2. HBsAg-negative, and HBcAb- and/or HBsAb-positive patients

- HBsAg becomes positive
- HBV DNA becomes positive in patients whose HBV DNA level is below the sensitivity of detection

In Japan, 1–3% of the adult population is reportedly HBsAg-positive and ~20–25% are HBsAg-positive with HBcAb and/or HBsAb positivity (4,6,12–16) (Table 1). Thus, ~25% of the population of Japan, or about one out of every four patients, are said to be at risk for HBV reactivation when undergoing chemotherapy. In addition, the risk of HBV reactivation is thought to depend on the degree of immunosuppression (the intensity of the chemotherapy, whether immunosuppressive agents are administered in combination, the type of malignant tumor and the immune response in each patient etc.) and the characteristics of the HBV infection (viral load, HBV serological state etc.). Regarding the degree of immunosuppression, associations have been reported with steroid combination chemotherapy, rituximab combination chemotherapy, hematopoietic stem cell transplantation, organ transplantation and the presence of malignant lymphoma; regarding the characteristics of the HBV infection, differences have been reported according to the serological state of HBV-associated markers (HBsAg, HBeAg) and the viral load of HBV DNA before chemotherapy (1–4,6).

HBsAg-Positive Patients

Numerous reports have described the reactivation of HBV in HBsAg-positive patients with almost every type of malignant tumor and who were treated with a wide variety of anticancer drugs; in these reports, the incidence of HBV reactivation in HBsAg-positive patients was ~20–50% (9,12,17–30) (Table 2). Moreover, a recent report indicated that when the molecularly targeted drug everolimus was used to treat HBsAg-positive patients with unresectable hepatocellular carcinoma, HBV reactivation occurred in 7 (59.1%) of the 22 patients (9). Thus, HBV reactivation in HBsAg-positive patients is observed relatively frequently in almost every type of malignant tumor and anticancer drugs, and caution is required irrespective of the type of cancer or the chemotherapy regimen.

Table 1. Frequencies of hepatitis B surface antigen, hepatitis B core antibody and hepatitis B surface antibody in patients from several countries

Authors	City, country	*n*	HBsAg(+)	HBcAb(+)	HBcAb(+) or HBsAb(+)
Yeo et al. (12)	Hong Kong, China	626	12.0%	ND	ND
Hui et al. (6)	Hong Kong, China	244	ND	62.0%	72.0%
Koo et al. (13)	Singapore, Singapore	233	7.2%	34.3%	ND
Ludwig et al. (14)	New York, the USA	3343	1.3%	9.0%	ND
Kusumoto et al. (4)	Nagoya, Japan	3874	1.5%	20.0%	23.2%
Hattori et al. (15)	Tokyo, Japan	1031	ND	16.9%	ND
Matsue et al. (16)	Chiba, Japan	261	3.4%	24.3%	ND
Our hospital	Chiba, Japan	863	2.9%	21.4%	25.2%

HBsAg, hepatitis B surface antigen; HBcAb, hepatitis B core antibody; HBsAb, hepatitis B surface antibody; ND, no data.

HBsAg-Negative Patients with HBcAb and/or HBsAb Positivity

In 2001, Dervite et al. reported, for the first time, a case of HBV reactivation in an HBsAg-negative patient who had received rituximab combination chemotherapy (5); in 2006, Hui et al. reported that when they used systemic chemotherapy to treat 244 HBsAg-negative patients with malignant lymphoma, HBV reactivation was observed in 8 (3.3%) of them, and all 8 of these patients were HBcAb-positive and/or HBsAb-positive (6). Also, in 2009, Yeo et al. reported that when systemic chemotherapy was used to treat 80 HBsAg-negative patients with malignant lymphoma, HBV reactivation was observed in 5 (6.25%) of them; all 5 of these patients were HBcAb- and/or HBsAb-positive, and rituximab and steroid combination chemotherapy had been performed in all 5 patients (7). Thus, it became evident that reactivation as a result of the combined use of chemotherapy with a high immunosuppressive effect, such as rituximab, or combination therapy with a steroid can occur even in HBsAg-negative patients with HBcAb and/or HBsAb positivity who were generally believed to have recovered from HBV infection and to have been virus-free (6,7,31–35) (Table 3). However, almost all these reports concerned patients with malignant lymphoma who had been treated with rituximab, and reports of HBV reactivation in patients with solid tumors or other treatment regimens have been rare.

Risk Factors for HBV Reactivation

The following risk factors for HBV reactivation have been reported: male gender, younger age, HBeAg positivity, high HBV DNA load, low titer of HBsAb at pretreatment, breast cancer, malignant lymphoma, concomitant steroid treatment, treatment with an anthracycline anticancer drug and treatment with rituximab (6,7,12,13,22,24–27,31–34,36–42) (Table 4).

Outcome of Patients Who Developed HBV Reactivation

The prognosis of patients who develop liver dysfunction because of HBV reactivation has been reported to be poor.

Downloaded from http://jjco.oxfordjournals.org/ at National Cancer Centre (JMLA) on January 17, 2013

Table 2. Main reports of HBV reactivation in HBsAg-positive patients

Regimen	Cancer type	No. of reactivations	No. of all patients	Proportion of reactivation (%)	Author	Year
DOX+CPA(AC)→CPA+MTX+5FU, etc	Breast	17	41	41	Yeo (17)	2003
5FU+CPA, etc	Breast	19	61	31.1	Yeo (18)	2004
DOX+CPA(AC) or 5-FU+DOX+CPA(FAC)	Breast	16	76	21	Yun et al. (19)	2011
CPA+DOX+5FU(CAF), DOX+CPA(AC)→DTX	Breast	23	111	20.7	Kim et al. (20)	2007
DOX+CPA(AC)→PTX or DTX	Breast	18	128	14.1	Sohn et al. (21)	2011
Everolimus	HCC	13	22	59.1	Dai et al. (9)	2011
5FU+CDDP-HAI, 5FU+EPI+MMC-HAI	HCC	8	33	24.2	Nagamatsu et al. (22)	2003
EPI+CDDP-TACE, CDDP-TACE	HCC	15	37	40.5	Jang (23)	2006
EPI+CDDP-chemolipiodolization	HCC	28	86	33.7	Jang (24)	2004
CDDP+DOX+5FU+IFN(PIAF) or DOX	HCC	37	102	36	Yeo (25)	2004
EPI+CPA+Etop (ACE)	Lymphoma	9	24	38	Cheng et al. (26)	2003
EPI+CPA+Etop+PSL (PACE)	Lymphoma	18	25	73	Cheng et al. (26)	2003
Various	Lymphoma	13	27	48	Lok (27)	1991
Various	Lymphoma	17	53	32	Leaw et al. (28)	2004
Various	Solid & hematol	1	46	2.2	Nishida et al. (29)	2012
Various	Solid & hematol	9	59	15.3	Eren et al. (30)	2009
Various	Solid & hematol	15	78	19.2	Yeo (12)	2000

Dox, doxorubicin; CPA, cyclophosphamide; MTX, methotrexate; 5FU, 5-fluorouracil; DTX, docetaxel; PTX, paclitaxel; CDDP, cisplatin; EPI, epirubicin; MMC, mitomycin C; IFN, interferon; Etop, Etoposide; PSL, prednisolone; HCC, hepatocellular carcinoma; Solid & hematol, solid tumors and hematological malignancies; HAI, hepatic arterial infusion chemotherapy; TACE, transcatheter arterial chemoembolization.

Table 3. Main reports of HBV reactivation in HBsAg-negative patients with HBcAb and/or HBsAb positivity

Regimen	Cancer type	No. of reativations	No. of all patients	Proportion of reactivation (%)	Author	Year
Rituximab-CHOP	Lymphoma	5	21	23.8	Yeo (7)	2009
Rituximab-CHOP	Lymphoma	3	52	5.8	Koo (31)	2011
Rituximab containing regimen	Lymphoma	4	95	4.2	Pei (32)	2010
Rituximab containing regimen	Lymphoma	2	74	2.7	Targhetta (33)	2008
Rituximab non-containing regimen	Lymphoma	2	245	0.8	Targhetta (33)	2008
Rituximab non-containing regimen	Lymphoma	2	51	3.9	Lok (27)	1991
Rituximab non-containing regimen	Lymphoma	2	62	3.0	Koo (31)	2011
Various+/−Rituximab	Lymphoma	8	173	4.6	Hui (6)	2006
Various+/−Rituximab	Lymphoma	1	67	1.5	Koo (13)	2010
Various+/−Rituximab	Lymphoma	2	48	4.1	Fukushima (34)	2009
Various	Myeloma	1	15	6.7	Yoshida (35)	2010

CHOP, cyclophosphamide + hydroxydaunorubicin + vincristine + prednisolone.

Yeo et al. used lamivudine to treat 32 patients who developed hepatitis as a result of HBV reactivation, but systemic chemotherapy had to be discontinued in 22 (69%) of the patients and 5 patients (16%) died of hepatic failure (43). In addition, Umemura et al. reported HBV reactivation in 23 patients, and 5 (22%) of these patients developed fulminant hepatitis, resulting in a liver-related mortality rate of 26% and a mortality rate of 100% among the fulminant patients. Meanwhile, 45 (9%) out of 529 patients with acute hepatitis B developed fulminant hepatitis, and the liver-associated mortality rate was 4 overall and 47% among the fulminant patients. Thus, the rate of conversion to fulminant hepatitis

Table 4. Risk factors for HBV reactivation in patients receiving chemotherapy

Host-related factors	Treatment-related factors
Younger age (12,13)	Use of steroid (26,36)
Male sex (13,27)	Use of anthracyclines (12,36,41)
Cancer type: malignant lymphoma (12,36)	Use of rituximab (6,7,32-34,42)
Cancer type: breast cancer (36,37)	
Cancer type: liver cancer (22,24)	
High HBV DNA level (36–38)	
HBeAg seropositive (12,22,24)	
High intrahepatic covalently closed circular DNA (cccDNA) (39)	
Low titer of HBsAb (31,40)	
Elevated serum ALT level (25)	

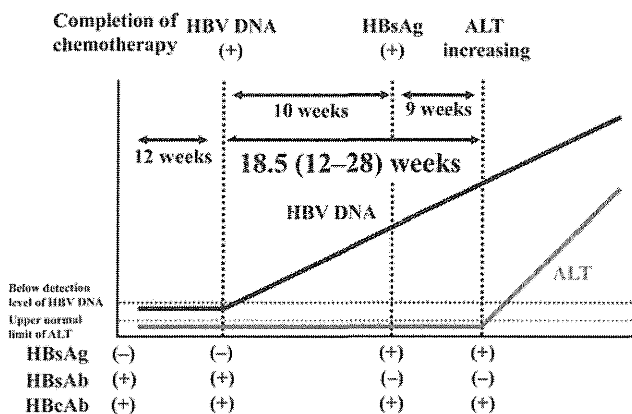

Figure 2. Pattern of hepatitis B viral reactivation in a hepatitis B surface antigen (HBsAg)-negative patient with hepatitis B core antibody (HBcAb) and/or hepatitis B surface antibody (HBsAb) positivity (7). Hepatitis due to HBV reactivation often developed after the completion of chemotherapy in an HBsAg-negative patient with HBcAb and/or HBsAb positivity. An increase in hepatitis B virus DNA has been reported to occur an average of 18.5 weeks before the onset of liver damage or hepatitis (7).

as a result of HBV reactivation and the liver-related mortality rate were significantly higher among patients with HBV reactivation than among patients with acute hepatitis (44). Thus, the prognosis of patients in whom HBV reactivation causes liver damage is extremely poor, and suppressing reactivation before it causes liver dysfunction is important.

Countermeasures Against HBV Reactivation

There are two approaches to preventing liver damage as a result of HBV reactivation: the prophylactic administration of an antiviral drug before chemotherapy, and the periodic monitoring of HBV DNA levels and the deferred pre-emptive administration of an antiviral drug when conversion to HBV DNA positivity is detected.

HBsAg-Positive Patients

Lau et al. randomly assigned 30 HBsAg-positive lymphoma patients who were scheduled to receive systemic chemotherapy into a prophylactic lamivudine group of 15 patients and a control group of 15 patients, and they reported that HBV reactivation occurred significantly less frequently in the lamivudine group (0%) than in the control group (eight patients, 53%) (45). In addition, a controlled study was conducted to evaluate the frequency of HBV reactivation by randomly assigning 76 patients with HBV-associated unresectable hepatocellular carcinoma to a prophylactic lamivudine therapy group and a control group when transcatheter arterial chemoembolization using epirubicin (50 mg/m^2) and cisplatin (60 mg/m^2) was performed (23). The results showed a reduction in the frequency of reactivation (lamivudine group versus control group: 2.8 versus 40.5%, $P < 0.001$) and the frequency of hepatitis as a result of reactivation (lamivudine group versus control group: 2.8 versus 29.7%, $P = 0.003$). Moreover, some meta-analyses of prophylactic lamivudine administration have already been reported (46,47), and the frequency of HBV reactivation, the proportion of deaths, the chemotherapy delay and the proportion of patients in whom chemotherapy was discontinued because HBV reactivation was shown to be lower in the prophylactic lamivudine groups. Thus, the prophylactic administration of antiviral drugs, such as lamivudine, has been shown to be useful in HBsAg-positive patients.

HBsAg-Negative Patients with HBcAb and/or HBsAb Positivity

The prevention of HBV reactivation has not been fully investigated in HBsAg-negative patients with HBcAb and/or HBsAb positivity. Previous reports show that in most of the patients who developed hepatitis, the hepatitis developed after the completion of chemotherapy. Moreover, an increase in HBV DNA has been reported to occur an average of 18.5 weeks before the onset of liver damage or hepatitis (6) (Fig. 2), and it may be possible to prevent liver damage arising from HBV reactivation even when the HBV DNA level is monitored once a month and deferred pre-emptive treatment with an antiviral drug is administered after the detection of the conversion to HBV DNA positivity. However, the usefulness of periodic HBV DNA monitoring has not been fully clarified, and confirmation using a well-designed prospective study is warranted. On the other hand, the prophylactic administration of an antiviral drug before chemotherapy in HBsAg-negative patients with HBcAb and/or HBsAb positivity is not recommended for economic reasons (48).

Downloaded from http://jjco.oxfordjournals.org/ at National Cancer Centre (JMLA) on January 17, 2013

HBV Reactivation Guidelines

The number of reports regarding HBV reactivation following chemotherapy has been gradually increasing, and countermeasures against HBV reactivation are urgently needed. Guidelines for the treatment of HBV reactivation following chemotherapy have been published by many groups: the American Association for the Study of Liver Disease (AASLD) Practice Guidelines in 2007 (49); the National Institute of Health (NIH) Consensus Development Conference Management of Hepatitis B in 2008 (50,51) and the European Association for the Study of the Liver (EASL) Clinical Practice Guidelines in 2009 (52). In 2009, the Guidelines for the Prevention of Immunosuppressive Therapy or Chemotherapy-induced Reactivation of HBV Infection was announced by the Ministry of Health, Labour and Welfare in Japan as a Joint report of the Intractable Liver Diseases Study Group of Japan and the Japanese Study Group for Standard Antiviral Therapy for Viral Hepatitis (48) (Fig. 3). All these guidelines are similar in principal (Table 2). When chemotherapy is going to be performed, the prophylactic administration of an antiviral drug in HBsAg-positive patients is recommended, and the periodic monitoring of the HBV DNA level and deferred pre-emptive administration of an antiviral drug after the detection of conversion to HBV DNA positivity is recommended for HBsAg-negative patients with HBcAb and/or HBsAb positivity. A summary of the guidelines that have been published by the Ministry of Health, Labour and Welfare in Japan (48) is presented below (Fig. 3).

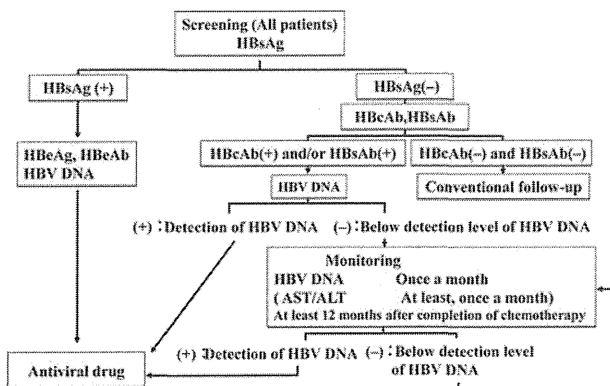

Figure 3. Japanese guidelines for the prevention of immunosuppressive therapy or chemotherapy-induced reactivation of hepatitis B viral infection (23). A screening examination for hepatitis B surface antigen (HBsAg) should be performed in every patient scheduled to receive chemotherapy. In patients with HBsAg-positive, Hepatitis B e antigen (HBeAg), HBe antibody (HBeAb) and hepatitis B virus (HBV) DNA levels should be measured, and an antiviral drug should, in principle, be prophylactically administered before the commencement of chemotherapy. In patients with HBsAg-negative, hepatitis B core antibody (HBcAb) and hepatitis B surface antibody (HBsAb) should be measured. If a patient tests positive for either HBcAb or HBsAb, the HBV DNA level should be quantitatively assayed. If the HBV DNA test is positive, an antiviral drug should be prophylactically administered. If the test is negative (below the sensitivity of detection), the HBV DNA level should be monitored monthly and the administration of an antiviral drug should be started after the detection of a conversion to positivity. If the patient is negative for both HBcAb and HBsAb, no risk of HBV reactivation exists, and periodic follow-up of the HBV DNA level is unnecessary.

Screening Examinations

A screening examination for HBsAg should be performed in every patient scheduled to receive chemotherapy, and patients with a high risk of HBV reactivation should be identified.

HBsAg-Positive Patients

HBeAg, hepatitis B e antibody (HBeAb) and HBV DNA levels should be measured, and the patient's HBV-related status should be confirmed prior to the start of chemotherapy. Since a high risk of HBV reactivation exists, an antiviral drug should, in principle, be prophylactically administered before the commencement of chemotherapy. However, because patients with chronic hepatitis or liver cirrhosis as well as HBV carriers are sometimes included among HBsAg-positive patients, a consultation with a hepatologist is also recommended.

HBsAg-Negative Patients

If a patient tests positive for either HBcAb or HBsAb, a risk of reactivation exists and the HBV DNA level should be quantitatively assayed. If the HBV DNA test is positive, an antiviral drug should be prophylactically administered. If the test is negative (below the sensitivity of detection), the HBV DNA level should be monitored monthly and the administration of an antiviral drug should be started after the detection of a conversion to positivity. As the rate of conversion to fulminant hepatitis and the liver-related mortality rate are high when an antiviral drug is administered after liver damage has occurred and the transaminases and total bilirubin levels have increased, the deferred pre-emptive administration of an antiviral drug before the onset of liver damage is important. If the patient is negative for both HBcAb and HBsAb, no risk of HBV reactivation exists, and periodic follow-up of the HBV DNA level is thought to be unnecessary.

Recommended Antiviral Drugs for Prophylactic Administration

The usefulness of lamivudine as a prophylactic antiviral drug to prevent HBV reactivation has been demonstrated in some of the above-mentioned randomized, controlled trials (23,45) and meta-analyses (46,47). However, because of problems regarding the therapeutic efficacy against HBV and the high frequency of HBV that is resistant to lamivudine, entecavir should be recommended for use in Japan (48).

Timing of the Termination of Prophylactic Antiviral Drug Administration

No clear evidence exists regarding the termination of the prophylactic administration of antiviral drugs. Once administration has been continued for 12 months after the completion of chemotherapy and a decrease in the ALT level to within the normal range and conversion to persistent HBV-DNA negativity have been achieved, the termination of the administration of the antiviral drug can be considered. However, HBV reactivation has sometimes been reported after the termination of the administration of an antiviral drug, and close follow-up is necessary for the next 12 months after the termination of antiviral drug administration. The recently reported recommendation regarding the safe withdrawal of antiviral drugs for chronic HBV patients (53) may also be referred to upon the termination of antiviral drug administration.

Problems Regarding HBV Reactivation

Guidelines for the prevention of immunosuppressive therapy or chemotherapy-induced reactivation of HBV infection have been announced (48), but the following issues should be noted: (i) patients who are HBsAg-negative with HBcAb and/or HBsAb positivity are regarded as a high-risk group, but the frequency of HBV reactivation, the types of anticancer drugs and the cancers that facilitate HBV reactivation, the clinical features of HBV reactivation and the optimal method of management etc. have not been sufficiently elucidated. (ii) Regular HBV DNA monitoring once a month and the deferred pre-emptive administration of an antiviral drug is recommended when performing chemotherapy in patients who are HBsAg-negative with HBcAb and/or HBsAb positivity, but the usefulness of such measures has not been clarified. Furthermore, the provisional clinical opinion of the American Society of Clinical Oncology (ASCO), which was

Table 5. Prospective studies on HBV reactivation

Study subjects							
Tumor type	HBsAg (+)	HBsAg (−), HBcAb and/or HBsAb (+)	No. of planned enrolled patients	Primary endpoint	Study design	No. for clinical trials registry	Country
All malignant diseases	Yes	No	180	Incidence of HBV reactivation under prophylactic anti-viral therapy	Randomized trial comparing prophylactic lamivudine versus entecavir	NCT01580202	Korea
All malignant diseases	Yes	No	70	Development of viral resistance to anti-viral therapy	Randomized trial comparing prophylactic lamivudine versus adefovir dipivoxil	NCT00489151	Hong Kong
Solid tumors	Yes	No	110	Incidence of chemotherapy interruptions	Randomized trial comparing immediate use versus deferred use of lamivudine	NCT00516945	Hong Kong
Lymphoma	No	Yes	90	Incidence of HBV reactivation	Randomized trial comparing prophylactic use versus therapeutic use of entecavir	NCT00926757	Taiwan
Lymphoma treated with rituximab-containing regimen	Yes	Yes	600	Incidence of HBV reactivation	Prospective observational trial	NCT01311232	Korea
Patients receiving immunosuppressive and/or anticancer drugs	Yes	Yes	530	Incidence of HBV reactivation under HBV DNA monitoring	Prospective observational trial	UMIN000002859	Japan
Lymphoma treated with rituximab-containing regimen	No	Yes	321	Incidence of HBV reactivation under HBV DNA monitoring	Prospective observational trial	UMIN000001299	Japan
Solid tumors	Yes	Yes	300	Incidence rate of HBV reactivation under HBV DNA monitoring and outcome of those who develop HBV reactivation	Prospective observational trial	UMIN000005369	Japan
Lymphoma treated with rituximab-containing regimen	No	Yes	150	Incidence of HBV reactivation under HBV DNA monitoring	Prospective observational trial	NCT00931229	Taiwan
Lymphoma treated with rituximab-CHOP	No	Yes	110	Incidence of HBV reactivation under lamivudine prophylaxis	Prospective interventional trial	NCT01210287	China

Downloaded from http://jjco.oxfordjournals.org/ at National Cancer Centre (JMLA) on January 17, 2013

published in 2010 (54), does not strongly recommend routine screening and the prophylactic use of antiviral drugs because the evidence was regarded as being insufficient to determine the net benefit. Thus, these guidelines were drafted in the absence of sufficient evidence of the ability to avoid the risk of HBV reactivation. At present, numerous well-designed prospective studies to clarify the present status of HBV reactivation are being conducted in Japan and in other countries (Table 5). In the future, the establishment of strong evidence regarding HBV reactivation is anticipated.

CONCLUSIONS

Because HBV reactivation is often observed following chemotherapy, even with recently developed molecularly targeted agents such as rituximab, and in patients who are HBsAg-negative with HBcAb and/or HBsAb positivity who are generally believed to have recovered from an HBV infection and to be free of the virus, HBV reactivation has attracted considerable attention in recent years. However, many issues regarding HBV reactivation have yet to be clarified, including how often reactivation actually occurs, which regimens involve a high risk of reactivation and how frequently monitoring should be performed. A variety of research is currently under way in both Japan and abroad, and strong evidence regarding HBV reactivation is anticipated in the future.

Funding

This work was supported in part by the National Cancer Center Research and Development Fund (23-A-30) and was supported in part by Grants-in-Aid for Cancer Research from the Ministry of Health, Labour, and Welfare, Japan.

Conflict of interest statement

None declared.

References

1. Vento S, Cainelli F, Longhi MS. Reactivation of replication of hepatitis B and C viruses after immunosuppressive therapy: an unresolved issue. *Lancet Oncol* 2002;3:333–40.
2. Lau GK. Hepatitis B reactivation after chemotherapy: two decades of clinical research. *Hepatol Int* 2008;2:152–62.
3. Yeo W, Johnson PJ. Diagnosis, prevention and management of hepatitis B virus reactivation during anticancer therapy. *Hepatology* 2006;43:209–20.
4. Kusumoto S, Tanaka Y, Mizokami M, et al. Reactivation of hepatitis B virus following systemic chemotherapy for malignant lymphoma. *Int J Hematol* 2009;90:13–23.
5. Dervite I, Hober D, Morel P. Acute hepatitis B in a patient with antibodies to hepatitis B surface antigen who was receiving rituximab. *N Engl J Med* 2001;344:68–9.
6. Hui CK, Cheung WW, Zhang HY, et al. Kinetics and risk of de novo hepatitis B infection in HBsAg-negative patients undergoing cytotoxic chemotherapy. *Gastroenterology* 2006;131:59–68.
7. Yeo W, Chan TC, Leung NW, et al. Hepatitis B virus reactivation in lymphoma patients with prior resolved hepatitis B undergoing anticancer therapy with or without rituximab. *J Clin Oncol* 2009;27:605–11.
8. Chen L, Shiah HS, Chen CY, et al. Randomized, phase I, and pharmacokinetic (PK) study of RAD001, an mTOR inhibitor, in patients (pts) with advanced hepatocellular carcinoma (HCC). *J Clin Oncol* 2009;27:(15s suppl) (Abstr 4587).
9. Dai CY, Shiah HS, Chang JY, et al. Change of serum HBV DNA titer and hepatitis flare-up after everolimus, a mTOR inhibitor, in HBsAg-seropositive advanced hepatocellular carcinoma. In: The 21st Conference of the Asian Pacific Association for Study of the Liver Proceeding P187 (abstract PP34–30).
10. Yim HJ, Lok AS. Natural history of chronic hepatitis B virus infection: what we knew in 1981 and what we know in 2005. *Hepatology* 2006;43:S173–S81.
11. Rehermann B, Ferrari C, Pasquinelli C, Chisari FV. The hepatitis B virus persists for decades after patients' recovery from acute viral hepatitis despite active maintenance of a cytotoxic T-lymphocyte response. *Nat Med* 1996;2:1104–8.
12. Yeo W, Chan PK, Zhong S, et al. Frequency of hepatitis B virus reactivation in cancer patients undergoing cytotoxic chemotherapy: a prospective study of 626 patients with identification of risk factors. *J Med Virol* 2000;62:299–307.
13. Koo YX, Tan DS, Tan IB, Tao M, Chow WC, Lim ST. Hepatitis B virus reactivation and role of antiviral prophylaxis in lymphoma patients with past hepatitis B virus infection who are receiving chemoimmunotherapy. *Cancer* 2010;116:115–21.
14. Ludwig E, Mendelsohn RB, Taur Y, et al. Prevalence of hepatitis B surface antigen and hepatitis B core antibody in a population initiating immunosuppressive therapy. *J Clin Oncol* 2010;28:15s(suppl; abstr 9009).
15. Hattori M, Ito Y, Yokoyama M, et al. Risk of hepatitis B virus reactivation in HBsAg-negative patients who receive chemotherapy. In: The 8th Annual Meeting of Japanese Society of Medical Oncology, Tokyo, March 18–19, 2010 (abstr O2–028).
16. Matsue K, Kimura S, Takanashi Y, et al. Reactivation of hepatitis B virus after rituximab-containing treatment in patients with CD20-positive B-cell lymphoma. *Cancer* 2010;116:4769–76.
17. Yeo W, Chan PK, Hui P, et al. Hepatitis B virus reactivation in breast cancer patients receiving cytotoxic chemotherapy: a prospective study. *J Med Virol* 2003;70:553–61.
18. Yeo W, Ho WM, Hui P, et al. Use of lamivudine to prevent hepatitis B virus reactivation during chemotherapy in breast cancer patients. *Breast Cancer Res Treat* 2004;88:209–15.
19. Yun J, Kim KH, Kang ES, et al. Prophylactic use of lamivudine for hepatitis B exacerbation in post-operative breast cancer patients receiving anthracycline-based adjuvant chemotherapy. *Br J Cancer* 2011;104:559–63.
20. Kim MK, Ahn JH, Kim SB, et al. Hepatitis B reactivation during adjuvant anthracycline-based chemotherapy in patients with breast cancer: a single institution's experience. *Korean J Intern Med* 2007;22:237–43.
21. Sohn BS, Ahn JH, Jung KH, et al. Updated longitudinal data on acute exacerbation of chronic hepatitis B in patients with breast cancer receiving anthracycline-based adjuvant chemotherapy: therapeutic vs. pre-emptive use of lamivudine. *Jpn J Clin Oncol* 2011;41:1059–66.
22. Nagamatsu H, Kumashiro R, Itano S, Matsugaki S, Sata M. Investigation of associating factors in exacerbation of liver damage after chemotherapy in patients with HBV-related HCC. *Hepatol Res* 2003;26:293–301.
23. Jang JW, Choi JY, Bae SH, et al. A randomized controlled study of preemptive lamivudine in patients receiving transarterial chemolipidolization. *Hepatology* 2006;43:233–40.
24. Jang JW, Choi JY, Bae SH, et al. Transarterial chemo-lipiodolization can reactivate hepatitis B virus replication in patients with hepatocellular carcinoma. *J Hepatol* 2004;41:427–35.
25. Yeo W, Lam KC, Zee B, et al. Hepatitis B reactivation in patients with hepatocellular carcinoma undergoing systemic chemotherapy. *Ann Oncol* 2004;15:1661–6.

26. Cheng AL, Hsiung CA, Su IJ, et al. Lymphoma Committee of Taiwan Cooperative Oncology Group. Steroid-free chemotherapy decreases risk of hepatitis B virus (HBV) reactivation in HBV-carriers with lymphoma. *Hepatology* 2003;37:1320–8.
27. Lok AS, Liang RH, Chiu EK, Wong KL, Chan TK, Todd D. Reactivation of hepatitis B virus replication in patients receiving cytotoxic therapy. Report of a prospective study. *Gastroenterology* 1991;100:182–8.
28. Leaw SJ, Yen CJ, Huang WT, et al. Preemptive use of interferon or lamivudine for hepatitis B reactivation in patients with aggressive lymphoma receiving chemotherapy. *Ann Hematol* 2004;83:270–5.
29. Nishida T, Hiramatsu N, Mizuki M, et al. Managing hepatitis B virus carriers with systemic chemotherapy or biologic therapy in the outpatient clinic. *Hepatol Res* 2012. doi:10.1111/j.1872-034X.2012.01073.x.
30. Eren OO, Artac M, Boruban MC, Yavas O, Arslan U, Basaranoglu M. Chemotherapy-induced hepatitis B virus reactivation in HbsAg positive cancer patients: a single center experience. *Med Oncol* 2009;26:386–92.
31. Koo YX, Tay M, Teh YE, et al. Risk of hepatitis B virus (HBV) reactivation in hepatitis B surface antigen negative/hepatitis B core antibody positive patients receiving rituximab-containing combination chemotherapy without routine antiviral prophylaxis. *Ann Hematol* 2011;90:1219–3.
32. Pei SN, Chen CH, Lee CM, et al. Reactivation of hepatitis B virus following rituximab-based regimens: a serious complication in both HBsAg-positive and HBsAg-negative patients. *Ann Hematol* 2010;89:255–62.
33. Targhetta C, Cabras MG, Mamusa AM, Mascia G, Angelucci E. Hepatitis B virus-related liver disease in isolated anti-hepatitis B-core positive lymphoma patients receiving chemo- or chemo-immune therapy. *Haematologica* 2008;93:951–2.
34. Fukushima N, Mizuta T, Tanaka M, et al. Retrospective and prospective studies of hepatitis B virus reactivation in malignant lymphoma with occult HBV carrier. *Ann Oncol* 2009;20:2013–7.
35. Yoshida T, Kusumoto S, Inagaki A, et al. Reactivation of hepatitis B virus in HBsAg-negative patients with multiple myeloma: two case reports. *Int J Hematol* 2010;91:844–9.
36. Yeo W, Zee B, Zhong S, et al. Comprehensive analysis of risk factors associating with Hepatitis B virus (HBV) reactivation in cancer patients undergoing cytotoxic chemotherapy. *Br J Cancer* 2004;90:1306–11.
37. Zhong S, Yeo W, Schroder C, et al. High hepatitis B virus (HBV) DNA viral load is an important risk factor for HBV reactivation in breast cancer patients undergoing cytotoxic chemotherapy. *J Viral Hepat* 2004;11:55–9.
38. Lau GK, Leung YH, Fong DY, et al. High hepatitis B virus (HBV) DNA viral load as the most important risk factor for HBV reactivation in patients positive for HBV surface antigen undergoing autologous hematopoietic cell transplantation. *Blood* 2002;99:2324–0.
39. Hui CK, Bowden S, Jackson K, et al. Clinical significance of intrahepatic hepatitis B virus covalently closed circular DNA in chronic hepatitis B patients who received cytotoxic chemotherapy. *Blood* 2005;105:2616–7.
40. Pei SN, Ma MC, Wang MC, et al. Analysis of hepatitis B surface antibody titers in B cell lymphoma patients after rituximab therapy. *Ann Hematol* 2012;91:1007–12.
41. Hsu CH, Hsu HC, Chen HL, et al. Doxorubicin activates hepatitis B virus (HBV) replication in HBV-harboring hepatoblastoma cells. A possible novel mechanism of HBV reactivation in HBV carriers receiving systemic chemotherapy. *Anticancer Res* 2004;24:3035–40.
42. Tsutsumi Y, Shigematsu A, Hashino S, et al. Analysis of reactivation of hepatitis B virus in the treatment of B cell non-Hodgkin's lymphoma in Hokkaido. *Ann Hematol* 2009;88:375–7.
43. Yeo W, Chan PK, Ho WM, et al. Lamivudine for the prevention of hepatitis B virus reactivation in hepatitis B s-antigen seropositive cancer patients undergoing cytotoxic chemotherapy. *J Clin Oncol* 2004;22:927–34.
44. Umemura T, Tanaka E, Kiyosawa K, et al. Mortality secondary to fulminant hepatic failure in patients with prior resolution of hepatitis B virus infection in Japan. *Clin Infect Dis* 2008;47:e52–6.
45. Lau GK, Yiu HH, Fong DY, et al. Early is superior to deferred preemptive lamivudine therapy for hepatitis B patients undergoing chemotherapy. *Gastroenterology* 2003;125:1742–9.
46. Martyak LA, Taqavi E, Saab S. Lamivudine prophylaxis is effective in reducing hepatitis B reactivation and reactivation-related mortality in chemotherapy patients: a meta-analysis. *Liver Int* 2008;28:28–38.
47. Loomba R, Rowley A, Wesley R, et al. Systematic review: the effect of preventive lamivudine on hepatitis B reactivation during chemotherapy. *Ann Intern Med* 2008;148:519–28.
48. Tsubouchi H, Kumada H, Kiyosawa K, et al. Prevention of immunosuppressive therapy or chemotherapy-induced reactivation of hepatitis B virus infection—Joint report of the Intractable Liver Diseases Study Group of Japan and the Japanese Study Group of the Standard Antiviral Therapy for Viral Hepatitis. *Kanzo* 2009;50:38–42 (In Japanese).
49. Lok AS, McMahon BJ. Chronic hepatitis B. *Hepatology* 2007; 45:507–39.
50. NIH Consensus Development Conference Statement on Management of Hepatitis B. NIH Consensus and State-of-the-Science Statements. Volume 25, Number 2. 20–22 October 2008.
51. Hoofnagle JH. Reactivation of hepatitis B. *Hepatology* 2009;49(suppl.):S156–65.
52. European Association for the Study of the Liver. EASL clinical practice guidelines: management of chronic hepatitis B. *J Hepatol* 2009;50:227–42.
53. Matsumoto A, Tanaka E, Suzuki Y, et al. Combination of hepatitis B viral antigens and DNA for prediction of relapse after discontinuation of nucleos(t)ide analogs in patients with chronic hepatitis B. *Hepatol Res* 2012;42:139–49.
54. Artz AS, Somerfield MR, Feld JJ, et al. American Society of Clinical Oncology provisional clinical opinion: chronic hepatitis B virus infection screening in patients receiving cytotoxic chemotherapy for treatment of malignant diseases. *J Clin Oncol* 2010;28:3199–202.

Downloaded from http://jjco.oxfordjournals.org/ at National Cancer Centre (JMLA) on January 17, 2013

Dynamics of Hepatitis B Virus Quasispecies in Association with Nucleos(t)ide Analogue Treatment Determined by Ultra-Deep Sequencing

Norihiro Nishijima[1], Hiroyuki Marusawa[1]*, Yoshihide Ueda[1], Ken Takahashi[1], Akihiro Nasu[1], Yukio Osaki[2], Tadayuki Kou[3], Shujiro Yazumi[3], Takeshi Fujiwara[4], Soken Tsuchiya[4], Kazuharu Shimizu[4], Shinji Uemoto[5], Tsutomu Chiba[1]

1 Department of Gastroenterology and Hepatology, Graduate School of Medicine, Kyoto University, Kyoto, Japan, 2 Department of Gastroenterology and Hepatology, Osaka Red Cross Hospital, Osaka, Japan, 3 Department of Gastroenterology and Hepatology, Tazuke Kofukai Medical Research Institute, Kitano Hospital, Osaka, Japan, 4 Department of Nanobio Drug Discovery, Graduate School of Pharmaceutical Sciences, Kyoto University, Kyoto, Japan, 5 Department of Surgery, Graduate School of Medicine, Kyoto University, Kyoto, Japan

Abstract

Background and Aims: Although the advent of ultra-deep sequencing technology allows for the analysis of heretofore-undetectable minor viral mutants, a limited amount of information is currently available regarding the clinical implications of hepatitis B virus (HBV) genomic heterogeneity.

Methods: To characterize the HBV genetic heterogeneity in association with anti-viral therapy, we performed ultra-deep sequencing of full-genome HBV in the liver and serum of 19 patients with chronic viral infection, including 14 therapy-naïve and 5 nucleos(t)ide analogue(NA)-treated cases.

Results: Most genomic changes observed in viral variants were single base substitutions and were widely distributed throughout the HBV genome. Four of eight (50%) chronic therapy-naïve HBeAg-negative patients showed a relatively low prevalence of the G1896A pre-core (pre-C) mutant in the liver tissues, suggesting that other mutations were involved in their HBeAg seroconversion. Interestingly, liver tissues in 4 of 5 (80%) of the chronic NA-treated anti-HBe-positive cases had extremely low levels of the G1896A pre-C mutant (0.0%, 0.0%, 0.1%, and 1.1%), suggesting the high sensitivity of the G1896A pre-C mutant to NA. Moreover, various abundances of clones resistant to NA were common in both the liver and serum of treatment-naïve patients, and the proportion of M204VI mutants resistant to lamivudine and entecavir expanded in response to entecavir treatment in the serum of 35.7% (5/14) of patients, suggesting the putative risk of developing drug resistance to NA.

Conclusion: Our findings illustrate the strong advantage of deep sequencing on viral genome as a tool for dissecting the pathophysiology of HBV infection.

Citation: Nishijima N, Marusawa H, Ueda Y, Takahashi K, Nasu A, et al. (2012) Dynamics of Hepatitis B Virus Quasispecies in Association with Nucleos(t)ide Analogue Treatment Determined by Ultra-Deep Sequencing. PLoS ONE 7(4): e35052. doi:10.1371/journal.pone.0035052

Editor: Antonio Bertoletti, Singapore Institute for Clinical Sciences, Singapore

Received November 17, 2011; **Accepted** March 8, 2012; **Published** April 16, 2012

Copyright: © 2012 Nishijima et al. This is an open-access article distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

Funding: This work was supported by JSPS Grant-in-aid for Scientific Research 21229009, 23390196, and Health and Labor Science Research Grants (H22-08) and Research on Hepatitis from the Ministry of Health, Labor and Welfare, Japan. (http://mhlw-grants.niph.go.jp/). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

* E-mail: maru@kuhp.kyoto-u.ac.jp

Introduction

Hepatitis B virus (HBV) is a non-cytopathic DNA virus that infects approximately 350 million people worldwide and is a main cause of liver-related morbidity and mortality [1–3]. The absence of viral-encoded RNA-dependent DNA polymerase proofreading capacity coupled with the extremely high rate of HBV replication yields the potential to rapidly generate mutations at each nucleotide position within the entire genome [4]. Accordingly, a highly characteristic nature of HBV infection is the remarkable genetic heterogeneity at the inter- and intra- patient level. The latter case of variability as a population of closely-related but nonidentical genomes is referred to as viral quasispecies [5,6]. It is well recognized that such mutations may have important implications regarding the pathogenesis of viral disease. For example, in chronic infection, G to A point mutation at nucleotide (nt) 1896 in the pre-core (pre-C) region as well as A1762T and G1764A mutations in the core-promoter region are highly associated with HBeAg seroconversion that in general results in the low levels of viremia and consequent clinical cure [7–9]. In contrast, acute infection with the G1896A pre-C mutant represents a high risk for fulminant hepatic failure [10,11]. Although these facts clearly illustrate the clinical implications of certain viral mutation, increasing evidence strongly suggests that

the viral genetic heterogeneity is more complicated than previously thought [12,13].

The major goals of antiviral therapy in patients with HBV infection are to prevent the progression of liver disease and inhibit the development of hepatocellular carcinoma [14]. Oral nucleos(t)ide analogue (NA) have revolutionized the management of HBV infection, and five such antiviral drugs, including lamivudine, adefovir, entecavir, tenofovir, and telbivudine, are currently approved medications [15,16]. These agents are well-tolerated, very effective at suppressing viral replication, and safe, but one of the major problems of NA therapy is that long-term use of these drugs frequently causes the emergence of antiviral drug-resistant HBV due to substitutions at specific sites in the viral genome sequences, which often negates the benefits of therapy and is associated with hepatitis flares and death [16,17]. It is unclear whether viral clones with antiviral resistance emerge after the administration of antiviral therapy or widely preexist among treatment-naïve patients.

There has been a recent advance in DNA sequencing technology [18]. The ultra-deep sequencers allow for massively parallel amplification and detection of sequences of hundreds of thousands of individual molecules. We recently demonstrated the usefulness of ultra-deep sequencing technology to unveil the massive genetic heterogeneity of hepatitis C virus (HCV) in association with treatment response to antiviral therapy [19]. On the other hand, there are a few published studies in which this technology was used to characterize genetic HBV sequence variations [20–22]. Margeridon-Thermet et al reported that the 454 Life Science GS20 sequencing platform provided higher sensitivity for detecting drug-resistant HBV mutations in the serum of patients treated with nucleoside and nucleotide reverse-transcriptase inhibitors [20]. Solmone et al also reported the strong advantage conferred by the same platform to detect minor variants in the serum of patients with chronic HBV infection [21]. Although in these previous studies low-abundant drug-resistant variants were successfully detected, the analyses were focused on the reverse-transcriptase region of circulating HBV in the serum and thus the whole picture of HBV genetic heterogeneity as well as the *in vivo* dynamics of HBV drug resistant variants in response to anti-viral treatment remains to be clarified. Moreover, intrahepatic viral heterogeneity in patients that achieved the clearance of circulating HBV is largely unknown.

By taking the advantage of an abundance of genetic information obtained by utilizing the Illumina Genome Analyzer II (Illumina, San Diego, CA) as a platform of ultra-deep sequencing, we determined the whole HBV sequence in the liver and serum of patients with chronic HBV infection to evaluate viral quasispecies characteristics. Moreover, we investigated the prevalence of rare drug-resistant HBV variants as well as detailed dynamic changes in the viral genetic heterogeneity in association with NA administration. Based on the abundant genetic information obtained by ultra-deep sequencing, we clarified the precise prevalence of HBV clones with G1896A pre-C mutations in association with HBe serostatus in chronically infected patients with or without NA treatment. We also detected a variety of minor drug-resistant clones in treatment-naïve patients and their dynamic changes in response to entecavir administration, demonstrating the potential clinical significance of naturally-occurring drug-resistant mutations.

Materials and Methods

Ethics Statement

The Kyoto University ethics committee approved the study, and written informed consent for participation in this study was obtained from all patients. The study was conducted in accordance with the principles of the Declaration of Helsinki.

Patients

The liver tissues of 19 Japanese patients that underwent living-donor liver transplantation at Kyoto University due to HBV-related liver disease were available for viral genome analyses. These individuals included 13 men and 6 women, aged 41 to 69 years (median, 55.2 years) and all but one were infected with genotype C viruses. Participants comprised 19 patients with liver cirrhosis caused by chronic HBV infection, including 14 antiviral therapy-naïve cases (chronic-naïve cases) and 5 cases receiving NA treatment, with either lamivudine or entecavir (chronic-NA cases) (Table 1). Serum HBV DNA levels were significantly higher in chronic-naïve cases than in chronic NA cases (median serum HBV DNA levels were 5.6, and <2.6 log copies/ml, respectively, Table 1). Liver tissue samples were obtained at the time of transplantation, frozen immediately, and stored at −80°C until use. Serologic analyses of HBV markers, including hepatitis B surface antigen (HBsAg), antibodies to HBsAg, anti-HBc, HBeAg, and antibodies to HBeAg, were determined by enzyme immunoassay kits as described previously [23]. HBV DNA in the serum before transplantation was examined using a polymerase chain reaction (PCR) assay (Amplicor HBV Monitor, Roche, Branchburg, NJ). To examine the dynamics of viral quasispecies in response to anti-HBV therapy, paired serum samples of 14 treatment-naïve patients before and after administration of daily entecavir (0.5 mg/day) were subjected to further analyses on viral genome.

Direct population Sanger sequencing

DNA was extracted from the liver tissue and serum using a DNeasy Blood & Tissue Kit (Qiagen, Tokyo, Japan). To define the consensus reference sequences of HBV in each clinical specimen, all samples were first subjected to direct population Sanger sequencing using the Applied Biosystems 3500 Genetic Analyzer (Applied Biosystems, Foster City, CA). Oligonucleotide primers for the HBV genome were designed to specifically amplify whole viral sequences as two overlapping fragments using the sense primer 169_F and antisense primer 2847_R to yield a 2679-bp amplicon (amplicon 1), and the sense primer 685_F and antisense primer 443_R to yield a 2974-bp amplicon (amplicon 2; Table S1). HBV sequences were amplified using Phusion High-Fidelity DNA polymerase (FINZYMES, Espoo, Finland). All amplified PCR products were purified using the QIAquick Gel Extraction kit (Qiagen) after agarose gel electrophoresis and used for direct sequencing. The serum of a healthy HBV DNA-negative volunteer was used as a negative control.

Viral genome sequencing by massively-parallel sequencing

Massively-parallel sequencing with multiplexed tags was performed using the Illumina Genome Analyzer II as described [19]. The end-repair of DNA fragments, addition of adenine to the 3′ ends of DNA fragments, adaptor ligation, and PCR amplification by Illumina PCR primers were performed as described previously [24]. Briefly, the viral genome sequences were amplified by high-fidelity PCR using oligonucleotide primers as described above, sheared by nebulization using 32 psi N2 for 8 min, and then the sheared fragments were purified and concentrated using a QIAquick PCR purification Kit (Qiagen). Nucleotide overhangs resulting from fragmentation were then converted into blunt ends using T4 DNA polymerase and Klenow

Table 1. Characteristics of patients with chronic HBV infection analyzed in this study.

	Chronic-naïve (N = 14)	Chronic-NA (N = 5)
Age†	55.5 (41–69)	55.0 (49–68)
Sex (male/female)	9/5	4/1
Alanine aminotransaminase (IU/l)†	41 (10–74)	30 (15–65)
Total bilirubin (mg/dl)†	0.9 (0.5–31.1)	1.7 (0.6–4.5)
Platelet count ($\times 10^4$/mm^3)†	12.7 (3.3–27.6)	5.1 (3.6–11.3)
HBV genotype		
B	1	0
C	13	5
Viral load (log copies /ml)†	5.6 (<2.6–8.8)*	<2.6 (<2.6–5.3)*
HBe-serostatus (HBeAg+/HBeAb+)	8/6	0/5
Fibrosis		
F0–F2	6	0
F3–F4	8	5
Activity		
A0–A1	7	3
A2–A3	7	2

†Values are median (range).
*P = 0.042.
doi:10.1371/journal.pone.0035052.t001

enzymes, followed by the addition of terminal 3′ A-residues. An adaptor containing unique 6-bp tags, such as "ATCACG" and "CGATGT" (Multiplexing Sample Preparation Oligonucleotide Kit, Illumina), was then ligated to each fragment using DNA ligase. We then performed agarose gel electrophoresis of adaptor-ligated DNAs and excised bands from the gel to produce libraries with insert sizes ranging from 200 to 350 bp. These libraries were amplified independently using a minimal PCR amplification step of 18 cycles by Illumina PCR primers with Phusion High-Fidelity DNA polymerase. The DNA fragments were then purified with a MinElute PCR Purification Kit (Qiagen), followed by quantification using the NanoDrop 2000C (Thermo Fisher Scientific, Waltham, MA) to make a working concentration of 10 nM. Cluster generation and sequencing was performed for 64 cycles on the Illumina Genome Analyzer II according to the manufacturer's instructions. The obtained images were analyzed and base-called using GA pipeline software version 1.4 with the default settings provided by Illumina.

Genome Analyzer sequence data analysis

Using the high performance alignment software "NextGene" (SoftGenetics, State College, PA), the 64 base-pair reads obtained from the Genome Analyzer II were aligned with the reference sequences of 3215 bp that were determined by direct population Sanger sequencing of each clinical specimen. Reads with 90% or more bases matching a particular position of the reference sequences were aligned. Furthermore, two quality filters were used for sequencing reads: the reads with a median quality score of more than 30 and no more than 3 uncalled nucleotides were allowed anywhere in the 64 bases. Only sequences that passed the quality filters, rather than raw sequences, were analyzed and each position of the viral genome was assigned a coverage depth, representing the number of times the nucleotide position was sequenced.

Allele-specific quantitative real-time PCR and semiquantitative PCR to determine the relative proportion of G1896A pre-C mutant

To determine the relative proportion of the G1896A pre-C mutant, allele-specific quantitative real-time PCR was performed based on the previously described method [25,26]. Oligonucleotide primers were designed individually to amplify the pre-C region of wild-type and the G1896A pre-C mutant HBV. Three primers were used for this protocol, two allele-specific sense primers, 1896WT_F (for wild-type) and 1896MT_F (for the G1896A pre-C mutant), and one common antisense primer, 2037_R (Table S1). Quantification of wild-type and the G1896A pre-C mutant was individually performed by real-time PCR using a Light Cycler 480 and Fast Start Universal SYBR Master (Roche, Mannheim, Germany) [27]. The relative proportion of the G1896A pre-C mutant was determined to calculate the G1896A pre-C mutant/total HBV ratios. Performance of this assay was tested using mixtures of two previously described plasmids, pcDNA3-HBV-wt#1 and pcDNA3-HBV-G1896A pre-C mutant [28]. Semiquantitative PCR was performed using primers described above, then agarose gel electrophoresis was performed.

Statistical analysis

Results are expressed as mean or median, and range. Pretreatment values were compared using the Mann-Whitney U-test or the Kruskal Wallis H-test. *P* values less than 0.05 were considered statistically significant.

The viral quasispecies characteristics were evaluated by analyzing the genetic complexity based on the number of different sequences present in the population. Genetic complexity for each site was determined by calculating the Shannon entropy using the following formula:

$$Sn = -\frac{\sum_{i=1}^{n} fi(\ln fi)}{N}$$

where n is the number of different species identified, fi is the observed frequency of a particular variant in the quasispecies, and N is the total number of clones analyzed [12,13]. The mean viral complexity in each sample was determined by calculating the total amounts of the Shannon entropy at each nucleotide position divided by the total nucleotide number (e.g., 3215 bases) of each HBV genome sequence.

Nucleotide sequence accession number

All sequence reads have been deposit in DNA Data Bank of Japan Sequence Read Archive (http://www.ddbj.nig.ac.jp/index-e.html) under accession number DRA000435.

Results

Validation of multiplex ultra-deep sequencing of the HBV genome

To differentiate true mutations from sequencing errors in the determined sequences, we first generated viral sequence data from the expression plasmid, pcDNA3-HBV-wt#1, encoding wild-type genotype C HBV genome sequences [28]. For this purpose, we determined the PCR-amplified HBV sequences derived from the expression plasmid using high-fidelity Taq polymerase to take the PCR-induced errors as well as sequencing errors into consideration. Viral sequences determined by the conventional Sanger method were used as reference sequences for aligning the amplicons obtained by ultra-deep sequencing. Three repeated ultra-deep sequencing generated a mean of 77,663 filtered reads, corresponding to a mean coverage of 38,234 fold at each nucleotide site (Table S2). Errors comprised insertions (0.00003%), deletions (0.00135%), and nucleotide mismatches (0.037%). The mean overall error rate was 0.034% (distribution of per-nucleotide error rate ranged from 0 to 0.13%) for the three control experiments, reflecting the error introduced by high-fidelity PCR amplification and by multiplex ultra-deep sequencing that remained after filtering out problematic sequences. We also confirmed that multiplex ultra-deep sequencing with and without the high-fidelity PCR amplification with HBV-specific primer sets showed no significant differences in the error rates on the viral sequence data (mean error rate 0.034% vs 0.043%). Accordingly, we defined the cut-off value in its current platform as 0.3%, a value nearly 1 log above the mean overall error rate.

Next, we performed additional control experiments to verify the detectability of the low abundant mutations that presented at a frequency of less than 0.3%. For this purpose, we introduced expression plasmids with a single-point mutation within that encoding a wild-type viral sequence with a ratio of 1:1000 and assessed the sensitivity and accuracy of quantification using high-fidelity PCR amplification followed by multiplex ultra-deep sequencing in association with the different coverage numbers (Table S3). Repeated control experiments revealed that the threshold for detecting low-abundant mutations at an input ratio of 0.10% among the wild-type sequences ranged between 0.11% and 0.24%, indicating that there was no significant difference in the detection rate or error rates under the different coverage conditions. Based on these results, the accuracy of ultra-deep sequencing in its current platform for detecting low-level viral mutations was considered to be greater than 0.30%.

Viral complexity of the HBV quasispecies in association with clinical status

To clarify HBV quasispecies in association with clinical status, we performed multiplex ultra-deep sequencing and determined the HBV full-genome sequences in the liver and serum with chronic HBV infection. First, we compared the sequences of the viral genome determined in the liver tissue with those in the serum and found no significant differences in the viral population between the liver and serum of the same individual. Indeed, the pattern and distribution of genetic heterogeneity of the viral nucleotide sequences in the liver tissue were similar to those observed in the serum of the same patient (Figure S1), suggesting that a similar pattern of viral heterogeneity was maintained in the liver and serum of patients with chronic HBV infection.

Next, we compared the viral heterogeneity in the liver of chronic-naïve and chronic-NA cases. A mean of 5,962,996 bp nucleotides in chronic-naïve cases and 4,866,783 bp nucleotides in chronic-NA cases were mapped onto the reference sequences, and an overall average coverage depth of 1,855 and 1.514 was achieved for each nucleotide site of the HBV sequences, respectively (Table 2). The frequencies of mutated positions and altered sequence variations detected in each viral genomic region are summarized in Table 2. The overall mutation frequency of the total viral genomic sequences was determined to be 0.87% in chronic-naïve cases and 0.69% in chronic-NA cases. Most genomic changes observed in viral variants were single base substitutions, and the genetic heterogeneity of the viral nucleotide sequences was equally observed throughout the individual viral genetic regions, including the pre-surface (preS), S, pre-core~core (preC-C), and X (Table 2). Consistent with the findings obtained from the viral mutation analyses, the overall viral complexity determined by the Shannon entropy value was 0.047 in chronic-naïve and 0.036 in chronic-NA cases, and the viral complexity was equally observed throughout the individual viral genetic region (Figure 1A). Among chronic-naïve cases, we observed no significant differences in the viral complexity in HBV DNA level, age, or degree of fibrosis (Figure 1B).

High sensitivity of the G1896A pre-C mutant to nucleos(t)ide analogues

Emergence of G1896A mutation in the pre-C region, and A1762T and G1764A mutations in the core-promoter region is well known to be associated with HBe-seroconversion [7–9]. We then evaluated the prevalence of these three mutations in the chronically HBV-infected liver, in association with HBe serologic status and the NA treatment history. In chronic-naive cases, 6 and 8 patients showed the pre- and post- HBeAg seroconversion status, respectively (Table 3). The mean prevalence of the G1896A pre-C mutant in HBeAg-positive cases was lower than that in the HBeAg-negative cases (27.4% and 46.5%, respectively). Importantly, however, 4 of 8 HBeAg-negative cases showed a relatively low prevalence of the G1896A pre-C mutant (Liver #8, #12, #13, #14), and all but one case (Liver #10) showed a high prevalence of the A1762T and G1764A mutations, irrespective of HBe serologic status and NA treatment history (Table 3). These findings suggested that other mutations except G1896A, A1762T and G1764A were also involved in the HBeAg seroconversion status. Notably, liver tissues of all but one (Liver #17) chronic-NA cases showed extremely low levels of the G1896A pre-C mutant (0.0, 0.0, 0.1, and 1.1%), suggesting the high sensitivity of the G1896A pre-C mutant to NA (Table 3).

To confirm the difference of the sensitivity to NA between the wild-type and the G1896A pre-C mutant, we examined the dynamic changes of the relative proportion of the G1896A pre-C mutant in the serum of 14 treatment-naïve patients before and after entecavir administration. Consistent with the findings obtained by ultra-deep sequencing, quantitative real-time PCR revealed that entecavir administration significantly reduced the proportion of the G1896A pre-C mutant in 13 of 14 cases (92.9%)

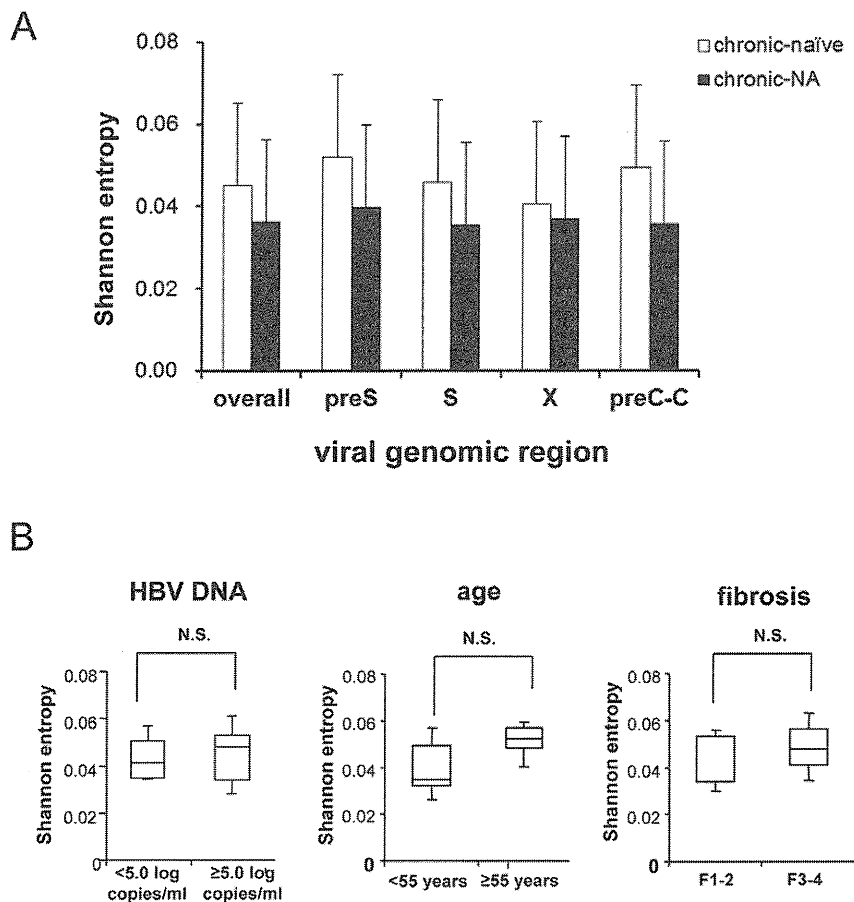

Figure 1. Viral complexity of the HBV quasispecies in association with clinical status. (A) The Shannon entropy values for each viral genomic region were determined in the liver of chronic-naïve and chronic-NA cases. (B) Among the chronic-naïve cases, the Shannon entropy values are shown for patients with serum HBV DNA levels less than 5.0 log copies/ml (<5.0) and greater than 5.0 log copies/ml (≥5.0) (left panel), patients under the age of 55 years (<55) and over the age of 55 (≥55) (middle panel), and patients with low (F1–2) and high (F3–4) liver fibrosis levels (right panel). preS: pre-surface, preC-C: precore~core N.S.: not significant.
doi:10.1371/journal.pone.0035052.g001

Table 2. The frequency of mutation rate and the Shannon entropy in each viral genome region.

	Liver	
	Chronic-naïve (N = 14)	**Chronic-NA (N = 5)**
Average aligned reads	93,172	76,043
Average aligned nucleotides	5,962,996	4,866,783
Average coverage	1,855	1,514
Mutation rate (%)		
Overall	0.87	0.69
preS	0.92	0.81
S	0.96	0.71
preC-C	1.05	0.72
X	0.63	0.61
Shannon entropy	0.047	0.036

Mutation rate (%): the ratio of total different nucleotides from the reference sequence to total aligned nucleotides.
preS: pre-surface, preC-C: pre-core~core.
doi:10.1371/journal.pone.0035052.t002

irrespective of their HBeAg serostatus, while the G1896A pre-C mutant were detectable in substantial proportion before treatment in all cases (Figure 2A, 2B and 2C; p = 0.001). These results further support the findings that HBV clones comprising the G1896A mutation were more sensitive to NA than those with wild-type sequences.

Prevalence of drug-resistant HBV clones in the liver of treatment-naïve patients

Increasing evidence suggests that drug-resistant viral mutants can be detected in the serum of treatment-naïve patients with chronic HBV infection [20,21]. Thus, we next determined the actual prevalence of spontaneously-developed drug-resistant mutants in chronically-infected liver of treatment-naïve patients to evaluate whether NA treatment potentiates the expansion of drug-resistant clones. The drug-resistant mutations examined included two mutations resistant to lamivudine and entecavir, four mutations resistant to entecavir, and three mutations resistant to adefovir [16,17]. Based on the detection rate of the low-level viral clones determined by the control experiments, we identified the drug-resistant mutants present in each specimen at a frequency of more than 0.3% among the total viral clones. Based on these criteria, at least one resistant mutation was detected in the liver of all of the chronic-naïve cases with chronic HBV infection (Table 4).

Table 3. The prevalence of G1896A mutation in the pre-C region, and A1762T and G1764A mutations in the core-promoter region in the liver of patients chronically infected with HBV.

	HBeAg/HBeAb	NA (duration of treatment)	Mutation Frequency					
			G1896A (Pre C)		A1762T (CP)		G1764A (CP)	
Chronic-naive								
Liver #1	+/−	-	640/1652	(38.7)	1647/1941	(84.9)	1683/1979	(85.0)
Liver #2	+/−	-	9/596	(1.5)	682/687	(99.3)	683/689	(99.1)
Liver #3	+/−	-	273/672	(40.6)	767/769	(99.7)	757/760	(99.6)
Liver #4	+/−	-	204/701	(29.1)	610/625	(97.6)	602/621	(96.9)
Liver #5	+/−	-	27/152	(17.8)	249/250	(99.6)	245/248	(98.8)
Liver #6	+/−	-	228/621	(36.7)	727/729	(99.7)	743/744	(99.9)
Liver #7	−/+	-	740/1193	(62.0)	1908/1913	(99.7)	1888/1913	(98.7)
Liver #8	−/+	-	111/1892	(5.9)	2321/2325	(99.8)	2335/2339	(99.8)
Liver #9	−/+	-	10935/10944	(99.9)	12019/12032	(99.9)	12163/12170	(99.9)
Liver #10	−/+	-	4554/4593	(99.2)	1/5191	(0)	4/5188	(0.1)
Liver #11	−/+	-	811/921	(88.1)	1234/1236	(99.8)	1226/1228	(99.8)
Liver #12	−/+	-	93/1265	(7.4)	1234/1234	(100)	1228/1229	(99.9)
Liver #13	−/+	-	83/877	(9.5)	1465/1529	(95.8)	1485/1549	(95.9)
Liver #14	−/+	-	0/717	(0)	1078/1410	(76.5)	1089/1414	(77.0)
Chronic-NA								
Liver #15	−/+	LAM (156w)	0/390	(0)	441/453	(97.4)	435/448	(97.1)
Liver #16	−/+	ETV (1w)	0/1399	(0)	1624/1632	(99.5)	1625/1630	(99.7)
Liver #17	−/+	LAM (144w)	345/816	(42.3)	988/991	(99.7)	994/994	(100)
Liver #18	−/+	LAM (98w)	2/3963	(0.1)	1015/1188	(85.4)	1190/1194	(99.7)
Liver #19	−/+	LAM (11w)	48/4214	(1.1)	3438/3456	(99.5)	3446/3462	(99.5)

Values in parenthesis show mutation frequency (%): the ratio of total mutant clones to total aligned coverage at each nucleotide sites.
NA: nucleotide analogue, pre C: precore, CP: core promoter, LAM: lamivudine, ETV: entecavir.
doi:10.1371/journal.pone.0035052.t003

The prevalence of the 9 drug-resistant mutations detected by ultra-deep sequencing in 14 chronic-naïve cases ranged from 0.3% to 30.0%, indicating that the proportion of resistant mutations substantially differed in each case. The most commonly detected mutation was M204VI (9 cases) and M250VI (11 cases), which were resistant to lamivudine and entecavir, and entecavir, respectively. Other mutations resistant to adefovir were detected in 7 (50.0%) and 3 (21.4%) cases at A181TV and N236T, respectively (Table 4).

Nine (64.2%) chronic-naïve cases possessed the M204VI mutants in their liver tissues and the proportion of mutant clones among the totally infected viruses ranged from 0.3% to 1.1% among the M204VI mutant-positive patients. In chronic-NA cases, 4 of 5 (80.0%) liver tissues harbored the M204VI mutants with the proportion among the totally infected viruses ranging from 0.4% to 18.7% (Table 4), while the mean serum HBV DNA was suppressed below 2.6 log copies/ml (Table 1). These results suggest that the mutant HBV clones comprising various drug-resistant mutations could latently exist even in the liver of NA treatment-naïve cases.

Expansion of drug-resistant HBV clones harboring M240VI mutations in response to NA administration

To clarify the risk of latent expansion of drug-resistant mutations due to NA treatment, we next examined the early dynamic changes of the prevalence of M204VI mutants in the serum of treatment-naïve patients in response to entecavir treatment. Ultra-deep sequencing provided a mean 40,791- and 38,823-fold coverage of readings, which were mapped to the M204VI nucleotide position at the YMDD sites of each reference sequence in patients before and after entecavir treatment.

Five of 14 (35.7%) patients harbored the M204VI mutations prior to entecavir treatment. Although the serum HBV DNA levels were significantly reduced in response to entecavir in all cases, the M204VI mutant clones were detected in 9 cases (64.3%) after entecavir administration (Table 5). Notably, one patient (Serum #3) who harbored the M240VI mutant clones at baseline had a relatively large expansion of drug-resistant clones among the total viral population in a time-dependent manner in response to entecavir treatment (Table 5). Similarly, M240VI mutant clones became detectable after entecavir administration in four patients (Serum #1, #7, #12, #13) that harbored no resistant mutants at baseline (Table 5). We found no correlation between the degree of the increase in the relative prevalence of M204VI mutant clones and that of the reduction in serum HBV DNA levels. Although only a limited number of patients exhibited a substantial increase in M204VI mutant clones after administration of anti-viral therapy, our findings might suggest that entecavir treatment latently causes selective survival of drug-resistant mutants in treatment naïve patients with chronic HBV infection.

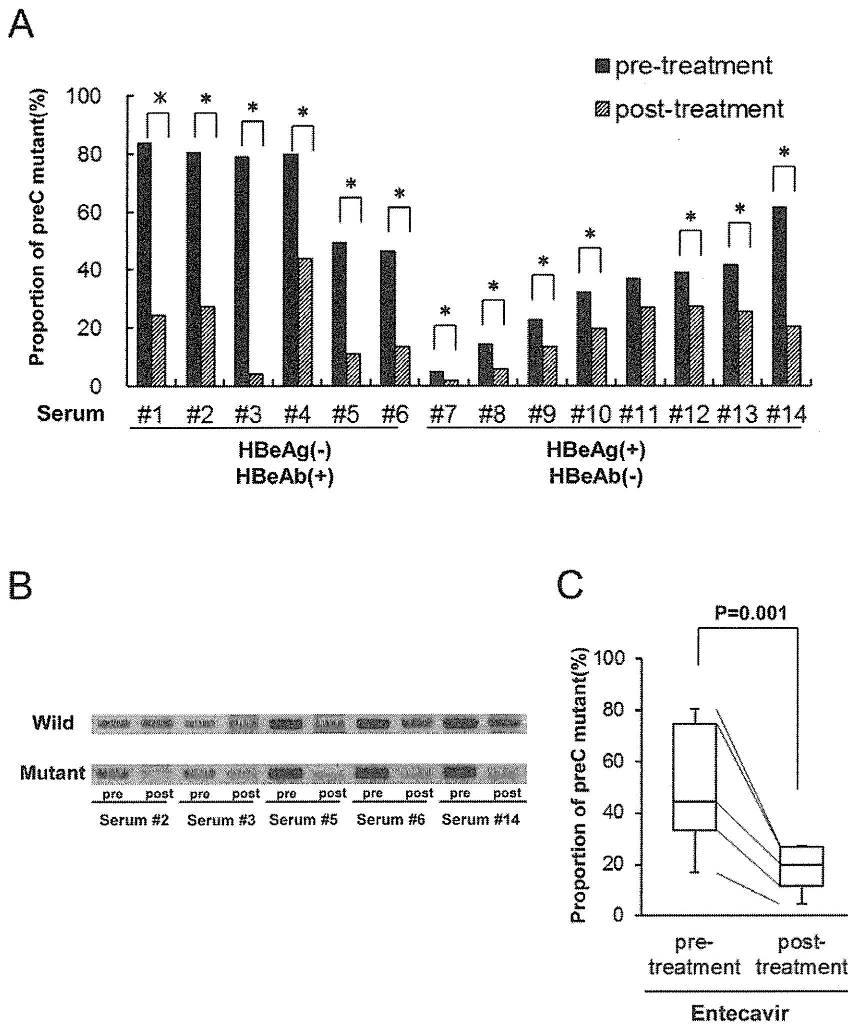

Figure 2. The reduction in the relative proportion of the G1896A pre-C mutant clones after entecavir administration. (A) The relative proportion of the G1896A pre-C mutant was determined in the serum of treatment-naïve patients pre- and post-entecavir administration using quantitative real-time PCR. Serum #1~6 were HBeAg-negative and HBeAb-positive, and Serum #7~14 were HBeAg-positive and HBeAb-negative before treatment. *: $p<0.05$ (B) Semiquantitative PCR analysis was performed using primers specific to the wild-type (upper panel) or G1896A pre-C mutant (lower panel) pre- and post-entecavir administration. A representative result from 5 cases is shown. (C) The relative proportion of the G1896A pre-C mutant was compared in 14 treatment-naïve patients between pre- and post-entecavir administration.
doi:10.1371/journal.pone.0035052.g002

Discussion

Direct population sequencing is the most common method for detecting viral mutations [29]. Conventional sequencing techniques, however, are not efficient for evaluating large amounts of genetic information of the viruses. Newly developed ultra-deep sequencing technology have revolutionized genomic analyses, allowing for studies of the dynamics of viral quasispecies as well as rare genetic variants of the viruses that cannot be detected using standard direct population sequencing techniques [30,31]. The sensitivity of ultra-deep sequencing analysis is primarily limited by errors introduced during PCR amplification and the sequencing reaction, thus it is a challenge to distinguish rare variants from sequencing artifacts. In the present study, we optimized the ultra-deep sequencing with a multiplex-tagging method and reproducibly detected variants within HBV quasispecies that were as rare as 0.3%. Based on this ultra-deep sequencing platform, we determined the abundant genetic heterogeneity of HBV at the intra- and inter-individual levels.

Because of its ability to handle abundant viral genome information, ultra-deep sequencing allowed us to evaluate low-abundant virus variants of patients with chronic HBV infection in detail. It is widely accepted that HBe seroconversion is highly associated with the emergence of G1896A pre-C and/or A1762T and G1764A core promoter mutant clones [7–9]. Unexpectedly, however, our results showed a diverse range of G1896A frequency (0–99.9%) in HBeAg-negative subjects and a high prevalence of core promoter mutations, irrespective of HBe serostatus. Consistent with our observation, previous studies utilizing conventional sequencing methods reported that the frequency of the G1896A pre-C mutant ranged from 12% to 85% [32]. All but one patient (Liver #10) showing a predominance of A1762T and G1764A were infected with genotype C, while patient#10 was infected with genotype B. Because A1762T and G1764A are reported to be significantly more frequent in genotype C [33], the difference in the prevalence of A1762T and G1764A in our study might be a reflection of the viral HBV genotype rather than HBe serostatus. Further investigation of the actual prevalence of these mutations

Table 4. The prevalence of the 9 drug-resistant mutations detected by ultra-deep sequencing derived from liver tissue.

	M204V/I		L180M		T184S/A/I/ L/G/C/M		S202C/G/I		I169T	
Drugs	**LAM/ETV**		**LAM/ETV**		**ETV**		**ETV**		**ETV**	
Chronic-naive										
Liver #1	**27/5421**	**(0.5%)**	2/3694	(-)	9/3886	(-)	5/5613	(-)	5/3784	(-)
Liver #2	**35/5344**	**(0.7%)**	0/538	(-)	1/563	(-)	17/6340	(-)	0/512	(-)
Liver #3	**13/1363**	**(1.0%)**	0/304	(-)	1/358	(-)	1/1379	(-)	0/264	(-)
Liver #4	11/5113	(-)	0/556	(-)	**2/547**	**(0.4%)**	11/5133	(-)	0/639	(-)
Liver #5	**2/117**	**(1.1%)**	0/409	(-)	1/380	(-)	1/189	(-)	1/474	(-)
Liver #6	12/8451	(-)	0/309	(-)	0/328	(-)	22/8457	(-)	0/334	(-)
Liver #7	**10/3098**	**(0.3%)**	1/1547	(-)	3/1477	(-)	8/3161	(-)	0/1621	(-)
Liver #8	**13/2442**	**(0.5%)**	1/2378	(-)	6/2312	(-)	1/2564	(-)	1/2507	(-)
Liver #9	**67/13879**	**(0.5%)**	2/5443	(-)	2/5107	(-)	6/13804	(-)	0/5650	(-)
Liver #10	16/7400	(-)	0/3524	(-)	3/3283	(-)	5/7113	(-)	0/3492	(-)
Liver #11	0/412	(-)	1/1328	(-)	**1/295**	**(0.3%)**	0/425	(-)	3/4729	(-)
Liver #12	**4/1098**	**(0.4%)**	1/1389	(-)	0/1272	(-)	2/1102	(-)	0/1544	(-)
Liver #13	**8/2476**	**(0.3%)**	1/2192	(-)	3/2085	(-)	4/2529	(-)	4/5029	(-)
Liver #14	5/3713	(-)	0/2009	(-)	4/1925	(-)	2/3820	(-)	5/3784	(-)
Chronic-NA										
Liver #15	0/339	(-)	0/49	(-)	0/49	(-)	0/338	(-)	0/40	(-)
Liver #16	**28/7278**	**(0.4%)**	0/4403	(-)	6/4053	(-)	14/7556	(-)	6/6084	(-)
Liver #17	**177/945**	**(18.7%)**	0/1059	(-)	0/1009	(-)	0/945	(-)	0/1051	(-)
Liver #18	**13/2655**	**(0.5%)**	0/1239	(-)	0/1185	(-)	**10/2708**	**(0.4%)**	0/1332	(-)
Liver #19	**80/6795**	**(1.2%)**	0/3168	(-)	2/2971	(-)	3/6734	(-)	0/3384	(-)

	M250V/I		A181T/V		N236T		P237H	
Drugs	**ETV**		**ADV**		**ADV**		**ADV**	
Chronic-naive								
Liver #1	**23/2719**	**(0.9%)**	10/3755	(-)	4/4210	(-)	2/4139	(-)
Liver #2	**9/2079**	**(0.4%)**	**2/549**	**(0.4%)**	1/1144	(-)	1/1188	(-)
Liver #3	**10/1699**	**(0.6%)**	**1/298**	**(0.3%)**	3/1636	(-)	1/1666	(-)
Liver #4	**3/388**	**(0.8%)**	**3/549**	**(0.5%)**	0/560	(-)	0/533	(-)
Liver #5	**2/91**	**(2.2%)**	1/409	(-)	0/55	(-)	0/60	(-)
Liver #6	0/214	(-)	**6/305**	**(2.0%)**	**1/294**	**(0.3%)**	0/257	(-)
Liver #7	**7/1289**	**(0.5%)**	4/1531	(-)	**24/2738**	**(0.9%)**	1/2692	(-)
Liver #8	2/1117	(-)	**689/2336**	**(29.5%)**	2/1713	(-)	0/1639	(-)
Liver #9	**27/7325**	**(0.4%)**	**38/5334**	**(0.7%)**	1/6607	(-)	4/6702	(-)
Liver #10	**12/3815**	**(0.3%)**	0/3454	(-)	**13/3245**	**(0.4%)**	2/3272	(-)
Liver #11	**1/199**	**(0.5%)**	1/972	(-)	0/251	(-)	0/251	(-)
Liver #12	**2/672**	**(0.3%)**	**408/1362**	**(30.0%)**	0/598	(-)	0/597	(-)
Liver #13	1/947	(-)	2/2160	(-)	0/1406	(-)	1/1374	(-)
Liver #14	**23/2719**	**(0.9%)**	10/3755	(-)	4/4210	(-)	2/4139	(-)
Chronic-NA								
Liver #15	**1/303**	**(0.3%)**	**2/49**	**(4.1%)**	0/377	(-)	0/384	(-)
Liver #16	1/922	(-)	0/4403	(-)	1/1597	(-)	3/1572	(-)
Liver #17	0/755	(-)	1/1050	(-)	0/698	(-)	**145/698**	**(20.8%)**
Liver #18	1/1464	(-)	2/1206	(-)	0/3156	(-)	0/3107	(-)
Liver #19	8/3834	(-)	**16/3128**	**(0.5%)**	0/3372	(-)	0/3428	(-)

(-): mutant clones less than 0.3% among total clones at each nucleotide sites.
LAM: lamivudine, ADV: adefovir, ETV: entecavir.
doi:10.1371/journal.pone.0035052.t004

Table 5. The prevalence of M204VI mutation at YMDD site in patients before and after entecavir administration.

	Entecavir treatment				
	Before		After		
	Prevalence of the mutated clones		Prevalence of the mutated clones		Period of NA treatment
Serum #3	222/32,238	(0.7%)	2,284/23,791	(9.6%)	2w
Serum #2	401/34,041	(1.2%)	266/25,301	(1.1%)	24w
Serum #5	521/48,723	(1.1%)	245/25,521	(1.0%)	56w
Serum #8	748/65,573	(1.1%)	336/28,702	(1.2%)	48w
Serum #9	312/30,599	(1.0%)	169/14,172	(1.2%)	56w
Serum #1	9/22,843	(-)	2,839/34,162	(8.3%)	8w
Serum #7	26/65,564	(-)	923/66,458	(1.4%)	4w
Serum #12	91/65,616	(-)	258/27,958	(0.9%)	24w
Serum #13	11/23,209	(-)	206/64,747	(0.3%)	32w
Serum #4	3/7,923	(-)	39/65,575	(-)	12w
Serum #6	52/65,582	(-)	77/55,273	(-)	16w
Serum #10	38/22,522	(-)	8/21,053	(-)	8w
Serum #11	47/43,853	(-)	5/16,520	(-)	16w
Serum #14	42/42,784	(-)	40/36,668	(-)	12w

Mutation frequency (%): the ratio of total mutant clones to total aligned coverage at each nucleotide sites.
(-): mutant clones less than 0.3% among total clones at each nucleotide sites.
doi:10.1371/journal.pone.0035052.t005

and the elucidation of other unknown mutations involved in HBe seroconversion are necessary for a better understanding of the underlying mechanisms of HBe seroconversion.

One thing to be noted is that the majority of the chronic-NA cases had extremely low levels of the G1896A pre-C mutant in their liver tissues, even though those cases were serologically positive for anti-HBe and negative for HBeAg. Moreover, entecavir administration significantly reduced the proportion of the G1896A pre-C mutant in the serum of the majority of patients irrespective of their HBeAg serostatus, while the G1896A pre-C mutant clones were detectable in a substantial proportion before treatment in all cases. These findings suggest that the G1896A pre-C mutant have higher sensitivity to NA than the wild-type viruses. Consistent with this hypothesis, several previous studies reported that NA is effective against acute or fulminant hepatitis caused by possible infection with the G1896A pre-C mutant [34,35]. Based on these findings, early administration of NA might be an effective strategy for treating patients with active hepatitis infected predominantly with the G1896A pre-C mutant.

Ultra-deep sequencing has a relatively higher sensitivity than conventional direct population sequencing and is thus useful for detecting drug-resistant mutations not detected by standard sequencing [20,21]. Recently, we revealed that drug-resistant mutants were widely present in treatment-naïve HCV-infected patients, suggesting a putative risk for the expansion of resistant clones to anti-viral therapy [19]. Here, we demonstrated that various drug-resistant HBV variants are present in a proportion of chronically HBV-infected, NA-naïve patients. Several studies using ultra-deep sequencing provided evidence that naturally-occurring drug-resistant mutations are detectable in treatment-naïve individuals with human immunodeficiency virus-1 infection [30,36,37]. Consistent with the cases of human immunodeficiency virus-1 infection, a few studies detected minor variants resistant to NA in the plasma of treatment-naïve patients with chronic HBV infection [20,21]. It remains unclear, however, whether these minor drug-resistant mutations have clinical significance. Our observation of the relative expansion of viral clones with the M204VI mutation during entecavir therapy in some cases indicates the possibility that preexisting minor mutants might provide resistance against NA through the selection of dominant mutant clones. Future studies with a larger cohort size are required to clarify the clinical implications of the latently existing low-abundant drug-resistant mutations.

The current ultra-deep parallel sequencing technology has limitations in the analyses of viral quasispecies. First, because the massively-parallel ultra-deep sequencing platform is based on a multitude of short reads, it is difficult to evaluate the association between nucleotide sites mapped to different genome regions in a single viral clone. Indeed, potential mutational linkages between the pre-C and reverse transcriptase regions were difficult to elucidate due to the short read length of the shotgun sequencing approach. Second, accurate analysis of highly polymorphic viral clones by ultra-deep sequencing is also difficult because the identification of mutations depends strongly on the mapping to the reference genome sequences.

In conclusion, we demonstrated that the majority of patients positive for anti-HBe and negative for HBeAg lacked the predominant infection of the G1896A pre-C mutant in the presence of NA treatment, suggesting that the G1896A pre-C mutant have increased sensitivity to NA therapy compared with wild-type HBV. We also revealed that drug-resistant mutants are widely present, even in the liver of treatment-naïve HBV-infected patients, suggesting that the preexisting low-abundant mutant clones might provide the opportunity to develop drug resistance against NA through the selection of dominant mutations. Further analyses utilizing both novel and conventional sequencing technologies are necessary to understand the significance and clinical relevance of the viral mutations in the pathophysiology of various clinical settings in association with HBV infection.

Supporting Information

Figure S1 Comparison of the viral complexity between the liver and serum of the same individual. Shannon entropy values throughout the whole viral genome of the liver and serum of the representative two cases are shown. (upper two panels, case #11; lower two panels, case #14). preC-C: pre-core~core, preS: pre-surface, P: polymerase.
(TIF)

Table S1 The oligonucleotide primers for amplifying HBV sequences in each clinical specimen.
(DOCX)

Table S2 Error frequency of Ultra-deep sequencing for the expression plasmid encoding wild-type genotype C HBV genome sequences by the three control experiments.
(DOCX)

Table S3 The sensitivity and accuracy of detecting the low abundant minor clones in association with the different coverage numbers.
(DOCX)

Acknowledgments

We thank Prof. G. Tsujimoto, Dr. F. Sato, Dr. Y. Matsumoto, Dr. Y. Endo, Dr. A Takai, Ms. Y. Nakagawa, Ms. K. Fujii and Ms. C. Hirano for ultra-deep sequencing analysis.

Author Contributions

Conceived and designed the experiments: NN HM. Performed the experiments: NN HM. Analyzed the data: NN HM YU AN TF ST KS TC. Contributed reagents/materials/analysis tools: NN HM YU YO TK SY SU. Wrote the paper: NN HM YU KT TC.

References

1. Dienstag JL (2008) Hepatitis B virus infection. N Engl J Med 359: 1486–1500.
2. Lok AS, McMahon BJ (2007) Chronic hepatitis B. Hepatology 45: 507–539.
3. Chang KM (2010) Hepatitis B immunology for clinicians. Clin Liver Dis 14: 409–424.
4. Murray JM, Purcell RH, Wieland SF (2006) The half-life of hepatitis B virions. Hepatology 44: 1117–1121.
5. Ngui SL, Teo CG (1997) Hepatitis B virus genomic heterogeneity: variation between quasispecies may confound molecular epidemiological analyses of transmission incidents. J Viral Hepat 4: 309–315.
6. Hollinger FB (2007) Hepatitis B virus genetic diversity and its impact on diagnostic assays. J Viral Hepat 14 Suppl 1: 11–15.
7. Akahane Y, Yamanaka T, Suzuki H, Sugai Y, Tsuda F, et al. (1990) Chronic active hepatitis with hepatitis B virus DNA and antibody against e antigen in the serum. Disturbed synthesis and secretion of e antigen from hepatocytes due to a point mutation in the precore region. Gastroenterology 99: 1113–1119.
8. Okamoto H, Yotsumoto S, Akahane Y, Yamanaka T, Miyazaki Y, et al. (1990) Hepatitis B viruses with precore region defects prevail in persistently infected hosts along with seroconversion to the antibody against e antigen. J Virol 64: 1298–1303.
9. Kramvis A, Kew MC (1999) The core promoter of hepatitis B virus. J Viral Hepat 6: 415–427.
10. Carman WF, Fagan EA, Hadziyannis S, Karayiannis P, Tassopoulos NC, et al. (1991) Association of a precore genomic variant of hepatitis B virus with fulminant hepatitis. Hepatology 14: 219–222.
11. Omata M, Ehata T, Yokosuka O, Hosoda K, Ohto M (1991) Mutations in the precore region of hepatitis B virus DNA in patients with fulminant and severe hepatitis. N Engl J Med 324: 1699–1704.
12. Domingo E, Gomez J (2007) Quasispecies and its impact on viral hepatitis. Virus Res. Netherlands. pp 131–150.
13. Fishman SL, Branch AD (2009) The quasispecies nature and biological implications of the hepatitis C virus. Infect Genet Evol 9: 1158–1167.
14. Kwon H, Lok AS (2011) Hepatitis B therapy. Nat Rev Gastroenterol Hepatol.
15. Dienstag JL (2009) Benefits and risks of nucleoside analog therapy for hepatitis B. Hepatology 49: S112–121.
16. Ghany MG, Doo EC (2009) Antiviral resistance and hepatitis B therapy. Hepatology 49: S174–184.
17. Zoulim F, Locarnini S (2009) Hepatitis B virus resistance to nucleos(t)ide analogues. Gastroenterology 137: 1593–1608.e1591–1592.
18. Margulies M, Egholm M, Altman WE, Attiya S, Bader JS, et al. (2005) Genome sequencing in microfabricated high-density picolitre reactors. Nature 437: 376–380.
19. Nasu A, Marusawa H, Ueda Y, Nishijima N, Takahashi K, et al. (2011) Genetic heterogeneity of hepatitis C virus in association with antiviral therapy determined by ultra-deep sequencing. PLoS One 6: e24907.
20. Margeridon-Thermet S, Shulman NS, Ahmed A, Shahriar R, Liu T, et al. (2009) Ultra-deep pyrosequencing of hepatitis B virus quasispecies from nucleoside and nucleotide reverse-transcriptase inhibitor (NRTI)-treated patients and NRTI-naive patients. J Infect Dis 199: 1275–1285.
21. Solmone M, Vincenti D, Prosperi MC, Bruselles A, Ippolito G, et al. (2009) Use of massively parallel ultradeep pyrosequencing to characterize the genetic diversity of hepatitis B virus in drug-resistant and drug-naive patients and to detect minor variants in reverse transcriptase and hepatitis B S antigen. J Virol 83: 1718–1726.
22. Han Y, Huang LH, Liu CM, Yang S, Li J, et al. (2009) Characterization of hepatitis B virus reverse transcriptase sequences in Chinese treatment naive patients. J Gastroenterol Hepatol 24: 1417–1423.
23. Ikeda K, Marusawa H, Osaki Y, Nakamura T, Kitajima N, et al. (2007) Antibody to hepatitis B core antigen and risk for hepatitis C-related hepatocellular carcinoma: a prospective study. Ann Intern Med 146: 649–656.
24. Ruike Y, Imanaka Y, Sato F, Shimizu K, Tsujimoto G (2010) Genome-wide analysis of aberrant methylation in human breast cancer cells using methyl-DNA immunoprecipitation combined with high-throughput sequencing. BMC Genomics 11: 137.
25. Louvel S, Battegay M, Vernazza P, Bregenzer T, Klimkait T, et al. (2008) Detection of drug-resistant HIV minorities in clinical specimens and therapy failure. HIV Med 9: 133–141.
26. Ntziora F, Paraskevis D, Haida C, Magiorkinis E, Manesis E, et al. (2009) Quantitative detection of the M204V hepatitis B virus minor variants by amplification refractory mutation system real-time PCR combined with molecular beacon technology. J Clin Microbiol 47: 2544–2550.
27. Matsumoto Y, Marusawa H, Kinoshita K, Endo Y, Kou T, et al. (2007) Helicobacter pylori infection triggers aberrant expression of activation-induced cytidine deaminase in gastric epithelium. Nat Med 13: 470–476.
28. Marusawa H, Matsuzawa S, Welsh K, Zou H, Armstrong R, et al. (2003) HBXIP functions as a cofactor of survivin in apoptosis suppression. EMBO J 22: 2729–2740.
29. Lok AS, Zoulim F, Locarnini S, Bartholomeusz A, Ghany MG, et al. (2007) Antiviral drug-resistant HBV: standardization of nomenclature and assays and recommendations for management. Hepatology 46: 254–265.
30. Hedskog C, Mild M, Jernberg J, Sherwood E, Bratt G, et al. (2010) Dynamics of HIV-1 quasispecies during antiviral treatment dissected using ultra-deep pyrosequencing. PLoS One 5: e11345.
31. Rozera G, Abbate I, Bruselles A, Vlassi C, D'Offizi G, et al. (2009) Massively parallel pyrosequencing highlights minority variants in the HIV-1 env quasispecies deriving from lymphomonocyte sub-populations. Retrovirology 6: 15.
32. Chowdhury A, Santra A, Chakravorty R, Banerji A, Pal S, et al. (2005) Community-based epidemiology of hepatitis B virus infection in West Bengal, India: prevalence of hepatitis B e antigen-negative infection and associated viral variants. J Gastroenterol Hepatol 20: 1712–1720.
33. Orito E, Mizokami M, Sakugawa H, Michitaka K, Ishikawa K, et al. (2001) A case-control study for clinical and molecular biological differences between hepatitis B viruses of genotypes B and C. Japan HBV Genotype Research Group. Hepatology 33: 218–223.
34. Yu JW, Sun LJ, Yan BZ, Kang P, Zhao YH (2011) Lamivudine treatment is associated with improved survival in fulminant hepatitis B. Liver Int 31: 499–506.
35. Lisotti A, Azzaroli F, Buonfiglioli F, Montagnani M, Alessandrelli F, et al. (2008) Lamivudine treatment for severe acute HBV hepatitis. Int J Med Sci 5: 309–312.
36. Simen BB, Simons JF, Hullsiek KH, Novak RM, Macarthur RD, et al. (2009) Low-abundance drug-resistant viral variants in chronically HIV-infected, antiretroviral treatment-naive patients significantly impact treatment outcomes. J Infect Dis 199: 693–701.
37. Lataillade M, Chiarella J, Yang R, Schnittman S, Wirtz V, et al. (2010) Prevalence and clinical significance of HIV drug resistance mutations by ultra-deep sequencing in antiretroviral-naïve subjects in the CASTLE study. PLoS One 5: e10952.

Cancer-Prevention and Early Detection

免疫抑制・化学療法により発症するB型肝炎の再活性化

Reactivation of hepatitis B virus by immunosuppressant or chemotherapy

池田　公史
Masafumi Ikeda
独立行政法人国立がん研究センター東病院 肝胆膵腫瘍科副科長

はじめに

免疫抑制剤や化学療法剤による治療を施行する際に，B型肝炎ウイルス(HBV)が再活性化し，時には劇症化する報告が，最近リツキシマブ(リツキサン®)併用化学療法などで散見され注目されている[1-4]。このHBVの再活性化を起こさせずに免疫抑制剤や化学療法剤による治療を十分に施行することが重要であり，そのためにもHBVの再活性化のリスク，対処方法を熟知しておくことが必要である。本稿では，免疫抑制や化学療法に伴う肝炎ウイルス，特にHBVによる再活性化に対するリスクや対応策，今後の課題を中心に概説する。

HBVの再活性化のリスクと特徴

肝臓は，抗がん剤またはその代謝物によりさまざまな肝障害が引き起こされる。なかでも，HBVを有する患者に免疫抑制剤や化学療法剤での治療を施行する際，急激なHBVの増殖が生じ，致死的な肝障害に陥ることもある。このように化学療法などによる免疫抑制状態などが誘因となり，HBVが急激に増殖することを再活性化という[5]。このHBVの再活性化は，HBs抗原陽性のキャリア／慢性肝炎の患者のみならず，HBs抗原は陰性でHBc抗体またはHBs抗体陽性の，いわゆる一過性感染してHBVは排除されたと考えられていた患者においても，HBVの再活性化のリスクがあることが判明している[1-4]。この再活性化は，一般的に次のように定義されている[5]。

・HBs抗原陽性例において
　① HBV DNAが10倍以上の上昇
　② HBe抗原陰性例で，HBe抗原が陽性化
・HBs抗原陰性で，HBc抗体またはHBs抗体陽性例において
　① HBs抗原が陽性化
　② HBV DNA検出感度以下の例でHBV DNAの陽性化

わが国の成人でのHBs抗原陽性の割合は1～2％，HBs抗体またはHBc抗体の陽性の割合は20～25％前後と報告されている(表1)。したがって，わが国で免疫抑制剤や化学療法剤で治療する際にHBVの再活性化のリスクがある症例は，4人に1人ぐらいの割合で存在するといわれている。

HBs抗原陽性例からのHBVの再活性化の報告

これまでにHBs抗原陽性例からのHBVの再活性化は，ほとんどすべてのがん腫，あらゆる抗がん剤で報告がある。おおよそ20～50％前後のHBVの再活性化の報告[5]があり，そのリスクファクターとして，男性，若年者，HBe抗原陽性，HBV DNA高用量，乳がんの患者，ステロイドの併用，アンスラサイクリン系の抗がん剤の使用，リツキシマブの使用，リンパ腫の患者などが挙げられてい

Presented by Medical*Online

表1 HBs抗原，HBc抗体，HBs抗体またはHBc抗体の陽性率

	n	HBs抗原陽性	HBc抗体陽性	HBs抗体または HBc抗体陽性	著者	報告年
香港	244	NA	62%	72%	Hui ら	2006
香港	626	12%	NA	NA	Yeo ら	2000
米国	3,343	1.3%	9.0%	NA	Ludwig ら	2010
名古屋	3,874	1.5%	20.0%	23.2%	Kusumoto ら	2009
東京	1,031	NA	16.9%	NA	Matsue ら	2010
千葉	261	3.4%	24.3%	NA	Hattori ら	2010
千葉(当院)	863	2.9%	21.4%	25.2%	Ikeda ら	2012

る。このようにHBs抗原陽性例からのHBVの再活性化は比較的高頻度に認められており，がん腫，化学療法のレジメンを問わずに注意を払う必要がある。

HBs抗原陰性で，HBc抗体陽性またはHBs抗体陽性例からのHBVの再活性化の報告

HBs抗原陰性，HBc抗体またはHBs抗体陽性で，従来，既往感染と考えられていた症例においても，HBVの再活性化が起こることが報告されている。2001年にDerviteらがリツキシマブ併用化学療法例において，HBs抗原陰性例でのHBV再活性化例をはじめて報告し[1]，2006年，HuiらがHBs抗原陰性の悪性リンパ腫244例に対して全身化学療法を施行したところ，8例(3.3%)にHBVの再活性化がみられ，全例でHBc抗体もしくはHBs抗体陽性であったと報告した[4]。また2009年に，YeoらはHBs抗原陰性の悪性リンパ腫80例に対して全身化学療法を施行したところ，5例(6.25%)にHBVの再活性化を認め，5例ともHBc抗体またはHBs抗体が陽性であり，その5例は全例でリツキシマブとステロイド併用化学療法を施行していたと報告した[6]。このように，リツキシマブなどの免疫抑制効果の高い化学療法剤やステロイドを併用することで，一過性感染で治癒したと考えられていたHBs抗原陰性で，HBs抗体陽性またはHBc抗体陽性例からも再活性化が起こることが判明した。したがって，HBV再活性化の高リスク群に，HBs抗原陽性例だけでなく，HBs抗原陰性で，HBc抗体陽性またはHBs抗体陽性例も含める必要がある。しかし，ほとんどがリツキシマブやステロイドを併用した悪性リンパ腫からの報告であり，固形がんやその他のレジメンからの報告はきわめて稀である。

HBVの再活性化症例の転帰

HBVの再活性化による肝障害を併発した症例の予後は不良と報告されている。YeoらはHBV再活性化による肝炎を併発した32例に対して，ラミブジンにて治療したが，全身化学療法の中止を余儀なくされた症例は22例(69%)で，5例(16%)は肝障害によって亡くなられたと報告した[7]。またUmemuraらは，HBVの再活性化を認めた23例のうち，劇症化した症例は5例(22%)で，肝関連死亡率は26%，劇症化例での死亡率は100%であったのに対して，急性肝炎の患者529例では劇症化した症例は45例(9%)で，肝関連死亡率は4%，劇症化例での死亡率は47%であり，HBVの再活性化による劇症化率ならびに肝関連死亡率，劇症化例での死亡率は有意に高率であったことを報告した[8]。このようにHBVの再活性化による肝障害を起こしてからでは予後は不良であり，肝障害を起こす前に再活性化を抑え込むことが重要である。

HBV再活性化の対策

HBVの再活性化による肝障害を予防するためには，抗ウイルス薬を予防投与する，またはHBV DNAをモニタリングして，HBV DNAの上昇を認めた際に抗ウイルス薬を予防

Presented by Medical*Online

投与するという2つの方針が考えられる。

1．HBs 抗原陽性例

Yeo らは，HBs 抗原陽性の悪性腫瘍患者に全身化学療法を行う際に，ラミブジンの予防投与を行った65例と予防投与を行わなかったヒストリカルコントロール193例を比較した検討で，HBV の再活性化はラミブジン予防投与群で4.6%，コントロール群で24.4%であり，ラミブジンの予防投与の有効性を報告した[7]。Lau らは，全身化学療法予定の HBs 抗原陽性の悪性リンパ腫30例を対象として，ラミブジン予防投与15例と非投与15例の2群にランダムに割り付けて検討した結果，HBV 再活性化の頻度はラミブジン予防投与群で0例（0%），ラミブジン非投与群で8例（53%）とラミブジン投与群で有意に低く，HBV の再活性化を起こした8例中7例で肝障害が生じたことを報告した[9]。また，肝動脈化学塞栓術予定の HBV 関連の切除不能肝細胞がん患者76例を対象に，エピルビシン50mg/m²とシスプラチン60mg/m²で肝動脈化学塞栓術を施行する際に，ラミブジンの予防投与群と非投与群の2群にランダムに割り付け，HBV の再活性化の頻度を評価する比較試験が行われた[10]。その結果，再活性化の頻度（予防投与群 vs. 非投与群：2.8% vs. 40.5%，$p<0.001$），再活性化による肝障害の頻度（2.8% vs. 2.97%，$p=0.003$），再活性化による肝不全の頻度（0% vs. 8.1%）は，ラミブジンの予防投与群で良好な結果であった。また，ラミブジンの予防投与に関するメタアナリシスもすでに報告されており[11]，ラミブジン予防投与群において，HBV の再活性化の頻度，再活性化による死亡割合，化学療法の遅延，化学療法の中止の割合は低いことが示されている。このように HBs 抗原陽性例には，ラミブジンをはじめとする抗ウイルス薬を予防投与することによる有用性が示されている[12]。

2．HBs 抗原陰性で，HBc 抗体陽性または HBs 抗体陽性例

HBs 抗原陰性で，HBs 抗体陽性または HBc 抗体陽性のいわゆる一過性感染後の症例に関しては，HBV の再活性化の予防に関する十分な検討は行われていない。これまでの報告では，肝炎発症例の多くが全身化学療法終了後の発症であった。また，肝障害・肝炎の出現より前に，HBV DNAの増加が平均18.5週ほど先行するといわれており[4]，HBV DNAを1ヵ月に1回モニタリングして，HBV DNA が陽性化してから抗ウイルス薬の投与を行っても，肝炎の重症化は予防可能と推察されている。しかし，この HBV DNA のモニタリングの有用性，安全性は明らかにされておらず，よくデザインされた前向き試験での検証が必要である。

HBV の再活性化のガイドライン

このように，HBV 再活性化の報告がしばしば行われるようになり，HBV 再活性化の対策が急務となり，海外から2007年に AASLD Practice Guideline[13]，2008年に NIH Consensus Development Conference Statement on Management of Hepatitis B[14]，2009年に EASL Clinical Practice Guideline[15]，そしてわが国からは，2009年に「免疫抑制・化学療法により発症するB型肝炎対策のガイドライン」が発表された[16]。上記のガイドラインの要約を（表2）に示す。どのガイドラインも趣旨は同様で，化学療法を施行する場合，HBs 抗原陽性例には抗ウイルス薬の予防投与を推奨しており，HBs 抗原陰性で，HBc 抗体陽性または HBs 抗体陰性の症例には，HBV DNA をモニタリングすることが推奨されている。本稿では，わが国でのガイドラインである「免疫抑制・化学療法により発症するB型肝炎対策のガイドライン」について解説する。

免疫抑制・化学療法により発症するB型肝炎対策のガイドライン（図1）[16]

免疫抑制剤や化学療法剤での治療を施行する場合，全例にスクリーニング検査として，HBs 抗原を測定し，HBV 再活性化の高リスク群を同定する。

1）HBs 抗原陽性の場合

HBe 抗原，HBe 抗体，HBV DNA 定量を測定し，治療前の状態を確認する。再活性化のリスクは高いので，原則，抗ウイルス薬として核酸アナログ製剤の予防投与を行う。ただし，HBs 抗原陽性例には，キャリアだけでなく慢性肝炎や肝硬変例も含まれることもあるため，肝臓専門医へのコンサルトも必要である。また，

Presented by Medical*Online