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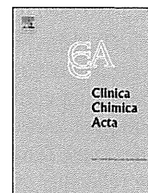

Comparison of LecT-Hepa and FibroScan for assessment of liver fibrosis in hepatitis B virus infected patients with different ALT levels

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ABSTRACT

Background: FibroScan is one of the noninvasive techniques based on the transient elastography that can assess the progression of liver fibrosis in chronic hepatitis patients in daily clinical practice. Recently, LecT-Hepa was validated as a serological glycomarker correlating well with the fibrosis stage determined by liver biopsy, and was superior to many other noninvasive biochemical markers and tests. We compared the reliability of LecT-Hepa with that of FibroScan for evaluation of liver fibrosis.
Methods: The effects of increased alanine aminotransferase (ALT) activities on LecT-Hepa and FibroScan were investigated.
Results: The areas under the receiver-operating characteristic curves, sensitivity and specificity for detecting cirrhosis, which is one of the outcomes of fibrosis estimation, were 0.82, 72.5% and 78.2% of LecT-Hepa, 0.85, 87.0% and 74.1% of FibroScan; these did not differ significantly. The count distribution of LecT-Hepa in non-cirrhosis group or cirrhosis group did not differ between the patients grouped according to their ALT levels, whereas that of FibroScan was substantially affected.
Conclusion: LecT-Hepa was confirmed as a reliable noninvasive test for the evaluation of liver fibrosis in hepatitis B virus-infected patients with comparable performance to that of FibroScan and proved to be unaffected by inflammation.

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1. Introduction

It is estimated that about 2 billion people worldwide have been infected with the hepatitis B virus (HBV), and >350 million of them have chronic HBV infection [1]. In China, a seroepidemiological survey of HBV infection in 2006 showed that the prevalence of hepatitis B surface antigen positivity was 7.18%. It was estimated that 93 million people were HBV carriers, of whom 30 million were patients with chronic hepatitis B (CHB) [2]. CHB may progress to cirrhosis and hepatocellular carcinoma. An accurate method for monitoring the progression of liver fibrosis is urgently needed for the prognosis and management of chronic liver diseases. Liver biopsy is generally considered as the gold standard for assessing hepatic histology in CHB [3–5]. However, it often has limitations due to its invasiveness, risk of complications, sampling errors, and interobserver variability [6–8]. Many noninvasive methods for replacing or complementing the liver biopsy have been developed in recent years [9–12]. FibroScan (transient elastography) and FibroTest (serological marker test) have been evaluated most frequently; these methods have similar diagnostic accuracies for predicting fibrosis staging from receiver-operating characteristic (ROC) curves [13–16]. FibroTest employs a narrow and complex algorithm for 5 biochemical markers (α2-macroglobulin, apolipoprotein A1, haptoglobin, γ-glutamyl transferase, and bilirubin), which requires extensive and specialized blood analysis [17]. Recently, we developed a novel diagnostic score named LecT-Hepa for convenient and rapid monitoring of liver fibrosis progression. It is based on glyco-alteration (e.g., fucosylation and desialylation) of serum α1-acid glycoprotein

Abbreviations: HBV, hepatitis B virus; CHB, chronic hepatitis B; PLT, platelet count; AGP, α1-acid glycoprotein; LSM, liver stiffness measurement; LC, liver cirrhosis; non-LC, non-cirrhosis; DSA, *Datura stramonium* agglutinin; MAL, *Maackia amurensis* lectin; AOL, *Aspergillus oryzae* lectin.

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(AGP), which is assessed using a triplex lectin–antibody immunoassay [18,19]. It has been demonstrated to be well correlated with the fibrosis stage determined by liver biopsy, and verified to be more efficient by comparing with other serological methods (hyaluronic acid, tissue inhibitor of metalloproteases-1, platelet count, APRI, Forns index, Fib-4 index, and Zeng's score) in a multicenter study [20]. Here, to evaluate the reliability of LecT-Hepa for assessing liver fibrosis, we compared the diagnostic performance of LecT-Hepa and FibroScan for distinguishing cirrhosis from non-cirrhosis in a large cohort of HBV-infected Chinese patients with different serum alanine aminotransferase (ALT) levels.

2. Materials and methods

2.1. Patients

A total of 239 patients who had been positive for hepatitis B surface antigen for at least 6 months were enrolled retrospectively from Ruijin Hospital (Shanghai, China) from March 2009 to May 2011. Patients who were coinfected with another hepatitis virus or HIV, or who had excessive alcohol intake (>20 g/d), hepatocellular carcinoma, or other causes of liver diseases were excluded. For all patients, serum biochemical parameters, including the levels of aspartate aminotransferase (AST) and ALT, as well as platelet (PLT), were assessed at the time of the liver stiffness measurement. Normal values for ALT and AST ranged between 10 and 64 IU/l and between 8 and 40 IU/l, respectively, which were determined based on the manufacturer's instructions and adjusted according to the results of validation test by medical laboratory of Ruijin Hospital. Serum samples were collected at the time of the liver stiffness measurement for detection of lectins and stored at −20 °C until analysis. The patients were divided into two groups: liver cirrhosis (LC) group and non-cirrhosis (non-LC) group. The diagnosis of cirrhosis was based on clinical and morphological criteria and ultrasonography according to standard definitions [21]. The institutional ethics committees of Ruijin Hospital of Shanghai Jiao Tong University approved this study, and the informed consent was obtained from all patients.

2.2. Liver stiffness measurement

Liver stiffness was measured by transient elastography using FibroScan (EchoSens, Paris, France). The measurement depth was between 25 mm and 65 mm. For each patient, 10 validated measurements were performed. The success rate was calculated as the number of validated measurements divided by the total number of measurements. The results were expressed in kilopascals. The median value was considered representative of the elastic modulus of the liver. Only procedures with 10 validated measurements and a success rate of at least 60% were considered reliable.

2.3. Automatic acquisition of quantitative glyco-alteration of AGP

Each individual serum (5 μl) that had been stored at −20 °C was diluted 10 fold with phosphate buffered saline containing 0.2% sodium dodecyl sulfate, and then heated at 95 °C for 20 min. AGP in the resulting solution was enriched by immunoprecipitation with biotinylated anti-AGP antibody using an automated protein purification system (ED-01; GP BioSciences Ltd., Tokyo, Japan). Each elution fraction (100 μl) was kept at −80 °C until a sandwich immunoassay was performed. Subsequent to the enrichment, fibrosis-specific glyco-alteration of AGP was quantified using simultaneous lectin–antibody sandwich immunoassays for three lectins: *Datura stramonium* agglutinin (DSA), *Maackia amurensis* lectin (MAL), and *Aspergillus oryzae* lectin (AOL), by a fully automatic chemiluminescence enzyme immunoassay system (HISCL-2000i; Sysmex Co., Kobe, Japan). The criterion formula of LecT-Hepa was as before described [19]:

$$\text{LecT-Hepa} = \text{Log}_{10}[\text{AOL/DSA}] \times 8.6 - [\text{MAL/DSA}].$$

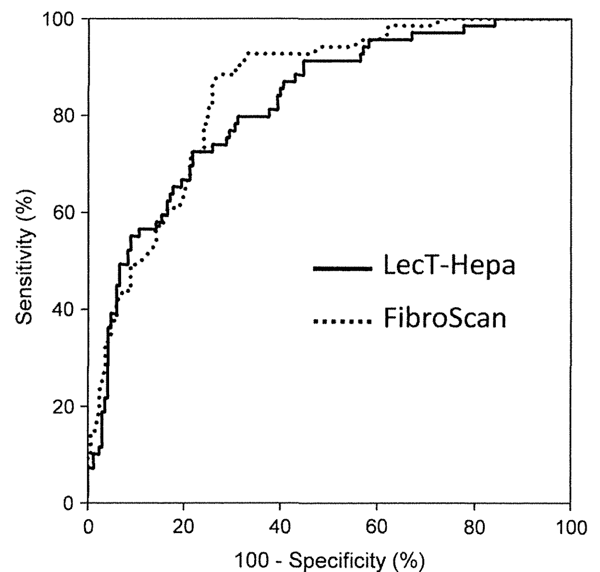
Table 1
Clinical characteristics of the patients.

Data	non-LC (n=170)	LC (n=69)	Significance non-LC vs LC
Age (y)	38.5±11.0	47.94±9.0	P<0.0001
Male sex (%)	126 (74.1%)	51 (73.9%)	–
AST (IU/l)	70.5±150.1	88.4±109.8	P=0.0002
ALT (IU/l)	111.6±213.7	88.5±116.1	P=0.1965
PLT ($\times 10^9$/l)	167.5±43.9	86.0±48.0	P<0.0001
FibroScan	10.3±8.8	27.0±19.1	P<0.0001
MAL/DSA	10.1±2.0	7.5±2.3	P<0.0001
AOL/DSA	5.1±13.5	24.0±47.6	P<0.0001

Patients were classified as non-LC or LC. LC, liver cirrhosis; non-LC, non-cirrhosis. Quantitative results are expressed as means±standard deviations or n (%).

2.4. Statistical analysis

Statistical calculations were performed using software from GraphPad Prism 5 (GraphPad, San Diego, CA). A P value of <0.01 (1%) was considered to be statistically significant. The diagnostic performance of the fibrosis markers and indices were assessed using ROC curves and were then expressed as diagnostic specificity, sensitivity,



	FibroScan	LecT-Hepa
AUC	0.85	0.82
(95% CI)	(0.797-0.897)	(0.763-0.877)
Se (%)	87.0	72.5
Sp (%)	74.1	78.2
PPV (%)	57.7	57.5
NPV (%)	93.3	87.5

Fig. 1. Receiver-operating characteristic curves of LecT-Hepa and FibroScan for distinguishing LC from non-LC. AUC, area under the receiver-operating characteristic curve; Se, sensitivity; Sp, specificity; PPV, positive predictive value; NPV, negative predictive value.

positive predictive value (PPV), negative predictive value (NPV) and area under the ROC curve (AUC) values (95% confidence interval [95% CI]).

3. Results

3.1. General characteristics

A total of 239 patients who showed evidence of chronic HBV infection and had undergone liver stiffness measurement were investigated. The mean age was 41.2 ± 11.3 y, and 177 (74%) of them were males. Among the all, 170 (71%) and 69 (29%) patients were diagnosed as non-LC and LC, respectively. Their characteristics are summarized in Table 1. Significant differences were found in Age ($P<0.0001$), AST ($P=0.0002$), PLT ($P<0.0001$), FiborScan ($P<0.0001$), MAL/DSA ($P<0.0001$), and AOL/DSA ($P<0.0001$) between the non-LC group and LC group, whereas ALT ($P=0.1965$) was not significantly different between the two groups.

3.2. Receiver-operating characteristic analysis

The overall diagnosis performances of LecT-Hepa and FibroScan were assessed using ROC curves. Fig. 1 shows the ROC curves for distinguishing LC from non-LC by both methods. The area under the ROC curve (95% CI) was 0.82 (0.763–0.877) for LecT-Hepa and 0.85 (0.797–0.897) for FibroScan. The overall diagnostic accuracies for LecT-Hepa and FibroScan were 77% and 78%, respectively. The obtained values for sensitivity, specificity, PPV, and NPV are shown in the bottom table of Fig. 1. There was no significant difference between both methods.

3.3. Effect of hepatic inflammation on the diagnostic cutoff values

Because the upper limit of the normal value for ALT level was 64 IU/l, the patients were categorized by the normal (≤64 IU/l) and elevated (>64 IU/l) ALT levels. According to this classification, 169 patients (71%) had the normal ALT level and 70 patients (29%) had the elevated ALT level. The proportions of patients with LC in the normal and elevated ALT levels were similar (28% of normal ALT patients and 30% of elevated ALT patients). Distribution of the values obtained by each test is shown in Fig. 2. Medians of these methods increased significantly between the non-LC group and LC group (all $P<0.0001$) in the both ALT levels. LecT-Hepa values in the non-LC group ($P=0.65$) and LC group ($P=0.02$) showed no significant difference between the two ALT categories (Fig. 2A). In contrast, the FibroScan value was obviously increased with the elevation of ALT levels ($P<0.0001$) even in the same diagnostic group (Fig. 2B). Thereby, we could distinguish the LC group in the normal ALT level from non-LC group in the elevated ALT level ($P<0.0001$) by LecT-Hepa, but could not by FibroScan ($P=0.05$). Collectively, the value of FibroScan was greatly affected by the ALT levels, whereas the value for LecT-Hepa was not influenced regardless of the ALT levels.

4. Discussion

This is the first study comparing LecT-Hepa with FibroScan. These results showed the obvious advantage of LecT-Hepa in comparison with FibroScan based on robustness against fluctuation of the ALT levels with a large cohort of HBV-infected Chinese patients at different ALT levels. Thus, the diagnostic performance of LecT-Hepa was the most reliable for monitoring the progression of hepatic fibrosis.

A recent paper showed that the majority of nucleoside-naïve patients with CHB who were treated with entecavir in the long-term cohort achieved substantial histological improvement and regression of fibrosis or cirrhosis [22], suggesting that a noninvasive test for the assessment of liver fibrosis in the treated patients is required during the follow-up. The liver biopsy is limited not only by its invasive nature, but also by its accuracy. A specimen collected in a standard liver biopsy using a short, narrow-gauge needle represents a very small portion of the whole liver mass, resulting in intra- and interobserver variability and sampling errors, which account for 25% of false-negative diagnoses of cirrhosis [23–25]. Therefore, a noninvasive marker that accurately reflects the condition of the whole liver is required.

At present, FibroScan is the most intensively evaluated noninvasive method for the assessment of liver fibrosis. Its diagnostic value is considered to be superior to that of biochemical markers [26]. However, several studies noted that liver stiffness measurements using FibroScan for patients with inflammation and acute liver damage overestimate the actual stage of fibrosis and may reduce the diagnostic accuracy [27,28]. In general, a high ALT level reflects a vigorous immune response

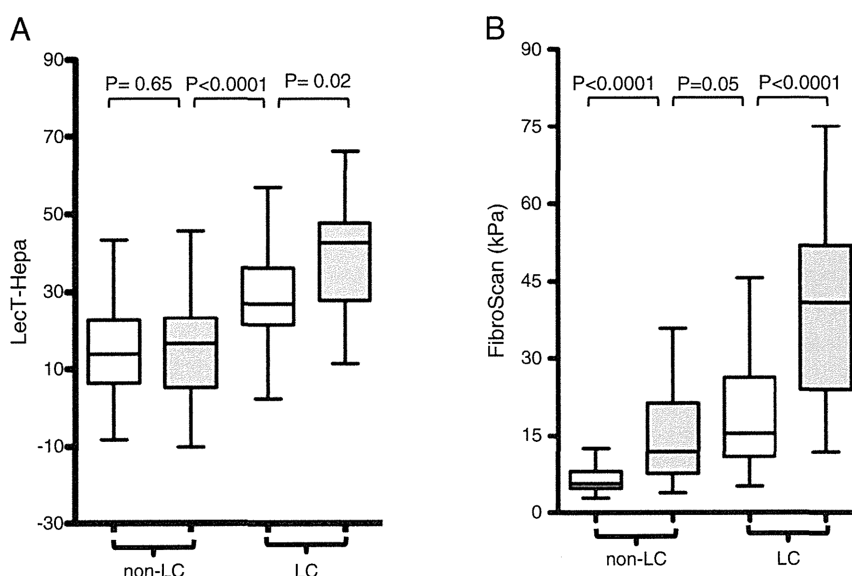


Fig. 2. Distribution of LecT-Hepa (A) and FibroScan (B) values in non-LC and LC patients with different ALT levels. The top and bottom of the whiskers are the 95th and 5th percentiles. The top and bottom of the boxes are the first and third quartiles. The size of the box represents the interquartile range within which 50% of the values are located. The line across the box indicates the median value. LC, liver cirrhosis; non-LC, non-cirrhosis. The open and gray boxes indicate normal (≤64 IU/l) and elevated (>64 IU/l) ALT levels, respectively.

to HBV and histological activity (i.e., necroinflammation). Our study obviously showed that the FibroScan values were substantially affected by ALT fluctuation. These results were also in accordance with the study of Kim et al., in which advanced fibrosis stage (F3-4) or cirrhosis showed a negative correlation with discordance between liver biopsy and FiborScan in assessing liver fibrosis in patients with CHB, and maximal activity grade 3–4 significantly influenced the liver stiffness measurement values in F3 and F4 [28]. In practice, hepatic activation and fibrosis stage should be estimated independently, as should histological diagnoses followed by a biopsy, such as the histological activity index scoring system. Thus, a marker that relies on an analysis of the specific protein content to monitor liver fibrosis should be robust against hepatic inflammation. In this context, we can explain that the reliability of LecT-Hepa is superior to that of FibroScan. LecT-Hepa has been already validated for estimating liver fibrosis using a large amount of serum specimens from patients with well-defined fibrosis stage by biopsy in a multicenter study [21]. This report led us to consider that LecT-Hepa can be a good substitute for liver biopsy. This is the reason we herein focused on the examination into the effect of hepatic inflammation on diagnosis of LC by LecT-Hepa.

In conclusion, we confirmed that LecT-Hepa is unaffected by inflammation. This suggested that LecT-Hepa is the most reliable and effective for the assessment of fibrosis progression in HBV-infected patients whose ALT levels are often flucturated and thus can be used for routine assessments of liver fibrosis in HBV-infected patients.

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Soluble MICA and a *MICA* Variation as Possible Prognostic Biomarkers for HBV-Induced Hepatocellular Carcinoma

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Abstract

MHC class I polypeptide-related chain A (MICA) molecule is induced in response to viral infection and various types of stress. We recently reported that a single nucleotide polymorphism (SNP) rs2596542 located in the *MICA* promoter region was significantly associated with the risk for hepatitis C virus (HCV)-induced hepatocellular carcinoma (HCC) and also with serum levels of soluble MICA (sMICA). In this study, we focused on the possible involvement of MICA in liver carcinogenesis related to hepatitis B virus (HBV) infection and examined correlation between the *MICA* polymorphism and the serum sMICA levels in HBV-induced HCC patients. The genetic association analysis revealed a nominal association with an SNP rs2596542; a G allele was considered to increase the risk of HBV-induced HCC ($P = 0.029$ with odds ratio of 1.19). We also found a significant elevation of sMICA in HBV-induced HCC cases. Moreover, a G allele of SNP rs2596542 was significantly associated with increased sMICA levels ($P = 0.009$). Interestingly, HCC patients with the high serum level of sMICA (>5 pg/ml) exhibited poorer prognosis than those with the low serum level of sMICA (≤5 pg/ml) ($P = 0.008$). Thus, our results highlight the importance of *MICA* genetic variations and the significance of sMICA as a predictive biomarker for HBV-induced HCC.

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Introduction

Hepatocellular carcinoma (HCC) reveals a very high mortality rate that is ranked the third among all cancers in the world [1]. HCC is known to develop in a multistep process which has been related to various risk factors such as genetic factors, environment toxins, alcohol and drug abuse, autoimmune disorders, elevated hepatic iron levels, obesity, and hepatotropic viral infections [2]. Among them, chronic infection with hepatitis B virus (HBV) is one of the major etiological factors for developing HCC with considerable regional variations ranging from 20% of HCC cases in Japan to 65% in China [3].

Interestingly, clinical outcome after the exposure to HBV considerably varies between individuals. The great majority of individuals infected with HBV spontaneously eliminate the viruses, but a subset of patients show the persistent chronic hepatitis B infection (CHB), and then progresses to liver cirrhosis and HCC through a complex interplay between multiple genetic and environmental factors [4]. In this regard, genome wide association studies (GWAS) using single nucleotide polymorphisms (SNPs) have highlighted the importance of genetic factors in the pathogenesis of various diseases including CHB as well as HBV-induced HCC [5,6,7,8,9,10,11,12,13]. Recently, we identified a genetic variant located at 4.7 kb upstream of the *MHC class I polypeptide-related chain A* (*MICA*) gene to be strongly associated with hepatitis C virus (HCV) -induced HCC development [14].

MICA is highly expressed on viral-infected cells or cancer cells, and acts as ligand for NKG2D to activate antitumor effects of Natural killer (NK) cells and $CD8^+$ T cells [15,16]. Our previous results indicated that a G allele of SNP rs2596542 was significantly associated with the lower cancer risk and the higher level of soluble MICA (sMICA) in the serum of HCV-induced HCC patients, demonstrating the possible role of MICA as a tumor suppressor. However, elevation of serum sMICA was shown to be associated with poor prognosis in various cancer patients [17,18,19,20].

Matrix metalloproteinases (MMPs) can cleave MICA at a transmembrane domain [21] and release sMICA proteins from cells. Since sMICA was shown to inhibit the antitumor effects of NK cells and CD8^{+} T cells by reduction of their affinity to binding to target cells [22,23], the effect of MICA in cancer cells would be modulated by the expression of MMPs. To elucidate the role of MICA in HBV-induced hepatocellular carcinogenesis, we here report analysis of the *MICA* polymorphism and serum sMICA level in HBV-induced HCC cases.

Materials and Methods

Study participants

The demographic details of study participants are summarized in Table 1. A total of 181 HCC cases, 597 CHB patients, and 4,549 non-HBV controls were obtained from BioBank Japan that was initiated in 2003 with the funding from the Ministry of Education, Culture, Sports, Science and Technology, Japan [24]. In the Biobank Japan Project, DNA and serum of patients with 47 diseases were collected through collaborating network of 66 hospitals throughout Japan. List of participating hospitals is shown in the following website (http://biobankjp.org/plan/member_hospital.html). A total of 226 HCC cases, 102 CHB patients, and 174 healthy controls were additionally obtained from the University of Tokyo. The diagnosis of chronic hepatitis B was conducted on the basis of HBsAg-seropositivity and elevated serum aminotransferase levels for more than six months according to the guideline for diagnosis and treatment of chronic hepatitis (The Japan Society of Hepatology, http://www.jsh.or.jp/medical/gudelines/index.html). Control Japanese DNA samples (n = 934) were obtained from Osaka-Midosuji Rotary Club, Osaka, Japan. All HCC patients were histopathologically diagnosed. Overall survival was defined as the time from blood sampling for sMICA test to the date of death due to HCC. Patients who were alive on the date of last follow-up were censored on that date. All participants provided written informed consent. This research project was approved by the ethics committee of the University of Tokyo and the ethics committee of RIKEN. All clinical assessments and specimen collections were conducted according to Declaration of Helsinki principles.

SNP genotyping

Genotyping platforms used in this study were shown in Table 1. We genotyped 181 HCC cases and 5,483 non-HBV control samples using either Illumina Human Hap610-Quad or Human Hap550v3. The other samples were genotyped at SNP rs2596542 by the Invader assay system (Third Wave Technologies, Madison, WI).

MICA variable number tandem repeat (VNTR) locus genotyping

Genotyping of the *MICA* VNTR locus in 176 HBV-induced HCC samples was performed using the primers reported previously by the method recommended by Applied Biosystems (Foster City, CA) [14]. Briefly, the 5′ end of forward primer was labeled with 6-FAM, and reverse primer was modified with GTGTCTT non-random sequence at the 5′ end to promote Plus A addition. The PCR products were mixed with Hi-Di Formamide and GeneScan-600 LIZ size standard, and separated by GeneScan system on a 3730×l DNA analyzer (Applied Biosystems, Foster City, CA). GeneMapper software (Applied Biosystems, Foster City, CA) was employed to assign the repeat fragment size (Figure S1).

Quantification of soluble MICA

We obtained serum samples of 111 HBV-positive HCC samples, 129 HCV-positive HCC samples, and 60 non-HBV controls from Biobank Japan. Soluble MICA levels were measured by sandwich enzyme-linked immunosorbent assay, as described in the manufacturer's instructions (R&D Systems, Minneapolis, MN).

Statistical analysis

The association between an SNP rs2596542 and HBV-induced HCC was tested by Cochran-Armitage trend test. The Odds ratios were calculated by considering a major allele as a reference. Statistical comparisons between genotypes and sMICA levels were performed by Kruskal-Wallis test (if more than two classes for comparison) or Wilcoxon rank test using R. Overall survival rate of the patients was analyzed by Kaplan-Meier method in combination with log-rank test with SPSS 20 software. The period for the survival analysis was calculated from the date of blood sampling to the recorded date of death or the last follow-up date. Differences with a P value of <0.05 were considered statistically significant.

Results

Association of SNP rs2596542 with HBV-induced HCC

In order to examine the effect of rs2596542 genotypes on the susceptibility to HBV-induced HCC, a total of 407 HCC cases and 5,657 healthy controls were genotyped. The Cochran Armitage trend test of the data revealed a nominal association

Table 1. Demographic details of subjects analyzed.

Subjects	Source	Genotyping platform	Number of Sample	Female (%)	Age (mean+/−sd)
Liver Cancer	BioBank Japan	Illumina Human Hap610-Quad	181	17.9	62.94±9.42
	University of Tokyo	Invader assay	226		
Control	BioBank Japan	Illumina Human Hap550v3	4549	47.95	55.19±12.5
	Osaka**	Illumina Human Hap550v3	934		
	University of Tokyo	Invader assay	174		
Chronic hepatitis B*	BioBank Japan	Invader assay	597	45.66	61.31±12.6
	University of Tokyo	Invader assay	102		

*Chronic hepatitis B patients without liver cirrhosis and liver cancer during enrollment.
**Healthy volunteers from Osaka Midosuji Rotary Club, Osaka, Japan.
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between HBV-induced HCC and rs2596542 in which a risk allele G was more frequent among HBV-induced HCC cases than an A allele (P = 0.029, OR = 1.19, 95% CI: 1.02–1.4; Table 2). To further investigate the effect of rs2596542 on the progression from CHB to HBV-induced HCC, we genotyped a total of 699 CHB cases without HCC. Although the progression risk from CHB to HBV-induced HCC was not statistically significant with rs2596542 (P = 0.197 by the Cochran Armitage trend test with an allelic OR = 1.3 (0.94–1.36); Table 2), we found a similar trend of association in which the frequency of a risk-allele G was higher among HBV-induced HCC patients than that of CHB subjects. Since we previously revealed that an A allele was associated with a higher risk of HCV-induced HCC with OR of 1.36 [14], the s2596542 alleles that increased the risk of HCC were opposite in HBV-induced HCC and HCV-induced HCC.

Soluble MICA levels are associated with SNP rs2596542

We subsequently performed measurement of soluble MICA (sMICA) in serum samples using the ELISA method in 176 HBV-positive HCC cases and 60 non-HBV controls. Nearly 30% of the HBV-induced HCC cases revealed the serum sMICA level of >5 pg/ml (defined as high) while the all control individuals except one showed that of ≤5 pg/ml (defined as low) ($P = 4.5 \times 10^{-6}$; Figure 1A). Then, we examined correlation between SNP rs2596542 genotypes and serum sMICA levels in HBV-positive HCC cases. Interestingly, rs2596542 genotypes were significantly associated with serum sMICA levels (P = 0.009; Figure 1B); 39% of individuals with the GG genotype and 20% of those with the AG genotype were classified as high for serum sMICA, but only 11% of those with the AA genotype were classified as high (AA+AG vs GG; P = 0.003) (Figure 1B). These findings were similar with our previous reports in which a G allele was associated with higher serum sMICA levels in HCV-induced HCC patients [14].

Negative association of variable number of tandem repeat (VNTR) with sMICA level

The *MICA* gene harbors a VNTR locus in exon 5 that consists of 4, 5, 6, or 9 repeats of GCT as well as a G nucleotide insertion into a five-repeat allele (referred as A4, A5, A6, A9, and A5.1, respectively). The insertion of G (A5.1) causes a premature translation termination and results in loss of a transmembrane domain, which may produce the shorter form of the MICA protein that is likely be secreted into serum [25]. However, the association of this VNTR locus with serum sMICA level was controversial among studies [14,26,27,28]. Therefore, we examined the association between the VNTR locus and sMICA level in HBV-induced HCC patients, and found no significant association (Figure S1 and S2), concordant with our previous report for HCV-induced HCC patients [14].

Soluble MICA levels are associated with survival of HCC patients

In order to evaluate the prognostic significance of serum sMICA levels in HCC patients, we performed survival analysis of HCC patients. A total of 111 HBV-infected HCC patients and 129 HCV-infected HCC patients were included in this analysis. The mean survival period for HBV- and HCV-infected patients with less than 5 pg/ml of serum sMICA were 67.1 months (95% CI: 61.1–73.1, n = 83), and 58.2 months (95% CI: 51.4–65.0, n = 85), respectively. On the other hand, for patients with more than 5 pg/ml of serum sMICA, the mean survival periods were 47.8 months (95% CI: 34.8–30.9, n = 28) for HBV-induced HCC patients and 59.5 months (95% CI: 51.9–67.1, n = 44) for HCV-induced HCC patients. The Kaplan-Maier analysis and log-rank test indicated that among HBV-induced HCC subjects, the patients in the high serum sMICA group showed a significantly shorter survival than those in the low serum sMICA (P = 0.008; Figure 2). In addition, we performed multi-variate analysis to test whether sMICA is an independent prognostic factor by including age and gender as covariates. The results revealed significant association of sMICA levels with overall survival (P = 0.017) but not with age and gender (Table S1).However, we found no association between the serum sMICA level and the overall survival in the HCV-induced HCC subjects (P = 0.414; Figure S3). Taken together, our findings imply the distinct roles of the *MICA* variation and sMICA between HBV- and HCV-induced hepatocellular carcinogenesis.

Vascular invasion in HBV-related HCC patients is associated with soluble MICA levels

Since sMICA levels were associated with the overall survival of HBV-related HCC patients, we tested whether sMICA levels affect survival through modulating invasive properties of tumors or size of the tumors. We tested the association between sMICA levels and vascular invasion in 35 HBV-related HCC cases, among whom 7 cases were positive and 21 cases were negative for vascular invasion. We found significant association between sMICA levels and vascular invasion (Figure 3; P = 0.014) in which 7 cases with positive vascular invasion showed high levels of sMICA (mean = 54 pg/ml) than 21 cases without vascular invasion (mean = 7.51 pg/ml). However, we found no association between tumor size and sMICA levels (P = 0.56; data not shown). These results suggest that sMICA may reduce the survival of HBV-related HCC patients by affecting the invasive properties of tumors.

Discussion

Several mechanisms such as HBV-genome integration into host chromosomal DNA [29] and effects of viral proteins including HBx [30] are shown to contribute to development and progression of HCC, while the immune cells such as NK and T cells function as key antiviral and antitumor effectors. MICA protein has been

Table 2. Association between HCC and rs2596542.

SNP	Comparison	Chr	Locus	Case MAF	Control MAF	P*	OR*	95% CI
rs2596542	HCC vs. Healthy control	6	*MICA*	0.294	0.332	0.029	1.19	1.02–1.4
rs2596542	HCC vs. CHB	6	*MICA*	0.294	0.320	0.197	1.13	0.94–1.36

Note: 407 HCC cases, 699 CHB subjects and 5,657 non-HBV controls were used in the analysis.
Chr.,chromosome; MAF, minor allele frequency; OR, odds ratio for minor allele; CI, confidence interval.
*Obtained by Armitage trend test.
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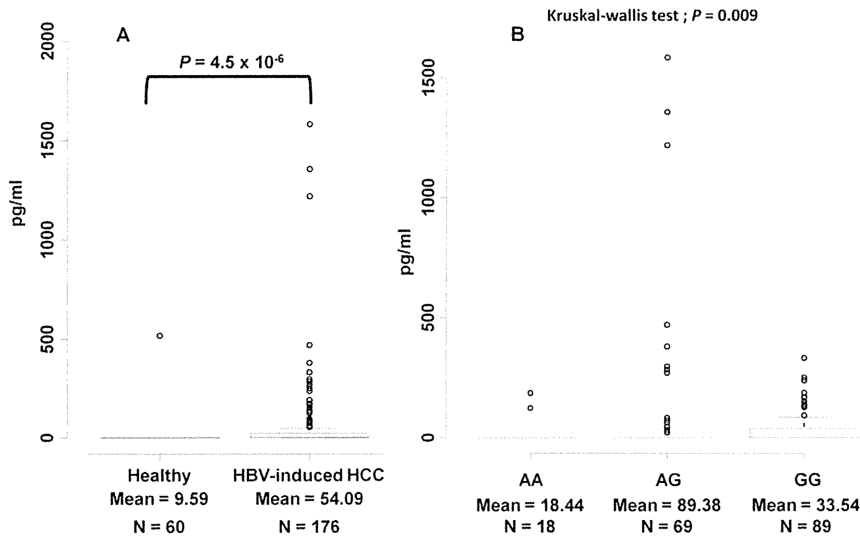

Figure 1. Soluble MICA levels are associated with HBV-related HCC. (A) Correlation between soluble MICA levels and HBV-induced HCC subjects. The y-axis displays the concentration of soluble MICA in pg/ml. The number of independent samples tested in each group is shown in the x-axis. Each group is shown as a box plot and the mean values are shown in the x-axis. The difference between two groups is tested by Wilcoxon rank test. The box plots are plotted using default settings in R. (B) Correlation between soluble MICA levels and rs2596542 genotype in HBV-positive HCC subjects. The x-axis shows the genotypes at rs2596542 and y-axis display the concentration of soluble MICA in pg/ml. Each group is shown as a box plot. $P = 0.027$ and 0.013 for AA vs. GG and AA vs. AG, respectively. The association between genotypes and sMICA levels was tested by Kruskal-wallis test, whereas the difference in the sMICA levels between AA and GG is tested by Wilcoxon rank test. The box plots are plotted using default settings in R.
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considered as a stress marker of gastrointestinal epithelial cells because of its induced expression by several external stimuli such as heat, DNA damage, and viral infections [31,32,33,34]. Here, we examined the association of rs2596542 and serum sMICA levels with HBV-induced HCC. Like in HCV-induced HCC [14], our results from ELISA revealed a significantly higher proportion

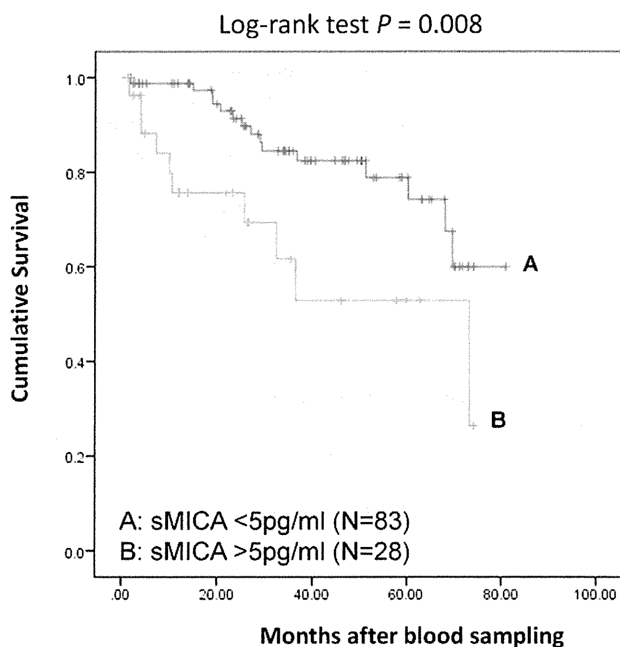

Figure 2. Kaplan-Meier curves of the patients with HBV-induced HCC. The patients were divided into two groups according to their sMICA concentration (high: >5 pg/ml and low: =<5 pg/ml). Statistical difference was analyzed by log-rank test. The y-axis shows the cumulative survival probability and x-axis display the months of the patients survival after blood sampling.
doi:10.1371/journal.pone.0044743.g002

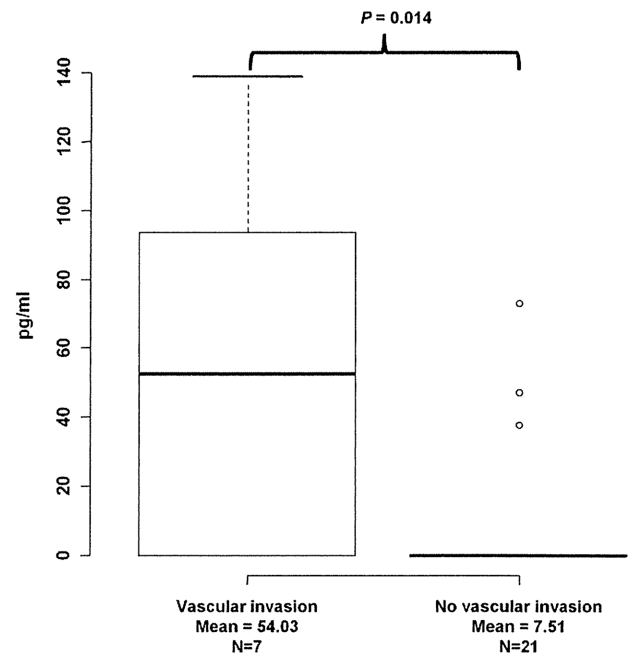

Figure 3. Correlation between soluble MICA levels and vascular invasion in HBV-induced HCC subjects. The y-axis displays the concentration of soluble MICA in pg/ml. The number of independent samples tested in each group is shown in the x-axis. Each group is shown as a box plot and the mean values are shown in the x-axis. The difference between two groups is tested by Wilcoxon rank test. The box plots are plotted using default settings in R.
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of high serum sMICA cases (nearly 30%) in the HBV-induced HCC group, compared to non-HBV individuals (1.7%). Moreover, the serum sMICA level was significantly associated with rs2596542, but not with the copy number differences of the VNTR locus, as concordant with our previous report [14].

Several studies have already indicated the roles of sMICA as prognostic markers for different types of malignant diseases [17,18,19,20]. Therefore, it is of medical importance to test whether serum sMICA levels can be used as a prognostic marker for patients with HCC. To our best knowledge, this is the first study to demonstrate the prognostic potential of sMICA for HBV-positive HCC patients; we found 19.3 months of improvement in survival among patients carrying less than 5 pg/ml of serum sMICA, compared to those having more than 5 pg/ml.

On the contrary, we found no significant correlation between sMICA levels and the prognosis of HCV-induced HCC cases. These opposite effects of *MICA* variation could be explained by the following mechanism. The individuals who carry the G allele would express high levels of membrane-bound MICA upon HCV infection and thus lead to the activation of immune cells against virus infected cells. On one hand, HBV infection results in increased expression of membrane-bound MICA as well as MMPs through viral protein HBx [35], which would result in the elevation of sMICA and the reduction of membrane-bound MICA. Since sMICA could block CD8+T cells, NK-CTL, and NK cells, higher sMICA would cause the inactivation of immune surveillance system against HBV infected cells. In other words, HBV may use this strategy to evade immune response and hence, higher levels of sMICA could be associated with lower survival rate among HBV-associated HCC. On the other hand, since HCV is not known to induce the cleavage of membrane bound MICA, individuals with low level membrane bound MICA expression (carriers of rs2596542-allele A) could be inherently susceptible for HCV-induced HCC. Thus, HBx-medicated induction of MMPs could partially explain the intriguing contradictory effect of MICA between HBV-induced HCC and HCV-induced HCC. Since we observed significant correlation of sMICA levels with vascular invasion, it may be the case that high levels of sMICA cause poor prognosis of HBV-related HCC cases by making tumors more aggressive and invasive. However it is important in future to determine the ratio of membrane-bound MICA to sMICA in case of HCV- and HBV-related HCC.

Interestingly, the immune therapy against melanoma patients induced the production of auto-antibodies against MICA [36]. Anti-MICA antibodies would exert antitumor effects through antibody-dependent cellular cytotoxicity against cells expressing membrane-bound MICA and/or activation of NK cells by inhibiting the sMICA-NKG2D interaction. However, further studies are necessary, using well-defined HBV-related HCC cohort, to investigate whether sMICA levels could be included as an additional factor to predict the survival rate among HBV-related HCC subjects. Taken together, our results indicate the potential of *MICA* variant and sMICA as prognostic biomarkers. Thus, MICA could be a useful therapeutic target for HBV-induced HCC.

Supporting Information

Figure S1 MICA repeat genotyping using capillary-based method. The alleles are annotated using GeneMapper software based on the size of the PCR product (185 bp = A4 allele, 188 bp = A5, 189 bp = A5.1, 191 bp = A6 and 200 bp = A9). The inset at the base of each peak shows the size of the PCR product with corresponding allele call by the software. The figure display all observed heterozygotes at A5.1 allele.
(TIF)

Figure S2 MICA VNTR alleles are not associated with soluble MICA levels. Each group is shown as a box plot. The difference in the sMICA values among each group is tested by Wilcoxon rank test. The box plots are plotted using default settings in R.
(TIF)

Figure S3 Kaplan-Meier curves of the patients with HCV-induced HCC. The patients were divided into two groups according to their sMICA concentration (<5 pg/ml or >5 pg/ml). Statistical difference was analyzed by log-rank test. The y-axis shows the cumulative survival probability and x-axis display the months of the patients survival after blood sampling.
(TIF)

Table S1 Clinical parameters of HBV-related HCC patients available for prognostic analyses.
(XLS)

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Author Contributions

Conceived and designed the experiments: VK KM YN. Performed the experiments: VK PHL YU HM ZD. Analyzed the data: VK PHL CT RM. Contributed reagents/materials/analysis tools: YN NK AT MK HS KT YT MS MM RT MO KK NK. Wrote the paper: VK PHL KM YN.

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Hepatology Research

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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

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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

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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/CDGBD	+	–	†	†	†	†	–	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.

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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.

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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

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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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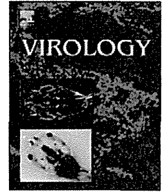

Interferon-α suppresses hepatitis B virus enhancer II activity via the protein kinase C pathway

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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).

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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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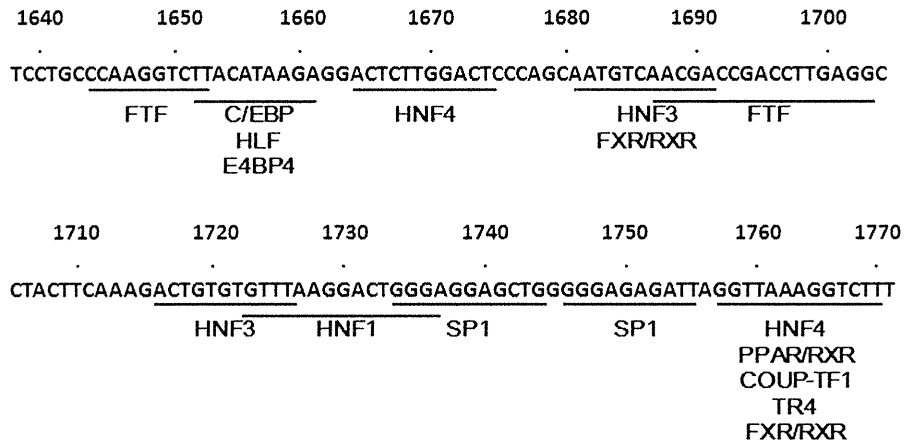

Fig. 1. Nucleotide sequences of the HBV En II region. The HBV sequence used in this study was of the *adw2* subtype (GenBank accession no. X02763). Numbering of the HBV sequence started at the unique *Eco*RI site. The underlined sequences represent the transcription factor binding sites mentioned in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