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Ⅳ. 研究成果の刊行物・別冊

J Gastroenterol
DOI 10.1007/s00535-010-0331-4

REVIEW

Reactivation of hepatitis B virus following rituximab-plus-steroid combination chemotherapy

Shigeru Kusumoto · Yasuhito Tanaka · Ryuzo Ueda · Masashi Mizokami

Received: 9 September 2010 / Accepted: 9 September 2010

Abstract Reactivation of hepatitis B virus (HBV) has been reported as a fatal complication following systemic chemotherapy or other immunosuppressive therapy. The risk of HBV reactivation differs according to both the patient's HBV infection status prior to systemic chemotherapy and the degree of immunosuppression due to chemotherapy. For establishing an optimal strategy for hepatitis prevention and treatment, it is necessary to understand the characteristics, the clinical course and the risk factors for HBV reactivation and to recognize the difference between hepatitis B surface antigen (HBsAg)-positive and -negative patients with HBV reactivation. Among the important viral risk factors, HBV-DNA level and HBV-related serum markers have been reported to be associated with HBV reactivation in addition to cccDNA, genotypes and gene mutations. Rituximab-plus-steroid combination chemotherapy has recently been identified as a host risk factor for HBV reactivation in hepatitis B core antibody (anti-HBc)-positive and/or hepatitis B surface antibody (anti-HBs) positive—but nonetheless HBsAg-negative—lymphoma patients. For these patients with resolved hepatitis B, preemptive therapy guided by serial HBV-DNA monitoring is a reasonable strategy to enable early diagnosis of HBV reactivation and initiation of antiviral therapy. In this review, we summarize the characteristics of HBV reactivation following rituximab-plus-steroid combination chemotherapy, mainly in HBsAg-negative lymphoma patients, and propose a strategy for managing HBV reactivation.

Keywords Reactivation · HBV · Rituximab

Abbreviations

Anti-HBc	Hepatitis B core antibody
Anti-HBs	Hepatitis B surface antibody
cccDNA	Covalently closed circular DNA
CHOP	Cyclophosphamide, doxorubicin, vincristine, prednisolone
HBsAg	Hepatitis B surface antigen
HBV	Hepatitis B virus
HSCT	Hematopoietic stem cell transplantation
R-CHOP	Rituximab, cyclophosphamide, doxorubicin, vincristine, prednisolone
RTD-PCR	Real-time detection PCR

Introduction

Reactivation of hepatitis B virus (HBV) has been reported not only in hepatitis B surface antigen (HBsAg)-positive patients following systemic chemotherapy, but also in a proportion of HBsAg-negative patients with anti-hepatitis B core antibody (anti-HBc) positivity and/or anti-hepatitis

S. Kusumoto · R. Ueda
Department of Medical Oncology and Immunology,
Nagoya City University Graduate School of Medical Sciences,
1 Kawasumi, Mizuho-chou, Mizuho-ku, Nagoya,
Aichi 467-8601, Japan

Y. Tanaka (✉)
Department of Virology and Liver Unit,
Nagoya City University Graduate School of Medical Sciences,
1 Kawasumi, Mizuho-ku, Nagoya 467-8601, Japan
e-mail: ytanaka@med.nagoya-cu.ac.jp

M. Mizokami
Research Center for Hepatitis and Immunology,
International Medical Center of Japan Konodai Hospital,
Ichikawa, Chiba, Japan

Published online: 06 October 2010

B surface antibody (anti-HBs) positivity [1–4]. Rituximab-plus-steroid combination chemotherapy has recently been identified as a risk factor for HBV reactivation in HBsAg-negative patients with malignant lymphoma [2, 3].

Rituximab is a chimeric human–mouse monoclonal antibody that targets the CD20 molecule [5]. Approximately 70–80% of malignant lymphomas are of B cell origin, and >90% of B cell lymphomas express CD20 on the cell surface. CD20 is an appropriate target molecule because it is not shed, modulated or internalized. The introduction of rituximab into the therapeutic regimen has dramatically improved the prognosis of CD20-positive lymphoma patients. The rituximab + cyclophosphamide, doxorubicin, vincristine, prednisolone (CHOP) combination chemotherapy, known as R-CHOP, is now the gold standard treatment for diffuse large B cell lymphoma worldwide [6]. The effectiveness of rituximab has recently been reported in refractory autoimmune diseases, including rheumatoid arthritis [7], thrombotic thrombocytopenic purpura [8] or idiopathic thrombocytopenic purpura [9], among others.

Many clinical trials have demonstrated the efficacy and safety of rituximab, as used in large numbers of patients, but it has also been recognized that attention must be paid to accompanying potential adverse events, which include late-onset neutropenia [10], progressive multifocal leukoencephalopathy [11] and HBV reactivation.

In this review, we summarize the characteristics of HBV reactivation following rituximab-plus-steroid combination chemotherapy, mainly in HBsAg-negative lymphoma patients, and propose a strategy for managing HBV reactivation.

Pathophysiology of HBV reactivation following systemic chemotherapy

In most immunocompetent hosts, HBV infection manifests as acute hepatitis [12]. Thereafter, HBsAg levels decrease to below the level of detection over some weeks as hepatitis B surface antibody (anti-HBs) titers increase. Most cases of acute hepatitis B completely resolve in adult patients. An individual is defined as having had a resolved HBV infection when he/she tests seropositive for anti-HBc and/or anti-HBs.

Because HBV replication mainly persists in the liver, even in patients with anti-HBs, for several years from the onset of acute hepatitis B [13], all individuals with a history of exposure to HBV are at risk of reactivation following systemic chemotherapy. Re-initiated HBV replication and increasing levels of serum HBV-DNA have been reported following systemic chemotherapy, especially those involving rituximab-containing regimens, even in patients with resolved HBV infections [1]. Under conditions of immunosuppression, HBV is more likely to increase rapidly and to infect many hepatocytes. With immune recovery after chemotherapy, immunocompetent cells attack the infected hepatocytes, causing recurrence of hepatitis B disease.

Risk factors associated with HBV reactivation: viral and host factors

As with chronic hepatitis B, the risk of HBV reactivation depends on the balance between replication of the virus and the immune response of the host. Thus, the risk of HBV reactivation differs according to the patient's HBV infection status prior to systemic chemotherapy as well as to the degree of immunosuppression due to chemotherapy.

Important viral factors, such as HBV-DNA viral load and HBV-related serum markers [presence or absence of HBsAg, hepatitis B e antigen (HBeAg), anti-HBc and anti-HBs] have been reported to be associated with HBV reactivation following anticancer therapy [3, 14–16] (Table 1). More recently, genotypes and mutations of HBV have been reported to be associated with reactivation [17, 18].

Important host factors associated with immunosuppression are steroid combination chemotherapy [19],

Table 1 Risk factors associated with HBV reactivation following systemic chemotherapy

Viral factors	Host factors
HBsAg	Combination therapy with steroid
HBeAg	Rituximab-plus-steroid combination therapy
Anti-HBc	Malignant lymphoma
Anti-HBs	Male gender
HBV-DNA levels	Absence of anti-HBs before chemotherapy
Covalently closed circular DNA	Decrease of anti-HBs titers during chemotherapy (in patients seropositive for anti-HBs before chemotherapy)
Occult HBV infection	
Genotype non-A (especially, genotype B)	
Gene mutation of precore and/or core promoter	

HBV Hepatitis B virus, *HBsAg* hepatitis B surface antigen, *HBeAg* hepatitis B e antigen, *anti-HBc* anti-hepatitis B core antibody, *anti-HBs* anti-hepatitis B surface antibody

rituximab-plus-steroid combination chemotherapy [2, 3], hematopoietic stem cell transplantation [20–23] (HSCT, allogeneic or autologous), as also shown in Table 1.

Viral risk factors: cccDNA, occult HBV infection, genotypes and gene mutations

Hepatitis B virus is known to be present in infected people as covalently closed circular DNA (cccDNA) located in hepatocyte nuclei and providing a stable template for replication [12]. It is this configuration that is believed to make HBV eradication so difficult. Therefore, the presence of cccDNA in the liver is considered to be an important risk factor in patients with HBV reactivation following systemic chemotherapy. The serum level of HBV core-related antigen (HBcrAg) has recently been reported to be correlated with the amount of cccDNA in the liver [24]. Consequently, quantification of this antigen would be expected to represent a predictive marker for HBV reactivation [25].

An HBsAg-negative individual is defined as having an occult HBV infection when HBV-DNA is nonetheless detectable in the blood using the real-time detection PCR (RTD-PCR) assay. Patients with occult HBV infection are thought to be at high risk for HBV reactivation, in the same way as HBsAg-positive patients [2, 26]. As a person with occult HBV infection is still anti-HBc-positive and/or anti-HBs-positive, the identification of such an occult HBV infection is possible by measuring HBV-DNA at screening when either marker is positive.

Ten genotypes have been detected with a sequence divergence of >8% in the entire HBV genome of about 3215 nucleotides (nt). These are designated by capital letters from A to J in the order of their documentation [27]. They have distinct geographical distributions, i.e., genotypes A and D are found mainly in Western countries, while genotypes B and C are prevalent in Asia, and they are associated with the severity of liver disease as well as response to antiviral therapy [28, 29]. Most recently, different genotypes were reported to be associated with different likelihoods of HBV reactivation [17, 18]. Thus, the non-A genotype, especially the B genotype, might represent an important risk factor in the setting of systemic chemotherapy.

Gene mutations in precore and core-promoter regions are very frequent in patients with fulminant hepatitis in Asia and the Middle East [30, 31]. These mutations, prevalent in genotype B and C viruses in Asia, might enhance viral replication, and thereby induce stronger immune responses, resulting in the development of fulminant hepatitis B. Most recently, these mutations have also been reported to be associated with the reactivation of HBV [18].

Host risk factors: loss of anti-HBs, use of rituximab, immune reconstitution

The anti-CD20 monoclonal antibody rituximab induces transient mature peripheral B cell depletion. In a multi-institutional phase II trial for patients with relapsed or refractory B cell lymphoma, rituximab was shown to almost completely deplete normal mature B cells for an average period of 6–9 months [5]. Recovery to normal levels may take 9–12 months. Because CD20 is not expressed by the antibody-forming plasma cells, hypogammaglobulinemia is rare, occurring in only 14% of patients receiving rituximab in clinical trials. Several large-scale randomized trials were subsequently conducted to assess the safety and efficacy of rituximab combination chemotherapy [6, 32]. In these trials, the addition of rituximab was not found to significantly increase infectious events. However, long-term treatment with rituximab, or HSCT together with rituximab, may result in the increased occurrence of hypogammaglobulinemia [33, 34] and may affect the clinical course, especially in terms of increased infectious events.

Decreased titers of anti-HBs have been reported to be closely associated with HBV reactivation [1, 35]. Reduced amounts of antibody are likely to contribute to reactivation in patients with resolved HBV infection. In a retrospective study on HBV reactivation in 80 HBsAg-negative lymphoma patients receiving R-CHOP-like or CHOP-like regimens, three important risk factors were identified, namely, male gender, absence of anti-HBs at diagnosis of lymphoma and the use of rituximab [3]. Multivariate analysis revealed that rituximab-plus-steroid combination chemotherapy was an important risk factor for HBV reactivation in 244 HBsAg-negative lymphoma patients who required chemotherapy [2].

HBV reactivation may also be associated with the immunological changes following either autologous or allogeneic HSCT [20–23, 36, 37]. The occurrence of acute or chronic graft versus host disease that requires the use of immunosuppressive drugs, such as steroids, cyclosporine or tacrolimus for long-term treatment, as well as delayed immune reconstitution may result in late onset HBV reactivation—especially in the allogeneic setting. In fact, some instances of HBV reactivation have been reported several years after allogeneic HSCT [37, 38]. It is therefore necessary to remain on the alert for such a late onset pattern.

Diagnosis and definition of HBV reactivation

The measurement of HBV-DNA and related serum markers, such as HBsAg, is essential for the diagnosis of HBV

reactivation. It remains important to rule out any other clinical conditions which may cause hepatitis.

As yet, there is no consensus on the definition of HBV reactivation. Among HBsAg-positive patients, it has been suggested that HBV reactivation should be defined as a one log or greater increase of HBV-DNA viral load compared to baseline, in addition to liver damage [39]. On the other hand, among HBsAg-negative patients, the definition of HBV reactivation has often been reported as the reappearance of HBsAg or the de novo detection of HBV-DNA in the blood [2, 3].

The clinician is advised to take the following precautions when considering a diagnosis of HBV reactivation:

1. A recent history of receiving a blood transfusion; consider a differential diagnosis of transfusion-transmitted hepatitis B.
2. Antibody titer may be decreased by immunosuppressive therapy or systemic chemotherapy [1, 35]; screen for HBV-related serum markers, such as anti-HBc and anti-HBs before initiating treatment.
3. Clinical course and prognosis of HBV reactivation are very different from acute hepatitis [40]; immediate initiation of antiviral therapy is extremely important for hepatitis due to HBV reactivation.

HBV reactivation in HBsAg-positive patients

Most cases of HBV reactivation associated with chemotherapy occur in HBsAg-positive patients, who are considered to be at high risk for HBV reactivation [41]. In particular, the use of steroids should be avoided in HBsAg-positive patients with lymphoma who are under chemotherapy and in those receiving HSCT. Prophylaxis with antiviral drugs for preventing HBV reactivation is important in these patients.

Before the rituximab era, 24–53% of HBsAg-positive patients on cancer chemotherapy experienced HBV reactivation. Accordingly, Yeo et al. [42] reported that HBV reactivation occurred in 47 of 193 (24%) of HBsAg-positive lymphoma patients who received systemic chemotherapy. Lok et al. [41] reported reactivation in 13 of 27 (48%) HBsAg-positive patients with malignant lymphoma following systemic chemotherapy. Lau et al. [43] conducted a randomized trial in 30 HBsAg-positive lymphoma patients either receiving or not receiving anti-viral drug prophylaxis during systemic chemotherapy. They found no reactivation in patients receiving prophylaxis, but eight of 15 (53%) patients without prophylaxis had HBV reactivation. More recently, Pei et al. [44] reported an extremely high incidence of HBV reactivation in HBsAg-positive lymphoma patients receiving rituximab-containing chemotherapy. Without prophylaxis in this setting, HBV reactivation occurred in eight of ten (80%) patients. The impact of rituximab on HBV reactivation in HBsAg-positive patients has not yet been studied. It remains necessary to collect clinical data on HBV reactivation in order to develop approaches to prevent it from occurring.

HBV reactivation in HBsAg-negative patients

Until recently, HBsAg-negative patients (including those with occult hepatitis B and resolved hepatitis B) were not recognized as being at risk for HBV reactivation when receiving conventional chemotherapy. This view is supported by the results of Lok et al. [41], who reported HBV reactivation in only 2.7% (2 of 72) of HBsAg-negative patients, which was far lower than the 48% of HBsAg-positive patients showing reactivation.

However, HBV reactivation has been sporadically reported in HBsAg-negative patients receiving rituximab-containing chemotherapy. The first case was reported by Devite et al. [1] in 2001. In 2006, Hui et al. [2] reported that of 244 HBsAg-negative lymphoma patients receiving systemic chemotherapy, eight (3.3%) developed HBV reactivation and all eight were either anti-HBc-positive and/or anti-HBs-positive. Moreover, the incidence of HBV reactivation in the rituximab-plus-steroid combination group was higher, namely, 12.2% (6/49 patients) than that in other combination therapy groups, in which it was only 1.0% (2/195 patients). Multivariate analysis demonstrated that rituximab-plus-steroid combination chemotherapy was a risk factor for HBV reactivation in HBsAg-negative patients. In that cohort, additional studies on archival samples showed a rising HBV-DNA viral load prior to hepatitis in all cases of reactivation, occurring at a median of 18.5 weeks (range 12–28) prior to overt hepatitis development. In 2009, Yeo et al. [3] also reported an HBV reactivation study in 80 HBsAg-negative patients with diffuse large B cell lymphoma receiving standard systemic chemotherapies, such as R-CHOP or CHOP-like regimens. HBV reactivation occurred in five (6.25%) of these patients, with four receiving antivirals and one patient dying of hepatitis. All 5 patients were anti-HBc-positive and anti-HBs-negative and had received R-CHOP. Thus, of 21 anti-HBc-positive patients receiving R-CHOP, five (23.8%) showed HBV reactivation. Therefore, not only HBsAg-positive patients, but also some HBsAg-negative patients, including anti-HBc-positive and/or anti-HBs-positive and/or HBV-DNA-positive patients, should be considered at high risk for HBV reactivation following rituximab-plus-steroid combination chemotherapy.

According to data collected by the Zenyaku Company of Japan, 111 Japanese patients developed serious hepatitis B

between September 2001 and May 2008 following rituximab-containing systemic chemotherapy [4]. These data include information gleaned retrospectively from medical practices, spontaneous reports to the company, reports at academic meetings and results of several investigational studies and clinical trials. The HBsAg status of 97 of these 111 patients prior to rituximab treatment was available: 47 (42%) were HBsAg-positive, and 50 (45%) were HBsAg-negative. The characteristics of HBV reactivation in these 50 HBsAg-negative patients are summarized below.

1. Of the 50 HBsAg-negative patients, only 11 also had a known anti-HBc status before rituximab therapy. All 11 were anti-HBc-positive, of which one and six patients were anti-HBs-positive and -negative, respectively.
2. Following treatment using steroid-containing regimens, such as R-CHOP, 40 patients developed HBV reactivation. Only four and three patients treated with regimens not containing steroids or receiving autologous peripheral blood stem cell transplantation, respectively, experienced HBV reactivation.
3. Among the HBsAg-negative patients, the incidence of fulminant hepatitis (20/50 patients, 40.0%) and mortality (25/50, 50.0%) was higher than that among the HBsAg-positive patients (10/47, 21.3% and 13/47, 27.7%, respectively).
4. Median time to onset of hepatitis from the last administration either of rituximab or chemotherapy was 9.6 weeks. Most of the HBsAg-negative patients developed hepatitis after completion of systemic chemotherapy, as anticipated. The most delayed case was reported to occur at 8.5 months in this cohort.

More recently, Fukushima et al. [45] conducted a prospective cohort study to monitor HBsAg on a monthly basis and HBV-DNA every 3 months in HBsAg-negative but anti-HBc-positive patients with malignant lymphoma both during and after systemic chemotherapy. They found that one of 24 patients developed HBV reactivation, which was diagnosed by an elevated HBV-DNA level—when the HBsAg was still negative. To the best of our knowledge, this study is the first clinical trial of HBV-DNA monitoring aimed at preventing HBV reactivation, and even with a limited number of cases at a single institution, it documents the potential benefit of early diagnosis by HBV-DNA monitoring, and thus earlier treatment.

Prevalence of the HBsAg-negative high-risk group for HBV reactivation (anti-HBc-positive and/or anti-HBs-positive)

According to a study of 3874 specimens collected consecutively over a 2-year period (2005, 2006) for the screening of viral infections before blood transfusion at the Nagoya City University Hospital, Japan, the frequency of HBsAg, anti-HBc and anti-HBs seropositivity was 1.5, 20 and 22%, respectively [4]. In this cohort, anti-HBc-positivity and/or anti-HBs-positivity reached 23.2% (899/3874 patients). In other countries, according to data mostly from single institutions, the frequency of HBsAg and anti-HBc seropositivity is 0.1 and 4.6% in the USA [46], 2.7–5.1 and 17.6–26.2% in Italy [47–49], 7.2 and 34.3% in Singapore [50], 23.2 and 44.2–62.0% in Hong Kong [2, 3] and 15.6 and 20.1% in China [51].

When interpreting data from other countries, it must be borne in mind that the prevalence of HBV infection varies greatly from country to country and in different areas. In Japan, if these anti-HBc-positive and/or anti-HBs-positive patients (23.2%) were actually to be at high risk for HBV reactivation following systemic chemotherapy including rituximab-plus-steroid combinations for malignant lymphoma, it would be necessary to follow up tenfold more patients than HBsAg-positive patients (1.5%), which represent the conventional high-risk group.

The characteristics of HBV reactivation in HBsAg-positive and -negative patients are summarized in Table 2. To establish an optimal strategy for hepatitis prevention and treatment, it is very important to recognize the difference between HBsAg-positive and -negative patients with HBV reactivation.

Management of HBV reactivation following systemic chemotherapy

Initiating antiviral treatment after hepatitis onset may be insufficient to control HBV reactivation. Yeo et al. [42] reported the results of a clinical trial in 32 patients given lamivudine as an antiviral drug for hepatitis due to HBV reactivation. Five patients (16%) died, and 22 (69%) needed to have their chemotherapy schedule modified. Based on the results of a retrospective study in Japan, Umemura et al. [40] reported that the incidence of fulminant hepatitis and mortality following HBV reactivation is high compared to the occurrence of acute hepatitis B. Therefore, it is necessary to identify high-risk groups in advance of chemotherapy, and it is crucial to start antiviral treatment immediately upon HBV reactivation—before hepatitis develops.

There are two current options to prevent HBV reactivation (1) prophylaxis with antiviral drugs, and (2) preemptive therapy starting at the time of detection of HBV-DNA in the blood. For HBsAg-positive patients undergoing systemic chemotherapy, prophylaxis with antiviral drugs is essential, as recommended by the latest American and Japanese guidelines [52, 53]. Antiviral drugs should also be administered to HBsAg-negative but HBV-DNA-positive

Table 2 Characteristics of HBV reactivation in HBsAg-positive and -negative patients

Characteristics	HBsAg-positive	HBsAg-negative high-risk group (anti-HBc-positive and/or anti-HBs-positive)
Seroprevalence of HBV infection	1.5% in Japan (Nagoya)	23.2% in Japan
	0.1% in USA (MDACC)	4.6% in USA[a]
	2.7–5.1% in Italy	17.6–26.2% in Italy[a]
	7.2% in Singapore	34.3% in Singapore[a]
	15.6% in China	20.1% in China[a]
	23.2% in Hong Kong	44.2–62.0% in Hong Kong[a]
Diagnosis and definition	A one log or greater increase in HBV-DNA level compared to baseline, with hepatitis	Reappearance of HBsAg and/or detection of HBV-DNA by RTD-PCR
Risk of HBV reactivation	20–50% on conventional chemotherapy	1.0–2.7% on conventional chemotherapy
	80% on rituximab-containing chemotherapy	12.2–23.3% on rituximab-plus-steroid combination
	>50% on HSCT	14–20% on HSCT
Risk factors on prechemotherapy	High viral load of HBV	Anti-HBs-negative
	HBeAg-positive	
	Liver cirrhosis or hepatocellular carcinoma	
Time of HBV reactivation	Most cases occur during and after chemotherapy, but HBsAg-positive cases with high viral loads may often occur at early stage of chemotherapy	Most cases occur at the end of chemotherapy and after completion of chemotherapy
		Median time to onset of hepatitis was 9.6 weeks
		The most delayed case was reported to occur at 8.5 months after chemotherapy (Zenyaku Company data)
		Some cases occurred at several years after HSCT
Rise in HBV-DNA prior to hepatitis	There are several patterns	Rising HBV-DNA occurred at a median of 18.5 weeks (range 12–28) prior to hepatitis due to HBV reactivation

HSCT Hematopoietic stem cell transplantation, *MDACC* MD Anderson Cancer Center, *RTD-PCR* real-time detection PCR

[a] The percentage shows the frequency of seropositivity for anti-HBc (not including cases seronegative for anti-HBc but seropositive for anti-HBs) in each cohort

patients who are potentially at an even greater risk for HBV reactivation. No standard management to prevent HBV reactivation has yet been established for HBsAg-negative patients seropositive for anti-HBc and/or anti-HBs. Nonetheless, an early diagnosis of HBV reactivation is critical to enable early initiation of active antiviral therapy. Preemptive therapy guided by serial HBV-DNA monitoring (monthly during and after chemotherapy for at least 1 year) is a reasonable strategy recommended by the latest Japanese guidelines [53]. If HBV-DNA becomes detectable, antiviral therapy should be started as soon as possible.

The latest CDC and Japanese guidelines recommend that patients receiving cytotoxic or immunosuppressive therapy should be tested for serologic markers of HBV infection, including HBsAg, anti-HBc and anti-HBs [53, 54]. HBV status should be established before any chemotherapy or immunosuppressive therapy is initiated because antibody titers may be reduced by the immunosuppressive action of the treatment. For patients positive for any HBV serological markers, the presence of HBV-DNA should be confirmed by RTD-PCR.

To date, most cases of HBV reactivation in HBsAg-negative patients have occurred in those who were anti-HBc-positive [3–4]. However, there is a report of an anti-HBc-negative but anti-HBs-positive patient developing HBV reactivation following rituximab-plus-steroid combination chemotherapy [2]. Therefore, for routine screening of these patients, both anti-HBc and anti-HBs should be tested as well as HBsAg.

Future perspectives regarding HBV reactivation

Most data on HBV reactivation in HBsAg-negative patients are limited to retrospective studies, so the exact risk of HBV reactivation has not been estimated precisely, and the

viral and host risk factors associated with HBV reactivation have not been analyzed thoroughly.

For HBsAg-negative patients, HBV reactivation is a complication not only of lymphoma treatment but also of treatments for other cancers [26, 55] as well as for autoimmune diseases [56]. Thus, establishing a standard strategy to prevent HBV reactivation is a very important and urgent issue.

A multicenter clinical trial in Japan is now ongoing to evaluate the efficacy and safety of preemptive therapy based on serial HBV-DNA monitoring in HBsAg-negative untreated B cell lymphoma patients seropositive for anti-HBc and/or anti-HBs during rituximab-plus-steroid combination chemotherapy (UMIN000001299).

Acknowledgments Financial support was provided by the Ministry of Health, Labour and Welfare of Japan (grant-in-aid H20-kanen-014 to S.K.).

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Journal of Medical Virology 83:412–418 (2011)

Virological and Clinical Characteristics on Reactivation of Occult Hepatitis B in Patients With Hematological Malignancy

Fuminaka Sugauchi,[1] Yasuhito Tanaka,[2]* Shigeru Kusumoto,[3] Kentaro Matsuura,[4] Masaya Sugiyama,[2] Fuat Kurbanov,[2] Ryuzo Ueda,[3] and Masashi Mizokami[5]

[1]*Department of Gastroenterology, Nagoya City Koseiin Medcial Welfare Center, Nagoya, Japan*
[2]*Department of Virology and Liver Unit, Nagoya City University Graduate School of Medical Sciences, Nagoya, Japan*
[3]*Department of Medical Oncology and Immunology, Nagoya City University Graduate School of Medical Sciences, Nagoya, Japan*
[4]*Department of Gastroenterology and Metabolism, Nagoya City University Graduate School of Medical Sciences, Nagoya, Japan*
[5]*The Research Center for Hepatitis and Immunology, National Center for Global Health and Medicine, Ichikawa, Japan*

The virological characteristics of hepatitis B virus (HBV) implicated in the reactivation of occult hepatitis B in patients who have received hematopoietic stem-cell transplantation or chemotherapy for the hematological malignancy are not well defined. Twenty-eight HBsAg-negative patients who received hematopoietic stem-cell transplantation and 138 HBsAg-negative patients treated for malignant lymphoma with chemotherapy including rituximab were enrolled. Three of the 28 patients (10.7%) received hematopoietic stem-cell transplantation and one of the 138 (0.72%) patients treated for malignant lymphoma with chemotherapy developed de novo HBV hepatitis. Anti-HBc was detected in four and anti-HBs in two patients. Genotype Bj was detected in two and C in two of they all possessed wild-type sequences in the core promoter region. A precore stop mutation (A1896) was detected in a patient with genotype Bj who developed fulminant hepatic failure. HBV DNA was detected in pretreatment HBsAg-negative samples in two of four patients, and the HBV genome sequence identified from sera before chemotherapy and at the time of de novo HBV hepatitis showed 100% homology. In an in vitro replication model, genotype Bj with the A1896 clone obtained from a fulminant case had a replication level much higher than clones obtained from de novo hepatitis B patients with genotype Bj or C with G1896. In conclusion, this is the first report demonstrating de novo hepatitis B from the reactivation of occult HBV infection confirmed by molecular evolutional analysis. The fulminant outcome of HBV reactivation can be associated with genotype Bj exhibiting high replication due to the A1896 mutation. ***J. Med. Virol. 83:412–418, 2011.*** © 2011 Wiley-Liss, Inc.

KEY WORDS: Hepatitis B virus; occult infection; reactivation; De novo; HBsAg

INTRODUCTION

Approximately 3 billion people, one half of the world population, have been exposed to hepatitis B virus (HBV), of whom approximately 350 million are infected persistently [Lee, 1997]. Reactivation of hepatitis B is a well-recognized complication in hepatitis B surface antigen (HBsAg)-positive patients when they undergo cytotoxic chemotherapy for malignant disease [Hoofnagle et al., 1982; Lok et al., 1991]. HBV reactivation has also been reported in patients with resolved HBV infection, as evidenced by the clearance of circulating HBsAg and the appearance of antibodies to hepatitis B core antigen (anti-HBc) with or without antibodies to hepatitis B surface antigen (anti-HBs) [Hui et al., 2006; Umemura et al., 2008; Kusumoto et al., 2009; Yeo et al., 2009]. In these patients, a low level of HBV replication has been shown to persist in the liver and in

Grant sponsor: Ministry of Education, Culture, Sports, Science and Technology, Japan.

*Correspondence to: Yasuhito Tanaka, MD, PhD, Department of Virology and Liver Unit, Nagoya City University Graduate School of Medical Sciences, Kawasumi, Mizuho, Nagoya 467-8601, Japan. E-mail: ytanaka@med.nagoya-cu.ac.jp

Accepted 21 October 2010

DOI 10.1002/jmv.21995

Published online in Wiley Online Library (wileyonlinelibrary.com).

peripheral-blood mononuclear cells for decades [Kuhns et al., 1992; Fong et al., 1993]. HBV reactivation has been reported in this setting after transplantation, immunosuppressive therapy, and allogeneic and autologous hematopoietic stem-cell transplantation, with the reappearance of HBsAg [Dhedin et al., 1998; Seth et al., 2002]. De novo hepatitis B is of particular concern in this subset of patients because it commonly results in severe liver dysfunction and fatal hepatitis [Sarrecchia et al., 2005].

Many HBV carriers exist in Asian countries and anti-HBc-seropositive occult carriers have been estimated at 20%–60% in their populations [Kiyosawa et al., 1994; Kusumoto et al., 2009]; however, the incidence, effective monitoring, and risk assessment for reactivation of HBV during hematopoietic stem-cell transplantation or cytotoxic chemotherapy need further investigation. In addition, the virological characteristics of HBV on the reactivation of occult hepatitis B are not well defined. In this study, we investigated the incidence of HBV reactivation and influences of HBV genotype and viral mutations on the reactivation of occult hepatitis B in patients who received hematopoietic stem-cell transplantation or cytotoxic chemotherapy for hematological malignancy.

METHODS

Patients

In this retrospective survey, 28 HBsAg-negative patients who received hematopoietic stem-cell transplantation for hematological malignancy and 138 HBsAg-negative patients treated for malignant lymphoma with rituximab plus cyclophosphamide, doxorubicin, vincristine, and prednisone (R-CHOP) at Nagoya City University Hospital were analyzed.

Hepatitis B virus reactivation was diagnosed when the HBsAg status changed from negative to positive after the initiation of chemotherapy or hematopoietic stem-cell transplantation, with an elevation of serum HBV DNA to more than 10^5 copies/mL. HBV DNA was retrospectively quantified by real-time polymerase chain reaction (PCR) using stored samples from patients with HBV reactivation as described below.

Serological Markers of HBV Infection

HBsAg, HBeAg, and the corresponding antibody (anti-HBe) were determined by enzyme immunoassay (EIA) (AxSYM; Abbott Japan, Tokyo, Japan) or chemiluminescence enzyme immunoassay (CLEIA) (Fujirebio, Tokyo, Japan). Anti-HBc of IgG classes was determined by radioimmunoassay (Abbott Japan).

Quantitation of Serum HBV DNA

Hepatitis B virus DNA sequences spanning the S gene were amplified by real-time detection polymerase chain reaction (RTD-PCR) in accordance with the previously described protocol with a slight modification; it has a detection limit of 100 copies/mL [Sugiyama et al., 2009].

Sequencing and Molecular Evolutionary Analysis of HBV

Nucleic acids were extracted from serum samples (100 μL) using the QIAamp DNA extraction kit (Qiagen, Hilden, Germany) and subjected to PCR for amplifying genomic areas bearing enhancer II/core promoter/precore/core regions [nt 1628–2364], as described previously [Sugauchi et al., 2002]. Amplicons were sequenced directly using the ABI Prism Big Dye ver. 3.1 kit in the AMI 3100 DNA automated sequencer (Applied Biosystems, Foster City, CA, USA). All sequences were analyzed in both forward and reverse directions. HBV genotypes were determined by molecular evolutionary analysis. Reference HBV sequences were retrieved from the DDBJ/EMBL/GenBank database and aligned by CLUSTAL X, and then genetic distances were estimated with the 6-parameter method in the Hepatitis Virus Database (http://s2as02.genes.nig.ac.jp/) [Shin et al., 2008]. Based on obtained distances, phylogenetic trees were constructed by the neighbor-joining (NJ) method with the mid-point rooting option. To confirm the reliability of the phylogenetic trees, bootstrap resampling tests were performed 1000 times.

Construction of Plasmid and Site-directed Mutagenesis of HBV DNA

Serum samples were obtained from three patients infected with genotype Bj and a patient with Ce. HBV DNA was extracted from 100 μL serum using QIAamp DNA blood kit (Qiagen). Four primer sets were designed to amplify two fragments covering the entire HBV genome. Amplified fragments were inserted into pGEM-T Easy Vector (Promega, Madison, WI) and cloned in DH5a competent cells (TOYOBO, Osaka, Japan). At least five clones of each fragment were sequenced and the consensus sequence determined. Among them, those containing the consensus sequence were identified and adopted as templates for further construction. Finally, a 1.24-fold amount of the HBV genome (nt 1413–3215/1–2185), sufficient to transcribe oversized pregenome and precore mRNA, was constructed into pUC19 vector (Invitrogen Corp., Carlsbad, CA). For site-directed mutagenesis, wild-type HBV was digested by *HindIII* and *EcoO65I* and ligated with the fragment carrying T1762/A1764 to produce 1.24-fold the amount of the genome carrying the precore stop-codon mutation.

Cell Culture and DNA Transfection

For the standard replication assay, 10-cm-diameter dishes were seeded with 1×10^6 Huh7 cells each. After 16 hr of culture, cells were transfected with 5 μg DNA construct using the FuGENE 6 transfection reagent (Roche Diagnostics, Indianapolis, IN) and harvested 3 days later. Transfection efficiency was measured by cotransfecting with 0.5 μg reporter plasmid expressing secreted alkaline phosphatase and estimating its enzymatic activity in the culture supernatant.

Southern Blot Hybridization

Hepatitis B virus DNA samples from cells at day 3 in culture were separated on 1.2% w/v agarose gel, transferred to a positive-charged nylon membrane (Roche Diagnostics), and hybridized with full-length HBV DNA labeled with alkaline phosphatase. Detection was performed with CDP-star (Amersham Biosciences, Piscataway, NJ), and signals were captured using the LAS-1000 image analyzer (Fuji Photo Film, Tokyo, Japan). Viral replication was compared between genotypes by quantifying dots of the single-strand band.

RESULTS

Clinical and Virological Features of Patients who Developed De Novo HBV

Three of the 28 patients (10.7%) received hematopoietic stem-cell transplantation and one of the 138 (0.72%) patients treated for malignant lymphoma developed de novo hepatitis B. The demographic and clinical features of the four patients who experienced HBV reactivation are summarized in Table I (cases A–D). All had normal liver functions before initiation of treatment. The mean age of the four patients with de novo hepatitis B was 49 ± 10 years and three were male. Two patients (cases A and C) were positive for anti-HBs, and three (cases A, B, and D) were positive for anti-HBc at the baseline of hematopoietic stem-cell transplantation or chemotherapy. Dynamics of serial serum alanine aminotransaminase (ALT), total bilirubin (T.Bil), prothrombin time (PT), HBV DNA levels, and the HBV serological status of the four patients are shown in Figure 1. Three patients except case A had abnormal ALT levels and all received an oral dideoxynucleotide (Lamivudine or Entecavir) as soon as HBV reactivation was suspected. Three patients (A, C, and D) recovered from the HBV reactivation, but patient B died from severe progressive liver failure despite intensive care.

Virological features are summarized in Table I. HBV genotype Bj was detected in 2 (50%) and C in 2 (50%) patients who all possessed wild-type sequences (A1762/G1764) in the core promoter region, although the precore stop mutation (G1896A) was detected in only one patient with genotype Bj who developed fulminant hepatic failure. None of the patients had PreS deletion or escape mutations in the S-region of HBV.

DNA Sequencing and Phylogenetic Analysis

Hepatitis B virus DNA was quantified retrospectively by RTD-PCR in stored samples of the four patients with HBV reactivation. Evidence of occult HBV infection at the time of the HBsAg-negative status (before commencement of chemotherapy and hematopoietic stem-cell transplantation) was detected by RTD-PCR in patients B and C. To determine the source of HBV infection, sera from patients B and C before commencement of chemotherapy or hematopoietic stem-cell transplantation (case B-1 and C-1) and at the time of HBV reactivation (case B-2 and C-2) were subjected to HBV core promoter and precore region (481 bp) sequencing. Sequences encompassing the core promoter to precore region obtained from sera before commencement of

TABLE I. Demographic, Biochemical and Virological Characteristics of Four Patients With HBV Reactivation

	Cases			
	A	B	C	D
Age (years)	36	47	60	52
Sex	Male	Male	Female	Male
Hematological malignancy	Malignant lymphoma	Acute myeloid leukemia	Multiple myeloma	Malignant lymphoma
Source of HSCT	Cord Blood (allogenic)	Bone marrow (allogenic)	Peripheral Blood (autologous)	None
Immune suppression therapy	Cyclosporin A	Tacrolimus, Prednisolone	Prednisolone	None
Chemotherapy	None	None	MCP	Rituximab-CHOP
HBV serology and DNA before therapy				
HBsAg	−	−	−	−
Anti-HBs	+	−	+	−
Anti-HBc	+	+	−	+
HBV-DNA	Negative	2.8 log copy/ml	2.2 log copy/ml	Negative
Peak levels of				
HBV-DNA	6.0 log copy/mL	8.6 log copy/mL	6.2 log copy/mL	6.3 log copy/mL
ALT (U/mL)	31	1435	938	1040
T. Bilirubin	0.6	18.5	0.9	4.8
ETV or LAM given	+	+	+	+
Outcome	Recovered	Died	Recovered	Recovered
HBV genotype	Bj	Bj	Ce	Ce
Core Promoter (1762/1764)	Wild	Wild	Wild	Wild
Pre Core (1896)	Wild	Mutant	Wild	Wild
PreS deletion	None	None	None	None

HSCT, hematopoietic peripheral stem cell transplantation; R-CHOP, rituximab, cyclophosphamide, doxorubicin, vincristine, prednisolone; MCP, ranimustine, cyclophosphamide, prednisolone; ETV, entecavir; LAM, lamivudine.

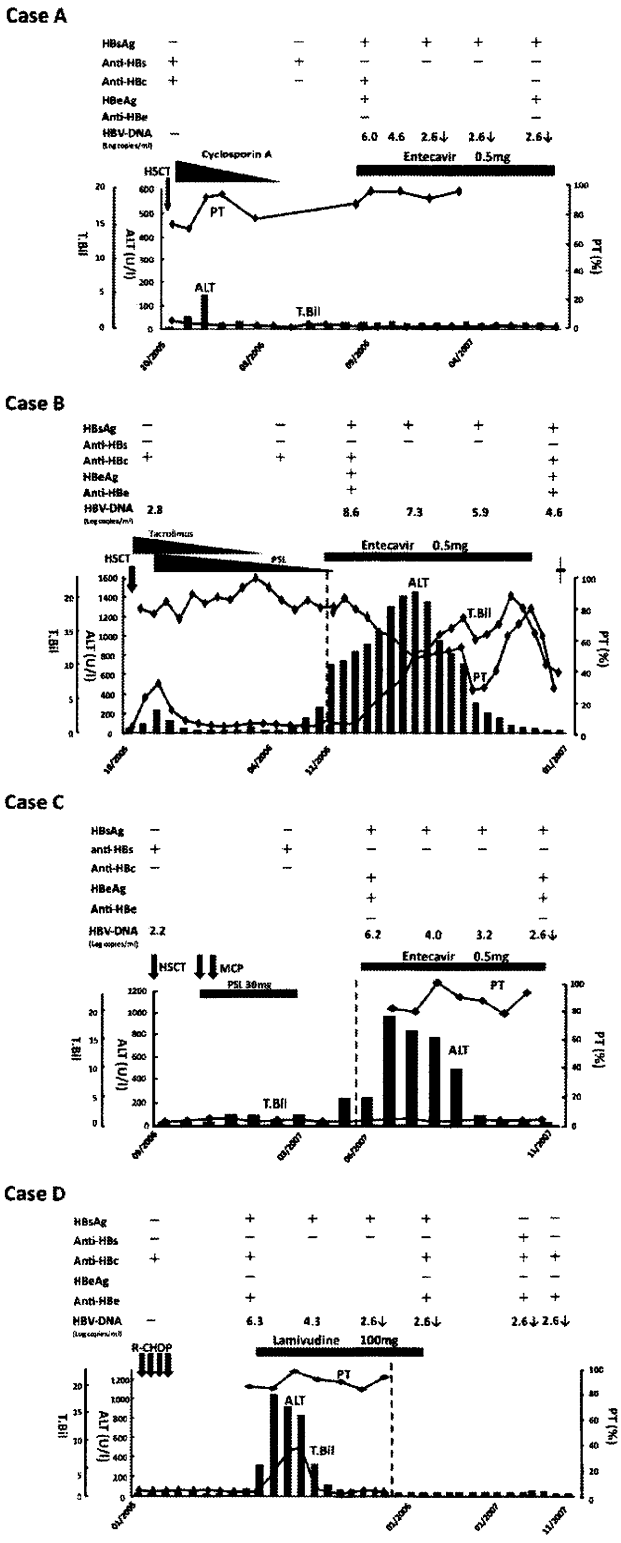

Fig. 1. Clinical course of 4 patients who developed reactivation of HBV. Changes of serial serum ALT, T.Bil, PT, HBV DNA, and HBV serology are shown. Hematopoietic stem-cell transplantation, hematopoietic peripheral stem cell transplantation; PSL, prednisolone; R-CHOP, rituximab, cyclophosphamide, doxorubicin, vincristine, prednisolone; MCP, ranimustin, cyclophosphamide, prednisolone.

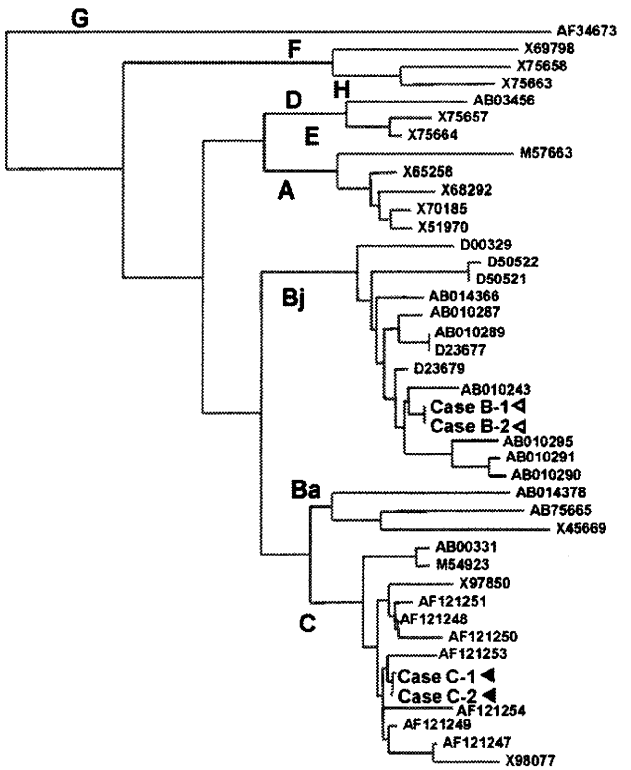

Fig. 2. Sera from cases B and C before chemotherapy or hematopoietic stem-cell transplantation (case B-1 and C-1) and at the time of HBV reactivation (case B-2 and C-2) were sequenced from the core promoter to precore region (481 bp). The phylogenetic tree demonstrated that patients B and C had de novo HBV hepatitis from reactivation of occult HBV infection.

chemotherapy and at the time of de novo HBV hepatitis showed 100% homology, and on the pylogenetic tree they were clustered together (Fig. 2). These results demonstrated that patients B and C had de novo HBV hepatitis from reactivation of occult HBV infection.

Wild-type HBV and Precore and Core Promoter Mutant Replication In Vitro

Figure 3 compares Southern blotting densities of HBV replicons constructed on bases of the following isolates: genotype Bj with PC wild-type clone obtained from case A (Bj: PC Wild), genotype Bj with PC mutant-type clone obtained from case B (Bj: PC Mutant) and genotype C with PC wild-type clone obtained from case C (C: PC Wild). The densities of the single-strand band were far higher for the Bj: PC Mutant clone obtained from patient B who had a fulminant outcome, indicating greatly enhanced replicative activity of the precore mutant in vitro.

DISCUSSION

The present study highlighted HBV reactivation in four patients who had resolved HBV infection evidenced by clearance of circulating HBsAg and the appearance of antibodies to anti-HBc with or without anti-HBs. Two patients had occult HBV infection at the time of the

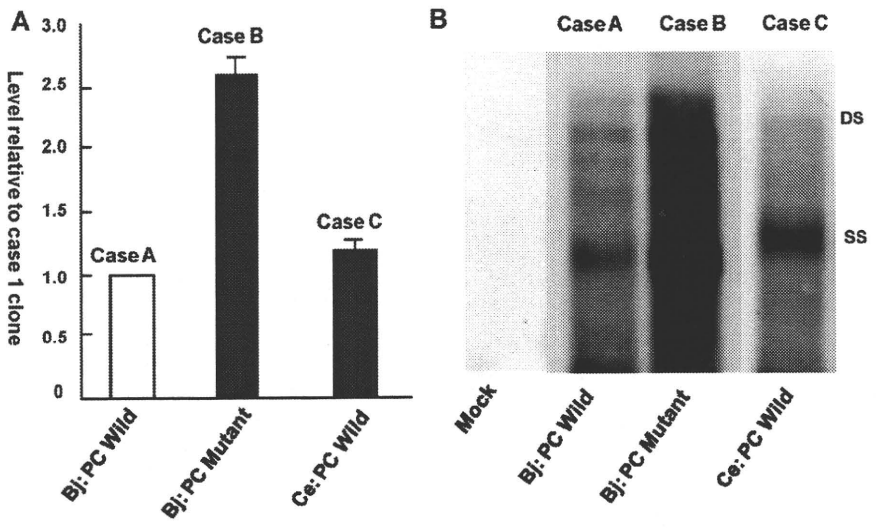

Fig. 3. **A**: Comparison of viral replication among patients in vitro. Southern blot analysis for replicative activity (intracellular HBV DNA) of genotype Bj with precore (PC) wild-type clone obtained from case A (Bj: PC Wild), genotype Bj with PC mutant-type clone obtained from case B (Bj: PC Mutant) and genotype C with PC wild-type clone obtained from case C (C: PC Wild). **B**: Quantitation of single-stranded HBV-DNA. DS, double strand; SS, single strand.

HBsAg-negative status before chemotherapy, as detected retrospectively by RTD-PCR. Sequencing of the HBV core promoter and precore region indicated complete matching between isolates from sera before commencement of chemotherapy and at the time of the manifestation of de novo hepatitis B. To our knowledge, this is the first report demonstrating de novo hepatitis B from the reactivation of occult HBV infection confirmed by molecular evolutionary analysis.

Hepatatis B Virus reactivation is known to occur in patients after solid organ transplant, allogenic, and autologous hematopoietic stem-cell transplantation, and after immunosuppressive therapy. Recently, HBV reactivation has been reported to occur in HBsAg-negative patients receiving rituximab-containing chemotherapy. In this study, one of the 138 (0.72%) lymphoma patients treated by R-CHOP developed de novo hepatitis B. As 20–25% of hospitalized HBsAg-negative patients were positive for anti-HBc and/or anti-HBs in our hospital, around 4% developed HBV reactivation, suggesting a high risk for this group. Hui et al. reported that 8 of 244 HBsAg-negative lymphoma patients receiving systemic chemotherapy developed new-onset hepatitis B (3.3%) [Hui et al., 2006]. These 8 patients were seropositive for either HBc or HBs antibody. Furthermore, the incidence of HBV reactivation in this cohort was higher in those receiving rituximab-plus-steroid combination than in those receiving other combination therapy: 12.2% (6 of 49) and 1.0% (2 of 195), respectively [Hui et al., 2006]. Multivariate analysis demonstrated that rituximab-plus-steroid combination chemotherapy is a risk factor for HBV reactivation. In another report Yeo et al. [2009] investigated 80 HBsAg-negative patients with diagnosed diffuse large B-cell lymphoma who were receiving R-CHOP or CHOP and had HBV reactivation as high as 6.25% (5/80). All five patients receiving R-CHOP had an anti-HBc-positive and anti-HBs-negative status at the treatment baseline. Thus, of 21 anti-HBc-positive lymphoma patients receiving R-CHOP in that study, five (23.8%) developed HBV reactivation [Yeo et al., 2009]. These observations strongly suggest that not only HBsAg-positive patients, but also HBsAg-negative patients with anti-HBc and/or anti-HBs and/or detectable serum HBV-DNA must be considered as a group at high risk for HBV reactivation following rituximab-plus steroid combination chemotherapy.

Eight genotypes have been classified by sequence divergence of >8% in the entire HBV genome composed of approximately 3200 nucleotides (nt), and designated A to H in the order of documentation [Miyakawa and Mizokami, 2003]. The genotypes have distinct geographical distribution and are associated with the severity of liver disease as well as response to antiviral therapies [Miyakawa and Mizokami, 2003]. Furthermore, subgenotypes have been reported for genotype A, B, and C, and named Aa (Asian/African type) and Ae (European type) [Sugauchi et al., 2004a], Bj (Japanese type) and Ba (Asian type) [Sugauchi et al., 2003; Sugauchi et al., 2004b], as well as Ce (east Asian type) and Cs (southeast Asian type) [Tanaka et al., 2005]. There are increasing lines of evidence that Aa and Ae, as well as Ba and Bj, influence the replication of HBV and have clinical relevance [Sugauchi et al., 2002; Tanaka et al., 2005]. In this study, the precore stop mutation (G1896A) was detected only in a patient with genotype Bj who developed fulminant hepatic failure. A recent cross-sectional study in which 23 patients with reactivation were compared with 529 acute hepatitis B patients in Japan, revealed that genotype B is more frequent among patients with HBV reactivation [Umemura et al., 2008]. Ozasa et al. [2006] compared 40 patients with fulminant and 261 with acute self-limited hepatitis, revealing that subgenotype Bj is associated

with the development of fulminant hepatitis. Furthermore, the frequency of both precore (G1896A) and core-promoter (A1762T/G1764A) mutations was significantly higher in patients with fulminant hepatitis than in those with acute self-limited hepatitis. These results suggested that HBV genotypes as well as gene mutations could be associated with the onset of fulminant hepatitis caused by HBV reactivation.

In this study, in vitro replication analysis demonstrated that the intracellular HBV DNA level of the wild-type genotype Bj was comparable with that of wild-type Ce, and precore (G1896A) mutation enhanced the replication capacity of genotype Bj (Fig. 3). Sugiyama et al. [2006] reported that the extracellular HBV DNA level of the genotype Bj clone replicating in vitro was much higher than other genotypes (even those with comparably high intra-cellular expression; i.e., subgenotype Ba and genotype C), suggesting that the Bj subgenotype might have a stronger inclination toward extracellular secretion. Such a high concentration of secreted viruses during subgenotype Bj infection in patients might cause the rapid and extensive infection of intact hepatocytes. Extremely high intracellular expressions of viral DNA were observed for the genotype Bj replicon with a precore stop-codon mutation reconstructed on the basis of an isolate from a patient with fulminant hepatitis, in agreement with previous in vitro and in vivo observations. Together, these clinical observations and experimental results strongly implicate the mutation in the precore region in fulminant manifestations of hepatitis.

In our patients, entecavir was chosen as the initial treatment rather than lamivudine. Entecavir achieves better HBV-DNA suppression and has a higher resistance barrier, which is important in a long treatment course in order to avoid the emergence of drug resistance; therefore, entecavir may be a promising drug for first-line treatment of the reactivation of occult hepatitis B.

Reactivation of HBV has also been described in the setting of bone marrow transplantation [Dhedin et al., 1998; Seth et al., 2002]. In the present study, de novo hepatitis B developed in 10.7% (3/28) of patients received hematopoietic stem-cell transplantation. In the recent report of a study on 137 consecutive patients (23 positive for HBsAg, 37 positive for anti-HBs, and 77 negative for HBV) who underwent hematopoietic stem-cell transplantation, hepatitis developed in 32 patients (23%), considered to be due to hepatitis B reactivation in 13 of the 32 patients [Kusumoto et al., 2009]. Hepatitis due to HBV reactivation was more common in HBsAg-positive patients than in HBsAg-negative patients (11 of 23 versus 2 of 114). It is concluded that in HBsAg-negative patients, the risk of HBV reactivation is higher for those receiving hematopoietic stem-cell transplantation (14–20%) than for those on conventional chemotherapy (1.0–2.7%) [Kusumoto et al., 2009].

In conclusion, this study showed that the fulminant outcome of the reactivation of occult hepatitis B in patients receiving hematopoietic stem-cell transplantation or cytotoxic chemotherapy for hematological malignancy was associated with genotype Bj, accompanied by high replication due to G1896A mutation.

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VOLUME 29 · NUMBER 4 · FEBRUARY 1 2011

JOURNAL OF CLINICAL ONCOLOGY

CORRESPONDENCE

Clinical Significance of Hepatitis B Virus (HBV) -DNA Monitoring to Detect HBV Reactivation After Systemic Chemotherapy

TO THE EDITOR: In *Journal of Clinical Oncology*, Niitsu et al[1] recently reported a prospective study to evaluate the risk of hepatitis B virus (HBV) reactivation in 51 HBV-recovered patients with diffuse large B-cell lymphoma who received rituximab-containing chemotherapy. Although no standard management of HBV reactivation was established for these patients, the study documented the potential benefit of early diagnosis by HBV-DNA monitoring, and thus, earlier treatment.

However, several issues concerning management of HBV reactivation are described in this report. First, Niitsu et al[1] reported that among patients who were positive for antibodies to the hepatitis B surface antigen (anti-HBs) and antibodies to the hepatitis B core antigen, the level of anti-HBs titer was checked once per week, and when the anti-HBs decreased to a level of less than 200 mU/mL, serum HBV-DNA was measured monthly. HBV reactivation in most patients who are negative for hepatitis B surface antigens and who have high anti-HBs titers has been reported to occur during periods after chemotherapy when anti-HBs titers are decreased. However, in a few cases, HBV reactivation may have occurred in patients with sustained high titers of anti-HBs, for instance, the patient who was reported to have maintained high titers of anti-HBs after rituximab-containing chemotherapy.[2] In this situation, escape mutants were demonstrated by sequence analysis, and that might have induced HBV reactivation, which suggests that simply monitoring anti-HBs titers may be insufficient to diagnose HBV reactivation at an early stage. Therefore, to prevent HBV reactivation, HBV-DNA monitoring is necessary in all patients who have recovered from HBV infection, both those without anti-HBs (or with low titers) as well as those with high anti-HBs titers.

Second, Niitsu et al[1] reported that the serum HBV-DNA load was determined by a quantitative reverse transcriptase polymerase chain reaction (detection value of 1.8 to 8.8 log copies/mL;COBAS AmpliPrep/COBAS TaqMan HBV-Test, Roche Diagnostics Japan, Tokyo, Japan). However, in October, 2006, when this study began, it was only possible to measure the HBV-DNA load in plasma with the TaqMan HBV-Test version 1.0.[3] Then, in 2009, a new version, TaqMan HBV-Test version 2.0,[4] became available worldwide, and the HBV-DNA load became measurable in serum. Therefore, it is prudent to note that there is an inconsistency with respect to sample selection for HBV-DNA measurements in the report by Niitsu et al.

Niitsu et al[1] also reported that patients were enrolled onto the trial for 3 years, and that HBV-DNA was determined during and after chemotherapy for 2 years. However, given that the median follow-up period for the patients who were enrolled onto this study was not indicated, it is likely that the evaluation of the risk of HBV reactivation was affected because the dropout rate during follow-up and in the event of death are competing risks.

This prospective study demonstrated the possibility of using HBV-DNA monitoring to make an early diagnosis of HBV reactivation. Thus, for patients who have recovered from HBV infection, preemptive antiviral therapy that begins when HBV-DNA is detected in the blood is a reasonable strategy.[5-7] However, the clinical evidence to date does not provide enough information to determine the optimal frequency and duration of such HBV-DNA monitoring. Well-designed clinical trials are needed to investigate the efficacy and safety of preemptive therapy guided by serial HBV-DNA monitoring.

Shigeru Kusumoto and Yasuhito Tanaka
Nagoya City University Graduate School of Medical Sciences, Nagoya, Japan

Masashi Mizokami
The Research Center for Hepatitis and Immunology, National Center for Global Health and Medicine, Ichikawa, Chiba, Japan

Ryuzo Ueda
Nagoya City University Graduate School of Medical Sciences, Nagoya, Japan

AUTHORS' DISCLOSURES OF POTENTIAL CONFLICTS OF INTEREST

Although all authors completed the disclosure declaration, the following author(s) indicated a financial or other interest that is relevant to the subject matter under consideration in this article. Certain relationships marked with a "U" are those for which no compensation was received; those relationships marked with a "C" were compensated. For a detailed description of the disclosure categories, or for more information about ASCO's conflict of interest policy, please refer to the Author Disclosure Declaration and the Disclosures of Potential Conflicts of Interest section in Information for Contributors.

Employment or Leadership Position: None **Consultant or Advisory Role:** None **Stock Ownership:** None **Honoraria:** None **Research Funding:** Shigeru Kusumoto, Chugai Pharmaceutical; Yasuhito Tanaka, Chugai Pharmaceutical **Expert Testimony:** None **Other Remuneration:** None

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DOI: 10.1200/JCO.2010.33.0332; published online ahead of print at www.jco.org on December 20, 2010

