表2. B 型肝炎の都道府県別届出医療機関数とその報告数 2009～2010 年

	届出医療機関数	報告数	1医療機関当たり報告数
北海道	3	3	1
青森	2	3	1-2
岩手	0	0	・・・
宮城	6	8	1-2
秋田	2	2	1
山形	1	2	1-2
福島	0	0	・・・
茨城	6	7	1-2
栃木	4	5	1
群馬	4	4	1
埼玉	12	13	1-2
千葉	4	5	1-2
東京	29	68	1-10
神奈川	14	24	1-4
新潟	0	0	・・・
富山	3	4	1-2
石川	1	1	1
福井	2	2	1
山梨	1	1	1
長野	1	1	1
岐阜	1	1	1
静岡	4	9	1-2
愛知	16	26	1-5
三重	1	2	2
滋賀	2	2	1
京都	7	8	1-2
大阪	24	32	1-4
兵庫	17	30	1-4
奈良	1	1	1
和歌山	0	0	・・・
鳥取	0	0	・・・
島根	2	2	1
岡山	4	16	5-6
広島	9	14	1-5
山口	1	1	1
徳島	0	0	・・・
香川	0	0	・・・
愛媛	5	5	1
高知	2	2	1
福岡	7	16	1-6
佐賀	1	1	1
長崎	2	4	1-3
熊本	3	3	1
大分	4	4	1
宮崎	8	13	1-4
鹿児島	1	1	1
沖縄	2	5	2-3
	219	351	

2011 年 1 月 7 日現在

厚生労働科学研究費補助金　肝炎等克服緊急対策研究事業
「B型肝炎ジェノタイプA型感染の慢性化など本邦における実態とその予防に関する研究」
平成22年度　分担研究報告書

ジェノタイプの異なるB型肝炎ウイルス表面抗原（HBsAg）の検出感度比較

研究分担者：水落利明　国立感染症研究所　血液・安全性研究部　室長

研究要旨：本邦で近年増加が見られるジェノタイプA型HBVの感染について、その実態を明らかにするためには、高感度で特異的、かつHBVジェノタイプの違いに左右されないHBsAg検出キットが求められる。本研究では、HBVのジェノタイプが異なることによるただ一カ所のアミノ酸置換により、HBsAgの検出感度が大きく変化することを明らかにした。このことは、HBsAgをそれらのジェノタイプに関わらず漏れなく検出できるようなキットを選択する上で重要な知見である。また、WHOが作成中であるジェノタイプ別のHBsAg国際標準品について、その候補検体を、国内で開発されたキットを用いて測定した結果、ジェノタイプに関わらず同等の感度で検出されることが示され、国際標準品として適することが示された。

A. 研究目的

B型肝炎ウイルス（HBV）表面抗原（HBsAg）は、HBV感染の有無を初期診断する上で重要なマーカーのひとつである。HBVには遺伝子配列の相違から、これまでにAからHまでの８種類の遺伝子型（ジェノタイプ）が知られている（注：ごく最近ジェノタイプJが報告されたため９種類となった）。これらの異なるジェノタイプのHBVによりコードされるHBsAgが、国内で承認を受けて販売されている様々なHBsAg検出用診断薬（キット）により同等の感度で検出されるかどうかについて我々は以前検討を行った。その結果、ほとんどのキットではジェノタイプに関わらず同等の感度でHBsAgを検出できることが示された。しかし、ある一種類のキット（Lumipulse II HBsAg：富士レビオ社製）においてはジェノタイプEとFのHBsAgに対する検出感度が他のジェノタイプのHBsAgに比べて明らかに低かった。本研究はこの原因を明らかにする目的で行った。

さらに、WHOからの依頼により、ジェノタイプの異なるHBsAg国際標準品候補検体について、国内で開発されたキットであるHISCL HBsAg（シスメックス社製）を用いて測定を行った。

B. 研究方法

HBsAgのアミノ酸配列をジェノタイプ別に比較したものが図１Aである。ここでジェノタイプEとFでは140番目のアミノ酸（S）が他のジェノタイプ（T）と異なることが示された。そこでこの箇所のアミノ酸を置換したHBsAg（E S140T, F S140T）を遺伝子工学的手法により作成した。これらのアミノ酸置換HBsAgと、ジェノタイプE, F wild typeのHBsAgについて、まずHBsAg定量キットとして国内承認を得ている

Architect HBsAg QT (アボットジャパン社)を用いて測定し、それを基準として 1.6IU/mL から２倍希釈で 0.1IU/mL までの希釈系列検体を作成した。そして Lumipulse II HBsAg キットによりそれら希釈検体の抗原量を測定した。また、このキットに改良を加えた Lumipulse Presto HBsAg キットを用いて同様の測定を行った。

WHO から送付されたジェノタイプ別（A, B, C, D, E, F, H）HBsAg 国際標準品候補検体について指示に従った希釈系列（1:20 から５倍希釈で 1:12,500 まで）を作成し、それらの抗原量を HISCL HBsAg (シスメックス社製)により測定した。

C. 研究結果

以前報告したように（Mizuochi et al. J. Virol. Methods 2006; 136:254-256.）Lumipulse II HBsAg は、ジェノタイプ E と F の HBsAg では、低抗原量（0.1IU/mL, 0.2IU/mL）の検体について陽性と判定できなかった（図１ B）。しかしアミノ酸置換した E S140T と F S140T については抵抗原量の検体でも陽性と判定した（図１ B）。一方、改良を行った Lumipulse Presto HBsAg を用いた場合には、E S140T および F S140T だけでなく、ジェノタイプ E および F の wild type の HBsAg でも低抗原量検体を陽性と判定した（図１ C）。これらの結果は、ひとつのアミノ酸残基が変化するだけで、HBsAg 検出キットの判定が陰性から陽性へと変わることを示している。つまり、これまで HBsAg の変異体（ミュータント）において指摘されていた HBsAg 検出キットによる判定の相違（Coleman et al. J. Med. Virol. 1999; 59:19-24.）と同様な現象が、HBV ジェノタイプの違いにおいても見られることが示された。

WHO から依頼された国際標準品候補検体についての検討結果を図２に示した。ここで明らかなように、HISCL HBsAg キットは、ジェノタイプの違いに関わらずほぼ同等な測定値（IU/mL）を示すことが確認された。

D. 考察

本研究により、HBsAg 内の一カ所のアミノ酸残基が変化することで、HBsAg 検出キットの検出感度が影響を受けることが示された。このような懸念はこれまでにも HBsAg 変異体の検出において指摘されていた。本研究ではジェノタイプの異なる HBV によりコードされた HBsAg の検出において、あるキットではジェノタイプ E と F の HBsAg については検出感度が低いことに注目し、その原因が一カ所のアミノ酸残基の違いにあることを明らかにした。更に HBsAg 検出に用いる抗体の組成を変化させることによってキットの感度を改善させることができた（図１ C : Lumipulse Presto HBsAg）。このような一カ所のアミノ酸残基の違いによる抗原検出系の感度低下は、HBsAg に限った問題ではない。例えば HCV core Ag においては、48 番目のアミノ酸残基が A から T に変わることにより、HCV core Ag キットの検出感度が低下することを既に我々は示している（Mohsan et al. J. Clin. Microbiol. 2009;47:4141-4143.）。

国内での蔓延が懸念されているジェノタイプ A の HBV 感染を早期に診断する上で HBsAg 検出キットの検出感度は重要である。現在国内で販売されているキットでは、ここで紹介した改良品を含め、ジェノタイプの違いによる検出感度の相違は最小限に留まっていることから、診断結果の信頼性は高いと考えられるだろう。

HBV ジェノタイプ別の HBsAg WHO 国際標準品確立にあたり、本研究で実施した各ジェノタ

イプ HBsAg 標準品候補検体の測定は、その目的に大いに貢献することができたと考える。

E. 結論

HBVのジェノタイプが異なることによるただ一カ所のアミノ酸置換が、HBsAg検出キットの感度に大きな影響を与えることを明らかにした。このことは、HBsAgをそれらのジェノタイプに関わらず漏れなく検出できるようなキットを選択する上で重要な知見である。また、WHOが作成中であるジェノタイプ別のHBsAg国際標準品について、その候補検体を、国内で開発されたキットを用いて測定した結果、ジェノタイプに関わらず同等の感度で検出されることが示され、国際標準品として適することが示された。

F. 健康危険情報

該当なし

G. 研究発表

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2.学会発表

なし

H.知的所有権の出願・取得状況

1.特許取得

なし

2.実用新案登録

なし

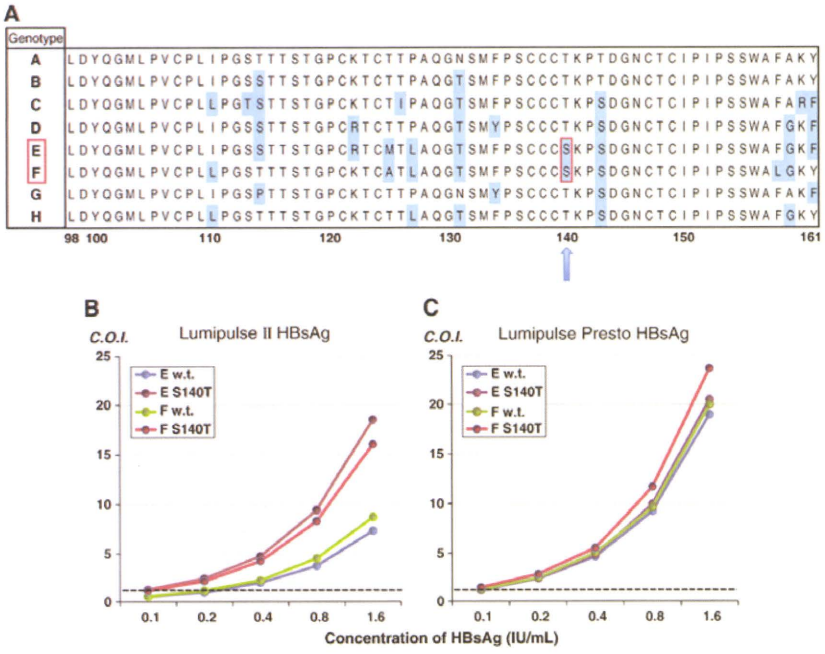

図1：アミノ酸残基置換がHBsAg検出キットの感度に及ぼす影響

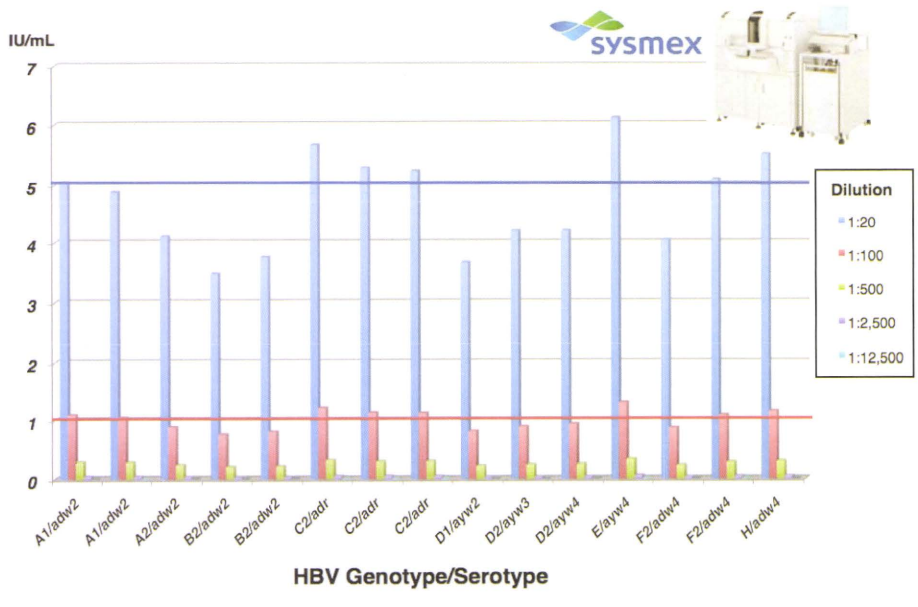

図2：ジェノタイプ別HBsAg WHO国際標準品候補検体の抗原量測定

Ⅲ．研究成果の刊行一覧

研究成果の刊行に関する一覧表

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Ⅳ. 研究成果の刊行物・別刷

Hepatitis B Virus and Its Genotypes: New development in Japan

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Key words HBV, Genotype, Infection

Hepatitis B virus (HBV) is a major public health problem, causing persistent infection in approximately 420 million people in the world. Recently, it was confirmed that HBV occurs in several genotypes with rather distinct geographical distribution. Here I summarize how this discovery changed the concept of acute hepatitis B in Japan.

In 1988, many researchers including our group conducted a molecular evolutionary analysis, which enabled the quantitative estimation of nucleotide sequence variations using all the known nucleotide sequences of hepadnavirus. We found that the mutation rate of HBV DNA was approximately 10,000 times as high as that of human genes and proposed that HBV could be classified into groups according to the nucleotide sequences (genotypes).[1] Currently, the presence of 10 genotypes, from A to J, has been confirmed globally.[2]

We collected approximately 3,000 specimens from all over the world to examine the global geographical distribution of HBV genotypes. The analysis revealed regional disparities; A and D genotypes being predominant in both Europe and North America, B and C in East Asia, D in North Africa, E in West Africa, and F and H in Latin America. This presumably reflects the adaptation of HBV to the evolutionary history of humans—that is, as human ancestors parted from chimpanzees and began evolving into *Homo sapiens* about 5 million years ago and dispersed to various parts of the world over the period of 200 thousand years, HBV also adapted to the environment of various regions around the globe. As a result, the difference in the nucleotide sequences among HBV genotypes has grown to more than 8.0%. This is considerably larger than the 0.3% difference among human individuals or the 1.2% difference between humans and chimpanzees in comparison.

On the other hand, clinical disparities of HBV infections among regions have long been recognized, even before the discovery of HBV genotypes. For example, HBV infections in Asia are transmitted mainly through mother-to-child infection, which frequently become chronic and develop into liver cancer. In Europe and North America, HBV typically spread as a sexually transmitted disease (STD) among adults, in which about 10% of the cases become chronic but rarely progress to liver cancer. On the other hand, childhood HBV infection predominates in Africa, which is highly likely to become chronic, and cases of liver cancer at younger ages are common. These differences in clinical picture had been explained by the difference in host immune competence among Caucasoid, Negroid, and Mongoloid races. However, the above-mentioned discovery of the 8% difference in the nucleotide sequence among HBV genotypes supports the idea that genotypes themselves are responsible for these clinical differences.

Japan is faced with the ever-increasing impacts of globalization. As many as 20 million Japanese people travel abroad each year, while 7 million people visit Japan from overseas. As a result,

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infections with uncommon genotypes of HBV are now being found in Japan, indicating the spread of genotypes that are not native to the country.[3] Even if only 10% of such people are to develop chronic conditions, they could serve as the core for the spread of foreign HBV genotypes in Japan. In fact, a case of HBV infection has been reported that was passed from a foreign female to a Japanese male and then to a Japanese female. A national survey has also revealed an increase of a genotype commonly seen in Europe and North America.[4]

These facts underscore the limitations of the current HBV infection control measures in Japan, which focus on mother-to-child infection, and indicate the need of HBV vaccination of all newborns. We must also begin to recognize HBV infection as an STD in this country as well, and consider starting universal vaccination for all babies and juveniles against HBV.

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

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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

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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.

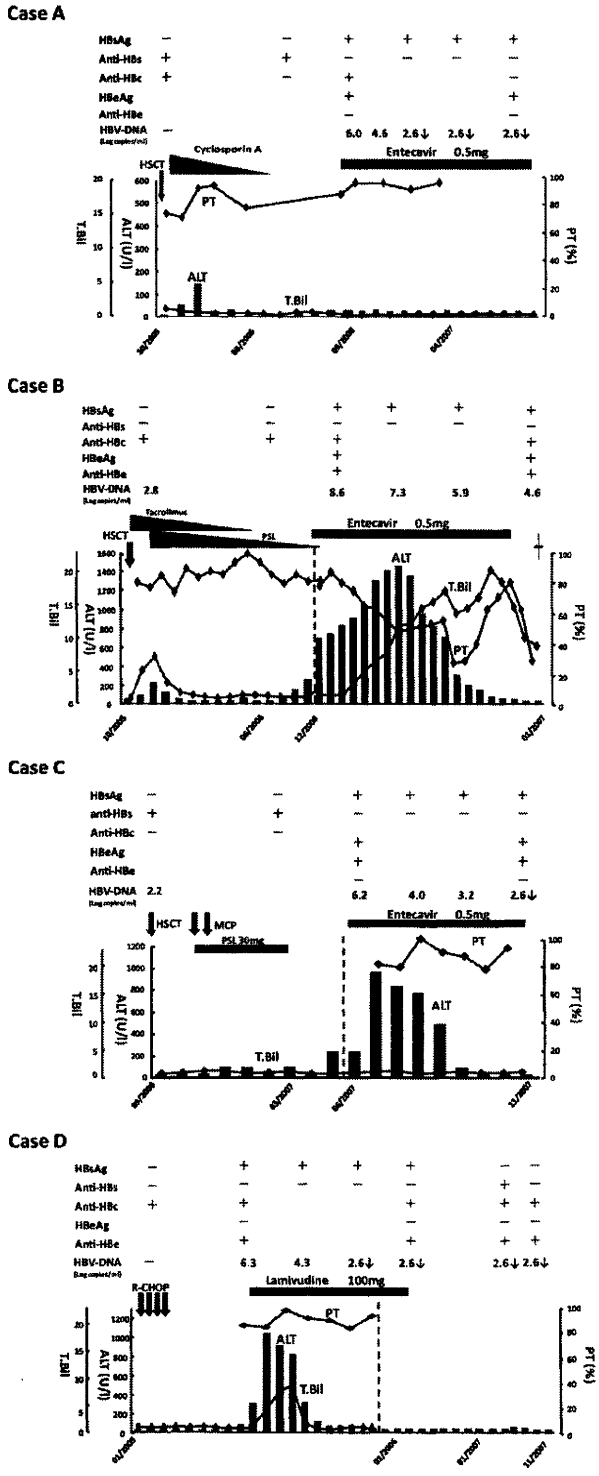

Fig. 1. Clinical course of 4 patients who developed reactivation of HBV. Changes of serial serum ALT, T.Bil, PT, HBV DNA, and HBV serology are shown. Hematopoietic stem-cell transplantation, hematopoietic peripheral stem cell transplantation; PSL, prednisolone; R-CHOP, rituximab, cyclophosphamide, doxorubicin,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

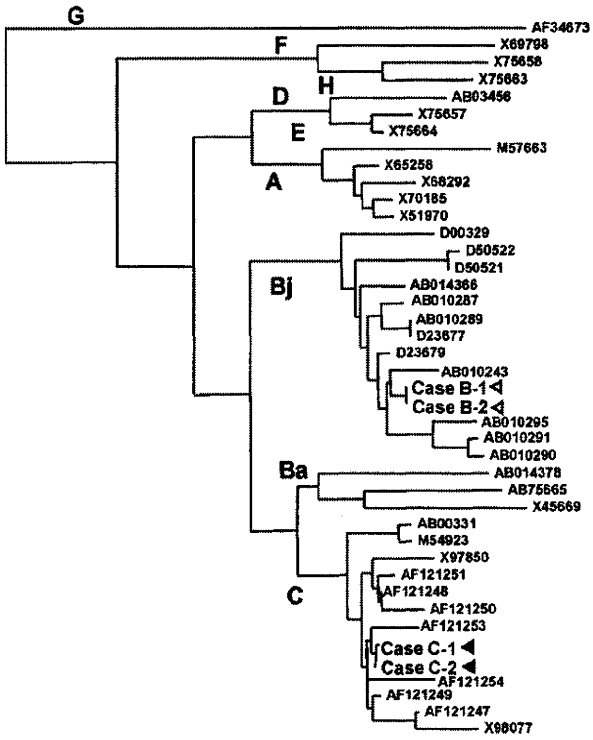

Fig. 2. Sera from cases B and C before chemotherapy or hematopoietic stem-cell transplantation (case B-1 and C-1) and at the time of HBV reactivation (case B-2 and C-2) were sequenced from the core promoter to precore region (481 bp). The phylogenetic tree demonstrated that patients B and C had de novo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

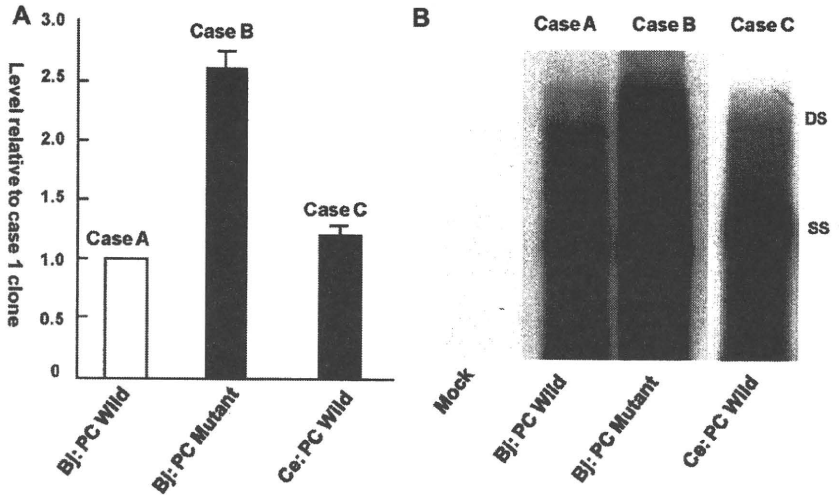

Fig. 3. A: Comparison of viral replication among patients in vitro. Southern blot analysis for replicative activity (intracellular HBV DNA) of genotype Bj with precore (PC) wild-type clone obtained from case A (Bj: PC Wild), genotype Bj with PC mutant-type clone obtained from case B (Bj: PC Mutant) and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