treatment with HDAC inhibitor, TSA (Fig. 5C). Interestingly, TSA strongly enhanced the reduction of cell viability in HepG2.2.15 cells. Acetylation of AR is regulated by TSA-sensitive HDACs [41]. It is well known that modification by acetylation enhances AR transactivation function at a subset of target promoters, and in particular those cell cycle control genes that induce cellular proliferation [42]. HDAC might also play an important role in HBV-related HCC and could serve as a candidate biomarker.

Recently, several selective HDAC inhibitors have been reported [43,44]. Further development of selective HDAC inhibitors might form part of a clinical strategy to target persistent viral infection and HBV-related HCC [45]. In the present study, we observed the alteration of the expression of endogeneous NRs by HBV, supporting the previous study [12], showing that multiple NRs expressed
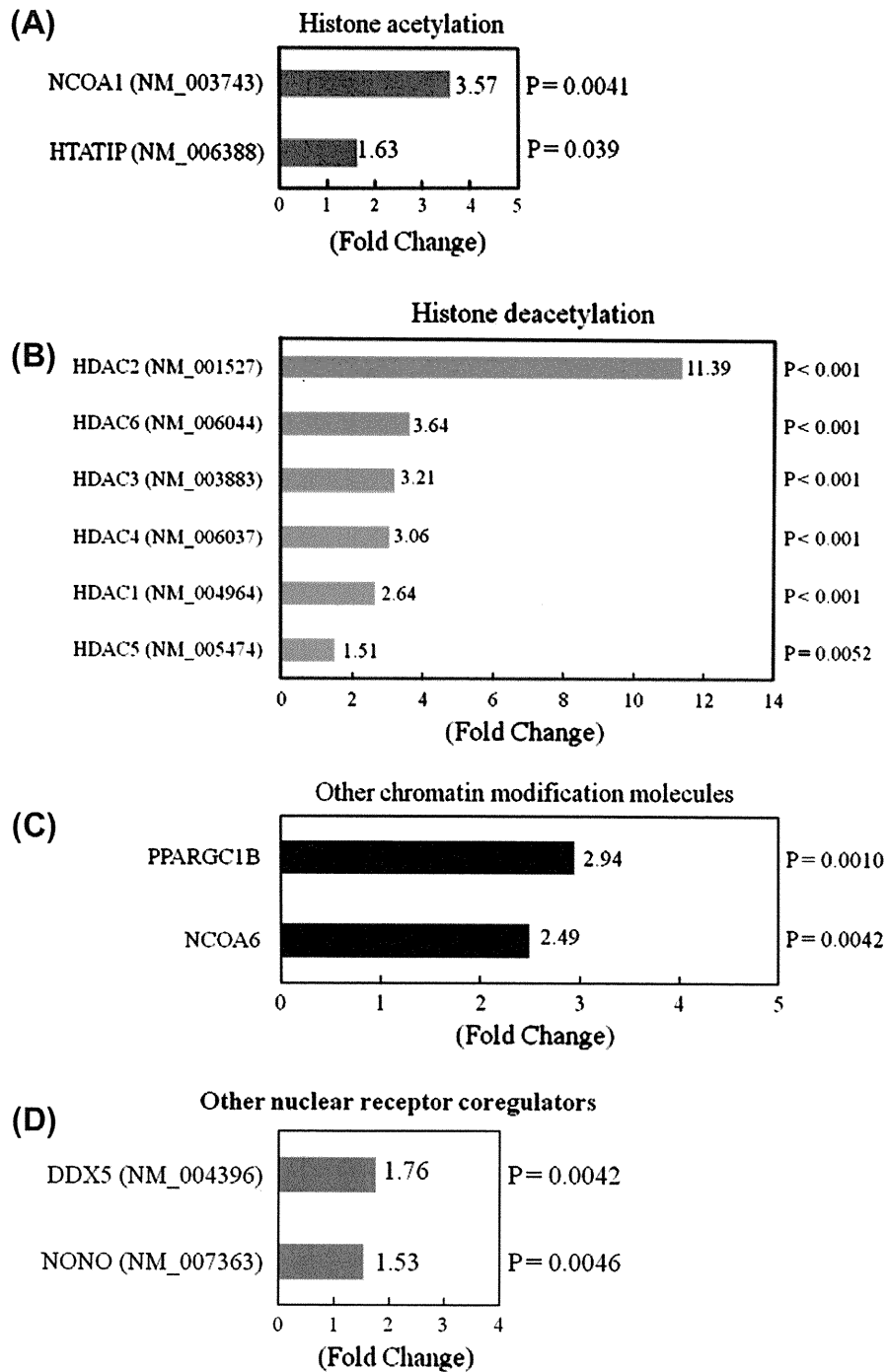
(A) Histone acetylation

NCOA1 (NM_003743)   3.57   P= 0.0041

HTATIP (NM_006388)   1.63   P= 0.039

0   1   2   3   4   5

(Fold Change)

Histone deacetylation

(B) HDAC2 (NM_001527)   11.39   P< 0.001

HDAC6 (NM_006044)   3.64   P< 0.001

HDAC3 (NM_003883)   3.21   P< 0.001

HDAC4 (NM_006037)   3.06   P< 0.001

HDAC1 (NM_004964)   2.64   P< 0.001

HDAC5 (NM_005474)   1.51   P= 0.0052

0   2   4   6   8   10   12   14

(Fold Change)

Other chromatin modification molecules

(C) PPARGC1B   2.94   P= 0.0010

NCOA6   2.49   P= 0.0042

0   1   2   3   4   5

(Fold Change)

Other nuclear receptor coregulators

(D) DDX5 (NM_004396)   1.76   P= 0.0042

NONO (NM_007363)   1.53   P= 0.0046

0   1   2   3   4

(Fold Change)

Fig. 4. Chromatin modification molecules (A–C) and other NR co-regulators (D) differentially expressed ($P < 0.05$) in HepG2.2.15 were classified into four categories according to their biological functions. (A) Histone acetylation, (B) histone deacetylation, (C) other chromatin modification molecules and (D) other NR co-regulators. The bar lengths were proportional to the ratios of gene expression levels between HepG2.2.15 and HepG2 indicated on the left of each bar: gene name and accession number. Positive and negative numbers indicated that the genes were upregulated or downregulated in HepG2.2.15, respectively.

by mammalian cell expression vectors activated HBV transcription and replication in human embryonic kidney 293T cell line.

NRs are keys for understanding the pathogenesis and pathophysiology of a wide range of hepatic disorders [46]. In conclusion, this study showed that NRs may play critical roles in regulating HBV replication during HBV infection of hepatocytes. Specific AR inhibitors or HDAC

inhibitors might shed new light on the treatment of patients with chronic HBV infection. Of course, the effectiveness of such potential therapies needed to be confirmed in vivo.

Conflict of Interest

No.
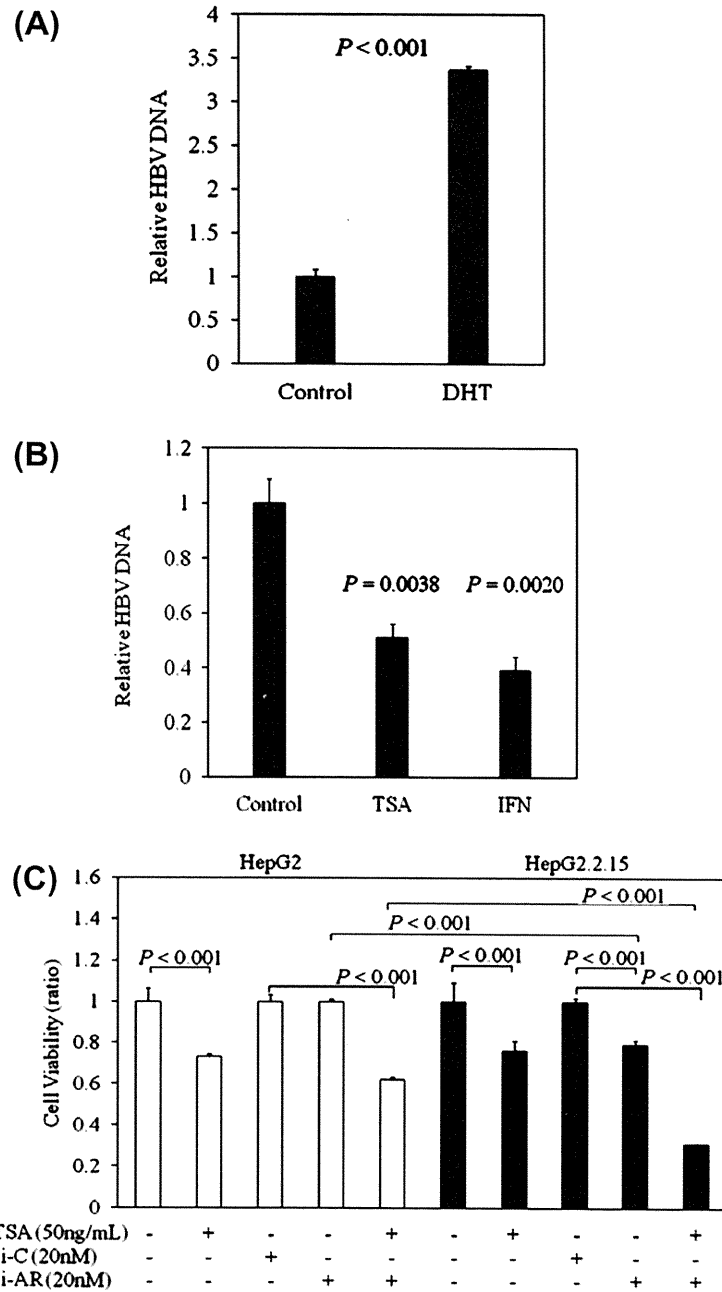
Fig. 5. Effects of 5α-androgen-17β-ol-3-one (DHT) and Trichostatin A (TSA) on HBV replication in HepG2.2.15 (A and B), and effects of knockdown of androgen receptor (AR) and TSA on cell viabilities of HepG2 and HepG2.2.15 (C). Cells were treated with 10nM DHT (A), 10 ng/mL TSA or 1000 U/mL interferon (IFN) alpha (B). Total DNA was isolated after 48 h of treatment, and real-time PCR was performed for hepatitis B virus (HBV) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) DNA expression. The results presented are based on the relative DNA level of GAPDH and are shown as mean ± SD from at least three independent experiments. (C) To evaluate cell growth and cell viability, dimethylthiazol carboxymethoxyphenyl sulfophenyl tetrazolium (MTS) assay was performed 48 h after electroporation of control siRNA (si-C) and siRNA targeting against AR (si-AR) with or without TSA treatment. Cells were treated with 50 ng/mL TSA for 24 h. Each untreated cell line was set as 1. Data are shown as mean ± SD from six independent experiments.

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Dysfunction of Autophagy Participates in Vacuole Formation and Cell Death in Cells Replicating Hepatitis C Virus[▽][§]

Shuhei Taguwa,[1][†] Hiroto Kambara,[1][†] Naonobu Fujita,[2] Takeshi Noda,[2] Tamotsu Yoshimori,[2] Kazuhiko Koike,[3] Kohji Moriishi,[4] and Yoshiharu Matsuura[1]*

Department of Molecular Virology, Research Institute for Microbial Diseases,[1] and Department of Genetics, Graduate School of Medicine,[2] Osaka University, Osaka 565-0871, Department of Gastroenterology, Graduate School of Medicine, University of Tokyo, Tokyo 113-8655,[3] and Department of Microbiology, Faculty of Medicine, Yamanashi University, Yamanashi 409-3898,[4] Japan

Hepatitis C virus (HCV) is a major cause of chronic liver diseases. A high risk of chronicity is the major concern of HCV infection, since chronic HCV infection often leads to liver cirrhosis and hepatocellular carcinoma. Infection with the HCV genotype 1 in particular is considered a clinical risk factor for the development of hepatocellular carcinoma, although the molecular mechanisms of the pathogenesis are largely unknown. Autophagy is involved in the degradation of cellular organelles and the elimination of invasive microorganisms. In addition, disruption of autophagy often leads to several protein deposition diseases. Although recent reports suggest that HCV exploits the autophagy pathway for viral propagation, the biological significance of the autophagy to the life cycle of HCV is still uncertain. Here, we show that replication of HCV RNA induces autophagy to inhibit cell death. Cells harboring an HCV replicon RNA of genotype 1b strain Con1 but not of genotype 2a strain JFH1 exhibited an incomplete acidification of the autolysosome due to a lysosomal defect, leading to the enhanced secretion of immature cathepsin B. The suppression of autophagy in the Con1 HCV replicon cells induced severe cytoplasmic vacuolation and cell death. These results suggest that HCV harnesses autophagy to circumvent the harmful vacuole formation and to maintain a persistent infection. These findings reveal a unique survival strategy of HCV and provide new insights into the genotype-specific pathogenicity of HCV.

Hepatitis C virus (HCV) is a major causative agent of blood-borne hepatitis and currently infects at least 180 million people worldwide (58). The majority of individuals infected with HCV develop chronic hepatitis, which eventually leads to liver cirrhosis and hepatocellular carcinoma (25, 48). In addition, HCV infection is known to induce extrahepatic diseases such as type 2 diabetes and malignant lymphoma (20). It is believed that the frequency of development of these diseases varies among viral genotypes (14, 51). However, the precise mechanism of the genotype-dependent outcome of HCV-related diseases has not yet been elucidated. Despite HCV's status as a major public health problem, the current therapy with pegylated interferon and ribavirin is effective in only around 50% of patients with genotype 1, which is the most common genotype worldwide, and no effective vaccines for HCV are available (35, 52). Although recently approved protease inhibitors for HCV exhibited a potent antiviral efficacy in patients with genotype 1 (36, 43), the emergence of drug-resistant mutants is a growing problem (16). Therefore, it is important to clarify the life cycle and pathogenesis of HCV for the development of more potent remedies for chronic hepatitis C.

HCV belongs to the genus *Hepacivirus* of the family *Flaviviridae* and possesses a single positive-stranded RNA genome with a nucleotide length of 9.6 kb, which encodes a single polyprotein consisting of approximately 3,000 amino acids (40). The precursor polyprotein is processed by host and viral proteases into structural and nonstructural (NS) proteins (34). Not only viral proteins but also several host factors are required for efficient replication of the HCV genome, where NS5A is known to recruit various host proteins and to form replication complexes with other NS proteins (39). In the HCV-propagating cell, host intracellular membranes are reconstructed for the viral niche known as the membranous web, where it is thought that progeny viral RNA and proteins are concentrated for efficient replication and are protected from defensive degradation, as are the host protease and nucleases (38).

Autophagy is a bulk degradation process, wherein portions of cytoplasm and organelles are enclosed by a unique membrane structure called an autophagosome, which subsequently fuses with the lysosome for degradation (37, 60). Autophagy occurs not only in order to recycle amino acids during starvation but also to clear away deteriorated proteins or organelles irrespective of nutritional stress. In fact, the deficiency of autophagy leads to the accumulation of disordered proteins that can ultimately cause a diverse range of diseases, including neurodegeneration and liver injury (12, 29, 30), and often to type 2 diabetes and malignant lymphoma (9, 32).

Recently, it has been shown that autophagy is provoked upon replication of several RNA viruses and is closely related to their propagation and/or pathogenesis. Coxsackievirus B3

* Corresponding author. Mailing address: Department of Molecular Virology, Research Institute for Microbial Diseases, Osaka University, 3-1, Yamadaoka, Suita-shi, Osaka 565-0871, Japan. Phone: 81-6-6879-8340. Fax: 81-6-6879-8269. E-mail: matsuura@biken.osaka-u.ac.jp.
† These authors contributed equally to this work.
§ Supplemental material for this article may be found at http://jvi.asm.org/.
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