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 [2–4]. 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).

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Evolution of hepatitis B genotype C viral quasi-species during hepatitis B e antigen seroconversion

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Background & Aims: Although the evolution of viral quasi-species may be related to the pathological status of disease, little is known about this phenomenon in hepatitis B, particularly with respect to hepatitis B e antigen (HBeAg) seroconversion.

Methods: Nucleotide sequences of the hepatitis B virus (HBV) *X/precore/core* region was analyzed at five time-points in four groups of chronic hepatitis B patients, interferon-induced seroconverters (IS, $N = 9$), interferon non-responders (IN, $N = 9$), spontaneous seroconverters (SS, $N = 9$), and non-seroconverters (SN, $N = 9$) followed during 60 months on an average. Only patients with genotype C were studied.

Results: Analysis of 1800 nucleotide sequences showed that there was no statistical difference between the nucleotide genetic distances of seroconverters (IS and SS; 6.9×10^{-3} substitutions (st)/site and 6.7×10^{-3} st/site, respectively) and those of non-seroconverters (IN and SN; 5.3×10^{-3} st/site and 3.8×10^{-3} st/site, respectively) before seroconversion. Compared to non-seroconverters (IN and SN; 5.1×10^{-3} st/site and 5.9×10^{-3} st/site, respectively), the sequence diversity of seroconverters (IS and SS; 10.9×10^{-3} st/site and 9.9×10^{-3} st/site, respectively) was significantly higher after seroconversion ($p < 0.05$), and was higher in seroconverters after seroconversion than before seroconversion ($p < 0.05$), while this changed very little in non-seroconverters during the observation period. Phylogenetic trees showed greater complexity in secoconverters than non-seroconverters. Parsimony-based estimation of the direction of sequence change between descendants and ancestors before HBeAg seroconversion, revealed higher frequencies of transversional A to T substitution in seroconverters (0.06 *vs.* 0.02, $p = 0.0036$) that coincided with the dynamics of quasi-species possessing A1762T mutation.

Conclusions: The distinctly greater viral diversity in HBeAg seroconverters after seroconversion could be related to escape mutants resulting from stronger selection pressure.

Introduction

Hepatitis B virus (HBV) is a major human pathogen which can cause severe hepatic disease, including chronic hepatitis, cirrhosis (LC), and hepatocellular carcinoma (HCC). Quasi-species comprises a complex and dynamic distribution of non-identical but related genomes [1]. The evolution of viral quasi-species has been reported as important in the pathogenesis of RNA viruses such as hepatitis C virus [2–6] and human immunodeficiency virus [7–10], but little is known about HBV. HBV is a hepatotropic, non-cytopathic DNA virus replicated by an error-prone polymerase through an RNA intermediate. Because of this feature, the replication of HBV lacks fidelity. This results in a complex distributions of genomes with naturally-acquired mutations or mutations selected by either antiviral therapy or the immune response of the host. HBV quasi-species have not been subjected to detailed investigation, especially in the context of hepatitis B e antigen (HBeAg) seroconversion (SC), an immunologically mediated event. Whether there is a causal relationship between HBV seroconversion and HBV quasi-species remains unclear. HBV-related disease is known to be mediated both virologically and immunologically. Several studies have depicted the dynamic evolution of HBV quasi-species during lamivudine resistance or multiple drug resistance. This highlights the importance of HBV molecular evolution in revealing the mechanism of drug resistance [11,12]. HBV-specific cytotoxic T-cells play a significant role in the control of replication of HBV, which has been well documented in the literature [13–16].

Precore/core protein is the target of immunologically mediated HBeAg seroconversion. When the *precore/core* gene in HBV DNA is transcribed and translated, HBeAg is produced and secreted into the circulation [17,18]. But the synthesis and secretion of HBeAg are aborted by the emergence of a point mutation from G to A at nucleotide (nt)1896 (G1896A). Convincing lines of evidence have indicated a close association between HBeAg/anti-HBe seroconversion and the emergence of *precore* and *core* promoter mutations [19,20].

Keywords: Chronic hepatitis B; Quasi-species; Hepatitis B e antigen seroconversion.

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Abbreviations: SC, seroconversion; ALT, alanine aminotransferase; CHB, chronic hepatitis B; HBV, hepatitis B virus; IFN, interferon; HBeAg, hepatitis B e antigen; HBsAg, hepatitis B surface antigen; IS, interferon-induced HBeAg seroconverters; IN, IFN non-responders; SS, spontaneous seroconverters; SN, non-seroconverters.

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The purpose of this study was to elucidate the evolution of HBV quasi-species during HBeAg seroconversion. The results might help us to better understand the pathogenic mechanisms of HBV. We selected patients with well-characterized clinical phenotypes and compared their viral diversity based on the nucleotide sequences of the *X*/precore/core region. *Precore* and *core* promoter mutations were also investigated in detail before and after HBeAg seroconversion.

Materials and methods

Patients

Sera from 36 chronic hepatitis B patients with well-characterized clinical follow-up for >5 years were selected from a chronic hepatitis B database (77 seroconverters and 67 non-seroconverters) at Chiba University Hospital. Only patients with genotype C (subtype C2) were studied to ensure that differences found in viral evolution were not due to genotypic variation. Nine patients in each group were selected randomly if they fulfilled the following criteria and had sufficiently long follow-up. The index group comprised patients with documented HBeAg seroconversion (spontaneous seroconverters, SS), with serum at the following time-points relative to HBeAg seroconversion: time-point I (−25.2 ± 6.2/months), time-point II (−11.6 ± 2.7/months), time-point III (1 ± 2.3/months), time-point IV (12.5 ± 3.3/months), and time-point V (25 ± 3.6 months). Untreated control patients included those who were followed for a similar period of time and were persistently HBeAg positive (non-seroconverters, SN), and they were matched for average age of seroconversion and time-point intervals of the SS group. A second index group of patients with interferon (IFN)-induced HBeAg seroconversion (IFN seroconverters, IS), with serum at the following time-points relative to HBeAg seroconversion: time-point I (−24.3 ± 3.1/months), time-point II (−11.2 ± 1.9/months), time-point III (1 ± 1.2/months), time-point IV (12.7 ± 1.7/months), and time-point V (25.4 ± 2.2/months). Control patients were persistently HBeAg-positive despite IFN therapy (IFN non-responders, IN). Controls were matched for the average age of seroconversion, sex and time-point intervals of the IS group.

HBeAg seroconversion was defined as the loss of HBeAg and the development of anti-HBe. The serial serum samples in this study were taken at five time-points for each patient, as described above. This study was approved by the Ethics Committee of Chiba University Hospital.

Serological examination

HBsAg, HBeAg and anti-HBe were determined by enzyme-linked immunosorbent assay (ELISA; Abbott Laboratory, Chicago, IL). HBV genotype was determined from the patients' sera by ELISA (HBV genotype EIA; Tokushu-Meneki Laboratory, Tokyo, Japan), based on the method described by Usuda et al. [21]. Serum HBV DNA levels were monitored using the Roche Amplicor Monitor test (Roche Diagnostics, Tokyo, Japan), which has a lower detection limit of 2.6 $\log_{10}$ copies/ml, at each time-point.

Cloning and sequencing

Total DNA was extracted from 200 μl of each serum sample using QIAamp DNA blood mini kits (Qiagen, Hilden, Germany) according to the manufacturer's instructions and eluted in 200 μl distilled water. Because HBeAg seroconversion is associated with a decrease in HBV DNA levels, nested PCR was performed for all the samples. The primers for the first round of PCR were 5′-TCG CAT GGA GAC CAC CGT GA-3′ (sense, nt1604–1623) and 5′-ATA GCT TGC CTG AGT GC-3′ (antisense, nt 2076–2060). The primers for the second round of PCR were 5′-CAT AAG AGG ACT CTT GGA CT-3′ (sense, nt 1653–1672) and 5′-GGA AAG AAG TCA GAA GGC-3′ (antisense, nt 1974–1957).

Amplification was performed with 2 μl of DNA template (extracted DNA from serum samples for the first round PCR and the first round PCR products for the second round PCR) in 50 μl reaction under the following conditions: an initial 2 min of denaturation at 94 °C and 36 cycles of 94 °C denaturation for 1 min, annealing at either 54 °C or 52 °C for 1 min, in the first and second round respectively, and 72 °C extension for 1 min. The last cycle was followed by a final extension at 72 °C for 10 min. A 473-base pairs fragment (nt 1604–2076) containing the *X/precore/core* region was amplified.

PCR reactions were followed by cloning using TOPO® TA cloning kits (Invitrogen, Carlsbad, CA). All PCR products were purified with QIAquick PCR Purification Kit (Qiagen, Hilden, Germany), then cloned into the TOPO vector, and transformed into *Escherichia coli*. At least 15 clones per one cloning for samples from PCR reactions proceeded subsequent to the electrophoretic size separation on 1.2% agarose gel. Ten positive clones per cloning for samples from each PCR reaction were sequenced using BigDye® Terminator and a 3730xl DNA Analyzer (Applied Biosystems, Foster City, CA). The cloning PCR and sequencing primers were M13-forward, 5′-GTA AAA CGA CGG CCA GT-3′, and M13-reverse, 5′-GGA AAC AGC TAT GAC CAT G-3′.

Sequence analysis

The DNAPARS program from PHYLIP v3.65 package, implemented in Simmonic Sequence Editor version 1.5 [22], was used for sequence analysis. To evaluate quasi-species-based evolution of HBV strains in chronic patients, sequences of clones ($N = 10$) isolated at each time-point ($N = 5$) from individual patients ($N = 36$) were subjected to alignment and used to generate one parsimonious ancestral sequence. Maximum nucleotide composition distances were evaluated pair-wise between the ancestral sequence and the sequences of each of the 10 clones with a mean value estimated for each patient at a given time-point (*MEGA* version 4 [23]). All patients were categorized into four groups with respect to seroconversion status and the mean distance value for each group was calculated for each time-point.

The differences in genetic distance among clinical groups and time-points, and diversity at each time-point, were analyzed using ANOVA (analysis of variance). Student's *t*-test was also performed to determine the average of genetic diversities in non-seroconverters. All graphical data are presented as means ± standard deviation (SD). Results were considered statistically significant at $p < 0.05$. The statistical analysis was performed with SPSS (2004; SPSS Inc., Tokyo, Japan).

Construction of phylogenetic trees

To examine the evolution of the viral sequence and whether this evolution was elicited by quasi-species or mutagenesis, phylogenetic trees were constructed using the Neighbor-Joining (NJ) model with the Simmonic Sequence Editor version 1.5, based on the genomic sequences of HBV. Moreover, to investigate viral genetic features possibly associated with seroconversion, sequences isolated at time-points 1 and 2 were further analyzed phylogenetically. Neighbor-Joining trees were constructed at time-points 1 and 2 (Fig. S1 and S2, respectively) using all groups of sequences.

Results

Baseline clinical characteristics of the patients and sequential levels of serum ALT and HBV DNA

The clinical and laboratory characteristics of all patients are listed in Table 1. The levels of alanine aminotransferase (ALT) and HBV DNA over time are illustrated in Fig. 1A and B, respectively. Serum ALT levels, a marker of hepatocyte damage, normalized after seroconversion and, for all groups except the interferon non-responders, were <40 IU/L at the end-point of observation. HBV DNA loads decreased markedly in seroconverters (<3 $\log_{10}$ copies/ml, $p < 0.0001$) but changed very little in non-seroconverters. It is noteworthy that, at the second year after seroconversion, serum HBV DNA loads increased in interferon-induced seroconverters compared to spontaneous seroconverters, without statistical significance ($p^H = 0.1087$) (Fig. 1B).

Viral nucleotide sequence diversity

Viral sequence diversity, phylogenetic trees, and mutation pattern based on 1800 HBV nucleotide sequences from clones of the *X/precore/core* region, were analyzed among selected patients.

Table 1. Baseline clinical features of patients.

	IFN Seroconverters (IS)	IFN Non-seroconverters (IN)	Spontaneous Seroconverters (SS)	Spontaneous Non-seroconverters (SN)
Age (y)	40 ± 8	40 ± 11	29 ± 10	34 ± 6
Male : Female	6:3	8:1	5:4	7:2
HBV DNA (log_{10} copies/ml)	6.8 ± 0.9	6.8 ± 1.0	6.8 ± 1.2	7.1 ± 0.8
ALT (IU/L)	88.3 ± 48.6	94.3 ± 144.4	89.8 ± 71.4	67.6 ± 48.7

Note: The IFN-induced group (seroconverters and non-responders) was older than the spontaneous group (seroconverters and non-responders). Males were the majority in all groups. Baseline serum HBV DNA and ALT levels are similar among the four groups. Data are shown as mean ± SD.

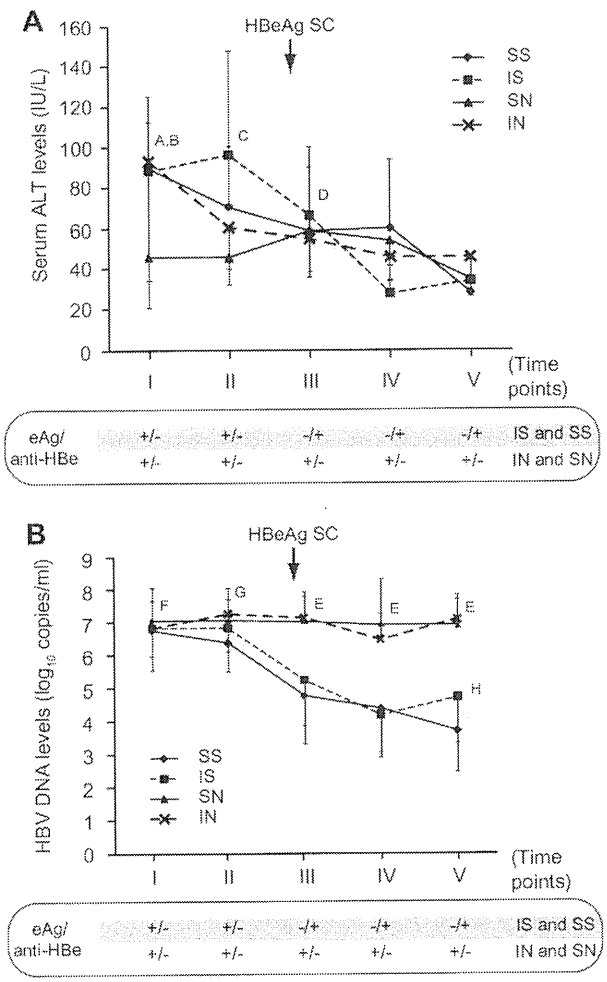

Fig. 1. Serum ALT and DNA levels in the four groups. The group of spontaneous seroconverters (SS) is a solid line diamond, IFN-induced seroconverters (IS) is a broken line square, IFN non-responders (IN) is a broken line asterisk, and non-seroconverters controls (SN) is a solid line triangle. (A) $p^A = 0.0234$ comparing time-point I with time-point IV, $p^B = 0.0028$ comparing time-point I with time-point V, $p^C = 0.007$ comparing time-point II with time-point V, $p^D = 0.0068$ comparing time-point III with time-point V. (B) $p^E < 0.0001$ comparing seroconverters with non-seroconverters, $p^F < 0.0001$ comparing time-point I with III, IV, V, $p^G < 0.0001$ comparing time-point II with the other time-points, $p^H = 0.1087$ at time-point V in seroconverters.

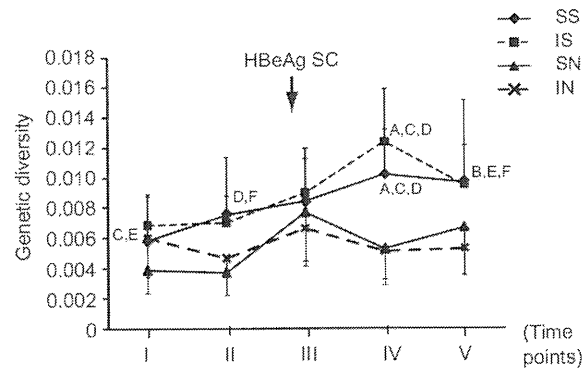

Fig. 2. Viral genetic diversity in the four groups. The group of spontaneous seroconverters (SS) is a solid line diamond, IFN-induced seroconverters (IS) is a broken line square, IFN non-responders (IN) is a broken line asterisk and non-seroconverters controls (SN) is a solid line triangle. $p^A < 0.0001$ comparing seroconverters with non-seroconverters at time-point IV, $p^B = 0.0301$ comparing seroconverters with non-seroconverters at time-point V, $p^C = 0.0013$ and $p^D = 0.0025$ comparing I and II with time-point IV in seroconverters. $p^E = 0.0121$ and $p^F = 0.021$ comparing time-points I and II with V in seroconverters.

Striking differences in nucleotide sequence diversity were revealed between seroconverters and non-seroconverters before and after seroconversion (Fig. 2). The nucleotide sequence diversity of seroconverters was similar to that of non-seroconverters before seroconversion. Analysis of genetic distance showed that the viral sequence diversity of seroconverters was significantly greater than that of non-seroconverters after seroconversion (Fig. 2, $p^A < 0.0001$ at time-point IV, $p^B = 0.0301$ at time-point V) and was greater in seroconverters after seroconversion than before (Fig. 2, $p^C = 0.0013$ and $p^D = 0.0025$), while almost no changes were observed in non-seroconverters during the observation period.

It is noteworthy that, in interferon-induced seroconverters at the last time-point of observation, the nucleotide sequence diversity was less, although this increased clearly at the first year after seroconversion. This tendency of reversed change at the last two time-points was also seen in HBV DNA loads (Fig. 1B), namely, increase or decrease of the genetic diversity accompanied by decrease or increase of the viral load in interferon-induced seroconverters. On the other hand, the nucleotide sequence diversity increased continuously in spontaneous seroconverters, accompanied by a concurrent decrease of viral loads (Fig. 1B) during the follow-up period. Amino acid sequence diversity had an almost

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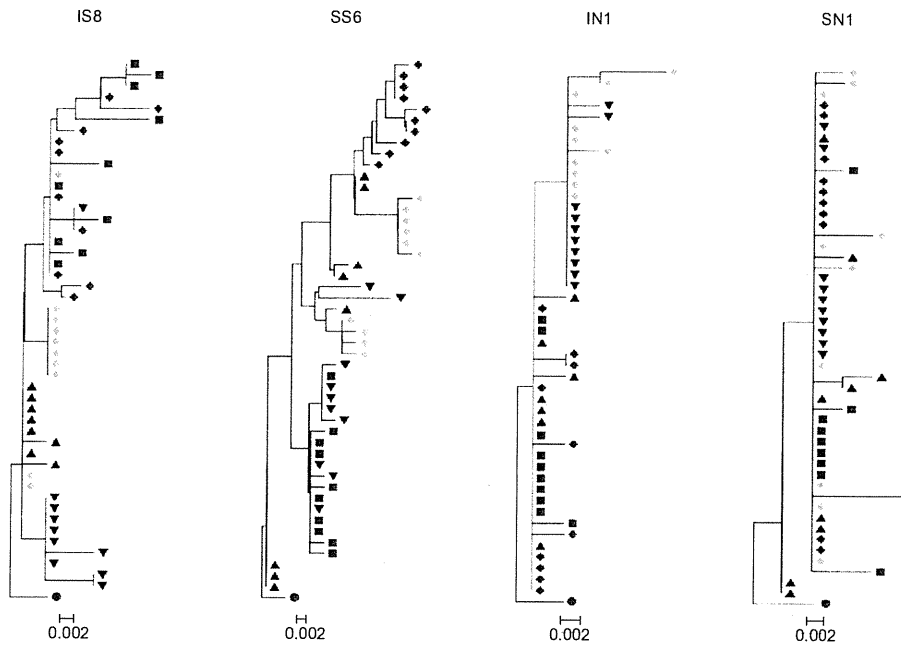

Fig. 3. Representative Neighbor-Joining phylogenetic trees of HBV sequences for each clinical group showing complex trees in seroconverters. HBV *X/precore/core* sequences from time-points I (purple filled triangle), II (blue filled inverted triangle), III (green filled square), IV (red filled diamond) and V (sky blue filled diamond) serum samples are analyzed phylogenetically and their positions are displayed on the trees. A sequence retrieved from the time-point I (red dot) of each group as outgroup in the trees, respectively. Scale bar represents 0.002% genetic variation. Seroconversion patients (IS, SS) show relatively complex branching patterns, forming clusters over time. With the pressure of seroconversion, the genetic diversity increased. In contrast, patients without seroconversion (IN, SN) were simply branching patterns and the genetic diversity in these patients changed very little over time.

identical pattern to that of DNA nucleotide sequence diversity (data not shown).

Construction of phylogenetic trees

Phylogenetic trees were complex for secoconverters and comparatively simple for non-seroconverters. In seroconverters (IS and SS), the arrangement and branch lengths of the trees were consistently more complex and longer than those for non-seroconverters. The genetic diversity was great after seroconversion in seroconverters (IS and SS) and less in non-seroconverters (IN and SN) (Fig. 3).

To investigate viral genetic features possibly associated with seroconversion, sequences isolated at time-points 1 and 2 (before seroconversion) were further analyzed phylogenetically. Trees were reconstructed using Neighbor-Joining, ML (data not shown), and PAML methods (data not shown). In general, no clusters were seen to be supported by robust bootstrap values for any group or particular patient quasi-species. This indicates that the region of the HBV genome studied does not contain patterns of variability sufficient for robust phylogenetic relation reconstruction. However, variability of branch lengths in the tree indicated that seroconversion patient groups exhibit greater diversity of the quasi-species compared to patients without seroconversion. This is in agreement with the genetic distance plot (Fig. 2), showing greater deviation from the mean values observed in patients with seroconversion. The IN group exhibited least deviation on the distance plot (Fig. 2) and shortest branch lengths on the trees (Fig. 3).

Interclonal differences of the quasi-species

To investigate whether a particular mutation pattern of evolution of the quasi-species is associated with seroconversion, we further analyzed the sequence changes in all patients at time-points 1 and 2, corresponding to the time before seroconversion. Parsimony-based ancestral sequences were generated using the Simmonic Sequence Editor. Aligned sequences of time-points 1 and 2 from a single patient were used as the input. Frequencies of changes in 12 types of mutations, including 4 transitions (CT, TC, AG, and GA) and 8 transversions (AT, TA, AC, CA, CG, GC, GT, and TG) were evaluated between generated descendants and ancestral sequences for each clone of the patient. Statistical *t*-test comparison of mean values of nucleotide changes between seroconversion and non-seroconversion groups is summarized in Table 2 and Supplementary Table 1.

Analysis of sequence changes indicated a higher frequency of transversional A to T in spontaneous seroconverters (SS *vs.* SN = 0.06 *vs.* 0.02, $p = 0.04$) and IFN-induced seroconverters (IS *vs.* IN = 0.05 *vs.* 0.01, $p = 0.05$) and A to C changes in IFN-induced seroconverters (IS *vs.* IN = 0.025 *vs.* 0.006, $p = 0.04$) before seroconversion. Comparison of seroconversion groups (SS and IS) indicated a higher frequency of transversional A to T mutation pattern ($p = 0.003$, Table 2) and the trend of G to A mutation is higher in seroconversion groups (SS and IS) (Table 2). Subsequently, alignments of the clones were generated. Visual inspection of the alignments indicated variation in the ratio of A1762T mutation in clones isolated from each patient at time-points 1 and 2 (Fig. 4). In contrast to non-seroconverters, seroconverters

Table 2. t-test comparison of mean values of nucleotide changes between seroconversion and non-seroconversion groups.

	Seroconversion (n = 18)	Non-seroconversion (n = 18)	*p*
CT	0.117033	0.103750	0.637023
TC	0.156706	0.201328	0.155252
AG	0.125483	0.148372	0.498916
GA	0.196722	0.124511	0.073433
AT	0.061194	0.022128	0.003665
TA	0.049372	0.045417	0.778612
AC	0.027944	0.012550	0.145158
CA	0.017128	0.011094	0.523868
CG	0.009439	0.007744	0.835337
GC	0.018167	0.014894	0.748267
GT	0.009839	0.019217	0.272185
TG	0.041783	0.035528	0.731324

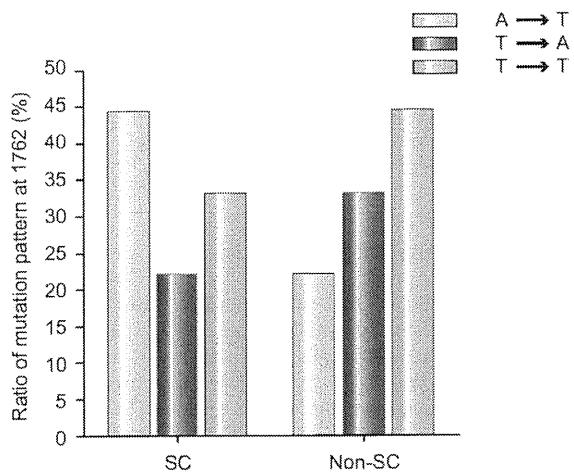

Fig. 4. The evolution of the *core* promoter mutation (A1762T) between seroconversion and control groups from time-point I to II. SC indicates seroconversion and non-SC, non-seroconversion. Alignment of the clones was carried out and the frequency of A1762T mutation in clones isolated from each patient at time-points 1 and 2 was determined. Subsequently, the evolutionary ratio of mutation from time-point I to II was calculated.

showed a higher frequency of A to T mutation pattern in the core promoter region from time-point I to II.

Core promoter (A1762T/G1764A) and precore (G1896A) mutations

Given that the *core* promoter/*precore* mutations influenced virus replication and HBeAg seroconversion, we analyzed the sequential change of *core* promoter (A1762T/G1764A)/*precore* (G1896A) mutations over time (Table 3). After seroconversion, patients with more than 50% *precore* mutant clone had higher HBV DNA loads than those with less than 50% of *precore* mutant clone (*precore* wild type) virus at time-point V [5.4 ± 1.3 (n = 5) *vs.* 3.8 ± 1.1 (n = 13), p = 0.0185] and 8 patients with a HBV DNA load less than 4.0 $\log_{10}$ copies/ml had all *precore* wild-type virus at time-point V (Table 3). Clinical progress of these patients was investigated over 10 years as median (range 1–20 years) after HBeAg seroconversion. HCC developed in 3 of 5 patients with *precore* mutant virus, compared to 1 of 13 patients with *precore* wild-type virus at time-point V (p = 0.017). On the other hand, 3 patients with ASC had all *precore* wild-type virus at time-point V (Table 3).

Discussion

In this study, analysis of 1800 nucleotide sequences from 36 HBV carriers showed that the viral diversity of seroconverters (IS and SS) after seroconversion was significantly greater than that of non-seroconverters (IN and SN) (Fig. 2, p <0.05) and was higher after seroconversion than before, in the seroconverters (Fig. 2, p <0.05). Phylogenetic analysis also generated complex trees for seroconverters and relatively simple trees for non-seroconverters. Analysis on interclonal differences in the quasi-specie showed a higher frequency of transversional A to T mutation pattern in seroconverters that coincided with the A1762T core promoter mutation. These findings suggested that HBeAg seroconversion involves dynamic shifts of the serum HBV quasi-species.

Osiowy et al. [24] examined viral quasi-species in eight HBeAg-negative patients at two time-points 25 years apart and obtained the evolutionary rate. Their results suggested that HBV diversity may be generated more rapidly than those estimated previously [25–29]. The higher evolutionary rate may be related to the seroconversion event driving quasi-specie complexity and diversification [24]. Our phylogenetic study showed that viral quasi-species populations appear to be replaced by new populations arising from a different clade after seroconversion.

Increased immune responses are accompanied by the reduction of viral loads and stronger immune pressure induces the selection of escape mutations, which leads to greater viral diversity [30]. According to this scenario, in our study, non-seroconverters have a high viral load and low quasi-species diversity and they obviously have a weak immune response.

Lim et al. [31] reported that viral genetic diversity in genotype B CHB patients was 2.4-fold greater in HBeAg seroconverters (spontaneous or IFN-induced) than in non-seroconverters before seroconversion. In this study of genotype C CHB patients, the nucleotide genetic distance was 1.49-fold greater in seroconverters (IS and SS) than in non-seroconverters before seroconversion but there was no statistical difference. This discrepancy might be due to the smaller region for analysis of genetic distance in our study than that of Lim et al. Another interpretation is that the host's immune response to the selection of mutant virus might differ between genotype B and genotype C. The natural course of CHB and the response to treatment could be affected by HBV genotype and there are some lines of evidence that indicate that the prevalence rates of *precore* and *core* promoter mutations vary among patients infected with HBV strains of different genotypes [32–34].

T-test comparison of mean values of nucleotide changes (Table 2) and linear logistic regression univariate analysis of mutations associated with seroconversion between seroconverters and non-seroconverters (data not shown) indicated a variation in the AT mutation pattern in the former (p = 0.003 and p = 0.006, respectively). This coincided with differences in the

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Table 3. *Core* promoter and *precore* mutations in seroconverters (IS and SS).

Patients	CP (ntA1762T/G1764A) (percent)			PC (ntG1896A) (percent)			DNA Loads (log_{10} copies/ml)			Histological diagnosis
	I	III	V	I	III	V	I	III	V	
IS1	100	100	100	0	0	70	5.7	3.8	4.8	CHB
IS2	100	100	100	90	100	90	7.6	7.2	7.6	HCC
IS3	70	100	100	10	0	10	6.5	5.2	5.5	CHB
IS4	90	100	10	0	10	0	7.6	6.2	3.3	CHB
IS5	100	40	20	0	0	0	7.6	3.6	4.1	CHB
IS6	70	90	90	20	10	90	5.7	4.1	4.5	HCC
IS7	100	100	90	0	10	10	7.2	3.1	3.4	LC
IS8	80	100	60	0	0	60	7.6	4.0	4.5	CHB
IS9	100	100	10	0	0	0	6.0	4.5	4.8	HCC
SS1	0	60	0	80	0	80	7.6	4.2	5.4	HCC
SS2	80	100	90	10	90	10	6.6	7.6	5.9	ASC
SS3	100	90	60	10	0	0	6.5	4.3	2.8	ASC
SS6	30	100	10	0	0	10	3.9	4.4	4.1	CHB
SS7	80	100	100	0	0	0	7.6	2.8	2.6	ASC
SS8	0	100	90	0	20	0	7.6	5.4	3.6	CHB
SS9	0	80	20	0	10	0	7.6	4.0	2.6	CHB
SS10	50	20	40	0	0	40	7.3	3.9	2.6	CHB
SS11	100	100	100	0	0	0	6.1	6.3	3.8	CHB

IS: interferon induced seroconverter; SS: spontaneous seroconverter; ASC: asymptomatic carriers; CHB: chronic hepatitis B; LC: cirrhosis; HCC: hepatocellular carcinoma.

ratio of T1762A quasi-species between seroconverters and non-seroconverters, indicating that it might be a marker preceding seroconversion in HBV/genotype C-infected patients as reported previously [35–37].

HBeAg seroconversion is an incomplete marker of immune control, although most patients experience some clinical benefit from it [38,39]. Previous studies have shown that the average rate of spontaneous HBeAg seroconversion in patients with chronic hepatitis B is about 10% per year [40,41]. HBeAg seroconversion associated with incomplete viral suppression may result in the emergence of the *precore* mutant and attendant chronic sequelae. Mutations in the *precore* and *core* promoter regions of the HBV genome have been reported in many HBeAg-negative CHB patients. Longitudinal studies found that the A1896 mutation emerges or is selected around the time of HBeAg seroconversion, and high *precore* mutant ratios have been associated with persistent hepatitis after anti-HBe seroconversion [42]. Patients who continued to have high HBV DNA titres after HBe seroconversion had a lower genetic heterogeneity but more often had the *precore* mutant.

The limitations of this study were, the small size of study group, only 10 clones per sample, and a small region for analysis of genetic distance. In addition, the *X/precore/core* region is a highly conserved region, investigation of another region of the HBV genome, such as the polymerase, might help us to better understand the evolution of quasi-species of HBV.

In conclusion, the distinctly greater viral diversity after seroconversion in HBeAg seroconverters could be related to increased HBV-specific T-cell responses and escape mutants which arise from selective pressure caused by host immune activity. Long-term follow-up is required to determine whether hepatitis B viral diversity decreases or remains at a high level. Further study will be needed to elucidate the relationship between seroconversion and viral quasi-species in relation to antiviral therapy.

Conflict of interest

The authors who have taken part in this study declared that they do not have anything to disclose regarding funding or conflict of interest with respect to this manuscript.

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Review Article

Management of hepatitis B: Consensus of the Japan Society of Hepatology 2009

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Recently, much progress has been made in the field of hepatitis B, such as natural history of the disease in relation to the amount of hepatitis B virus (HBV) DNA, genotypes of HBV influencing the natural course and treatment effects, mutations of HBV influencing the severity of the disease and development of hepatocellular carcinoma, and antiviral treatment such as nucleos(t)ide analogues and pegylated interferon. To make the consensus for the diagnosis, management and treatment of hepatitis B, a meeting was held during 45th annual meeting of Japan Society of Hepatology (JSH) in June 2009. In the meeting, recommendations and informative statements were discussed on the following subjects: (i) natural history of HBV infection; (ii) clinical implication of HBV genotypes; (iii) HBV mutations and their potential impact on pathogenesis of HBV infection; (iv) indications for antiviral treatment of chronic hepatitis B; (v) nucleos(t)ide analogues for chronic hepatitis B; and (vi) interferon therapy for chronic hepatitis B. The presenters reviewed the data on these subjects and proposed the consensus statements and recommendations. These statements were discussed among the organizers and presenters, and were approved by the participants of the meeting. In the current report, the relevant data were reviewed and the 12 consensus statements and nine recommendations on chronic hepatitis B were described.

Key words: genotype, hepatitis B virus, interferon, mutation, natural history, nucleotide analogue

Hepatitis B virus (HBV) is one of the most distributed viruses which infect humankind. More than 3 billion people, one half of the world's population, have been exposed to HBV during their life.[1] Acute infection in adults is self-limited in general whereas infection during early childhood will develop into persistent chronic infection in most individuals.[2] More than 400 million people worldwide are chronically infected with HBV and are at risk of developing life-threatening complications including liver cirrhosis and hepatocellular carcinoma (HCC).[1] HBV is a major public health problem worldwide especially in East Asia and Africa. In Japan, approximately 1.5 million people are infected with HBV and it is one of the major causes of HCC and chronic hepatic failure. Other complications of HBV infection include fulminant hepatitis and acute liver failure.

The consensus meeting for diagnosis, management and treatment for hepatitis B was held during the 45th annual meeting of the Japan Society of Hepatology (JSH) in June 2009 (Congress President: M Kudo), where the recommendations and informative statements were discussed. Although the JSH consensus meeting of hepatitis B had been held four times so far, recommendations were hitherto published only in Japanese and this is the first report in English. Established

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information for pathogenesis and contributing factors for disease progression which was agreed by the organizers and presenters are shown as "consensus statements", and clinically useful consensus are shown as "recommendations". The quality of recommendations or informative statements are required to show a "level" (assessing strength or certainty) of evidence and "grading" of recommendations or assessment according to a standard reporting system of clinical guidelines.[3]

NATURAL HISTORY OF HBV INFECTION

AN EVALUATION OF studies on the natural history of HBV infection was done using the scoring system proposed by MacMahon *et al.*[4] in the present analysis because scoring systems for treatment studies cannot always be applied directly to those using natural history. The proposed scoring system consists of levels 1 (1a, 1b), 2 (2a, 2b, 2c), and 3. Level 1a is defined as a population-based longitudinal cohort study with a hepatitis B surface antigen (HBsAg) negative comparison group. Level 1b is identical to level 1a, but with no comparison group. Level 2a is defined as a clinic-based longitudinal cohort study, level 2b is a population-based or clinic-based cohort nested case–control study, and level 2c is a cross-sectional clinic-based study. Level 3 is defined as an observation study case series.

The natural history of chronic HBV infection can be classified into several phases based on levels of alanine aminotransferase (ALT), hepatitis B e-antigen (HBeAg) status, amounts of HBV DNA, and estimated immunological states.[4–9] A representative classification of these phases is shown in Table 1. In the immune tolerance phase, HBeAg is positive, serum levels of ALT are normal, histological activities of hepatitis are absent or minimal, and levels of HBV DNA are elevated. The immune tolerance phase is thought to occur most frequently in individuals who are infected through perinatal transmission, and this phase usually lasts until adolescence or young adulthood.[10–12]

The chronic hepatitis B phase is characterized by elevated ALT and HBV DNA levels. In this phase, the host's immune system recognizes HBV as being foreign and initiates an immune response that results in hepatitis. In cases who are HBeAg positive, active hepatitis can be prolonged and may result in cirrhosis. However, chronic hepatitis B eventually transitions into an inactive phase with a loss of HBeAg positivity in the majority of patients. Seroconversion to anti-HBe and the fall of serum HBV DNA to low levels result in the disappearance of disease activity, despite persisting HBsAg and low levels of HBV DNA.[13–16] Seroconversion rates range 7–16% per year according to reports with higher evidence levels (levels 1b, 2a).[16–19] Factors associated with seroconversion are age (level 1b),[20] ALT levels (level 1b), occurrence of acute exacerbation of hepatitis (level 1b),[19,21] and genotype (level 2c).[22,23]

The seroconversion of HBeAg results in the transition from hepatitis phase to inactive carrier phase, which is generally thought to be a benign course for HBV carrier, but sometimes hepatitis can be reactivated spontaneously.[24] Patients experiencing reactivation undergo another transition, with increases in HBV DNA and ALT levels and disease activity without reappearance of HBeAg.[24] This phase is referred to as HBeAg negative chronic hepatitis B. Occasional severe hepatitis B flare-ups with middle range HBV DNA levels (3–8 log copies/mL) occur in this phase.[8,25] HBeAg negative chronic hepatitis B is caused by mutant strains of HBV unable to produce HBeAg,[25,26] and tends to develop into cirrhosis and complicate HCC more than HBeAg positive chronic hepatitis B.[27–30]

Table 1 Phases in the natural history of HBV carriers (modified from [4])

Phase	Hepatitis	Blood			Liver
		DNA	HBeAg	HBsAg	cccDNA
Immune tolerance	–	8–11	+	+	+
HBeAg positive Chronic hepatitis	Usually Persistent	6–10	+	+	+
HBeAg negative Chronic hepatitis	Often Fluctuating	3–8	–	+	+
Inactive carrier	–	<4	–	+	+
Recovery	–	–	–	–	+

HBV DNA: log copies/mL. cccDNA, covalently close circular DNA; HBeAg, hepatitis B e-antigen; HBsAg, hepatitis B surface antigen; HBV, hepatitis B virus.

Many factors that are associated with the development of HCC have been reported so far. Higher age (level 1a), male sex (level 1a), presence of cirrhosis (level 2a) and familial cluster of carriers (level 2c) are reported as host factors.[31,32] Viral factors include high viral load (level 1b),[33–36] existence of pre-core and core promoter mutations (level 2a), genotype C and high ALT levels (level 1b). High viral load should be considered as a factor in patients over 35–40 years of age. Co-infection with hepatitis C virus, hepatitis D virus or HIV (level 2a), drinking habit (level 2c) and exposure to aflatoxin (level 2c) are reported as social and environmental factors.[37–39] Other lifestyle-related factors, such as smoking habit, obesity and complications from diabetes mellitus, have been documented as well.

Consensus 1
In patients with chronic hepatitis B, seroconversion of HBeAg usually results in the transition from hepatitis phase to inactive carrier phase, which generally has low HBV replication and normal ALT levels. However, reactivation of chronic hepatitis can spontaneously occur without the reappearance of HBeAg. At this point, active hepatitis continues and the risk of complicating cirrhosis and HCC is high in patients with HBeAg negative chronic hepatitis B. (Level 1b.)

In the inactive carrier phase, HBV replication is continuously suppressed as a result of predominantly host immunological pressure against HBV. Patients in the inactive carrier phase generally have a benign course because active hepatitis subsides and the risk of HCC decreases.[19,20,24,40] However, regular follow up is required because reactivation of HBV sometimes occurs spontaneously or as a result of immunosuppressive therapy.[19,24]

Hepatitis B surface antigen is known to fall to undetectable levels in some inactive carriers. This HBsAg negative phase, referred to as the recovery phase, has no hepatitis and a low risk of HCC. Still, caregivers must be aware that patients who are old or cirrhotic have a relatively higher risk of HCC.[41,42] Disappearance of HBsAg in the recovery phase does not indicate complete eradication of HBV because the HBV genome remains as covalently close circular DNA (cccDNA) in the nucleus of hepatocytes.

Consensus 2
2-1 HBV can not be completely eradicated using any currently existing treatment measures. (Level 2a.)
2-2 Patients in the inactive carrier or recovery phase have a benign clinical course. However, regular follow up of such patients is required because reactivation of hepatitis B and ensuing HCC can occur. (Level 1b, 2a.)

Clinicians have to consider two types of hepatitis B reactivation: one during the inactive carrier phase and the other in the recovery phase.[4] Both types of reactivation have been attributed with increasing incidence to strong immunosuppressive therapies. De novo hepatitis B, a reactivation of hepatitis B in the recovery phase, tends to develop into fulminant hepatitis, which has a very high mortality rate.[43–46] Thus, establishment of effective measures to prevent reactivation of hepatitis B is necessary.

Consensus 3
3-1 Reactivation of hepatitis B can occur during the inactive carrier or recovery phases and stems mainly from strong immunosuppressive treatment courses. (Level 2a.)
3-2 Recent advances in medical care have increased the use of immunosuppressive agents and thus the incidence of hepatitis B reactivation. (Level 2a.)
3-3 Reactivation of hepatitis B tends to develop into fulminant hepatitis. (Level 2a.)

Recommendation 1
In addition to the loss or seroconversion of HBeAg, a substantial decrease in HBV viral load and subsequent disappearance of hepatitis are the primary targets in the treatment of patients with chronic hepatitis B. (Level 1b.)

Recommendation 2
The main goals of HBV carrier treatment are patients in the inactive carrier and recovery phases. However, caregivers should be aware that reactivation of hepatitis B and complication of HCC can occur even in these benign phases. (Level 1b.)

Recommendation 3
Reactivation of hepatitis B due to immunosuppressive therapy tends to develop into severe hepatitis, thus requiring the establishment of effective preventative measures. (Level 2a.)

CLINICAL IMPLICATION OF HBV GENOTYPES

DISTINCT CLINICAL AND/OR virological characteristics of the HBV infection have been reported in different geographical parts of the world and are increasingly associated with host factors, environmental factors and the genetic diversity of the infecting virus.[47] HBV is classified into at least eight genotypes (A–H) based on an intergroup divergence of 8% or more in the complete nucleotide sequence and a number of subgenotypes (Aa/A1, Ae/A2, Bj/B1, Ba/B2, Cs/C1, Ce/C2, D1, D2, and so forth) that are currently known to have distinctive association with ethnic and/or geographical distribution.[48]

Association between HBV genotype and clinical manifestation

Acute hepatitis

The universal vaccination program against HBV has significantly reduced the number of new infection cases in most countries with levels of endemicity estimated from intermediate to high.[49] However, efficiency of universal vaccination in countries with a low level of endemicity still remains controversial. Japan is one of the countries with a low level of endemicity and mainly vertical (mother to baby) transmission route.[50] In Japan, HBV vaccination in combination with HBV immunoglobulin treatment is the only recommended measure for infants born to HBsAg positive mothers. Studies in Japan indicated genotype C (subgenotype Ce/C2) to be the major type in the country and genotype B (subgenotype Bj/B1) is the second distributed. Surveillance studies have shown a recent trend toward increase in number of acute hepatitis B infection among young adults mainly through sexual contacts.[51,52] Although most cases are associated with genotype C infection, there is a continuous trend toward increase in prevalence of genotype A among acute hepatitis cases.[51,53–56] Patients infected with genotype C have been known to be rarely associated with development of chronic persistence after acute infection in immune competent adults in Japan (1%) in contrast to the higher rates of those infected with genotype A (6–23%).[53,54] A recent multicenter study in Japan indicated a trend among chronic hepatitis B patients toward increase in prevalence of genotype A (from 1.7% in 2002 to 3.5% in 2006), whereas other genotypes remained stable at their prevalence during the same period.[57] The shift in genotype prevalence with the increase of genotype A among chronically infected carriers can be explained by higher risk of genotype A to develop persistence. This is consistent with higher rates of chronic persistence after acute infection in adults in European countries where genotype A is prevalent (10%).[48,58] This is also consistent with results of *in vitro* and *in vivo* comparisons of different genotype strains showing different dynamics of replication: slow for genotype A and rapid by genotype C.[59,60] The surveillance study indicated that all patients treated with lamivudine (LVD) recovered from acute hepatitis, whereas none of the three patients who developed a chronic outcome had received antiviral treatment during their acute phase of infection, indicating that LVD might be able to prevent the chronic outcome.[54] Cumulatively, these data indicate the clinical importance of routine genotyping for acute hepatitis B patients.

Fulminant hepatitis

One of the most serious complications of acute HBV infection is fulminant hepatitis. In Japan, the annual number of fulminant hepatitis reported was approximately 400 cases, with approximately half of these caused by HBV infection. Despite its rather low incidence, fulminant hepatitis is a national problem because the mortality rate is extremely high.[61] It is important to understand factors predisposing for development of fulminant hepatitis. Viral factors associated with the development of fulminant hepatitis are mutations in the core promoter (T1762/A1764)[62] and the pre-core region (A1896).[54,63,64] However, these findings were not consistent with studies in Europe and the USA.[65–67] A large-scale cross-sectional study in Japan revealed association between genotype B (subgenotype Bj/B1) infection and development of fulminant hepatitis; on the other hand, no cases of fulminant hepatitis were registered among those infected with genotype A (subgenotype Ae/A2).[54] Differences in genotypes circulating in Asia and Europe/USA may indicate that distinct viral factors are playing roles in manifestation of infection by different genotype.

Chronic hepatitis

Chronic HBV infection is the most common cause of HCC in Asia.[68] Efficient surveillance and early diagnosis of development of this life complication requires risk stratification of chronic hepatitis B patients. Older age, male sex and liver cirrhosis are well recognized factors associated with increased risk of HCC.[69,70] In addition, recent large-scale population-based and clinical case–control studies carried out in Asia, have shown that infecting virus factors associated with a high risk of HCC, include HBV DNA levels,[71,72] HBV basal core promoter mutations,[35] genotype C (vs B),[22,36,73,74] and sub-

genotype Ce/C2.[71,75] There are data indicating that genotype C infection associated with a higher viral load than genotype B.[76] Association of genotype F with HCC was found to be higher than that of genotype C in Alaskan natives.[77,78] Unfortunately, there are few prospective studies examining other HBV genotypes for association with adverse outcomes. Genotype A (subgenotype Aa/A1) was found in association with development of HCC in young adults in South Africa.[79,80] However, very high rates of detection of subgenotype Aa/A1 among asymptomatic carriers suggest contribution of environmental factors (aflatoxin contained in food) for the development of HCC. In comparison with Aa/A1, HCC associated with Ae/A2 is found primarily in older individuals. In addition, the rate of complications, including HCC, for those infected with subgenotype Ae/A2 appears to be less than that found in those infected with genotype D, C or F1.[77,81] A prospective study in Spain showed that genotype A (presumably Ae/A2) infection was associated with a significantly higher cumulative rate of sustained biochemical remission, HBV DNA and HBsAg clearance in patients with chronic HBV infection than genotype D infection.[81]

Consensus 4

4-1 Recently, there is an increase of HBV genotype A proportion among acute hepatitis B infection cases in Japan. (Level 3.)

4-2 HBV genotype A acute infection has a tendency to evolve in chronic hepatitis compared to genotype B/C. (Level 3.)

4-3 Antiviral therapy of acute infection might be efficient in prevention of chronic carrier stage. (Level 3.)

4-4 Genotype C compared with genotype B is associated with higher risk of outcome in HCC in chronic carriers. (Level 2a, grade B.)

4-5 Genotype A compared with genotype D and F in chronic carriers is associated with better prognosis in terms of spontaneous ALT normalization and DNA clearance. (Level 2a, grade B.)

HBV MUTATIONS AND THEIR POTENTIAL IMPACT ON PATHOGENESIS OF HBV INFECTION

THE HBV GENOME consists of double-stranded DNA, 3200 bp in length. HBV replicates through reverse transcription of a RNA intermediate, the pregenome RNA, different from all known mammalian DNA viruses. HBV infection is characterized by high levels of virus production, however, the HBV reverse transcriptase is an error-prone enzyme lacking proof-reading capacity, resulting in a large number of nucleotide substitutions during replication. The misincorporation rate has been estimated to be of the order of 10^{10} incorrect nucleotide incorporations per day. As a result, HBV has a quasispecies distribution in infected patients.

Naturally occurring mutations identified in the HBV genome are more prevalent in patients with chronic hepatitis than in HBeAg positive asymptomatic carriers. Among them, several specific mutations have been shown to be associated with the pathogenesis of HBV infection.

HBeAg seroconversion

A HBV strain harboring stop codon mutation in the precore region was first reported in anti-HBe positive patients with chronic hepatitis.[25] The precore region located upstream of the core region is involved in the production and secretion of HBeAg protein. HBeAg is secreted into blood after removal of N-terminal 19 amino acids (a.a.) and C-terminal 34 a.a. from HBeAg precursor protein composed of precore and core regions. Nucleotide substitution of G to A at nt 1896 confers stop codon (TAG) mutation from tryptophan (TGG) at codon 28 in the precore region, resulting in a failure to produce HBeAg protein.[82–84] Although controversial, 10 genotypes have been identified tentatively so far[85] and genotypes affect the occurrence of stop codon mutation in the precore region. The stop codon mutation in the precore region (G1896A) is rarely encountered in HBV genomes of genotype A, some of genotype C and F, because they possess C at position 1858 that makes a pair with G at position 1896 in the stem-loop structure of the *cis*-encapsidation signal.[86]

The HBV core promoter regions located upstream of core region are involved in the transcription of precore mRNA and pregenomic RNA. Nucleotide substitution of A to T at nt 1762 combined with substitution of G to A at nt 1764 in the core promoter region give rise to a reduced transcription of precore mRNA and increased level of viral DNA, resulting in a decreased production of HBeAg protein and enhanced viral replication.[87–89]

Consensus 5

Nucleotide substitution G1896A confers stop codon mutation in the precore region. Nucleotide substitution A1762T combined with substitution G1764A in

the core promoter region give rise to a reduced transcription of precore mRNA. These nucleotide changes in combination with a reduction of HBeAg caused by suppressed replication of HBV are closely associated with HBeAg seroconversion. (Level 2b, grade B.)

Association between HBV mutations and clinical manifestation

Fulminant hepatitis

Precore and core promoter mutations are very frequent in patients with fulminant hepatitis from Asia[62,63,90] and the Middle East.[64] However, these mutations were not detected in those from Western countries.[65,67,91,92] This difference could be attributable to the difference of genotype prevalence, frequent genotype Ae and rare Bj in Western countries.[86] The patients infected with the former genotype rarely have precore mutant virus, while the latter frequently have the mutant virus. Stop codon mutation in the precore region is inhibited in genotype A because of C at position 1858 that makes a pair with G at position 1896 in the stem-loop structure of the *cis*-encapsidation signal.[93]

Ozasa *et al.* analyzed the difference of host and viral factors between 40 patients with fulminant hepatitis B and 256 with acute self-limited hepatitis B in a multicenter cross-sectional study,[54] and showed that precore stop codon mutation of G1896A and genotype Bj are associated with fulminant hepatitis in Japan. They also reported the marked enhancement of viral replication by introducing either G1896A or A1762T/G1764A mutation into the Bj clone in *in vitro* transfection study. Because this type of HBV mutant is found not only in patients with fulminant hepatitis but also in asymptomatic HBV carriers,[94] the interaction between the virus and the host's immune response might influence the outcome of HBV infection.

In addition to the mutants mentioned above, pre-S2 defective virus or HBV defective in secretion because of surface gene mutations are reported in patients with fulminant hepatitis. These mutant viruses showed a characteristic feature of virus retention in hepatocytes and misassembly with high replication capacity.[95–97]

HCC development

Evidence has been accumulating over the past decade that the risk of developing cirrhosis and HCC is influenced by the patient's viral status, such as genotype, viral load and genomic mutations. Naturally occurring mutations have been identified in the structural and non-structural genes as well as the regulatory elements of the virus, and these mutations are more prevalent in patients with chronic hepatitis than in HBeAg positive asymptomatic carriers.[98]

A double mutation, A1762T/G1764A in the basal core promoter region has been found in patients with advanced liver disease and HCC. Several case–control studies,[30,35,99–102] retrospective cohort studies[103,104] and one prospective cohort study[105] confirmed this finding, while some conflicting results were also reported in the case–control studies[106,107] and one prospective study.[108]

The role of deletions in the pre-S region of the HBV genome has been shown to be associated with the development of progressive liver diseases including HCC. Several case–control studies confirmed this finding.[27,107–110] A further mapping study of the pre-S region showed that all the deletion regions encompassed T- and B-cell epitopes and most of them lost one or more functional sites including the polymerized human serum albumin-binding site.[109] Deletion of these functional sites may cause intracellular retention of HBV envelope proteins and viral particles and contribute to more progressive liver damage and HCC development.

In addition to these common mutations, several other mutations, C1653T in the enhancer II region, T1753C/A/G in the core promoter region, and G1317A/T1341C/A/G in enhancer I region, have been reported to be associated with the development of HCC in some case–control studies.[30,107,111]

Consensus 6
There is some evidence that emergence of HBV genomic mutations arising during the course of chronic infection influence the outcome of chronic liver disease. Among them, core promoter mutations A1762T/G1764A might have a potential for developing progressive liver disease and HCC. (Level 2a, grade B.)

HBsAg escape mutant

The HBsAg mutant was first described in a child born to a HBsAg positive mother who developed acute hepatitis B in spite of vaccination and passive immunization against HBV.[112] This viral strain contained a substitution of glycine to arginine at position 145 (sG145R) and was able to escape the immune surveillance, resulting in an infection despite the presence of anti-HBs antibodies, vaccine escape mutant. Similar mutants have been detected all over the world.[113–115]