performed. Especially, further investigation is needed to clarify the relationship between the change in hepatitis B surface antigen levels during treatment and HCC incidence in patients with HBV infection.

In conclusion, in the consecutive surveillance for HCC after the initiation of ETV treatment, monitoring the change in AFP levels at 24 weeks is essential, especially among patients of advanced age or with cirrhosis.

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Original Article

Managing hepatitis B virus carriers with systemic chemotherapy or biologic therapy in the outpatient clinic

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Aim: The number of outpatients receiving systemic chemotherapy in Japan has recently increased. We retrospectively examined whether hepatitis B virus (HBV) carriers were safely treated and managed with systemic chemotherapy or biologic agents as outpatients at our oncology center.

Methods: A total of 40 115 consecutive infusion chemotherapy or biologic therapies were administrated to 2754 outpatients in the Chemotherapy and Oncology Center at Osaka University Hospital from December 2003 to March 2011. We first studied the prevalence of outpatients with hepatitis B surface antigen (HBsAg), and then retrospectively evaluated a database to determine the frequencies of testing for other HBV-related markers and the incidence of developing hepatitis or HBV reactivation in patients positive for HBsAg. As a control for comparison, we also examined these same factors in patients with hepatitis C virus antibody (anti-HCV).

Results: The majority of physicians at our hospital screened for HBsAg (95%) and anti-HCV (94%) prior to administrating chemotherapy. Of the 2754 outpatients, 46 (1.7%) were positive for HBsAg and 90 (3.3%) were positive for anti-HCV. Fifteen patients that were HBsAg positive were treated with lamivudine or entecavir prior to chemotherapy. None of the patients with HBsAg taking a prophylactic antiviral developed hepatitis, and only one breast cancer patient without prophylactic antiviral treatment (1/31 [3.2%]) developed hepatitis due to HBV reactivation.

Conclusion: HBV reactivation occurred in outpatients without prophylactic antiviral treatment, but the incidence was relatively low.

Key words: biologic therapy, chemotherapy, hepatitis B virus reactivation, outpatient

INTRODUCTION

HEPATITIS B IS one of the world's most common and serious infectious diseases. It is estimated that more than one-third of the world's population has been exposed to the hepatitis B virus (HBV) and that there are approximately 350 million chronic carriers worldwide, 75% of whom live in South-East Asia and the Western Pacific regions.[1–4] In Japan, approximately 26 million people have been exposed to HBV. Of those who have been exposed, 1.5 million people are estimated to be chronic carriers.[5] Generally, one-fifth of all HBV carriers develop chronic hepatitis, cirrhosis and primary hepatocellular carcinoma. The majority of HBV patients are, however, clinically inactive.

Among HBV-related liver diseases, HBV reactivation is now a well-recognized complication in HBV inactive carriers who receive cytotoxic chemotherapy for cancer. HBV reactivation was first described in patients with lympho- and myeloproliferative disorders by Wands *et al.*[6] in 1975. Wands *et al.*[6] demonstrated that patients with hepatitis B antigen (HBAg) developed hepatitis with a marked increase in the HB-Ag titer during chemotherapy. The reactivation condition ranges from asymptomatic self-limiting anicteric hepatitis to severe, potentially fatal, progressive decompensated hepatitis. In addition, HBV reactivation during or after chemotherapy or other immunosuppressive therapy

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was recently reported as de novo HBV-related hepatitis even in previously exposed HBV patients without hepatitis B surface antigen (HBsAg), particularly in cases using rituximab.[7]

Based on this background, a guideline for preventing HBV reactivation during and after cytotoxic or immunosuppressive therapies was proposed in 2009 and revised in 2011 by two collaborative study groups from the Japanese Ministry of Health, Labor and Welfare, which included measures not only for HBV carriers, but also for patients without HBsAg.[8] The guideline was intended to identify patients with the potential for HBV reactivation. Therefore, HBsAg screening is recommended for all patients scheduled for chemotherapy or other immunosuppressive therapy. If a patient is positive for HBsAg, prophylaxis is recommended, in addition to testing for hepatitis B e-antigen (HBeAg), antibody to hepatitis B e-antigen (anti-HBe) and HBV DNA. On the other hand, if a patient is negative for HBsAg, testing for anti-hepatitis B core (HBc) and anti-HBs is recommended. If a patient is positive for either or both anti-HBs and anti-HBc, then testing for HBV DNA is recommended. If a patient is positive for HBV DNA, prophylaxis is recommended. If a patient is negative for HBV DNA, monthly monitoring of HBV DNA and aspartate aminotransferase (AST)/alanine aminotransferase (ALT) is recommended, and should be continued for at least 12 months after the end of chemotherapy.[8]

The number of outpatients undergoing cancer chemotherapy has recently increased due to the advances in cytotoxic agents and supportive therapies. Moreover, there has been an increase in the number of patients with inflammatory bowel disease or rheumatoid arthritis requiring immunosuppressive therapy, such as biologic agents (e.g. anti-tumor necrosis factor agents). In Japan, the increase in immunosuppressive therapies has led to a shift in hospital care to outpatient therapy since 2002 for heath insurance reasons. The corresponding data for HBsAg positive outpatients requiring these immunosuppressive therapies are, however, not known. In this study, we retrospectively examined whether asymptomatic HBV carriers were safely treated and managed with systemic chemotherapy or immunosuppressive therapies in the outpatient setting.

METHODS

Patients

THIS WAS A retrospective study in a single institute. A total of 40 115 consecutive infusion treatments in 2754 outpatients (1122 men, 1632 women) with cancer or autoimmune disease, such as rheumatoid arthritis or Crohn's disease, treated with cytotoxic or biologic agents in the Chemotherapy and Oncology Center for outpatients at Osaka University Hospital from December 2003 to March 2011 were enrolled. Patients receiving second-line or more chemotherapy were also included.

Methods

The cytotoxic or biologic infusion agents were administrated to each patient according to the standard protocol for the specific tumor type or disease commonly treated within health insurance parameters in Japan. Oncology center staff and pharmacists basically reviewed all protocols before treatment. Medical records of all patients with HBsAg were retrospectively reviewed for this study. As a control, the records of patients with hepatitis C virus antibody (anti-HCV) were examined. If the patients were positive for HBsAg or anti-HCV, their medical records were additionally reviewed to determine whether they were tested for anti-HBs, anti-HBc, HBeAg, anti-HBe and HBV DNA, or administrated antiviral drugs before treatment. HBsAg, anti-HBs, anti-HBc, HBeAg and anti-HBe were measured by chemiluminescent immunoassay, but both HBeAg and anti-HBe were measured by chemiluminescent enzyme immunoassay until 5 May 2005. HBV DNA was measured by polymerase chain reaction (PCR) until 30 September 2009 and then real-time PCR. For the antiviral drugs, data collected included not only cases that received the drug for prophylaxis, but also cases in which treatment for chronic hepatitis was already administrated before treatment. Collected data were entered into a database that did not include any identifying information about the respondents. The follow-up period was defined as the period from the first visit in our center for outpatients to the last visit at Osaka University Hospital.

The study was approved by the Clinical Investigation and Research board of Osaka University Hospital (#11202, 10 December 2011). The study was performed in accordance with the Declaration of Helsinki, as revised in 2008.

Definitions of hepatitis and HBV reactivation

Hepatitis was defined as a more than threefold increase in serum ALT of the upper limit of normal on two consecutive determinations. Patients who had been clinically diagnosed with hepatitis due to drug or tumor involvement were excluded from this study. HBV reactivation was defined as an increase of more than 1 log

copy/mL of serum HBV DNA, or the serum HBV DNA turned from negative to positive.

Statistical analysis

Statistical analysis was performed with JMP software ver. 9.02 (SAS Institute). Data are expressed as the mean ± standard deviation and probability value. The χ^2-test was used for the analysis of categorical variables. Probability values of less than 0.01 were considered statistically significant.

RESULTS

Baseline characteristics

THE MAJORITY OF physicians treating patients in our outpatient clinic screened for HBsAg (2607/2754, 95%) and anti-HCV (2586/2754, 94%) prior to administrating treatments. Of 2754 outpatients, 46 patients (1.7%) were positive for HBsAg and 90 (3.3%) were positive for anti-HCV. Two patients were positive for both HBsAg and anti-HCV. Table 1 shows the patient characteristics and Table 2 shows the laboratory data for patients with HBsAg or anti-HCV at the first infusion treatment at our outpatient clinic. The median follow-up period was 21 months (range, 2–102). Of 46 patients positive for HBsAg, 35 (76%), 14 (30%), 19 (41%), 24 (52%) and 25 (54%) patients were tested for anti-HBs, anti-HBc, HBe-Ag, anti-HBe and HBV DNA, respectively. Of 90 patients positive for anti-HCV, 24 (27%), 19 (21%), 23 (26%), seven (8%) and two (2%) patients were tested for anti-HBs, anti-HBc, HBe-Ag, anti-HBe and HBV DNA, respectively (Table 4). Two patients with both HBsAg and anti-HCV were tested for HBV DNA.

Table 1 Patient characteristics

	Patients with HBsAg ($n = 46$)	Patients with anti-HCV ($n = 90$)
Age	59 ± 10	66 ± 10
Sex (M/F)	16/30	55/35
Number of treatments	10 (1–210)	11 (1–62)
Agents for treatment		
Cytotoxic agents	44	87
Immunosuppressive agents	2	3
Type of cancer or basic disease		
Breast cancer	20	13
Gastrointestinal cancer	8	26
Hepato-biliary-pancreatic cancer	7	22
Hematologic malignancy	7	10
Lung cancer	2	7
Renal cancer	1	1
Rheumatoid arthritis	1	1
Prostatic cancer	0	5
Gynecologic cancer	0	2
Others	0	3
Tumor infiltration of the liver	17	18

HBsAg, hepatitis B surface antigen; anti-HCV, hepatitis C virus antigen.

Table 2 Patients' baseline laboratory data at first visit

	Patients with HBsAg ($n = 46$)	Patients with anti-HCV ($n = 90$)
WBC (/μL)	5110 ± 2015	4920 ± 1825
Hb (g/dL)	12.2 ± 2.1	12.0 ± 1.7
Plt (/μL)	20.3 ± 7.9	19.9 ± 9
AST (U/L)	23 ± 9	34 ± 32
ALT (U/L)	20 ± 11	27 ± 30
T.Bil (mg/dL)	0.3 ± 0.04	0.7 ± 0.3

ALT, alanine aminotransferase; AST, aspartate aminotransferase; Hb, hemoglobin; HBsAg, hepatitis B surface antigen; anti-HCV, hepatitis C virus antigen; Plt, platelets; T.Bil, total bilirubin; WBC, white blood cells.

Of the 46 patients positive for HBsAg, 15 had been treated with lamivudine or entecavir prior to chemotherapy or biologic therapies (33%). Of these 15, nine had been treated prophylactically (cases 1–9; Table 3), and the others had already been treated for chronic hepatitis B (case 10–15; Table 3) before their fist visit to the oncology center. They were all tested for HBV DNA before treatment and then monitored for HBV DNA. The method of monitoring for HBV DNA, however, basically depended on each physician and was not uniform. On the other hand, 31 patients (67%) with HBsAg underwent chemotherapy or biologic therapy without antiviral prophylaxis (Table 4). Of these 31, 10 were tested for HBV DNA before treatment and five of the 10 tested positive for HBV DNA.

Of the 46 patients positive for HBsAg, 20 patients had breast cancer, six of whom were treated with prophylactic antiviral medication (30%) and five of the six patients were positive for HBV DNA prior to chemotherapy. Of the other 14 patients without prophylaxis, four were tested for HBV DNA and 10 were not. Of the four patients tested for HBV DNA, one was positive. One of the 10 not tested developed HBV reactivation (case 35; Tables 3 and 5). There were eight patients with gastrointestinal cancer, none of whom was treated with prophylactic antiviral medication, although four were

Table 3 Details of patients with HBsAg

Case	No. of treatments	Follow-up period (months)	Sex	Age, years	Type of cancer or basic disease	First agent at the center	HBsAg	Anti-HBs	HBeAg	Anti-HBe	Anti-HBc	HBV DNA, log copies/ml	Anti-HCV	Corticosteroid use	Antiviral prior to chemotherapy of biologics	Hepatitis	Reactivation
1	8	2	M	56	Malignant lymphoma	Rituximab	+	−	†	+	+	4.3	−	Present	Lamivudine	None	None
2	20	11	F	47	Breast cancer	Paclitaxel	+	−	−	+	†	3.3	−	Present	Entecavir	None	None
3	10	32	F	44	Leukemia	Rituximab	+	−	−	+	+	3.3	−	Present	Entecavir	None	None
4	4	31	M	66	Malignant lymphoma	Rituximab	+	−	+	†	†	Negative	−	Present	Entecavir	None	None
5	11	28	F	62	Breast cancer	Paclitaxel	+	−	−	+	+	Negative	−	Present	Entecavir	None	None
6	23	26	F	79	Breast cancer	Navelbine	+	−	†	+	+	2.1	−	Present	Entecavir	Present	None
7	21	25	F	66	Breast cancer	Docetaxel	+	−	−	+	+	2.3	−	Present	Entecavir	None	None
8	14	22	F	43	Breast cancer	FEC	+	−	†	+	†	<2.1	−	Present	Entecavir	None	None
9	9	16	F	60	Breast cancer	Paclitaxel	+	−	†	+	+	3.5	−	Present	Entecavir	None	None
10	19	15	M	71	Bile duct cancer	Gemcitabine	+	−	†	+	†	2.1	−	Present	Lamivudine	None	None
11	6	33	F	60	Malignant lymphoma	Rituximab	+	−	−	+	†	3	−	Present	Lamivudine + adefovir	None	None
12	8	60	F	73	Malignant lymphoma	VDS + MTX	+	−	†	†	†	Negative	−	Present	Entecavir	None	None
13	5	44	F	35	Malignant lymphoma	CHOP	+	−	†	†	+	Negative	−	Present	Entecavir	None	None
14	4	33	F	69	Macroglobulinemia	Rituximab	+	−	−	+	+	Negative	−	Present	Entecavir	None	None‡
15	6	2	M	60	Bile duct sarcoma	CDDP + gemcitabine	+	−	−	−	−	Negative	−	Present	Entecavir	None	None
16	6	102	M	65	Esophageal cancer	Paclitaxel	+	−	†	†	†	†	−	Present	None	Present	None
17	210	19	M	61	RCC	IL-2	+	−	−	†	†	†	−	None	None	None	None
18	8	4	F	56	Breast cancer	FEC	+	†	†	†	†	†	−	Present	None	None	None
19	18	15	F	52	Colon Cancer	FOLFIRI	+	†	−	†	†	†	−	None	None	None	None
20	12	85	F	51	Breast cancer	Paclitaxel	+	−	†	†	†	Negative	−	Present	None	None	None
21	16	7	M	49	Gastric cancer	Paclitaxel	+	†	†	†	†	†	−	Present	None	None	None
22	14	5	F	51	Brest cancer	Paclitaxel	+	†	†	†	†	†	−	Present	None	None	None
23	14	69	F	74	Bile duct cancer	Gemcitabine	+	†	†	†	†	†	†	None	None	None	None
24	3	61	F	64	Lung cancer	Paclitaxel	+	†	†	†	†	†	−	Present	None	None	None
25	5	66	F	59	Breast cancer	FEC	+	−	−	+	+	Negative	−	Present	None	None	None
26	8	4	M	68	Gastric cancer	Paclitaxel	+	−	−	+	+	Negative	−	Present	None	None	None
27	20	11	F	36	Pancreatic NET	Dacarbazine	+	−	†	+	+	4.4	−	None	None	None	None
28	3	4	M	55	Gastric cancer	Paclitaxel	+	−	†	+	†	3.2	−	Present	None	None	None
29	18	52	M	58	Colon cancer	5-FU + LV	+	−	−	+	†	†	−	None	None	None	None
30	14	53	F	59	Breast cancer	Paclitaxel	+	†	†	†	†	†	−	Present	None	None	None
31	25	9	F	52	Breast cancer	Paclitaxel	+	−	−	†	†	†	−	Present	None	None	None
32	198	53	F	44	Breast cancer	Paclitaxel/herceptin	+	−	−	+	†	3.9	−	Present	None	None	None
33	70	20	F	59	Breast cancer	5-FU + MTX	+	−	−	+	+	†	−	Present	None	None	None
34	11	13	F	72	Gastric cancer	Paclitaxel	+	−	†	+	†	†	−	Present	None	None	None
35	23	48	F	46	Breast cancer	FEC	+	−	†	†	†	†	−	Present	None	Present	Present
36	22	47	M	60	Reumatoid arthritis	Infliximab	+	−	†	†	†	<2.1	−	None	None	None	None
37	4	45	F	68	Breast cancer	FEC	+	†	†	†	†	†	−	Present	None	None	None
38	11	8	M	47	Bile duct cancer	Gemcitabine	+	+	−	+	†	7.2	−	Present	None	None	None
39	4	39	F	58	Breast cancer	Paclitaxel	+	−	†	+	†	Negative	−	Present	None	None	None
40	14	16	M	70	Bile duct cancer	Gemcitabine/CDDP	+	−	†	†	†	†	+	Present	None	None	None
41	7	21	M	52	Lung cancer (NSCLC)	Pemetrexed/CBDCA	+	−	†	†	†	†	−	Present	None	None	None
42	2	4	M	65	Esophageal cancer	Docetaxel	+	−	−	+	†	Negative	−	Present	None	None	None
43	3	17	M	64	HCC	5-FU	+	−	−	+	+	†	+	None	None	None	None
44	12	15	F	64	Breast cancer	Herceptin	+	†	†	†	†	†	−	Present	None	None	None
45	B	8	F	71	Breast cancer	Docetaxel	+	†	†	†	†	†	−	Present	None	None	None
46	14	12	F	69	Breast cancer	Abraxane	+	†	†	†	†	†	−	Present	None	None	None

†Untested.
‡Case 14: past history of HBV reactivation.
Corticosteroid use: as chemotherapeutic regimens (including use for anti-emetics).
HBV DNA: before prophylactic antiviral or start at chemotherapy.
5-FU, 5-fluorouracil; CDDP, cisplatin; CBDCA, carboplatin; CHOP, cyclophosphamide/adriamycin/vindesine/predonine; FEC, 5-FU/epirubicin/cyclophosphamide; FOLFIRI, 5-FU/levofolinate/irinotecan; HBc, hepatitis B core; HBeAg, hepatitis B e-antigen; HBs, hepatitis B surface; HBsAg, hepatitis B surface antigen; HBV, hepatitis B virus; HCC, hepatocellular carcinoma; HCV, hepatitis C virus; IL-2, interleukin-2; LV, levofolinate; MTX, methotrexate; NET, neuroendocrine tumor; NSCLC, non-small cell lung cancer; RCC, renal cell carcinoma; VDS, vindesin.

Table 4 Patients' hepatitis viral marker

Viral marker status	Patients with HBsAg ($n = 46$)	Patients with anti-HCV ($n = 90$)
HBsAg		
Positive/negative/untested	46/0/0	2/86/2
(%)	(100/0/0)	(2/96/2)
Anti-HBs		
Positive/negative/untested	1/34/11	8/16/66
(%)	(2/74/24)	(9/18/76)
Anti-HBc		
Positive/negative/untested	13/1/32	8/11/71
(%)	(28/2/70)	(9/12/79)
HBeAg		
Positive/negative/untested	1/18/27	0/23/67*
(%)	(2/39/59)	(0/26/74)
Anti-HBe		
Positive/negative/untested	23/1/22	4/3/83*
(%)	(50/2/48)	(4/3/93)
HBV DNA		
<2.1/≤2.1 log copies/mL /untested	12/13/21	1/1/88*
(%)	(26/28/46)	(1/1/98)
Anti-HCV		
Positive/negative/untested	2/40/4	90/0/0
(%)	(4/87/9)	(100/0/0)
HCV RNA		
Positive/negative/untested	0/0/46	21/6/63*
(%)	(0/0/100)	(23/7/70)

*$P < 0.001$. Frequency of antibody testing between patients with HBsAg vs anti-HCV.
HBc, hepatitis B core; HBeAg, hepatitis B e-antigen; HBs, hepatitis B surface; HBsAg, hepatitis B surface antigen; HBV, hepatitis B virus; HCV, hepatitis C virus.

tested for HBV DNA and two of those tested positive. Seven patients had hepato-biliary-pancreatic cancer, and two of these had already received antiviral drugs before being treated for cancer (cases 10 and 15; Table 3). The other five, however, were not treated with prophylactic antiviral drugs, even though two of these were tested for HBV DNA and both were positive (cases 27 and 38; Table 3).

Seven patients positive for HBsAg had hematologic malignancies, and all were treated with antiviral drugs. Three of them were started on antiviral drugs as prophylaxis against HBV reactivation before treatment, but four patients had already received antiviral drugs before treatment for hematologic malignancies (cases 11–14; Table 3). One patient had a past history of HBV reactivation before this chemotherapy (case 14; Table 3).

Hepatitis and HBV reactivation (Tables 3 and 5)

There were three patients with HBsAg who had hepatitis during and after chemotherapy (cases 6 [ALT, 188 U/L], 16 [ALT, 205 U/L] and 35 [ALT, 487 U/L]; Table 3 [6.5%]), two of whom (cases 16 and 35 [4.6%]) showed more than fivefold increases in serum ALT of the upper limit of normal. None of them met the diagnostic criteria for acute liver failure in Japan.[9] Two of them (cases 6 and 16) were clinically judged to be caused by drugs or alcohol from history taking and laboratory data, one of whom did not show an increase of serum HBV DNA. Only one breast cancer patient (a 47-year-old woman) without prophylactic antiviral treatment (1/31 [3.2%]), however, developed hepatitis and was clinically diagnosed with hepatitis due to HBV reactivation (case 35; Tables 3 and 5), although the definition of HBV reactivation was not strictly applied because her HBV DNA level was not tested before visiting our outpatient clinic. She underwent surgery for breast cancer, including a sentinel lymph node biopsy, on April 2008, and then received adjuvant chemotherapy for breast cancer on May 2008. Serological examination indicated that she was positive for HBsAg, but negative for HBeAg, and anti-HBs, anti-HBc, anti-HBe and HBV-DNA were not tested before chemotherapy. Her chemotherapeutic regimen comprised FEC (5-fluorouracil, 500 mg/m^2; epirubicin, 100 mg/m^2; cyclophosphamide,

Table 5 Viral reactivation

	Patients with HBsAg ($n = 46$)	Patients with anti-HCV ($n = 90$)
With prophylactic antiviral	15	0
Without prophylactic antiviral	31	90
Development of hepatitis related to viral reactivation	1† (without antiviral)	0

†Case 35.
HBsAg, hepatitis B surface antigen; HCV, hepatitis C virus.

500 mg/m^2) with administration of corticosteroids. She received six cycles of FEC every 3 weeks on schedule. On day 40 after she finished the last cycle, she was aware of general fatigue and jaundice. On day 46, she was admitted to the hospital with hepatitis B. Blood tests on admission showed: AST, 508 U/L; ALT, 487 U/L; total bilirubin, 8.5 mg/dL; direct bilirubin, 6.7 mg/dL; prothrombin time, 79% (International Normalized Ratio, 1.10), NH_3 122 μg/dL; and HBV DNA, 5.3 log/copies. She received glycyrrhizinic acid by i.v. injection and then entecavir (0.5 mg/day). A liver biopsy was performed on day 11 after admission and pathologically proven viral hepatitis; her Histological Activity Index (HAI) score was 10 (interface hepatitis, 3; intralobular degeneration, 3; portal inflammation, 1; fibrosis, 3). Her liver function gradually improved and she was discharged from the hospital on day 18 after admission. The liver function tests returned to normal within 6 weeks and HBV DNA was negative 8 weeks after admission.

DISCUSSION

HEPATITIS B VIRUS reactivation is now a well-recognized complication associated with the use of immunosuppressive chemotherapy in HBV carriers. HBV reactivation depends on both the intensity of the immunosuppressive agents and factors related to HBV or a host's immune balance. Therefore, the clinical consequences vary from asymptomatic elevation of hepatic enzymes to severe hepatitis and death from fulminant hepatitis. The prevalence of HBV reactivation ranges widely and is reported to occur in 20–78% of infected patients who undergo systemic chemotherapy for non-hepatic malignancies.[10,11] Initiation of antiviral prophylaxis prior to chemotherapy and its continuation until restitution of normal host immunity is important to prevent hepatitis B reactivation.[12]

Hepatitis B virus reactivation can occur by different mechanisms. First, glucocorticoids directly stimulate HBV gene expression *in vitro*[13] because the HBV genome has a specific glucocorticoid response element.[14] Second, steroid, cytotoxic or immunosuppressive agents induced the breakdown of the host's immune balance, leading to HBV replication and sometimes severe hepatitis.

In fact, HBV reactivation may occur during or after completion of the full course of chemotherapy. Several anticancer immunosuppressive agents have been associated with HBV reactivation. Corticosteroids and anthracyclines are most frequently associated with HBV reactivation.[15–17] Anthracycline has been demonstrated *in vitro* to stimulate HBV DNA secretion from HepG2-derived 2.2.15 cells in a dose-dependent manner.[18] Until recently, most of the cases with HBV reactivation were reported in patients with hematological malignancies, particularly lymphoma. HBV reactivation, however, is increasingly observed in patients with solid tumors, particularly breast cancer. Kim *et al.*[19,20] and Yeo *et al.*[19] reported that patients with HBsAg and breast cancer during adjuvant anthracycline-based chemotherapy developed acute hepatitis related to HBV reactivation (20.7% and 24%, respectively). A previous multivariate analysis indicated that a diagnosis of lymphoma or breast cancer was significantly related to HBV reactivation.[15]

The most important precaution to prevent HBV reactivation is the oncologist's knowledge of HBV reactivation. In Japan, a recommendation for the prevention of HBV reactivation was published in January 2009[8] and revised in 2011. The guideline is intended to identify patients with the possibility of developing HBV reactivation. The guideline recommends that all patients scheduled for chemotherapy or other immunosuppressive therapy be screened for HBsAg and tested further for anti-HBc and anti-HBs, even if negative for HBsAg. The present study demonstrates a consensus for oncologists in our institute to test for HBV or HCV in the serum of patients scheduled for chemotherapy. In fact, around 95% patients were tested for HBsAg or anti-HCV, even before this recommendation, but HBV DNA was only tested in 52% patients positive for HBsAg. This finding suggests that little attention is paid to HBV reactivation.

It is reported that 20% of oncologists in the USA do not check HBV serology, and 30% of oncologists test for HBV serology only when liver tests are abnormal.[21] These findings are consistent with another study of HBV reactivation among oncologists in Canada. Some chemotherapeutic agents such as anthracyclines are well known to induce cardiotoxicity. Lee *et al.*[22] reported that all patients scheduled for cardiotoxic chemotherapy underwent left ventricular function testing (100%), but only 14% of them were tested for HBsAg. Based on these reports, HBV reactivation is not commonly tested for by oncologists throughout the world, even though the percentage of HBV carriers was less in the USA and Canada compared to that in Japan.

In our retrospective study, HBV reactivation was relatively less frequent than in previous reports. The HBV reactivation might be less frequent in outpatient clinic patients than previously speculated. We speculated that

some bias might cause relatively less frequent HBV reactivation in this study due to its nature as a retrospective study. First, as many as 46% of patients with HBsAg were not examined for HBV DNA before treatment and then some patients were not regularly monitored for HBV DNA. Although the Japanese guideline recommended measuring serum HBV DNA monthly for at least 12 months after the discontinuation of chemotherapy,[8] there was a lack of data after the discontinuation of chemotherapy in some cases because of changing hospitals for palliative therapy. These may affect relatively less frequent HBV reactivation. This finding is, however, reasonable considering that oncologists have not been sufficiently aware of HBV reactivation until recently.

In conclusion, none of the patients with HBsAg who were treated with antiviral therapy developed hepatitis. HBV reactivation occurred in HBsAg positive outpatients without prophylactic antiviral treatment, but the incidence was relatively low in selected patients with non-hematological malignancies. Educational intervention is needed to prevent reactivation of HBV, and screening for HBV viral markers should be performed before starting chemotherapy.

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Interferon-α suppresses hepatitis B virus enhancer II activity via the protein kinase C pathway

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ABSTRACT

HBV has two enhancer (En) regions each of which promotes its own transcription. En II regulates production of pregenomic RNA, a key product of HBV replication, more strongly than En I. Although IFN-α has been found to suppress En I activity, its effect on En II activity has not been examined. Here we used luciferase assay to demonstrate that IFN-α suppresses En II activity. Analysis with several deletion/mutation constructs identified two major segments, nt 1703–1727 and nt 1746–1770, within the En II sequence as being responsible for the suppressive effects of IFN-α. Pre-treatment with protein kinase C (PKC) inhibitors blocked this effect regardless of the expression levels of phospho-STAT1 and Mx upon IFN-α stimulation. These results indicate that IFN-α suppresses En II activity via the PKC pathway, which may be an alternative suppressive pathway for HBV replication. (136 words).

Introduction

Hepatitis B virus (HBV) causes acute and chronic hepatitis in humans, and chronic infection is closely associated with the development of liver cirrhosis and hepatocellular carcinoma (Lok and McMahon, 2009). HBV has a partially double-stranded 3.2-kb DNA genome (relaxed circular (RC) DNA) in its nucleocapsid. When HBV invades host cells, RC-DNA is converted into a plasmid-like covalently closed circular DNA (cccDNA) inside the nucleus. From the cccDNA, the 3.5-, 2.4-, 2.1-, and 0.8-kb mRNAs are transcribed by cellular RNA polymerase II (Beck and Nassal, 2007). Among these RNAs, 3.5-kb pregenomic RNA (pgRNA) serves as the template of reverse transcription for synthesis of negative-strand DNA. Thus, transcription of pgRNA from cccDNA is one of the key steps in HBV replication.

In the HBV genome, there are four promoters (CP, SPI, SPII, and XP) and two transcriptional enhancer regions. Both enhancers stimulate transcription from the promoters (Antonucci and Rutter, 1989; Moolla et al., 2002; Su and Yee, 1992; Vannice and Levinson, 1988; Yee, 1989). Enhancer I (En I), which is located upstream of the X gene, activates transcription in a relatively cell-independent manner (Vannice and Levinson, 1988). In contrast, enhancer II (En II) (Fig. 1), located just upstream of CP, specifically activates transcription in hepatocytes (Wang et al., 1990; Yee, 1989; Yuh and Ting, 1990). Hepatocytes selectively express transcription factors which activate En II activity, such as HNF1 (Wang et al., 1998), HNF3 (Johnson et al., 1995; Li et al., 1995), HNF4 (Guo et al., 1993; Raney et al., 1997), CCAAT/enhancer binding protein (C/EBP) (López-Cabrera et al., 1990, 1991; Yuh and Ting, 1991) and FTF (Ishida et al., 2000; Li et al., 1998). This characterizes En II as a hepatocyte-specific *cis*-acting element. A previous report showed that, upon transfection with HBV genome, human hepatic cells, but not non-hepatic cells, were able to express pgRNA (Sureau et al., 1986). For this reason, En II is considered to regulate the production of pgRNA more strongly than En I (Yee, 1989).

Interferon-α (IFN-α) has been used as an anti-viral agent against HBV. It suppresses HBV viral load and ameliorates hepatic inflammation (Jonas et al., 2010; Liaw, 2009). Type I IFN activates the Janus kinase (JAK) bound to the cytoplasmic domain of its receptor. JAK phosphorylates transcription factors such as signal transducers and activators of transcription (STAT) 1 and STAT2. Phosphorylated STAT1 and STAT2 bind to IFN regulatory factor 9 (IRF9). These transcription factors form a complex, IFN-stimulated gene factor 3 (ISGF3). This complex binds to IFN stimulation response element (ISRE) in the promoter region of various genes, and activates interferon-stimulated genes (ISGs) (Der et al., 1998). Some of the ISGs including RNA-activated protein kinase (PKR), 2′,5′-oligoadenylate synthetases (OAS), and Mx have been shown to possess antiviral activity. ISG induction by type I IFN is considered to be the main pathway to suppressing viral replication.

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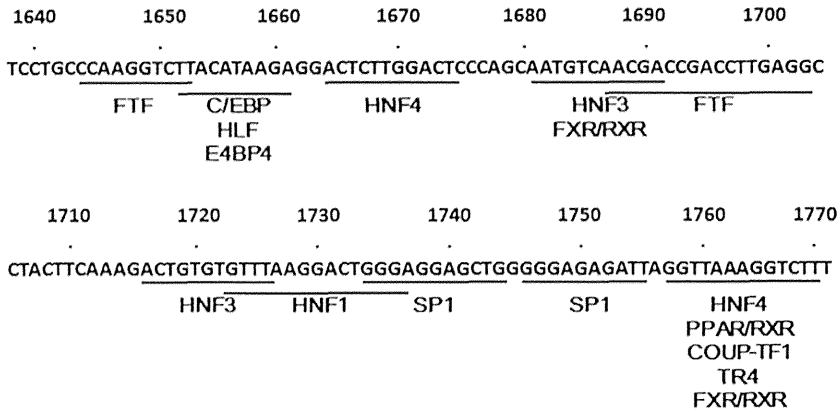

Fig. 1. Nucleotide sequences of the HBV En II region. The HBV sequence used in this study was of the *adw2* subtype (GenBank accession no. X02763). Numbering of the HBV sequence started at the unique *Eco*RI site. The underlined sequences represent the transcription factor binding sites mentioned in previous reports.

Type I IFN has been reported to inhibit HBV En I and core promoter activities (Nakao et al., 1999; Romero and Lavine, 1996; Schulte-Frohlinde et al., 2002; Tur-Kaspa et al., 1990). Nakao et al. demonstrated that IFN-α suppressed En I transcriptional activity by the binding of ISGF3 to the ISRE-like sequence in En I region (Nakao et al., 1999). However, there has been no study on the effect of IFN-α on HBV En II activity. In this study, we demonstrated that IFN-α suppressed En II activity via activation of PKC. Notably, STAT1 activation and ISG induction may be dispensable for IFN-α-mediated suppression of En II activity. This might shed light on understanding the inhibition of HBV replication by IFN-α.

Results

En II activity is down-regulated by IFN-α

We constructed a luciferase gene expression vector by inserting the En II sequence (nt 1640 to 1771) into pGL4LUC (pGL4LUC-En II). Huh-7 cells were transfected with pGL4LUC or pGL4LUC-En II, treated with or without IFN-α, and luciferase activities were evaluated. Insertion of En II increased the luciferase activity (about 228-fold) (Fig. 2A). IFN-α down-regulated the luciferase activity of pGL4LUC-En II, but did not affect that of pGL4LUC (Fig. 2B). This result suggested that IFN-α inhibited the activity of En II, and we examined the time course of IFN-α-induced suppression of En II activity. The suppressive effect of IFN-α on En II activity appeared at 3 h after administration of IFN-α, peaked at 6–12 h, and was gradually attenuated (Fig. 2C). Next, dose-response analysis showed that the En II activity was down-regulated by IFN-α in a dose-dependent manner, with the maximal suppressive effect at 300–1000 IU/m (Fig. 2D). We also examined the IFN-α-mediated suppression of En II activity in other hepatoma cell lines, PLC/PRF/5 and Hep3B. IFN-α significantly suppressed En II activities in both these cell lines (Fig. 2E). We next assessed whether or not IFN-α regulated HBV transcription in the HBV genome transfected cells by RT-PCR. HBV-RNA levels were significantly reduced by IFN-α (Fig. 2F). These results indicate that IFN-α suppresses HBV En II activity as well as its expression at a transcriptional level.

Both nt 1703–1727 and nt 1746–1770 within the En II region are required for suppression of En II activity by IFN-α

To determine the region responsible for the inhibitory effect of IFN-α on En II activity, we divided the En II sequence into six segments (Fig. 3A), and constructed plasmids containing En II sequences with deletion of each segment (pGL4LUC-En II-D1∼6). Huh-7 cells were transfected with these deleted constructs, treated with IFN-α, and then assayed for luciferase activity. None of the deletions could restore the suppressive activity by IFN-α (Fig. 3B), suggesting that there are several responsible regions for the IFN-α-induced suppression of En II activity. Next, we constructed plasmids containing four iterations of each segment within the En II sequence in tandem (pGL4LUC-En II-T1∼6) to examine the contribution of individual short fragments. IFN-α significantly suppressed the activities of pGL4LUC-En II-T2, T3, T4, T5 and -T6 in luciferase assay. Among them, the activities of pGL4LUC-En II-T4 and -T6 showed the largest suppression by IFN-α (Fig. 3C). On the basis of this result, we constructed a luciferase reporter vector with deletions of both segment 4 (nt 1703–1727) and segment 6 (nt 1746–1770) (pGL4LUC-En II-D4+6). The activity of this dual-deleted construct did not show a significant change due to IFN-α (Fig. 3D). These results suggest that both nt 1703–1727 and nt 1746–1770 within the En II region are required for the suppression of En II activity by IFN-α.

IFN-α-mediated suppression of En II activity is dependent on JAK activation

IFN-induced signal transduction occurs through the sequential activation of JAKs and STATs (Darnell et al., 1994). We examined the role of JAK in the inhibition of En II activity. JAK inhibitor alone did not affect En II activity. But the pre-treatment of the cells with JAK inhibitor completely blocked the suppressive effect of IFN-α on En II activity (Fig. 4A). The effect of JAK inhibitor was confirmed by the reducion of Mx induction in Western blot analysis (Fig. 4B). This result demonstrates that JAK activation is necessary for the IFN-α-induced suppression of En II activity.

The PKC pathway is involved in IFN-α-mediated suppression of En II activity

Previous reports demonstrated that type I IFN activated various kinases such as MAPK family members (MEK/ERK and p38 MAPK) (David et al., 1995; Goh et al., 1999), PI3K/Akt (Uddin et al., 1995), JNK (Caraglia et al., 1999) and protein kinase C (PKC) (Uddin et al., 2002). Here we examined the involvement of alternative pathways by pre-treatment with inhibitors for various kinases, including MEK, p38 MAPK, PI3K/Akt, JNK and PKC. The name of each inhibitors and its target kinase is commented in Table 1. As shown in Fig. 5A, only staurosporine, a PKC inhibitor, blocked the inhibitory effect of IFN-α, and other inhibitors did

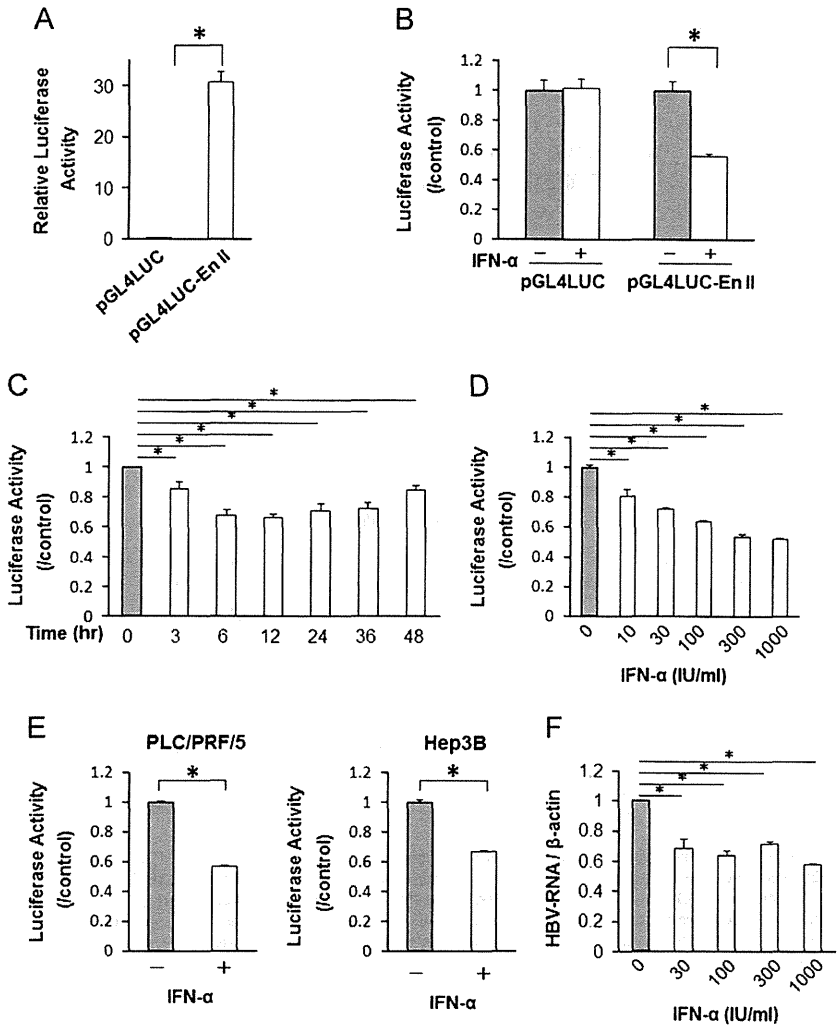

Fig. 2. Suppression of HBV En II transcriptional activity and reduction of HBV-RNA by IFN-α. A, B. Huh-7 cells were transfected with pGL4LUC or pGL4LUC-En II or incubated with or without IFN-α (100 IU/ml). After 24 h, the activity of firefly luciferase was evaluated. C. Huh-7 cells were transfected with pGL4LUC-En II, and incubated with IFN-α (100 IU/ml). Luciferase activities were evaluated at the indicated times. D. Huh-7 cells were transfected with various concentrations (0–1000 IU/ml) of IFN-α for 12 h and luciferase activities were evaluated. E. PLC/PRF/5 cells (left panel) and Hep3B (right panel) cells were transfected with pGL4LUC-En II, and incubated with or without IFN-α (300 IU/ml). Luciferase activities were evaluated. F. Huh-7 cells were transfected with pHBV1.5, and treated with IFN-α at various concentrations (0–1000 IU/ml). At 72 h after IFN-α treatment, cells were harvested, and the abundances of HBV-RNA were evaluated by quantitative RT-PCR. The HBV-RNA level of the IFN-α treated cells was normalized with that of non-treated cells. $^*p < 0.05$. "/control" on the vertical axis means the ratio of luciferase activity of IFN-α treated cells normalized with that of non-treated cells.

not. Since staurosporine is a PKC inhibitor showing broad-spectrum activity (Marte et al., 1994), we also examined other inhibitors specific for PKC isoforms. Previous reports demonstrated that IFN-α activated PKC-α/β and PKC-δ (Pfeffer et al., 1990; Uddin et al., 2002). Indeed, activation of PKC-α/β and PKC-δ by IFN-α was confirmed by immunoblot analysis (Fig. 5B). Thus, we examined the PKC inhibitors rottlerin and Gö6976 (Gschwendt et al., 1994; Martiny-Baron et al., 1993). All PKC inhibitors blocked the suppression of En II activity by IFN-α (Fig. 5C). These results suggest that several isoforms of PKC are involved in the IFN-α-mediated suppression of En II activity. We also examined STAT1 activation and ISGs induction by IFN-α in cells pre-treated with these PKC inhibitors using immunoblot analysis (Fig. 5D). Expression levels of phospho-STAT1 and Mx differed among these PKC inhibitors. Staurosporine and Gö6976 slightly diminished the activation of STAT1, but rottlerin did not. This result suggests that PKC isoforms might not strongly regulate activation of STAT1. Rottlerin, a specific inhibitor for PKC-δ, inhibited the induction of Mx, which agreed with previous findings (Kaur et al., 2005). Staurosporine and Gö6976 did not suppress Mx expression. Taken together, all these PKC inhibitors blocked the suppression of En II activity by IFN-α regardless of the expression levels of phospho-STAT1 and Mx. These results suggest that STAT1 activation and ISG induction may be dispensable for the IFN-α-mediated suppression of En II activity. Next, we examined the effect of phorbol 12-myristate 13-acetate (PMA), a PKC activator (Castagna et al., 1982; Griner and Kazanietz, 2007). PMA suppressed En II activity (Fig. 5E), and PMA stimulation did not result in STAT1 phosphorylation and Mx induction (Fig. 5F), suggesting that suppression of En II by PMA is independent of STAT1 activation and ISG induction. On the basis of these findings, we conclude that IFN-α suppresses En II activity via the PKC pathway, which may not involve STAT1 activation and ISG induction.

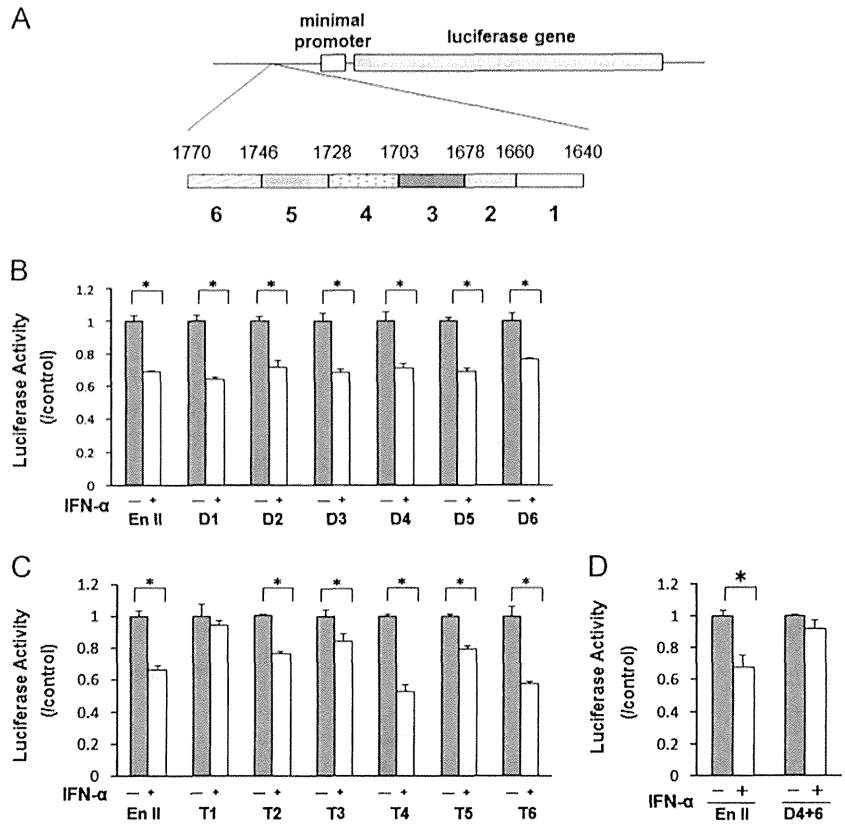

Fig. 3. Deletion/mutational analysis to identify the responsive sequence for the suppressive effect of IFN-α on En II. A. Scheme of pGL4LUC-En II and six segments defined within the En II sequence. The En II sequence was integrated just upstream of the minimal promoter of pGL4LUC. B. Huh-7 cells were transfected with the reporter vectors with deletion of each segment (pGL4LUC-En II-D1~6), incubated with 300 IU/ml IFN-α for 12 h, and luciferase activities were evaluated. C. Plasmids containing four iterations of each segment within En II sequence in tandem (pGL4LUC-En II-T1~6) were generated and luciferase activities were evaluated similarly. D. Plasmid with deletion of both nt 1703–1727 and nt 1746–1770 (pGL4LUC-En II-D4+6) was constructed and luciferase activities were evaluated similarly. $*p < 0.05$. "/control" on the vertical axis means the ratio of luciferase activity of IFN-α treated cells normalized with that of non-treated cells.

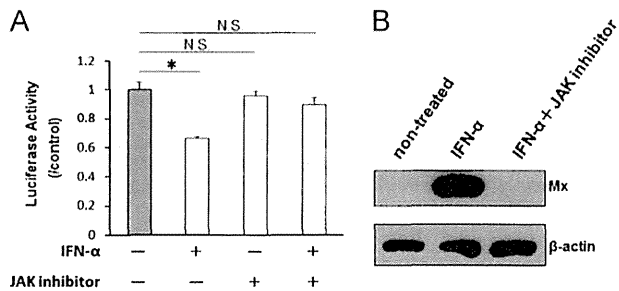

Fig. 4. Involvement of JAK activation in the IFN-α-induced suppression of En II activity. A. Huh-7 cells were transfected with pGL4LUC-En II and treated with JAK inhibitor (1 μM) for 1 h. The cells were then incubated with IFN-α(150 IU/ml) for 12 h, followed by luciferase assay. B. Huh-7 cells were pre-treated with JAK inhibitor for 1 h, and then incubated with IFN-α(150 IU/ml) for 12 h, followed by immunoblot analyses to detect Mx protein. $*p < 0.05$. "/control" on the vertical axis means the ratio of luciferase activity of IFN-α treated cells normalized by that of non-treated cells.

Table 1
A comment of the inhibitors and its target kinase.

PD98059	MEK inhibitor
SB203580	P38MAPK inhibitor
LY294002	PI3K inhibitor
Akt-1-1/2	Akt inhibitor
SP600125	JNK inhibitor
Staurosporine	PKC inhibitor with broad spectrum
Rottlerin	Inhibitor specific for PKC-δ
Gö6976	Inhibitor specific for Ca^{2+}-dependent PKC isoforms

Knockdown of a single transcription factor does not influence IFN-α-induced suppression of En II activity

We anticipated that IFN-α suppressed En II activity by functional down-regulation of some transcription factor(s) phosphorylated in a PKC-dependent manner. Among transcription factors which bind the En II region, previous reports showed that Specificity Protein 1 (Sp1) (Mahoney et al., 1992; Pal et al., 1998; Rafty and Khachigian, 2001), Retinoid X Receptor α (RXRA) (Delmotte et al., 1999) and C/EBP (Mahoney et al., 1992) were inactivated by PKC. Thus, we examined the En II response to IFN-α after knockdown of these transcription factors. C/EBP, RXR and Sp1 expression was efficiently reduced by siRNA (Fig. 6A). We observed no significant change in the suppression of En II activity compared with control siRNA (Fig. 6B). This result suggests that several transcription factors (including unknown proteins) might be involved in the IFN-α-mediated suppression of En II activity.

Discussion

In the present study, we demonstrated that IFN-α suppressed HBV En II activity. The inhibition by IFN-α of En II activity could be blocked by pre-treatment with PKC inhibitors, and this

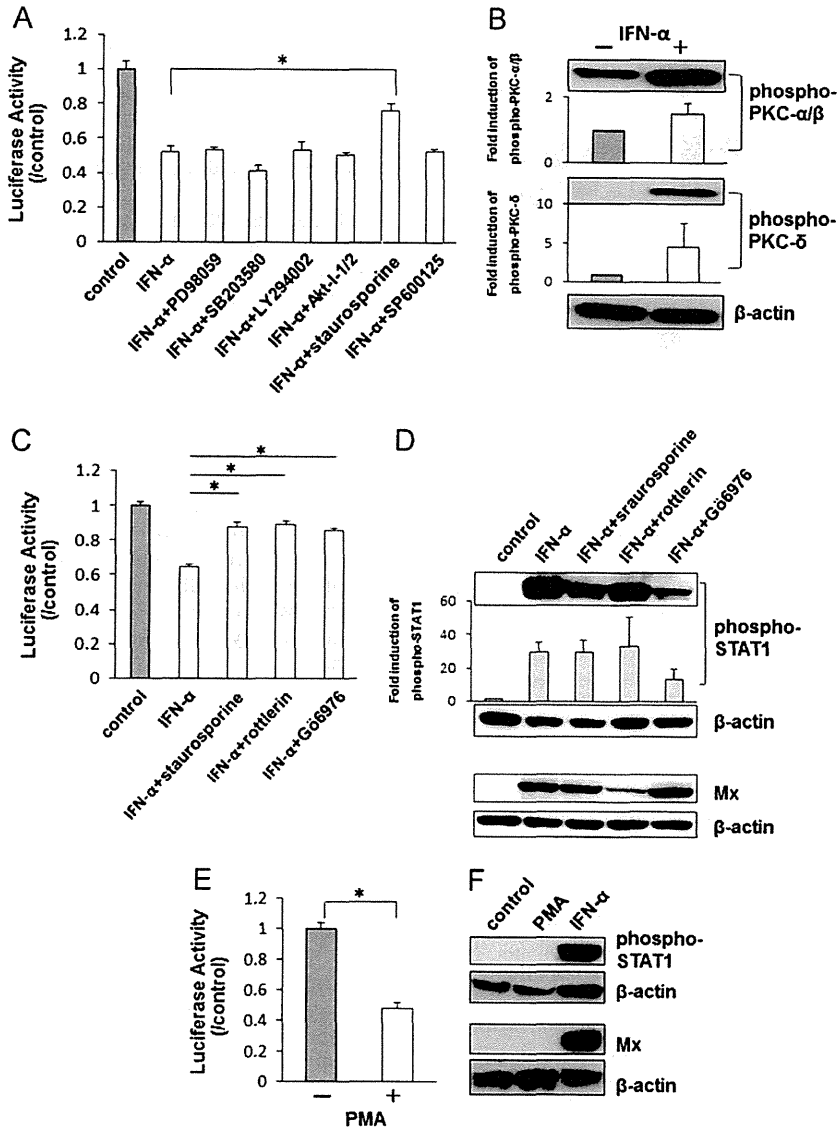

Fig. 5. PKC-dependent suppression of En II activity by IFN-α. A and C. Huh-7 cells were transfected with pGL4LUC-En II, treated separately with each kinase inhibitor for 1 h. The cells were then treated with IFN-α(1000 IU/ml) for 12 h, and luciferase activities were evaluated. B. Huh-7 cells were treated with IFN-α(1000 IU/m) for 12 h. Immunoblot analyses were performed to detect phosphorylated PKC-α/β and phosphorylated PKC-δ. Quantitative analysis of the expression level of phopho- PKC-α/β and -δ was performed by using ImageJ. Each level was normalized with that of IFN-α-non-treated cells. D. Huh-7 cells were harvested at 30 min to detect phosphorylated STAT1 and at 12 h to detect the expression of Mx after administration of IFN-α (1000 IU/m), and immunoblot analyses were performed. Quantitative analysis of the expression level of phopho-STAT1 was performed by using ImageJ. Each level was normalized with that of IFN-α-non-treated cells. E. Huh-7 cells were transfected with pGL4LUC-En II, treated with PMA (100 nM) for 12 h, and luciferase activities were evaluated. F. Huh-7 cells were treated with PMA (100 nM) or IFN-α(1000 IU/m). The cells were harvested at 30 min to detect phosphorylated STAT1 and at 12 h to detect the expression of Mx, and immunoblot analyses were performed. *$p < 0.05$. "/control" on the vertical axis means the ratio of luciferase activity of IFN-α/ PMA treated cells normalized with that of non-treated cells.

blocking effect may not involve STAT1 activation and ISG induction. The latter, ISG induction via the JAK-STAT pathway, has been considered to be the main mechanism suppressing viral replication. Our findings suggest a pathway for IFN-α repression of HBV transcription other than ISG induction.

PKCs are involved in a wide variety of cell functions and signal transduction pathways regulating cell migration and polarity, proliferation, differentiation and cell death (Nishizuka, 1988). In the PKC family, there are at least ten isoforms which can be divided into three sub-groups based on their structural characteristics and cofactor requirements. These include the classical PKC (cPKC: α, βI, βII, and γ), the novel PKC (nPKC: δ, ε, η and θ), and the atypical PKC (aPKC: ζ and ι/λ) (Azzi et al., 1992; Breitkreutz et al., 2007; Kikkawa et al., 1989). IFN-α can activate multiple PKC isoforms: not only PKC-δ, but also PKC-α/β (Pfeffer et al., 1990), PKC-ε (Pfeffer et al., 1991), and PKC-θ (Srivastava et al., 2004). Despite the variety of PKC isoforms, most phosphorylate similar sequences (Breitkreutz et al., 2007). Both the PKC-α/β inhibitor (Gö6976) and PKC-δ inhibitor (rottlerin) blocked the inhibitory effect of IFN-α on En II activity. Thus, it was speculated that each PKC isoform might be similarly involved in suppressing of En II activity.

Other studies have examined the role of the PKC pathway in HBV replication. Kang et al. (2008) reported that PKC-mediated phosphorylation increased capsid assembly and stability (von Hahn et al., 2011), and von Hahn et al. (2011) reported that the

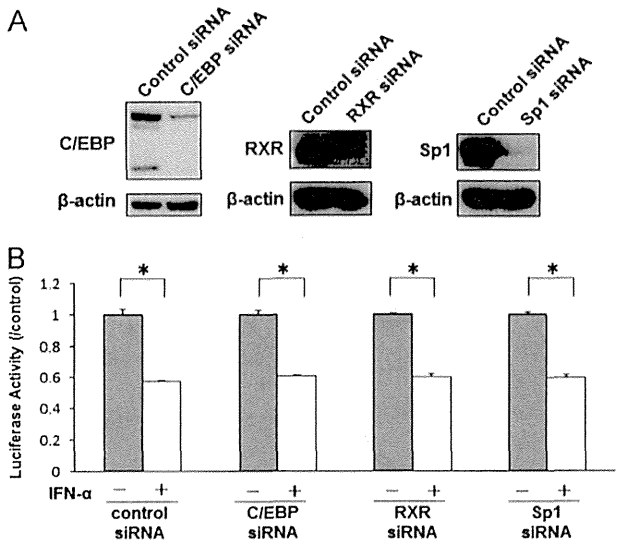

Fig. 6. IFN-α-mediated suppression on En II activity with knockdown of C/EBP, RXR and Sp1. A. Huh-7 cells were transfected with 10 nM siRNA (negative control or specific for C/EBP, RXR and Sp1). Immunoblot analyses for expressions of C/EBP, RXR, Sp1 and β-actin were performed at 48 h post siRNA transfection. B. Huh-7 cells were transfected with 10 nM siRNA (negative control or specific for C/EBP, RXR and Sp1). On the next day, si-RNA treated cells were transfected again with pGL4LUC-En II. On the following day, these transfected cells were incubated with IFN-α(1000 IU/ml) for 12 h, and luciferase activities were evaluated. "/control" on the vertical axis means the ratio of luciferase activity of IFN-α treated cells normalized with that of non-treated cells.

pan-PKC inhibitor sotrastaurin did not affect HBV replication. While the role of PKC in the HBV life cycle is still controversial, our findings suggest that PKC isoforms activated by IFN-α play inhibitory roles in HBV transcription by down-regulation of En II activity. As von Hahn et al. reported, sotrastaurin alone did not affect HBV replication. But, based on our present data about another pan-PKC inhibitor, staurosporine, we speculate that sotrastaurin may also block the inhibitory effect of IFN-α on En II activity.

We showed that knockdown of a single transcription factor did not influence the IFN-α-mediated suppression of En II activity, suggesting that several transcription factors might be involved in this suppression. We also showed that both segment 4 (nt 1703–1727) and segment 6 (nt 1746–1770) within the En II region are required for the IFN-α-induced suppression of En II activity. Although these two regions seem to be more important than the others, all the deleted version of reporter constructs showed almost completely similar suppression activities (Fig. 3B). We speculate that there may be some transcription factors which affect both the segment 4 and 6. Even if one of these regions is deleted, some factors may affect the other region, and result in the suppression of En II activity. Further study will be needed to clarify the mechanism.

Indeed, there are no identified transcription factors which could bind both segment 4 and 6. Only two transcription factors (HNF1 and 3) were reported to bind segment 4 (Johnson et al., 1995; Wang et al., 1998), and there have been no reports indicating that IFN-α or PKC inactivates HNF1 or 3. We also examined the expression levels of HNF1 and 3 of the IFN-α treated and the non-treated cells by RT-PCR. There was no significant difference in the expression of these transcription factors between the IFN-α treated and the non-treated cells (Nawa et al., unpublished data). Thus, we speculate that HNF1 or 3 might not be involved in the IFN-α mediated suppression of En II activity. There may be unknown transcription factors in the PKC pathway.

Previous reports showed that IFN-α suppressed En I activity (Nakao et al., 1999; Tur-Kaspa et al., 1990). Nakao et al. (1999) indicated that this occurred due to the binding of ISGF3 to an ISRE-like motif within the En I region. However, Rang et al. (2001) demonstrated that IFN-α reduced HBV-RNA levels derived from both HBV genome wild type and mutated ISRE-like motifs. This result contradicted the Nakao's result that the activity of the En I mutated ISRE-like motif was not suppressed by IFN-α. Schulte-Frohlinde et al. (2002) reported that IFN-α suppressed HBV core promoter regulated transcriptional activity, even when the ISRE-like motif of En I was deleted. The results of Rang et al. and Schulte-Frohlinde et al. suggest that IFN-α might suppress the activity of regions other than En I. In the present study, we demonstrated that IFN-α suppressed En II activity via the PKC pathway. En II might be one of the candidate regions down-regulated by IFN-α within the HBV genome.

Since En II activates viral transcription only in hepatocytes, it is responsible for the hepatocyte-specific gene expression of HBV. There had been no study on the effect of IFN-α on En II activity. Our study clarified that the PKC pathway is involved in the IFN-α-mediated suppression of En II activity, but may not involve ISG induction. Our result should aid in establishing better treatment with IFN-α against HBV infection. As we could not determine the molecule which inhibits En II activity by IFN-α, further study is needed to clarify this molecule and to control hepatitis B by IFN-α treatment.

Materials and methods

Plasmids

The HBV sequence used in this study was of the *adw2* subtype (GenBank accession no. X02763). Numbering of the HBV sequence started at the unique *Eco*RI site. The En II region in this study was defined as nt 1640–1771 of HBV sequence (Fig. 1) (Ishida et al., 2000). To construct pGL4LUC-En II, a plasmid containing the HBV En II region, the DNA fragment was amplified with PCR and inserted between *Hind* III and *Nhe* I site of pGL4 Luciferase Reporter Vector (pGL4LUC) (Promega, Madison, WI). The PCR primers were as follows: 5′-CCAAGCTTCTGCCCAAGGTC-3′ and 5′-CCCGCTAGCAAAGACCTTTAACCTAATCTCCTCC-3′. The constructs of the En II sequence with various deletions were generated by modifying pGL4LUC-En II using the QuikChange Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA). The constructs containing four tandem repeats of short fragment in En II sequence were generated by inserting duplexes of synthesized oligonucleotides into the multi-cloning site of pGL4LUC. All of the En II sequences were inserted in the antisense orientation to evaluate their enhancer activity.

Plasmid pHBV1.5 containing a 1.5-fold-overlength genome of HBV-DNA (GenBank accession no. AF305422) has been described previously (Bruss and Ganem, 1991).

Cell lines and reagents

The human hepatocellular carcinoma cell lines Huh-7, PLC/PRF/5, and Hep3B were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (Sigma-Aldrich, St. Louis, MO) in a humidified incubator at 5% CO_2 and 37 °C. Human natural IFN-α was kindly provided by Hayashibara Biochemical Laboratories, Inc. (Okayama, Japan).

The inhibitors/activators and the final concentrations used were: JAK inhibitor I (1 μM), PD98059 (10 μM), SB203580 (10 μM), LY294002 (10 μM), Akt-I-1/2 (5 μM), staurosporine (10 or 20 nM), rottlerin (5 μM), Gö6976 (1 μM), SP600125 (10 μM)

(Calbiochem, San Diego, CA), phorbol 12-myristate 13-acetate (PMA) (100 nM) (Sigma-Aldrich, St. Louis, MO).

Plasmid transfection and luciferase assay

Huh-7 cells were co-transfected with the firefly luciferase plasmid and pGL4-RL-tk, an expression vector of renilla luciferase, which was used as an internal control, using FuGENE HD reagent (Roche Applied Science, Indianapolis, IN) according to the manufacturer's protocol. Activities of firefly luciferase and renilla luciferase were measured using the Dual-Glo Luciferase Assay System (Promega, Madison, WI), and then relative luciferase activity was calculated by normalizing firefly luciferase activity to renilla luciferase activity.

RNA extraction

Total RNA was isolated from cells using ISOGEN (Nippon Gene, Tokyo, Japan) according to the manufacturer's protocol. The isolated RNA was treated with DNase I (Promega, Madison, WI) to avoid contamination with transfected plasmid, and then purified with a mixture of phenol, chloroform, and isoamylalcohol (pH 7.9), followed by ethanol precipitation.

Western blot analysis

Cultured cells were lysed with a lysis buffer (1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, and protein inhibitor cocktail (Nacalai Tesque), in PBS, pH 7.4). Equal amounts of protein were electrophoretically separated by polyacrylamide gel and transferred onto PVDF membrane. For immunodetection, the following antibodies were used: anti-STAT1 antibody, anti-phospho-STAT1 antibody, anti-phospho-PKC-α/β II (Thr 638/641) antibody, anti-phospho-PKC-δ (Thr 505) antibody, anti-C/EBP antibody, anti-RXR antibody, anti-Sp1 antibody, anti-β-actin antibody from Cell Signaling Technology (Beverly, MA), and anti-Mx antibody from Abcam (Cambridge, UK). The signals of phosphorylated proteins such as phospho-PKC-α/β, -δ and phospho-STAT1 were analyzed quantitatively using image analyzing software (ImageJ; version 1.45).

Small RNA interference

Stealth Select RNAi specific for STAT1 (HSS 10273) was purchased from Invitrogen (Carlsbad, CA). Silencer Select siRNA specific for C/EBP (ID: S2890), RXR (ID: S12386) and Sp1 (ID: S13319) were purchased from Ambion (Austin, TX). Stealth RNAi Negative Control Low GC Duplex (Invitrogen, Carlsbad, CA) was used as a control for the off-target effect following Stealth Select RNAi delivery. The transfections were carried out using Lipofectamine RNAiMAX (Invitrogen, Carlsbad, CA) according to the reverse transfection protocol.

Real-time reverse-transcription PCR

For cDNA synthesis, 1 μg of total RNA was reverse-transcribed using High Capacity RNA-to-DNA Master Mix (Applied Biosystems, Foster City, CA). cDNA, equivalent to 20 ng RNA, was used as a template for real-time reverse-transcription PCR (RT-PCR) using Applied Biosystems 7900HT Fast Real-Time PCR System (Applied Biosystems, Foster City, CA). mRNA expressions of C/EBP, FTF, HNF1, HNF3, and HNF4 were measured using TaqMan Gene Expression Assays and were corrected with the quantified expressions level of β-actin mRNA. Assay IDs for the genes were as follows: C/EBP (Hs00269972_s1), FTF (Hs00187067_m1), HNF1 (Hs00167041_m1), HNF3 (Hs00232754_m1), and HNF4 (Hs01023298_m1).

For the detection of pgRNA and pre-C mRNA, the primers and the probes were designed as follows according to a previous study (Laras et al., 2002): the sense primer was 5′-TCTTGTACATGTCCCACTGTTCAA-3′ (nt 1843–1866); the anti-sense primer was 5′-AATGCCATGCCCCAAAGC-3′ (nt 1890–1909); the probe was 5′-FAM-CTCCAAGCTGTGCCTT -3′ (nt 1869–1884). Since they were within precore/core coding sequence, only the total abundance of pgRNA and pre-C RNA could be detected.

Statistical analysis

Data were presented as mean ± SD. Differences between two groups were determined using Student's t-test for unpaired observations. $p < 0.05$ was considered statistically significant.

Disclosures

All authors have nothing to disclose.

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VIRAL HEPATITIS

HLA-DP genes polymorphisms associate with hepatitis B surface antigen kinetics and seroclearance during nucleot(s)ide analogue therapy

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Keywords
hepatitis B surface antigen – *HLA-DP* – lamivudine – nucleot(s)ide analogue

Abbreviations
ADV, adefovir dipivoxil; ALT, alanine aminotransferase; ETV, entecavir; GWAS, genome-wide association study; HBeAg, hepatitis B e antigen; HBsAg, hepatitis B surface antigen; HBV DNA, hepatitis B virus deoxyribonucleic acid; HBV, hepatitis B virus; HLA, human leucocyte antigen; HR, hazard ratio; IFN, interferon; LAM, lamivudine; NA, nucleos(t)ide analogues; SNP, single nucleotide polymorphism; VBT, virological breakthrough; VR, virological response.

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Abstract

Background & Aims: Genome-wide association studies (GWAS) recently indicated that polymorphisms in the human leucocyte antigen *(HLA)-DP* genes were associated with risk of persistent hepatitis B virus (HBV) infection and clearance of HBV, but the effect of *HLA-DP* gene polymorphisms on the effect of antiviral therapy was unknown. We here investigated whether such polymorphisms were associated with decreases in HBsAg levels and seroclearance in patients who received long-term lamivudine (LAM) treatment. *Methods:* Japanese patients (202) who were hepatitis B e antigen positive at baseline, received LAM as first-line treatment, and consented to *HLA-DP* genotyping (*HLA-DPA1* rs3077 and HLA-DPB1 rs9277535) were categorized into two cohorts, viz., a cohort who achieved virological response without rescue therapy (cohort 1) and those who did so with rescue therapy (cohort 2). *Results:* Serum HBsAg levels declined significantly between year 3 and 9 from baseline among cohort 1 patients possessing ≥2 A-alleles at rs3077 and rs9277535. The percentages of such patients in cohort 1 patients with decreases in HBsAg ≥0.5 log IU/ml were higher than those with <2 A-alleles (71.8% [28/39] vs. 38.9% [23/59]; $P = 0.004$). However, there was no significant difference in cumulative HBsAg seroclearance rates between patients with ≥2 and those with <2 A-alleles in cohort 1. In cohort 2, HBsAg seroclearance rates were higher in patients with ≥2 A-alleles than in those with <2 A-alleles ($P = 0.003$). *Conclusion:* We found an association between *HLA-DP* polymorphisms and decreases in HBsAg levels and seroclearance among HBeAg-positive patients treated with LAM.

More than 2 billion people worldwide have been exposed to hepatitis B virus (HBV) and about 350 million people are chronically infected, the majority of whom are in Asia (75%). The prevalence of HBV in Japan is 0.8%, which is lower than that in other Asian countries, such as Taiwan (>10%) and China (1–3). Recently, oral nucleot(s)ide analogues (NAs) have been used as a mainstay therapeutic strategy against chronic hepatitis B. Such antiviral agents, including lamivudine (LAM), entecavir (ETV), telbivudine, adefovir dipivoxil (ADV) and tenofovir disoproxil fumarate, inhibit viral replication. These NAs vary in both the strength and the rapidity with which they suppress HBV DNA (4–10). Sustained viral suppression by NA therapy can improve liver fibrosis and clinical outcomes of patients (11, 12). LAM was the first NA to be approved for treating chronic hepatitis B in Japan, followed by ADV and ETV.

Responses to antiviral treatments can be evaluated by monitoring serum HBV DNA levels, and hepatitis B e (HBeAg) and hepatitis B surface antigen (HBsAg) and antibody levels. Serum HBsAg levels have been recognized as one of the predictive markers of the prognosis and effect of antiviral therapy. Some studies recently reported the rates of HBsAg seroclearance and HBsAg

kinetics after pegylated interferon (PegIFN) and/or NAs therapy (13–15). These studies demonstrated that HBsAg seroclearance is associated with HBV genotype, baseline HBsAg levels, and the decrease in HBsAg early during treatment. However, it remains unclear whether host factors are associated with treatment-related HBsAg seroclearance.

Genome-wide association studies (GWAS) have been well applied in the field of viral hepatitis, and several studies have reported that the human leucocyte antigen (*HLA*)-*DP* locus, located on chromosome 6, is associated with the risk of persistent infection with HBV (16). A few studies have reported that the *HLA-DP* locus is also associated with HBV clearance (17–20). Two single nucleotide polymorphisms (SNPs) in a region including *HLA-DPA1* and *HLA-DPB1* are strongly associated with persistent HBV infection (*HLA-DPA1* rs3077 and *HLA-DPB1* rs9277535). The minor alleles (A-alleles) of both rs3077 and rs9277535 seem to have protective effects against chronic hepatitis B (16). Although there have been two reports on the association between the *HLA-DP* locus and antiviral therapy for chronic hepatitis B, further investigation of association of *HLA-DP* SNPs with the effect of antiviral therapy is warranted (21, 22).

We therefore hypothesized that the minor alleles of *HLA-DPA1* rs3077 and *HLA-DPB1* rs9277535 may have an impact on HBsAg kinetics and seroclearance during NA therapy.

In this study, we investigated whether polymorphisms in *HLA-DP* genes are associated with reduction in HBsAg titres and seroclearance in chronic HBeAg-positive hepatitis B patients who received long-term LAM treatment and subsequently achieved favourable sustained viral responses.

Patients & methods

Study population

Over a period of 12 years (September 1995 to September 2007), 949 consecutive patients, chronically mono-infected with HBV (confirmed HBsAg positivity over a period of at least 6 months), were treated with LAM monotherapy at the Department of Hepatology, Toranomon Hospital, Metropolitan Tokyo. The indication for antiviral therapy was abnormal ALT levels accompanying the increase in HBV DNA levels (over 4 log copies/ml). However, in cases where ALT levels were normal, patients with advanced fibrosis were also administrated LAM. We selected 791 patients as study subjects after we excluded all those who had been treated with LAM for <6 months, were co-infected with hepatitis C virus, had not provided sufficient serum samples, and/or had insufficient clinical records. No patient was co-infected with human immunodeficiency virus in this cohort. Of these 791 patients, 441 were HBeAg positive and 350 were HBeAg negative at baseline.

HLA-DP SNPs were analysed in 253 of 441 patients who are HBeAg positive. Ninety eight of 253 patients achieved viral response (VR: HBV DNA <600 copies/ml) and subsequently maintained low viral load (HBV DNA <1 log copies/ml from nadir; cohort 1). Over time, 136 of these 253 individuals experienced an increase in HBV DNA (≥1 log copies/ml; e.g. because of virological breakthrough [VBT]), and as a result, 133 (98%) individuals were provided with ADV treatment (10 mg) added to LAM, as a rescue therapy. Of the 133, 104 patients achieved VR with rescue therapy and subsequently maintained a low viral load (HBV DNA <1 log copies/ml from nadir; cohort 2). Thus, in total, 202 patients were enrolled in this retrospective cohort study (Fig. 1). All of these patients are Japanese. Written informed consent was obtained from each patient. This study protocol conformed to the ethical guidelines of the 1975 Declaration of Helsinki and was approved *a priori* by the institution's human research committee.

Clinical data collection and follow-ups

Data on patient characteristics, biochemistry, haematology, virology, histology and previous treatments were collected and registered in our institute's database at the time of patient enrolment. Prior to beginning LAM therapy, the presence of family history of HBV infection was surveyed in all patients. Data on the treatment dose and duration of previous IFN therapy were collected from our hospital's IFN therapy database or requested from other hospitals as necessary.

At least every 1–3 months, liver function and virological markers of HBV infection were measured in all patients. All serum HBsAg titres were measured from frozen serum samples collected at 6 months, 1, 3, 5 years, and once annually for 6–10 years, and then stored at −80°C. The time-point of HBsAg clearance was defined by the measurement in consecutive available serum samples before HBsAg undetected. A genotypic analysis of drug resistance was performed in cases of insufficient virological response or VBT, defined as an increase in serum HBV DNA levels ≥1 log above the nadir measured after the initial virological response. Cirrhosis was diagnosed by laparoscopy, liver biopsy, or clinical data, such as imaging modalities and portal hypertension. The primary outcome for this study was seroclearance and significant reduction in HBsAg. The endpoint of the follow-up was HBsAg clearance or last visit before March 2013.

Markers of HBV infection

Serum HBsAg titres were measured using ARCHITECT HBsAg QT assay kits (Abbott Laboratories, Tokyo, Japan), which have a lower limit of detection of 0.05 IU/ml and an upper limit of detection of 250 IU/ml. To expand the upper range from 250 to 125 000 IU/ml, serum samples exceeding the scale were

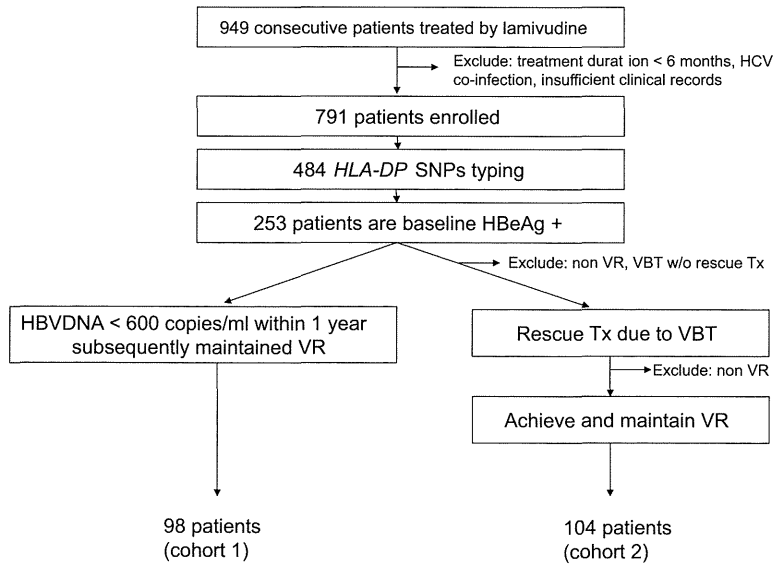

Fig. 1. Schematic of study protocol. HBeAg, hepatitis B e antigen; HCV, hepatitis C virus; HLA, human leucocyte antigen; SNP, single nucleotide polymorphism; VBT, virological breakthrough; VR, virological response.

diluted stepwise to 1:20 and 1:500 with ARCHITECT diluents as the product document described. HBeAg was determined by enzyme-linked immunosorbent assay with a commercial kit (HBeAg EIA; Institute of Immunology, Tokyo, Japan). HBV DNA was quantified using the Amplicor monitor assay (Roche Diagnostics, Tokyo, Japan), which has a dynamic range over 2.6–7.6 log copies/ml, or COBAS TaqMan HBV v.2.0 (Roche Diagnostics), which has a dynamic range over 2.1–9.0 log copies/ml. A commercial kit (HBV Genotype EIA; Institute of Immunology) was used to determine HBV genotypes serologically by using the combination of epitopes expressed on the pre-S2 region product, which is specific for each of the seven major genotypes (A–G). YMDD mutants (rt M204I/V) were determined by polymerase chain reaction-based enzyme-linked minisequence assay with a commercial kit (Genome Science Laboratories, Tokyo, Japan).

HLA-DP SNPs typing

SNPs in *HLA-DPA1* (rs3077) and *HLA-DPB1* (rs9277535), located on chromosome 6, were genotyped by TaqMan assay or Invader assay, as previously described (16). *IL28B* genotype was assayed for the rs8099917 SNP using TaqMan assay or Invader assay.

Statistical analyses

Categorical data were compared between groups using chi-square or Fisher's exact tests. Continuous variables with a non-parametric distribution were analysed with Mann–Whitney *U*-tests, while those with a parametric distribution were analysed with Student's *t*-tests. Cox regression analyses were used to assess which variables were significantly associated with HBsAg clearance. All baseline factors that were found to be significantly associated with HBsAg clearance by univariate analysis were entered into a multivariate analysis. Independent baseline factors associated with clearance of HBsAg were calculated using a stepwise Cox regression analysis. Cumulative HBsAg clearance rates were analysed using the Kaplan–Meier method; differences in the resulting curves were tested using log-rank tests. Cochran–Armitage trend tests were performed for the association between HBsAg seroclearance and an increase in the number of A-alleles. Significance was defined as $P < 0.05$ for all two-tailed tests. Data analysis was performed with IBM SPSS version 19.0 software (IBM Corp., Armonk, NY, USA) and R software version 2.13 (R Foundation for Statistical Computing, Vienna, Austria; www.r-project.org).

Results

Patient characteristics and clinical course

Eighteen of 202 patients successfully cleared HBsAg. Of these, 11 belonged to cohort 1, and seven to cohort 2. Table 1 provides a comparison of the baseline characteristics between patients who were and were not able to successfully clear HBsAg (all patients, cohort 1 and cohort 2).

In cohort 1, baseline characteristics that were significantly associated with HBsAg clearance included HBV genotype and high HBV DNA levels; in cohort 2, such