wound healing in the presence of TNF-α in vitro (Figure 1*C*). As shown in Figure 1*D* and 1E, RIPK2 mRNA and protein expression were efficiently decreased in HepG2 cells transfected with RIPK2 siRNA (siRIPK2), but not control (siC). RIPK2 silencing reduced hepatic wound closure 1.8-fold, which was associated with a 2-fold decrease in IL-6 production, known to be an important cytokine for the regeneration of liver [11], and a 2.3-fold decrease in interleukin 8 production (Figure 1*F–H*). Importantly, RIPK2 silencing did not affect cell viability (data not shown).

Given that HBeAg downregulates RIPK2 expression (Figure 1*A* and 1*B*), we examined whether HBeAg has an effect on hepatic wound healing in the presence of TNF-α (Figure 1*I*). As expected, we observed that both cell migration

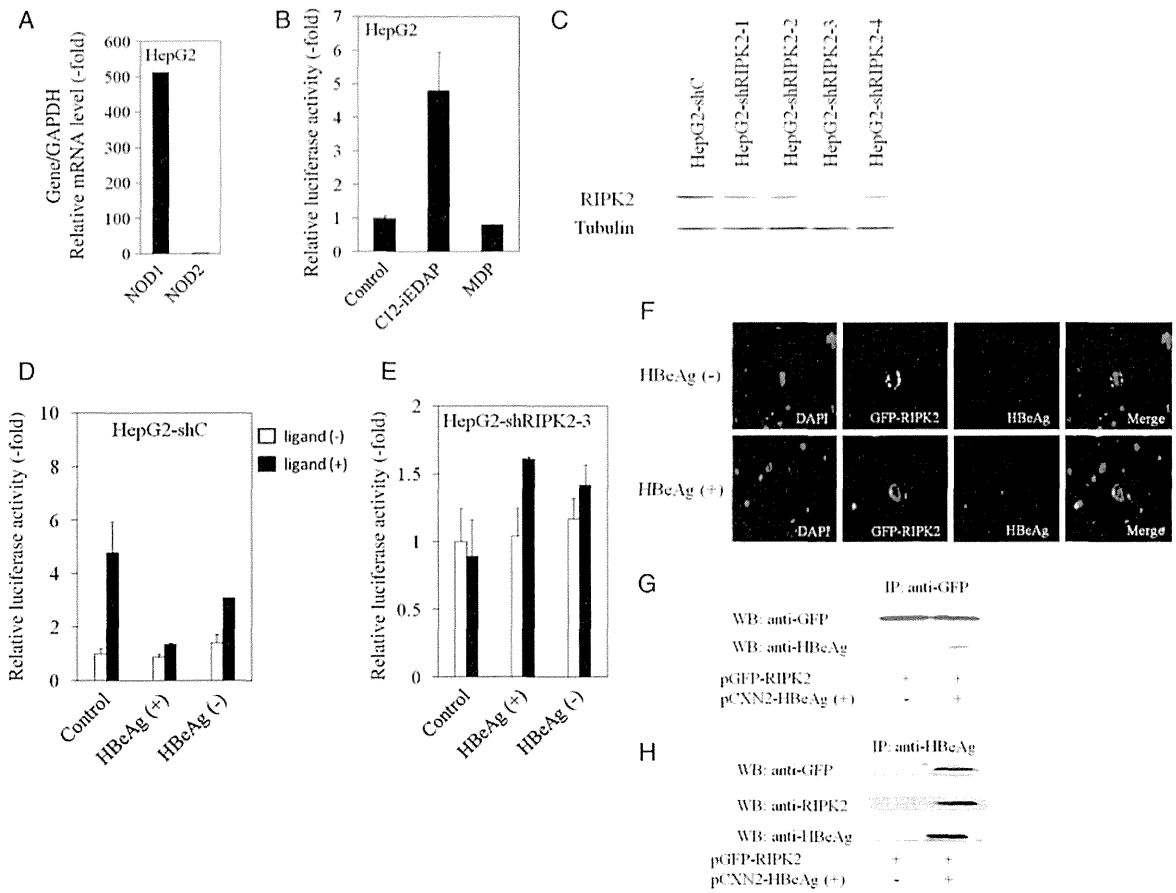

Figure 2. The nucleotide-binding oligomerization domain–containing protein 1 (NOD1) ligand C12-iEDAP induces NF-κB activation, knockdown of receptor-interacting serine/threonine protein kinase 2 (RIPK2) inhibits NOD1 ligand–induced NF-κB activation in HepG2 cells, and hepatitis B virus e antigen (HBeAg) interacts with RIPK2. *A*, Real-time reverse transcription–polymerase chain reaction analysis of NOD1 and NOD2 messenger RNA expression in HepG2. NOD1 and NOD2 expression levels were normalized to GAPDH expression levels. *B*, NF-κB–driven luciferase activity in HepG2 cells stimulated with the NOD1 ligand C12-iEDAP or the NOD2 ligand muramyl dipeptide (MDP) in HepG2. *C*, Western blot analysis of RIPK2 and tubulin expression in HepG2 cells stably transfected with control small hairpin RNA (shRNA; HepG2-shC) or with RIPK2 shRNA (HepG2–shRIPK2-1/2-4) expressing plasmids. *D* and *E*, HepG2-shC (*D*) and HepG2–shRIPK2-3 (*E*) cell lines were transiently transfected with pCXN2, pCXN2-HBeAg(+), or pCXN2-HBeAg(−) plasmids together with pNF-κB–luc. Cells were treated for 4 hours, with or without NOD1 ligand C12-iEDAP (2.5 μg/mL), and luciferase activity was determined. Primers specific for NOD1 (sense primer: 5′-ACTACCTCAAGCTGACCTAC-3′; antisense primer: 5′-CTGGTTTACGCTGAGTCTG-3′), for NOD2 (*sense primer*: 5′-CCTTGCATGCAGGCAGAAC-3′; *antisense primer*: 5′-TCTGTTGCCCCAGAATCCC-3′), and for other genes as described previously were purchased from Sigma [4]. *F*, HBeAg specifically colocalizes with RIPK2. COS7 cells were transiently cotransfected with 0.1 μg pCXN2-HBeAg(+) or pCXN2-HBeAg(−) together with 0.3 μg pGFP–human RIPK2. HBeAg was revealed with anti-HBeAg primary antibody and Alexa-Fluor-548 secondary antibody. *G* and *H*, HEK293T cells were transiently transfected with or without GFP-RIPK2 and HBeAg-expressing plasmids. Cellular extracts were precleared with protein G–Sepharose, and interacting complexes were immunoprecipitated (IP) with either anti-GFP (*G*) or anti-HBeAg (*H*) antibodies. Immunocomplexes were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis, and proteins were visualized by immunoblotting (WB) with indicated antibodies. Results are representative of 3 independent experiments.

and RIPK2 mRNA expression were reduced in HBeAg-positive HepG2 cells, compared with HBeAg-negative cells (1.5-fold decrease; Figure 1*J* and 1*K*). These results suggest that HBeAg impairs hepatic cell migration–dependent RIPK2 expression. Among NF-κB–targeting genes, expression of vimentin mRNA was impaired in HepG2-shRIP2 and in HBeAg-positive HepG2 (data not shown), and vimentin might be one of the candidates for impairment of their migrations [12].

RIPK2 Plays an Important Role in NF-κB Activation Induced by NOD1 Ligand, and HBeAg Blocks This Pathway

HepG2 cells express NOD1 but not NOD2 at the mRNA level (Figure 2*A*). In agreement with this finding, NF-κB was activated in HepG2 cells exposed to NOD1 ligand C12-iEDAP (level of activation, 4.8-fold, compared with untreated control) but not in those exposed to NOD2 ligand MDP (Figure 2*B*). As for Huh7 cells, activation of NF-κB was not detected following exposure to C12-iEDAP or MDP (data not shown). These results suggest that C12-iEDAP triggered NF-κB activation through NOD1 in HepG2 cells, which is consistent with findings from a previous study [9].

We examined whether knockdown of RIPK2 has an effect on NOD1-induced NF-κB activation in HepG2 cells. First, we established HepG2 cell lines that constitutively expressed RIPK2-shRNA (HepG2-shRIPK2-1/2-4) or control-shRNA (HepG2-shC) (Figure 2*C*). The HepG2–shRIPK2-3 cell line, which expresses the lowest levels of RIPK2, and the HepG2-shC cell line were treated for 4 hours, with or without C12-iE-DAP, before measurement by the NF-κB–driven luciferase assay (Figure 2*D* and 2*E*). C12-iEDAP triggered NF-κB activation in HepG2-shC (Figure 2*D*) but not in HepG2–shRIPK2-3 (Figure 2*E*), indicating that RIPK2 plays an important role in NF-κB activation induced through NOD1 triggering.

To assess the influence of HBeAg in that pathway, we measured NOD1-mediated NF-κB activity in HepG2-shC and HepG2–shRIPK2-3 cell lines transiently transfected with HBeAg-expressing plasmids. As shown in Figure 2*D*, HBeAg expression in HepG2-shC abolished C12-iEDAP–induced NF-κB activation.

HBeAg Interacts With RIPK2 and Colocalizes With RIPK2

RIPK2 mediates activation of transcription factors, such as NF-κB, following its activation, which is initiated by membrane-bound or intracytosolic receptors, such as TLR, NOD1, and NOD2 [7, 13, 14]. Confocal microscopy analysis of cells transfected with GFP-RIPK2 revealed subcellular localization of RIPK2 (data not shown). To compare the localization of RIPK2 with that of HBeAg, cells were cotransfected with pGFP–human RIPK2 with pCXN2-HBeAg(+) or pCXN2-HBeAg(−). After 48 hours, cells were stained with mouse monoclonal anti-HBe antibody. Confocal microscopy suggested subcellular colocalization of RIPK2 with HBeAg (Figure 2*F*). Reinforcing this assumption, GFP-RIPK2 coimmunoprecipitated with HBeAg (Figure 2*G*), while HBeAg coimmunoprecipitated with RIPK2 (Figure 2*H*) in transiently transfected cells with RIPK2- and HBeAg-expressing plasmids.

DISCUSSION

In the present study, we have shown the expression of NOD1 and NOD1 ligand–induced NF-κB activation in HepG2 cells and that RIPK2 plays an important role in NOD1 ligand–induced NF-κB activation. NF-κB activation plays an essential role in the production of inflammatory cytokines such as IL-6, which HBeAg could suppress in hepatocytes [4]. We have also shown that HBeAg inhibits RIPK2 expression and interacts with RIPK2, which may represent 2 mechanisms through which HBeAg blocks NOD1 ligand–induced NF-κB activation, thus contributing to the pathogenesis of chronic HBV infection and establishing viral persistence, although further studies including clinical situations might be needed.

HBeAg can be secreted by hepatocytes. Yet, it has been reported that as much as 80% of the precore protein p22 remains localized to the cytoplasm rather than undergoing further cleavage that allows its secretion as mature HBeAg [15]. Our present study showed subcellular colocalization of HBeAg with RIPK2 (Figure 2*F*). In addition to HBeAg protein in cell culture medium, we observed similar inhibition of NF-κB activation (data not shown).

Overall, we provided a novel molecular mechanism whereby HBeAg modulates innate immune signal-transduction pathways through RIPK2. Elsewhere, it was also reported that HBeAg impairs cytotoxic T-lymphocyte activity [2]. HBeAg inhibits RIPK2 expression and interacts with RIPK2, decreasing NF-κB activation and inflammatory cytokine production in hepatocytes. Taken together, HBeAg could impair both innate and adaptive immune responses to promote chronic HBV infection.

Notes

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Potential conflicts of interest. All authors: No reported conflicts.

All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest. Conflicts that the editors consider relevant to the content of the manuscript have been disclosed.

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Reactivation of Hepatitis B Virus in Patients With Undetectable HBsAg Undergoing Chemotherapy for Malignant Lymphoma or Multiple Myeloma

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Despite increasing reports of hepatitis B virus (HBV) reactivation in hematological malignancies, its incidence, and risk factors are still obscure. The aim of this study was to clarify the frequency and risk factors of HBV reactivation in hepatitis B surface antigen (HBsAg) undetectable patients with malignant lymphoma or multiple myeloma, during or after chemotherapy. A total of 109 patients with undetectable HBsAg undergoing chemotherapy for malignant lymphoma or multiple myeloma were enrolled in this study. Anti-hepatitis B surface (anti-HBs) and anti-hepatitis B core (anti-HBc) were checked before treatment, and HBV DNA in sera was quantified monthly during and after chemotherapy. Out of 109 patients, 42 (38.5%) had anti-HBs and 59 (54.1%) had anti-HBc. Among the 59 anti-HBc positive patients, four patients (4/59, 6.8%) showed HBV reactivation during 20.5 median follow-up months. In all four patients with HBV reactivation, peripheral lymphocyte counts before chemotherapy were lower than those without HBV reactivation ($P = 0.033$). HBV reactivation occurred during and after chemotherapy containing rituximab for non-Hodgkin lymphoma. Four patients, who had HBV reactivation, did not develop de novo hepatitis due to HBV reactivation and were able to undergo chemotherapy against malignant lymphoma as scheduled. Monitoring of HBV DNA in sera is useful for the early diagnosis of HBV reactivation, and preemptive therapy is an useful alternative to prevent hepatitis due to HBV reactivation. Patients must be monitored periodically for HBV-DNA levels during and after chemotherapy. ***J. Med. Virol. 85:1900–1906, 2013.*** © 2013 Wiley Periodicals, Inc.

KEY WORDS: reactivation; hepatitis B virus; chemotherapy; lymphocyte

INTRODUCTION

Reactivation of the hepatitis B virus (HBV) is a well-recognized complication following systemic chemotherapy for hematological malignancies [Francisci et al., 2010; Yagci et al., 2010; Sugauchi et al., 2011]. HBV infection has a wide clinical spectrum. Therefore different serologic markers or combinations of markers are used to identify different phases of HBV infection and to determine whether a patient has acute or chronic HBV infection, is immune to HBV as a result of prior infection or vaccination, or is susceptible. During acute or chronic hepatitis B infection, hepatitis B surface antigen (HBsAg) can be detected in high levels in serum. The presence of hepatitis B surface antibody (anti-HBs) is generally interpreted as an indication of recovery and immunity from HBV infection. Anti-HBs also develops in a person who has been successfully vaccinated against hepatitis B. Total hepatitis B core antibody (anti-HBc) appears at the onset of symptoms in acute hepatitis B and persists for life. The presence of anti-HBc indicates previous or ongoing infection with HBV in an undefined time frame. Therefore, in the past, anti-HBc and/or anti-HBs positive patients

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without HBsAg was thought to be eradicated of HBV in the host after resolution of HBV infection. However, recently it has become known that a low level of virus replication continues in hepatocytes even after the end of the acute HBV infection [Yotsuyanagi et al., 1998]. The covalently closed circular DNA (cccDNA) persist for many years in the liver of patients, HBsAg and HBV DNA in the blood has fallen to an undetectable level. Immunosuppressive drugs interfere with the cellular and humoral arm of the immune system controlling hepatocellular HBV infection. In healthy patients, host immunity is able to control HBV in most of the cases. This changes in an immune impaired patient, whether it is due to immunosuppressive drugs or monoclonal antibodies like rituximab. The humoral arm is profoundly affected by rituximab which depletes the body's antibody producing B cells. Other immunosuppressive drugs also impair the innate immune response. Therefore, reactivation of HBV following systemic chemotherapy can develop not only in HBsAg positive patients with a sustained HBV infection, but also in HBsAg undetectable patients with a past history of HBV infection [Wu et al., 2009; Cheung et al., 2010; Matsue et al., 2010; Wursthorn et al., 2010]. Rituximab and corticosteroids are especially well known to cause reactivation of HBV in patients without HBsAg in sera [Kusumoto et al., 2011]. Rituximab is a human monoclonal antibody derived from chimeric mice that inhibits an immune response by attacking CD20 positive B cells [Hiddemann et al., 2005]. Corticosteroids block cytokine synthesis and act as immune-suppressing drugs [Auphan et al., 1995] and, in addition, stimulate HBV DNA, mRNA, and protein production in a stable expression system [Tur-Kaspa and Laub, 1990]. These agents are used for the treatment of malignant lymphoma or other hematological malignancies. Multiple myeloma is recognized as a disease with a risk of HBV reactivation, due to its treatment regime with a high dose of corticosteroids [Yoshida et al., 2010]. To date, there has been increasing reports of HBV reactivation in patients treated with chemo/immunosuppressive therapy including the agents noted above [Hui et al., 2006; Matsubara et al., 2009; Shinkai et al., 2010]. However, the incidence or risk factors of HBV reactivation remain unclear because only a few prospective cohorts have presented for this new clinical entity. This study aimed to assess the incidence and risk factors of HBV reactivation, and analyzing the clinical course of HBV reactivation that occurred in the patients with malignant lymphoma or multiple myeloma during and after treatment.

PATIENTS AND METHODS

Study Patients

Consecutive patients with undetectable HBsAg who received chemotherapy for malignant lymphoma or multiple myeloma from January 2007 to October 2010 were included in this study. After admission, all patients underwent a physical examination and blood chemistry analysis. The study patients consisted of 109 patients (60 male, 55%; 49 female, 45%). The median age was 68-years-old, with a range of 22–91 years. Ninety-six patients (88.1%) had malignant lymphoma and 13 (11.9%) had multiple myeloma. Diagnosis of subtypes in malignant lymphoma included Diffuse large B-cell lymphoma (n = 54, 56.3%), Follicular lymphoma (n = 22, 22.9%), Marginal zone B-cell lymphoma (n = 7, 7.3%), Mantle cell lymphoma (n = 2, 2.1%), Burkitt lymphoma (n = 2, 2.1%), Intravascular large B-cell lymphoma (n = 1, 1.0%), Lymphoplasmacytoid lymphoma (n = 1, 1.0%), Peripheral T-Cell lymphoma (n = 1, 1.0%), Angioimmunoblastic T-Cell Lymphoma (n = 1,1.0%), and Hodgkin lymphoma (n = 5, 5.2%).

Determination of HBV Serological Markers and HBV DNA Quantification

On admission, all patients were screened for HBsAg in sera using a commercially available kit (Architect, Abbott Japan, Tokyo, Japan). Patients with undetectable HBsAg were enrolled in this study. Before treatment of hematological malignancies, patients were tested for anti-HBc, anti-HBs and blood parameters, and were then followed up by monthly monitoring of HBV DNA loads in sera or plasma and blood parameters. All serial sera were stored at −40° C. HBV DNA levels were quantified using Amplicor (range from below 2.6 to 7.6 log copies/ml; Roche Diagnostics, Tokyo, Japan) up to December 2007 and real-time TaqMan PCR (range from below 1.8 to 8.8 log copies/ml; Roche Diagnostics) since then. HBsAg with a highly sensitive chemiluminescent enzyme immunoassay (CLEIA) [Shinkai et al., 2010] was checked in stored sera sampled from patients with HBV reactivation retrospectively.

Definition of HBV Related Hepatitis and HBV Reactivation

Hepatitis was defined as a serum level of alanine aminotransferase (ALT) threefold higher than the normal upper limit of two consecutive determinations, 5 days apart, in the absence of the clinical and laboratory features of acute hepatitis A, hepatitis C, hepatitis E, or other systematic infections [Matsue et al., 2010]. The definition of HBV reactivation was the detection of HBV DNA in sera, including when the DNA load was not quantifiable but a PCR signal was detectable.

Statistical Analysis

In order to assess the risk factors of HBV reactivation, Fisher's exact test was applied for categorical variables, and Mann–Whitney's *U*-test was used for numerical variables. Receiver operating characteristic (ROC) curve was constructed to evaluate the

TABLE I. Clinical Characteristics of HBsAg Undetectable Patients Undergoing Chemotherapy for ML or Multiple Myeloma

Sex (M/F)	60/49
Age of years, median (range)	67.9 (22–91)
Follow-up period, median (range)	20.5 months (1.0–58.5)
Anti-HBc positive	59
Anti-HBs positive	42
Diagnosis	
Multiple myeloma	13
Diffuse large B cell lymphoma	54
Follicular lymphoma	22
Marginal zone B cell lymphoma	7
Burkitt lymphoma	2
T-cell lymphoma	2
Hodgkin lymphoma	5
Others	4
No. of rituximab administration	81
No. of glucocorticoids administration	108

diagnostic ability of HBV reactivation using a measured variable. A *P*-value less than 0.05 was considered significant. The best cutoff was defined as the point on the ROC curve closest to the upper left corner. All statistical analyses were performed using SPSS18 (IBM).

RESULTS

Patient Characteristics

The background characteristics of patients are shown in Table I. Out of 109 patients, 59 (54.1%) had anti-HBc, 42 (38.5%) had anti-HBs, and 47 (43.1%) had neither. Thirty-nine (35.7%) had both anti-HBc and anti-HBs. The number of patients with multiple myeloma were 13, and 96 patients had malignant lymphoma. Of all patients with malignant lymphoma, Hodgkin lymphoma was diagnosed in 5 patients, and non-Hodgkin lymphoma was confirmed in 91 patients. Diffuse large B-cell lymphoma was the dominant subtype of lymphoma. Rituximab was administered in 81 patients and glucocorticoids were used in 108 patients. None were treated by autologous peripheral blood stem cell transplantation or allogenic stem cell transplantation.

Consequences of HBV Serology

Among the 109 patients with undetectable HBsAg at the follow-up period of 20.5 median months (1.0–58.5), 4 (3.7%) showed the emergence of HBV DNA in sera, and were therefore diagnosed as HBV reactivation. They had never received a blood transfusion. The background characteristics and clinical features in patients with HBV reactivation are shown in Table II. None of the 50 patients without anti-HBc revealed HBV reactivation. In contrast, out of the 59 anti-HBc positive patients, 4 (6.8%) became positive for HBV DNA in sera. Among 20 anti-HBc positive and anti-HBs negative patients, 3 (15.0%) patients developed HBV reactivation, and only 1 of the 39 (2.6%) positive for both had an emergence of HBV DNA in sera. Sufficient anti-HBs antibodies in sera among HBV-resolved patients might reduce the incidence of HBV reactivation.

All four patients who developed HBV reactivation had lymphoma and were treated with rituxmab and glucocorticoids containing chemotherapy (Table II).

TABLE II. Clinical Characteristics of HBV Reactivation Patients

	Case 1	Case 2	Case 3	Case 4
Age/sex	75/F	70/M	66/M	83/F
Diagnosis	DLBCL	DLBCL	FL	BL
Stage[a]	IIIB	IIIB	IIA	IVB
Anti-HBc/HBs	+/–	+/–	+/+	+/–
Treatment	R-CHOP like	R-CHOP like	R-CHOP	R-MTX/CPM/VCR/ ADM/DEXA/ETP
Period from initiation of treatment (days)	42	46	398	148
Period from last rituximab (days)	3	26	159	23
Frequency of rituximab administration	5	1	12	8
During or after treatment	During treatment	During treatment	After treatment	During treatment
HBV DNA on the reactivation point (log copy/ml)	3.6	<1.8+[b]	3.6	<1.8+[b]
Peak HBV DNA (log copy/ml)	7.6	2.7	3.6	<1.8+[b]
HBV genotype	C	Ba	Not detected	Not detected
HBV pre-core	Wild	Wild	Not detected	Not detected
HBV core promoter	Wild	Mutant	Not detected	Not detected
Antiviral treatment	+	+	–	–
Outcome	Alive	Alive	Alive	Alive

DLBCL, diffuse large B-cell lymphoma; FL, follicular lymphoma; BL, Burkitt lymphoma; R-CHOP, rituximab, pirarubicin, cyclophosphamide, vincristine, and prednisone; R-CHOP, rituximab, pirarubicin, cyclophosphamide, vincristine, and prednisone; R-MTX/CPM/VCR/ADM/DEXA/ETP, rituximab, methotrexate, cyclophosphamide, vincristine, adriamycin, dexamethasone, and etoposide; ALT, alanine aminotransferase.
[a]Ann Arbor staging.
[b]The DNA load was not quantifiable, but a PCR signal was detectable.

Reactivation of HBV occurred during the course of chemotherapy in three cases, and after maintenance therapy with rituximab in only one case (Fig. 1). Although there is no apparent correlation between HBV reactivation and complete blood count data prior to chemotherapy (data not shown), baseline counts of peripheral lymphocytes were associated with an incidence of HBV reactivation ($P = 0.033$). Nadir levels in peripheral lymphocytes for all subjects during treatment and baseline levels of immunoglobulin G were also assessed. However, none of the parameters were confirmed to be associated with the incidence of HBV reactivation, except for peripheral lymphocytes (Table III). The ROC analysis for the prediction of reactivation using lymphoid counts before treatment showed the area under the curve (AUC) to be 0.814, with the best cut-off to be 860/μl. In four cases with HBV reactivation, based on high sensitive HBsAg assay, HBsAg was examined from stored sera at the time of HBV reactivation, but none was detectable. The detailed clinical course are; (1) Case 1, initially negative for both HBsAg and anti-HBs, became positive for HBV DNA 42 days after the initiation of treatment. She had received chemotherapy using multiple agents such as rituximab, pirarubicin, cyclophosphamide, vincristine, and prednisone (R-CHOP like regimen). After an elevation in quantified HBV DNA in sera, the patient was treated with entecavir, at 1mg per day. HBV DNA was immediately undetectable without hepatitis. (2) Case 2 had detectable HBV-DNA 46 days after the initiation of an R-CHOP like regimen. One month after the transient emergence of HBV DNA in serum (signal positive, but not quantified), the HBV DNA became naturally undetectable. However, 6 weeks later, HBV DNA became detectable again, and after the confirmation of a sustained increase in the HBV DNA load, entecavir was continuously given. Thereafter, he showed a decrease in the HBV DNA load below the lower limit for detection without hepatitis, and ALT level became within normal range. (3) Case 3 had maintenance therapy with rituximab after the CHOP regimen. He showed an increase in the HBV DNA load at over 3 log copies/ml just once, 159 days after maintenance therapy with rituximab, but HBV DNA became undetectable again naturally. Although he had an anti-HBs titer of 601.2 mIU/ml before chemotherapy, the titer decreased to 500.8 mIU/ml at the showing of HBV reactivation. (4) Case 4 was not positive for quantified HBV DNA, but had a transient replication signal of HBV DNA in serum at day 148 by real time PCR. These two cases did not present with continuous viremia of HBV, and, as such, antiviral drugs were not administered. Although four cases showed HBV reactivation, they did not develop de novo hepatitis due to HBV reactivation and were able to undergo chemotherapy against malignant lymphoma as scheduled.

DISCUSSION

HBV reactivation is known as a significant complication of chemotherapy for hemodyscrasia [Francisci et al., 2010; Yagci et al., 2010; Sugauchi et al., 2011].

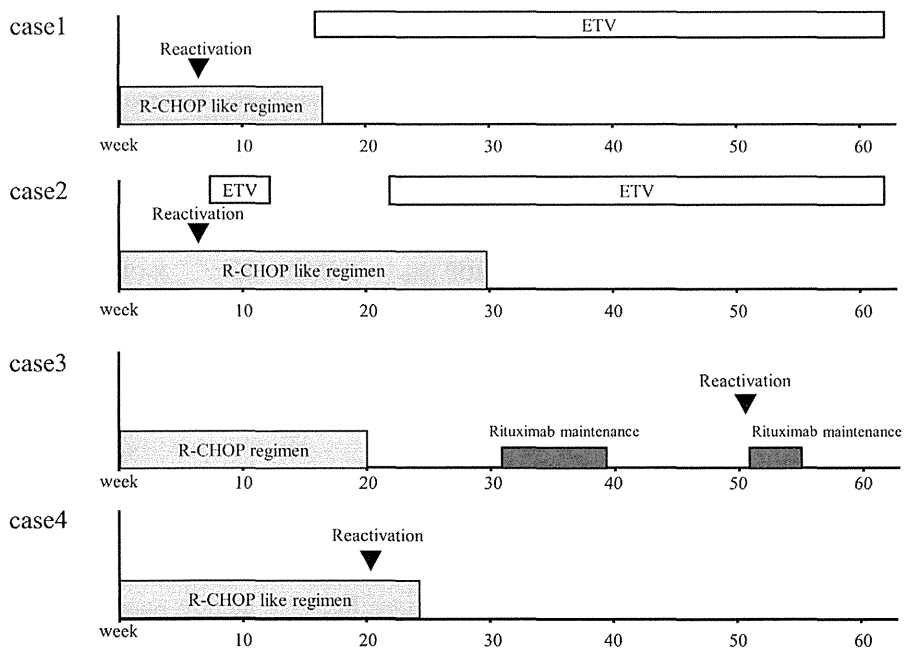

Fig. 1. Reactivation of HBV occurred during the course of chemotherapy in three cases, and after maintenance therapy with rituximab in only one case.

TABLE III. Comparison of Background Between Patients With or Without HBV Reactivation

			Reactivation proportion		
	Reactivation	Non-reactivation	Estimates(%)	95%CI(%)	*P*-value[a]
Number	4	105	3.7	(1.0–9.1)	
Sex(M/F)	2/2	58/47	3.3/4.1	(0.4–11.5)/(0.5–14.0)	1.000
Age[b], mean	73.5	67.7			0.420
Anti-HBc positive	4	55	6.8	(1.9–16.5)	0.123
Anti-HBs positive	1	41	2.4	(0.0–12.6)	1.000
Diagnosis					
Multiple myeloma	0	13	0.0	(0.0–24.7)	1.000
Diffuse large B cell lymphoma	2	52	3.8	(0.5–12.8)	1.000
Follicular lymphoma	1	21	4.8	(0.1–22.8)	1.000
Marginal zone B cell lymphoma	0	7	0.0	(0.0–41.0)	1.000
Burkitt lymphoma	1	1	50.0	(1.3–98.7)	0.072
T-cell lymphoma	0	2	0.0	(0.0–84.2)	1.000
Hodgkin lymphoma	0	5	0.0	(0.0–52.2)	1.000
Others	0	4	0.0	(0.0–60.2)	1.000
No. of rituximab administration	4	84	4.5	(1.3–11.2)	1.000
No. of glucocorticoid administration	4	104	3.7	(1.0–9.2)	1.000
Lymphocyte before chemotherapy median[b] (/μl) (range)	776 (460–1368)	1363 (274–10156)			0.033
Nadir lymphocyte during and after chemotherapy median[b] (/μl) (range)	133 (8–217)	247 (0–1729)			0.130
Immunoglobulin G before chemotherapy median[b] (mg/dl) (range)	1237 (1103–1479)	1421 (82–9085)			0.733

CI, confidence interval.
[a]Fisher's exact test.
[b]Medians and ranges are presented, compared by Mann–Whitney test.

Recently, the risk for development of HBV reactivation after chemotherapy in HBsAg undetectable patients has been reported [Wu et al., 2009; Cheung et al., 2010; Matsue et al., 2010]. Hui et al. [2006] described that 6 of 49 patients with undetectable HBsAg with malignant lymphoma receiving rituxmab plus corticosteroid chemotherapy developed new onset hepatitis B, and the risk factor was rituximab plus corticosteroid administration. Yeo et al. [2009] noted that 5 of 21 HBsAg undetectable, anti-HBc positive patients with diffuse large B-cell lymphoma who were treated with rituximab combination chemotherapy had reactivated HBV, and the risk factors were male, anti-HBs negative, and rituximab combination chemotherapy. It was recently reported that HBV reactivation had occurred in HBsAg undetectable multiple myeloma patients who underwent chemotherapy; 1 of 61 HBsAg undetectable multiple myeloma patients had reactivated HBV following chemotherapy [Yoshida et al., 2010]. However, additional prospective study would be required to know the precise frequency and risk factors for HBV reactivation among patients treated for malignant lymphoma or multiple myeloma. In addition, HBV reactivation was reported to be associated with the presence of anti-HBc and anti-HBs in baseline sera [Hui et al., 2006; Yeo et al., 2009], but the other factors associated with HBV reactivation have not yet been described.

In this study, all four cases with HBV reactivation were positive for anti-HBc before chemotherapy, and three of them were negative for anti-HBs (Table III). However, because of the limitation of size of samples in this study, this study could not evaluate the significance of anti-HBc for HBV reactivation ($P = 0.06$). This might be one of the key results of this study, but it could also be a chance finding. Therefore, serological markers including the titrations of anti-HBc and anti-HBs should be analyzed for the purpose of determining their relationship with HBV reactivation in larger scaled studies.

Out of four patients with HBV reactivation, two patients were treated with entecavir because they showed a persistent increase in the HBV DNA load. In contrast, in the remaining two patients, one patient showed a temporary replication signal of HBV DNA by real-time PCR, and another patient revealed a slight increase in the DNA load during a close follow-up. As HBV DNA of the latter two cases immediately became undetectable by real-time PCR, they were not given antiviral drugs. None of the four patients with HBV reactivation presented de novo hepatitis due to HBV reactivation. All cases were able to receive chemotherapy for underlying diseases as scheduled initially. Although there has been no proposal for the optimal time point for initiation of an anti-HBV treatment for this disease setting, preemptive therapy should be started immediately in patients with sustained viral replication quantified by real-time PCR.

In the present study, periodical quantitation of HBV DNA was useful for monitoring active

replication of HBV in patients receiving chemotherapy. HBsAg was also measured in serial sera in all cases of HBV reactivation, using novel CLEIA which was reported to be highly sensitive. However, HBsAg was not detected in any serum obtained from patients with HBV reactivation, indicating insufficient sensitivity of the assay for detecting HBV reactivation. As shown in the Japanese guidelines [Hirohito Tsubouchi et al., 2009], at present, routine measurement of HBV DNA levels would be preferable to an assay for HBsAg for the purpose of the early diagnosis of HBV reactivation.

Additionally, baseline lymphocyte counts in patients who had HBV reactivation were significantly less than those in patients who did not, although there was no difference in the nadir level of peripheral lymphocytes between patients who developed HBV reactivation and those who did not during or after chemotherapy. Based on these results, it is possible that lower levels of baseline peripheral lymphocytes might have a correlation with the occurrence of reactivated HBV in patients with malignant lymphoma or multiple myeloma. Although there is no similar data, further clinical studies are needed to evaluate the association between HBV reactivation and differential count of lymphocytes. In hepatocytes of chronic hepatitis B patients, cellular and humoral immunity could be associated with viral clearance, especially cytotoxic T lymphocytes (CTL) and natural killer (NK) cells which have roles to suppress proliferation of HBV. Gu et al. [2009] showed that serum HBV DNA levels in chronic hepatitis B patients were correlated to the frequency of HBV-specific CTL. Li et al. [2011] also reported that patients with acute hepatitis B possess a higher frequency of HBV-specific CTL than chronic hepatitis B patients. These reports may indicate that the HBV-specific CTL would be associated with suppression of HBV proliferation. This study could not evaluate differential counts of lymphocytes, and functional analyses of CTL. Further studies with CTL would provide a better understanding of the mechanism of this condition.

In conclusion, among the 59 anti-HBc positive patients with malignant lymphoma or multiple myeloma, four patients (6.8%) showed HBV reactivation during and after chemotherapy. HBV reactivation did not occur among patients without anti-HBc in this study. Monitoring of HBV DNA in sera is useful for the early diagnosis of HBV reactivation, and preemptive therapy is a useful alternative to prevent hepatitis due to HBV reactivation. Patients must be monitored periodically for HBV-DNA levels during and after chemotherapy.

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No association for Chinese HBV-related hepatocellular carcinoma susceptibility SNP in other East Asian populations

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Abstract

Background: A recent genome-wide association study (GWAS) using chronic HBV (hepatitis B virus) carriers with and without hepatocellular carcinoma (HCC) in five independent Chinese populations found that one SNP (rs17401966) in *KIF1B* was associated with susceptibility to HCC. In the present study, a total of 580 HBV-derived HCC cases and 1351 individuals with chronic hepatitis B (CHB) or asymptomatic carrier (ASC) were used for replication studies in order to evaluate the reported association with HBV-derived HCC in other East Asian populations.

Results: We did not detect any associations between rs17401966 and HCC in the Japanese cohorts (replication 1: OR = 1.09, 95 % CI = 0.82-1.43; replication 2: OR = 0.79, 95 % CI = 0.54-1.15), in the Korean cohort (replication 3: OR = 0.95, 95 % CI = 0.66-1.36), or in the Hong Kong Chinese cohort (replication 4: OR = 1.17, 95 % CI = 0.79-1.75). Meta-analysis using these cohorts also did not show any associations with P = 0.97.

Conclusions: None of the replication cohorts showed associations between rs17401966 and HBV-derived HCC. This may be due to differences in the genetic diversity among the Japanese, Korean and Chinese populations. Other reasons could be the high complexity of multivariate interactions between the genomic information and the phenotype that is manifesting. A much wider range of investigations is needed in order to elucidate the differences in HCC susceptibility among these Asian populations.

Keywords: Hepatitis B, hepatocellular carcinoma, candidate SNP, replication study, genome-wide association study

Background

Hepatitis B (HB) is a potentially life-threatening liver infection caused by the hepatitis B virus (HBV), and approximately 360 million people worldwide are thought to be chronically infected with HBV. The clinical course of HBV infection is variable, including acute self-limiting infection, fulminant hepatic failure, inactive carrier state and chronic hepatitis with progression to cirrhosis and hepatocellular carcinoma (HCC). Although some HBV carriers spontaneously eliminate the virus, 2-10 % of individuals with chronic HB (CHB) develop liver cirrhosis every year, and a subset of these individuals suffer from liver failure or HCC. Around 600,000 new HCC cases are diagnosed annually worldwide, with HCC being relatively common in Asia-Pacific countries and sub-Saharan Africa; more than 70 % of HCC patients are diagnosed in Asia (with 55 % in China) [1]. However, HCC is relatively uncommon in the USA, Europe and Australia [1,2]. The majority of HCC develops in patients with cirrhosis, which is most often attributable

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to chronic HBV infection followed by chronic HCV in the Asia-Pacific region [3].

A recent genome-wide association study (GWAS) using Japanese CHB cases and controls confirmed that 11 SNPs in a region including *HLA-DPA1* and *-DPB1* were associated with CHB [4]. Moreover, a GWAS using chronic HBV carriers with and without HCC in five independent Chinese populations reported that one SNP (rs17401966) in *KIF1B* was associated with HCC susceptibility [5]. In the present study, we performed replication studies using Japanese, Korean and Hong Kong Chinese cases and controls in order to evaluate the reported association with HBV-derived HCC in other East Asian populations.

Results

We performed SNP genotyping of rs17401966 located in the *KIF1B* gene for the purpose of replication analysis of the previous GWAS report [5]. Four distinct cohorts were used for these replication analyses (Table 1). We first examined two independent Japanese case–control samples including 179 cases and 769 controls from Biobank Japan (replication 1), and 142 cases and 251 controls from various hospitals (replication 2). We did not detect any associations between rs17401966 and HCC in the Japanese cohorts (replication 1: OR = 1.09; 95 % CI = 0.82-1.43, replication 2: OR = 0.79; 95 % CI = 0.54-1.15). We further examined Korean case–control samples comprising 164 cases and 144 controls (replication 3) and Hongkongese 94 HCC cases and 187 CHB controls (replication 4), but again did not detect any association (replication 3: OR = 0.95; 95 % CI = 0.66-1.36, replication 4: OR = 1.17; 95 % CI = 0.79-1.75). Logistic regression analysis adjusted for age and gender also did not show any association (P_{log} = 0.65, 0.27, 0.11, 0.56 for each replication panel). Moreover, we conducted meta-analysis to combine these studies, also not detect any association (P_{meta} = 0.97).

Discussion and conclusions

Zhang et al. [5] reported that SNP rs17401966 was significantly associated with HBV-related HCC (joint OR = 0.61). They conducted a GWAS using 348 cases and 359 controls in a population in Guangxi in southern China, and selected 45 SNPs for the replication study based on the results ($P < 10^{-4}$). In the first replication study, they used 276 cases and 266 controls from Beijing in northern China, and 5 SNPs showed the same direction of association as in the GWAS ($P < 0.05$). They performed a further replication study (of 507 cases and 215 controls) in Jiangsu in eastern China and only one SNP showed the same trend ($P = 3.9 \times 10^{-5}$). Guangdong and Shanghai samples from southern and eastern China were used for further replication studies. The association yielded a p-value of 1.7×10^{-18} on meta-analysis.

We performed four replication analyses using Japanese, Korean and Hong Kong Chinese samples (Table 1). Although sample size of each cohort is smaller than that of the previous GWAS, we conducted mete-analysis of all our study. The result did not show any association between rs17401966 and HBV-derived HCC (P_{meta} = 0.97).

This may be due to differences in genetic diversity among Japanese, Korean and Chinese populations. A maximum-likelihood tree of 126 populations based on 19,934 SNPs showed that Japanese and Korean populations form a monophyletic clade with a 100 % bootstrap value [6]. However, Chinese populations form a paraphyletic clade with two other populations. This indicates that Japanese and Korean populations are genetically closer to one another than the Chinese population.

Table 1 Association between rs17401966 and HBV-derived HCC

cohort	sample size	cases			controls				OR		
	(cases/controls)	GG	AG	AA	GG	AG	AA	HWE p	(95 % CI)	P [a]	P_{het} [b]
replication 1	179/769	13	61	105	45	261	463	0.599	1.09	0.578	
(Japan 1)		(7.2)	(34.1)	(58.7)	(5.9)	(33.9)	(60.2)		(0.82-1.43)		
replication 2	142/251	5	46	91	14	91	146	1	0.79	0.212	
(Japan 2)		(3.5)	(32.4)	(64.1)	(5.6)	(36.2)	(58.2)		(0.54-1.15)		
replication 3	164/144	17	59	88	15	55	74	0.616	0.95	0.790	
(Korea)		(10.4)	(36.0)	(53.6)	(10.4)	(38.2)	(51.4)		(0.66-1.36)		
replication 4	94/187	10	39	44	13	80	94	0.767	1.17	0.432	
(Hong Kong)		(10.6)	(41.5)	(46.8)	(6.9)	(42.8)	(50.3)		(0.79-1.75)		
Meta-analysis [c]									0.996	0.965	0.423
									(0.84-1.18)		

[a]P value of fisher's exact test for allele model.
[b]Result of Breslow-Day test.
[c]Results of meta-analysis were calculated by the Mantel-Haenzel method.

We did not find any association with Hong Kong Chinese cohort (P = 0.43). Moreover, a study using 357 HCC cases and 354 HBV-positive non-HCC controls in Hong Kong Chinese did not show any significant difference (P = 0.91) [7]. Previous population studies have revealed that various Han Chinese populations show varying degrees of admixture between a northern Altaic cluster and a southern cluster of Sino-Tibetan/Tai-Kadai populations in southern China and northern Thailand [6]. Although Hong Kong is located closed to the Guangdong (cohort 3 of Zhang et al study), there is great heterogeneity for rs17401966 between Hong Kong cohorts (our study and Chan's study [7]) and Guangdong cohort (our study versus Zhang's study: $P_{het} = 0.0066$; Chan's study versus Zhang's study: $P_{het} = 0.035$). This result suggests the existence of other confounding factors, which can differentiate the previous study in China and this study.

One of the possible reasons could be the high complexity of multivariate interactions between the genomic information and the phenotype that is manifesting. HCC development is a multiple process which links to causative factors such as age, gender, environmental toxins, alcohol and drug abuse, higher HBV DNA levels, and HBV genotype variations [8]. The eight HBV genotypes display distinct geographical and ethnic distributions. Genotypes B and C are prevalent in Asia. Specific variations in HBV have been associated with cirrhosis and HCC. These variations include in particular mutations in pre-core region (Pre-C), in basal core promoter (BCP) and in ORF encoding Pre-S1/Pre-S2/S and Pre-C/C. Because there is an overlap between Pre-C or BCP mutations and genotypes, these mutations appear to be more common in genotype C as compared to other genotypes [9].

Aflatoxins are a group of 20 related metabolites and Aflatoxin B1 is the most potent naturally occurring chemical liver carcinogen known. Aflatoxin exposures multiplicatively increase the risk of HCC in people chronically infected with HBV, which illustrates the deleterious impact that even low toxin levels in the diet can have on human health [10–12]. Liu and Wu estimated population risk for aflatoxin-induced HCC around the world [13]. Most cases occur in sub-Saharan Africa, Southeast Asia and China, where populations suffer from both high HBV prevalence and largely uncontrolled exposure to aflatoxin in food. But we could not obtain the information of these confounding factors from both of the previous GWAS study and this study. A much wider range of investigations is thus needed in order to elucidate the differences in HCC susceptibility among these Asian populations.

Methods

Samples

Case and control samples used in this study were collected from Japan, Korea and Hong Kong listed in supplementary Additional file 1: Table S1. A total of 179 cases and 769 control subjects were analyzed in the first replication study. DNA samples from both CHB controls and HBV-related HCC cases used in this study were obtained from the BioBank Japan at the Institute of Medical Science, the University of Tokyo [14]. Among the BioBank Japan samples, we selected HBsAg-seropositive CHB patients with elevated serum aminotransferase levels for more than six months, according to the guidelines for diagnosis and treatment of chronic hepatisis from The Japan Society of Hepatology (http://www.jsh.or.jp/medical/gudelines/index.html). The mean (and standard deviation; SD) age was 62.0 (9.4) years for the cases and 54.7 (13.5) years for the controls. The second Japanese replication sample sets for the cases (n = 142) and controls (n = 251) study were obtained from 16 hospitals. The case samples for the second replication included 142 HCC patients and the controls included 135 CHB patients and 116 asymptomatic carriers (ASC). The mean (SD) age was 61.3 (10.2) years for the cases and 56.2 (10.9) years for the controls. The Korean replication samples were collected from Yonsei University College of Medicine. The third replication set was composed of 165 HCC patients and 144 CHB patients. The mean (SD) age was 52.2 (8.9) and 37.3 (11.3) years for the cases and controls, respectively. The samples in Hong Kong were collected from the University of Hong Kong, Queen Mary Hospital. The fourth replication set was composed of 94 HCC patients and 187 CHB patients. The mean (SD) age was 58.0 (10.5) and 56.9 (8.3) years for the cases and controls, respectively. All participants provided written informed consent. This research project was approved by the Research Ethics Committees at the Institute of Medical Science and the Graduate School of Medicine, the University of Tokyo, Yonsei University College of Medicine, the University of Hong Kong, Nationa Center for Global Health and Medicine, Hokkaido University Graduate School of Medicine, Teine Keijinkai Hospital, Iwate Medical University, Saitama Medical University, Kitasato University School of Medicine, Musashino Red Cross Hospital, Kanazawa University Graduate School of Medicine, Shinshu University School of Medicine, Nagoya City University Graduate School of Medical Sciences, Kyoto Prefectural University of Medicine, National Hospital Organization Osaka National Hospital, Kawasaki Medical College, Tottori University, Ehime University Graduate School of Medicine, and Kurume University School of Medicine.

SNP Genotyping

For the first replication samples, we genotyped rs17401966 using PCR-based Invader assay (Third Wave Technologies, Madison, WI) [15], and for the second, third and fourth replication samples, we used TaqMan genotyping assay (Applied Biosystems, Carlsbad, CA). In the TaqMan SNP

genotyping assay, PCR amplification was performed in a 5-μl reaction mixture containing 1 μl of genomic DNA, 2.5 μl of KAPA PROBE FAST qPCR Master Mix (Kapa Biosystems, Woburn, MA), and 40 x TaqMan SNP Genotyping Assay probe (ABI) for this SNP. QPCR thermal cycling was performed as follows: 95°C for 3 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. The SNP call rate of each replication panel was 100 %, 100 %, 99.7 % and 99.6 %.

Statistical analysis

We performed Hardy-Weinberg equilibrium test for the case and control samples in each replication study. Fisher's exact test was applied to two-by-two contingency tables for three different genetic models; allele frequency, dominant and recessive model. Odds ratios and confidence intervals were calculated using the major alleles as references. Meta-analysis was conducted using the Mantel-Haenszel method. Heterogeneity among studies was examined by using the Breslow-Day test. Genotype-phenotype association for the SNP rs17401966 was assessed using logistic regression analysis adjusted for age and gender in plink 1.07 (http://pngu.mgh.harvard.edu/~purcell/plink/index.shtml).

Additional file

Additional file 1: Table S1. Samples used in this study.

Abbreviations

HB: Hepatitis b; HBV: Hepatitis b virus; HCC: Hepatocellular carcinoma; CHB: Chronic hepatitis b; HCV: Hepatitis c virus; GWAS: Genome-wide association study; ASC: Asymptomatic carrier.

Competing interests

The authors declare that they have no competing interests.

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Original Article

Risk of hepatocellular carcinoma in cirrhotic hepatitis B virus patients during nucleoside/nucleotide analog therapy

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Aim: Some patients develop hepatocellular carcinoma (HCC) during nucleoside/nucleotide analog (NA) therapy even if alanine aminotransferase (ALT) or hepatitis B virus (HBV) DNA levels are sufficiently reduced. The aim of this study is to identify the risk factors of development of HCC during NA therapy.

Methods: Six hundred and two patients were analyzed who were continuously receiving NA for chronic HBV infection. The patients who developed HCC previously or within 1 year of therapy were excluded. In the patients studied, the median duration of therapy was 90 months. A total of 492 patients had chronic hepatitis (CH) and 110 had liver cirrhosis (LC).

Results: In 602 patients, the rate of normalization of ALT, loss of serum HBV DNA and development of HCC were 90.4%, 55.4%, and 6.1%, respectively. The significant risk factors of development of HCC were LC status and duration of therapy. The annual incidence of HCC in LC patients was 2.53%/year, compared with 0.34%/year in CH patients. When the relation between the incidence of HCC and the response to therapy was evaluated, in patients with normalization of ALT level, loss of HBV DNA by real-time polymerase chain reaction or hepatitis B e-antigen seroconversion, the incidences of HCC was reduced to some extent. However, none of the patients who achieved hepatitis B surface antigen (HBsAg) seroclearance during NA therapy developed HCC.

Conclusion: LC status was the significant risk factor of development of HCC during NA therapy. However, none of the patients who showed HBsAg seroclearance developed HCC. The ultimate goal of therapy for reduced risk of HCC may be HBsAg seroclearance.

Key words: hepatitis B surface antigen seroclearance, hepatitis B virus DNA, hepatocellular carcinoma, liver cirrhosis, nucleoside/nucleotide analog therapy, risk factors

INTRODUCTION

HEPATITIS B VIRUS (HBV) can cause chronic hepatitis (CH), liver cirrhosis (LC) and hepatocellular carcinoma (HCC).[1–3] To prevent progression of liver diseases, nucleoside/nucleotide analogs (NA), such as lamivudine, adefovir or entecavir, are used widely for antiviral therapy of chronic HBV infection.[4–6] During NA therapy, alanine aminotransferase (ALT) and HBV DNA levels are often reduced within normal ranges or under the detection limit. However, it is true that some patients develop HCC during NA therapy.[7]

The conventional goals of antiviral therapy for patients with chronic HBV infection should be hepatitis B e antigen (HBeAg) seroconversion, normalization of ALT level and loss of serum HBV DNA. Ultimately, loss of hepatitis B surface antigen (HBsAg), namely HBsAg seroclearance, is desirable. However, HBsAg seroclearance during NA therapy is very rare, especially in Asian countries.[8] It was reported that low serum HBsAg levels were associated with a low risk of HCC in patients with low HBV DNA levels.[9] Hence, the surrogate goals of NA therapy could be normalization

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of ALT levels, loss of HBV DNA and HBeAg seroconversion.

Recent studies have reported that the incidence of development of HCC in patients receiving NA was significantly reduced, compared with non-therapy patients.[10,11] In addition, it was shown that a high proportion of patients with LC during NA therapy carried a higher risk of HCC, compared with those with CH.[11–13] However, the relationship between the risk of HCC during NA therapy and the responses of NA therapy is not clear.

The aim of the present study was to identify the risk factors for the development of HCC during NA therapy and the relation with the responses to the therapy.

METHODS

Patients

SEVEN HUNDRED AND seventy-two patients receiving NA therapy were recruited retrospectively from the 15 hospitals in the Japanese Red Cross Liver Network. All patients were HBsAg positive for more than 1 year, serum HBV DNA positive before NA therapy, and negative for anti-hepatitis C virus or anti-HIV. Of 772 patients, 25 were excluded because they developed HCC before commencement of NA therapy or developed HCC within 1 year of NA therapy. In addition, 145 patients were excluded because of lack of data of quantitative HBsAg levels. Therefore, 602 patients were analyzed in this study.

Of 602 patients, 492 had CH and 110 had LC. Two hundred and ten patients were diagnosed by liver biopsy with their written informed consent, and the other patients were diagnosed by clinical findings, laboratory data and computed tomography or magnetic resonance imaging. Of 602 patients, 405 patients were receiving entecavir, 56 were receiving lamivudine, 67 switched from lamivudine to entecavir and 74 switched from lamivudine alone to lamivudine and adefovir. All patients were receiving NA continuously for more than 1 year until the end of follow up.

Methods

To detect development of HCC during NA therapy, all patients underwent ultrasound, computed tomography or magnetic resonance imaging for screening HCC at least every 6 months.

The duration of NA therapy was defined as the months from the start of therapy to the final month of the continuous therapy. If HCC was detected during therapy, the duration was stopped at the month of detection of HCC.

The normal ALT level was defined as less than 40 IU/L in this study. The serum HBV DNA level was determined by real-time polymerase chain reaction (PCR) or transcription-mediated amplification (TMA).

Loss of HBV DNA was defined as negative state by real-time PCR. In only 53 patients, HBV DNA were determined by TMA. Thus, analysis of the relationship between the incidence of HCC and loss of HBV DNA was evaluated in the patients determined only by real-time PCR.

Hepatitis B surface antigen levels were quantitatively determined by chemiluminescent immunoassay (CLIA) or chemiluminescence enzyme immunoassay (CLEIA). The upper limits of detection of CLIA or CLEIA were 2000 or 250 IU/mL, respectively. The lower limits of detection of CLIA or CLEIA were 0.05 or 0.03 IU/mL, respectively. Thus, in this study, HBsAg seroclearance was defined as HBsAg levels of less than 0.05 IU/mL by CLIA or less than 0.03 IU/mL by CLEIA.

Because only approximately 35% of all the patients were diagnosed by liver biopsy, we used two other methods to confirm the risk of HCC in cirrhotic patients with advanced liver fibrosis. The first method was a stratification by platelet count less than or $10 \times 10^4/\mu L$ or more.[14,15] In addition, the other method was a stratification by FIB-4 index, less than or 3.25 or more. These platelet count or FIB-4 index were considered to be good indicators of cirrhosis or advanced liver fibrosis.[16,17]

This study was designed and performed in accordance with the provision of the Declaration of Helsinki and Good Clinical Practice Guidelines, and was approved by the institutional review board in all attending hospitals.

Statistical analyses were carried out using the Wilcoxon rank sum test, the χ^2-test or Fisher's exact test in the univariate analyses, and by the Cox proportional hazard model in the multivariate analysis. Statistical significance level was set at $P < 0.05$.

RESULTS

THE BASELINE CHARACTERISTICS of the patients studied are shown in Table 1. The median age was 52 years (range, 21–79), and the male : female ratio was 381:221. The median duration of NA therapy was 90 months (range, 12–204). The ratio of CH : LC disease status was 492:110, and the family history of yes : no : unknown HCC was 64:375:163. The laboratory findings were: median ALT, 69 IU/L (range, 9–2821); median platelet count, $16.1 \times 10^4/\mu L$ (range,

Table 1 Baseline characteristic of the patients studied

Characteristics	$n = 602$
Age (years)	52 (21–79)
Sex (M:F)	381:221
Nucleotide analogs (Ent : Lam→Ent : Lam : Lam + Ade)	405:67:56:74
Duration of nucleotide analog therapy (months)	90 (12–204)
Disease (CH : LC)	492:110
Family history of HCC (yes : no : unknown)	64:375:163
AST (IU/L)	58 (14–1752)
ALT (IU/L)	69 (9–2821)
Albumin (mg/dL)	4.2 (1.7–5.5)
Platelet count ($\times 10^4/\mu L$)	16.1 (3.1–41.7)
HBV genotype (A : B : C : D : NT)	4:23:231:2:342
HBeAg status (positive : negative)	295:305
HBV DNA level (log copies/mL)	6.8 (2.3–9.1)

Median (range).
Ade, adefovir; ALT, alanine aminotransferase; AST, aspartate aminotransferase; CH, chronic hepatitis; Ent, entecavir; HBV, hepatitis B virus; HBeAg, hepatitis B e-antigen; HBV, hepatitis B virus; HCC, hepatocellular carcinoma; Lam, lamivudine; LC, liver cirrhosis; N, no; NT, not tested; Y, yes.

3.1–41.7); HBV genotype A : B : C : others : unknown, 4:23:231:2:342; HBeAg positive : negative status, 295:305; and median HBV DNA level, 6.8 log copies/mL (range, 2.3–9.1).

Of 602 patients at the final follow up during NA therapy, 90.4% showed a normal ALT level, 55.4% lost serum HBV DNA, 16.3% showed HBeAg seroconversion, 2.2% revealed HBsAg seroclearance and 6.1% developed HCC during therapy.

Risk factors of development of HCC during NA therapy

The risk factors associated with the development of HCC during therapy were identified to be pretreatment disease status (LC) ($P < 0.001$), duration of NA therapy ($P < 0.001$), ALT levels ($P < 0.001$) and platelet counts ($P < 0.001$) by univariate analyses (Table 2). By multivariate analyses, LC status and duration of therapy were demonstrated to be the most significant risk factors ($P < 0.001$ and $P < 0.001$, respectively).

Cumulative incidence of development of HCC

The cumulative incidence of the development of HCC was analyzed by the Kaplan–Meier method (Fig. 1a).

Table 2 Significant risk factors related with development of HCC during NA therapy

Characteristics	Development of HCC during therapy		Analysis	
	Yes $n = 37$ (6.1%)	No $n = 565$ (93.9%)	Univariate P	Multivariate P
Age (years)	57.2 (41–76)	51.5 (21–79)	0.036	0.446
Sex (M : F)	25:12	356:209	0.725	
Disease (CH : LC)	13:24	479:86	<0.001	<0.001
Duration of NA therapy (months)	37 (12–98)	94.6 (12–204)	<0.001	<0.001
Family history of HCC (yes : no)	6:21	58:354	0.509	
Pretreatment data				
ALT (IU/L)	55 (17–274)	72 (9–2821)	<0.001	0.401
Platelet count ($\times 10^4/\mu L$)	10.9 (4.0–30.3)	16.4 (3.1–41.7)	<0.001	0.146
HBeAg status (positive : negative)	17:20	280:285	0.798	
HBV DNA level (log copies/mL)	6.6 (2.5–8.9)	6.8 (2.3–9.1)	0.090	
HBsAg level >250 IU/mL (Y : N : NT)	24:6:7	410:61:94	0.519	
Final data during therapy				
ALT (<40 : ≥40 IU/L)	28:9	501:64	0.098	
HBeAg seroconversion in HBeAg positive patients (Y : N)	2:6	46:106	0.749	
Loss of HBV DNA by real-time PCR (Y : N)	14:17	247:193	0.330	
HBsAg seroclearance (Y : N)	0:37	13:552	0.351	

ALT, alanine aminotransferase; AST, aspartate aminotransferase; CH, chronic hepatitis; HBeAg, hepatitis B e-antigen; HBsAg, hepatitis B surface antigen; HBV, hepatitis B virus; HCC, hepatocellular carcinoma; LC, liver cirrhosis; N, no; NA, nucleoside/nucleotide therapy; NT, not tested; PCR, polymerase chain reaction; Y, yes.

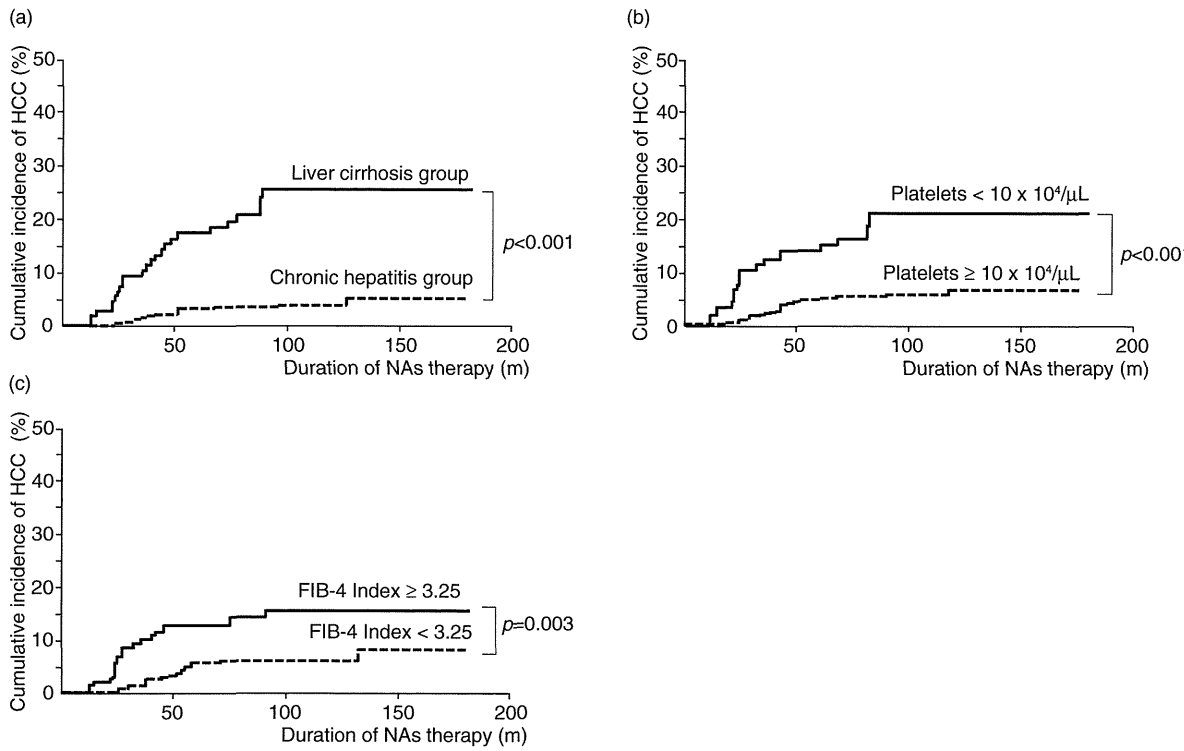

Figure 1 Cumulative incidence of development of hepatocellular carcinoma (HCC) during nucleoside/nucleotide analog (NA) therapy by Kaplan–Meier analysis. (a) Stratification by clinical or histological diagnosis, liver cirrhosis (LC) or chronic hepatitis (CH). (b) Stratification by platelet count. (c) Stratification by the FIB-4 Index. All these analyses indicated that cirrhotic patients carried a higher risk of HCC than non-cirrhotic patients.

The incidence of HCC was significantly higher in the LC group than in the CH group ($P < 0.001$). The annual incidence of development of HCC in the LC group and the CH group was 2.53%/year and 0.34%/year, respectively.

Only approximately 35% of the patients were diagnosed by liver biopsy. Hence, we employed two other methods to confirm the higher risk of HCC in cirrhotic patients with advanced liver fibrosis. By the first method of a stratification according to platelet count ($\geq 10 \times 10^4/\mu L$ or $<10 \times 10^4/\mu L$), the cumulative incidence of HCC was significantly high in the patients with a platelet count of less than $10 \times 10^4/\mu L$, compared with those with a platelet count of $10 \times 10^4/\mu L$ or more ($P < 0.001$) by the Kaplan–Meier analysis (Fig. 1b). Second, when the incidence of HCC was stratified by the FIB-4 index, the patients with a FIB-4 index of 3.25 or more had a significantly high risk, compared with those with a FIB-4 index of less than 3.25 ($P = 0.003$) (Fig. 1c).

Relationship between response to NA and incidence of HCC

To identify the goal of NA therapy for suppression of development of HCC, the relationship between response to NA therapy and incidence of HCC is important. First, when the relationship between normalization of ALT (<40 IU/L) and incidence of HCC was compared, there was no significant difference in the incidence of HCC between the abnormal ALT group and the normalized ALT group in patients with CH and those with LC (Fig. 2a). Second, when the relationship between loss of serum HBV DNA by real-time PCR and incidence of HCC was compared, there was no significant difference between positive and negative HBV DNA groups in CH patients and LC patients (Fig. 2b).

Third, when the relationship between HBeAg seroconversion and incidence of HCC was compared in the patients with positive HBeAg at commencement of the

therapy, there was no significant difference between the group with HBeAg seroconversion and the group without HBeAg seroconversion (Fig. 2c).

When the patients achieved these three goals of NA therapy, namely, normalization of ALT, loss of HBV DNA and HBeAg seroconversion, the incidence of HCC reduced only to some extent.

Finally, the relationship between HBsAg seroclearance and incidence of HCC was compared, none of the patients who achieved HBsAg seroclearance developed HCC in this study (Fig. 2d).

Relationship between duration of NA therapy and incidence of HCC

Duration of NA therapy was a significant risk factor associated with the development of HCC by multivariate analysis (Table 2). Thus, the relationship between duration of NA therapy and incidence of HCC was studied by receiver–operator curve (ROC) analysis. The area under the ROC was 0.802 (95% confidence interval [CI], 0.749–0.856; $P < 0.001$). Duration of therapy of less than 57 months was demonstrated to be the nearest cut-off value with a sensitivity of 76.1% and specificity of 76.3%. When compared with the groups with therapy duration of 57 months or more, and less than 57 months, a significantly higher incidence of development of HCC was observed in the group with therapy duration of less than 57 months than the group with therapy duration of 57 months or more in CH patients and LC patients ($P < 0.001$).

DISCUSSION

IN THE PRESENT study, we indicated that LC patients have a significantly higher risk of development of HCC than CH patients during NA therapy; and that the risk of HCC still existed even if the conventional goals of therapy like normalization of ALT, loss of serum HBV DNA or HBeAg seroconversion were achieved during therapy. However, it was demonstrated that none of the patients who achieved HBsAg seroclearance during therapy developed HCC in this study. It was confirmed that the ultimate goal of antiviral therapy for patients with chronic HBV infection should be HBsAg seroclearance.

Generally, patients with chronic HBV infection are at a high risk of development of HCC.[18] Even during antiviral therapy, a proportion of patients develop HCC.[7,10,11] Furthermore, some patients whose serum HBV DNA levels are under the detection limit level and ALT levels are within normal range develop HCC.

It has been widely stated previously that the conventional goals of therapy for patients with chronic HBV infection are normalization of ALT level, loss of serum HBV DNA and HBeAg seroconversion.[19–21] Of course, the ultimate goal of the therapy should be HBsAg seroclearance. However, HBsAg seroclearance is not common during antiviral therapy. Therefore, achievable conventional goals like normalization of ALT, serum HBV DNA and HBeAg seroconversion are usually pursued.

To achieve these goals, knowing the risk factors of development of HCC during NA therapy is important. We identified LC status and short duration of therapy to be the most significant factors associated with HCC during therapy. That is, the incidence of HCC during therapy in CH patients with a favorable virological response was very low, compared with cirrhotic patients with a favorable response.

Recently, it was reported that the risk of HCC in the patients undergoing NA therapy was reduced, compared with untreated patients, and that LC status was a significant factor of HCC during NA therapy.[10,11] Therefore, NA therapy is thought to be useful for reducing risk of development of HCC in CH and LC patients. However, some risk of HCC during NA therapy was noted in only LC patients.

In our study, the number of patients whose histological diagnosis was confirmed by liver biopsy was limited. The remainder of patients were diagnosed by clinical findings and CT or MRI. Thus, to confirm that LC status was a risk factor for HCC, all patients were analyzed by stratification of platelet count and FIB-4 index.[14–17] It was indicated that lower platelet count or higher FIB-4 index were strongly associated with cirrhotic condition. Using these two additional methods, cirrhotic patients with advanced liver fibrosis were confirmed to have a higher risk of HCC than non-cirrhotic patients.

So far, the relationship between response to NA therapy and risk of HCC is still unclear. Some previous papers have reported that low HBV DNA and normal ALT were associated with lower risk of development of HCC.[9,22–24] In this study, it was demonstrated that in CH patients who achieved normalization of ALT, loss of serum HBV DNA and HBeAg seroconversion, the risk of HCC was reduced remarkably, whereas the risk of HCC was not related to virological response to the therapy in LC patients. Hence, we should observe LC patients carefully for development of HCC during NA therapy, irrespective of a good virological response.

Because this was a multicenter study, HBsAg levels were determined by CLIA or CLEIA. These two methods