Table 2. Detailed Demographic Characteristics and Serological and Virological Data for the Donor and Recipient Pairs

Donor			Donor index sample HBV markers		Look-back sample HBV DNA, IU/mL	Donor sample at the time of further investigations			Recipient			Blood component transfused	Recipient HBV markers			Transfusion-follow-up sample collection (months)	HBV status
Code	Sex/age, years	Anti-HBc	Anti-HBs (u/L)	HBV DNA (IU/mL)		Anti-HBc	Anti-HBs (U/L)	HBV DNA (IU/mL)	Code	Sex/age, years	Immuno deficiency		Anti-HBc	Anti-HBs (U/L)	HBV DNA (IU/mL)		
Rx15	M/59	Pos	Neg[a]	16		Pos	32.7	UD[b]	Rx93	M/70	No	RC[c]	Neg	Neg	UD	35	Susceptible
									Rx97	M/72	Yes	Plt	Pos	>1000	UD	33	Recovered HBV[a]
					19				**Rx69**	**F/76**	**No**	**RC**	**Pos**	**52**	**9.3**	**25**	**OHB**
									Rx73	F/77	No	RC	Pos	87	UD	49	Recovered HBV
									Rx77	F/58	Yes	FFP	Neg	Neg	UD	48	Susceptible
									Rx95	F/52	No	Plt	Pos	299	UD	38	Recovered HBV[a]
									Rx82	F/34	No	FFP	Neg	102	UD	32	Vaccinated
									Rx76	F/69	No	Plt	Pos	175	UD	49	Recovered HBV
									Rx100	M/6	No	Plt	Neg	Neg	UD	51	Susceptible
									Rx96	**F/51**	**No**	**RC**	**Pos**	**30**	**14**	**60**	**OHB**
									Rx83	M/19	No	Plt	Neg	Neg	UD	50	Susceptible
									Rx84	F/22	No	RC	Neg	131	UD	46	Vaccinated
									Rx85	F/59	Yes	Plt	Pos	22	UD	52	Recovered HBV
Rx28	M/48	Pos	30	UD		Pos	80.1	2.79	Rx49	F/46	Yes	Plt	Neg	Neg	UD	44	Susceptible
Rx34	M/58	Pos	38	UD	5	Pos	80.5	4.79	Rx47	F/75	No	RC	Pos	367	UD	20	Recovered HBV[a]
					14				Rx46	F/63	No	RC	Pos	Neg	UD	23	Recovered HBV
					14				Rx45	M/4	Yes	Plt	Neg	Neg	UD	24	Susceptible
Rx24	F/48	Pos	430	UD	UD	Pos	866.5	UD	Rx92	M/22	No	RC	Neg	64	UD	34	Vaccinated
									Rx99	M/3	No	FFP	Neg	Neg	UD	36	Susceptible
									Rx91	M/65	No	Plt	Neg	14	UD	47	Vaccinated
									Rx75	F/35	No	FFP	Neg	Neg	UD	63	Susceptible
					UD				Rx81	F/60	No	RC	Neg	17	UD	33	Vaccinated
Rx19	M/50	Pos	19	UD	UD	Pos	75.4	UD	Rx102	M/6	No	FFP	Neg	54	UD	23	Vaccinated
									Rx74	M/57	No	FFP	Neg	Neg	UD	40	Susceptible
					26				Rx98	F/31	No	RC	Neg	74	UD	31	Vaccinated
					UD				**Rx70**	**F/55**	**No**	**RC**	**Pos**	**Neg**	**33**	**23**	**CHB**
									Rx68	F/87	No	Plt	Pos	137	UD	46	Recovered HBV
Rx23	F/46	Pos	39	10	21	Pos	85.5	1.81	Rx94	M/60	No	RC	Neg	Neg	UD	25	Susceptible
Rx36	M/61	Pos	18	6.2		Pos	67.2	UD	Rx39	M/53	No	RC	Neg	>1000	UD	48	Vaccinated[a]
					7				Rx66	M/12	Yes	Plt	Neg	412	UD	26	Vaccinated
									Rx40	M/47	Yes	FFP	Pos	>1000	UD	39	Recovered HBV[a]
									Rx44	M/48	Yes	Plt	Pos	Neg	UD	43	Recovered HBV

Table 2. (*Continued*)

Donor	Donor index sample HBV markers			Look-back sample HBV DNA, IU/mL	Donor sample at the time of further investigations			Recipient			Blood component transfused		Recipient HBV markers		Transfusion-follow-up sample collection (months)	HBV status
				27				Rx50	M/17	No	RC	Neg	175	UD	19	Vaccinated
								Rx43	F/42	Yes	FFP	Pos	77	UD	41	Recovered HBV
Rx33	F/59	Pos	Neg	UD	UD	Pos	85.2	UD	Rx38	F/50	Yes	RC	Neg	Neg	UD	15	Susceptible
					UD				Rx41	M/20	No	RC	Neg	21	UD	22	Vaccinated
									Rx42	**F/38**	**Yes**	**Plt**	**Pos**	**Neg**	**$7.3x10^7$**	**48**	**CHB + HBV reaetivation**
									Rx48	M/53	Yes	RC	Pos	Neg	UD	52	Recovered HBV
Rx58	M/56	Pos	Neg	8.5		Pos	24.5	1.12	Rx67	M/56	Yes	Plt	Pos	>1000	UD	52	Recovered HBV[a]
					17				Rx90	F/30	No	RC	Pos	26	UD	15	Recovered HBV
									Rx89	F/29	No	RC	Neg	11	UD	39	Vaccinated
									Rx88	M/41	No	Plt	Neg	Neg	UD	39	Susceptible
									Rx71	F/49	No	Plt	Pos	708	UD	32	Recovered HBV[a]
Rx63	M/49	Pos	Neg	11		Pos	Neg	UD	Rx78	F/50	No	CRYO	Neg	89	UD	38	Vaccinated
					8				Rx87	M/54	Yes	RC	Pos	138	UD	19	Recovered HBV
					26				Rx72	M/66	No	RC	Neg	Neg	UD	16	Susceptible
					UD				Rx86	F/68	No	Plt	Neg	Neg	UD	25	Susceptible
									Rx80	M/40	No	RC	Neg	Neg	UD	35	Susceptible
									Rx79	M/34	Yes	RC	Neg	Neg	UD	30	Susceptible

NOTE. **Bolded** recipient data indicates those found HBV DNA positive in the follow-up sample. CRYO, cryoprecipitate; FFP, fresh frozen plasma; HBV, hepatitis B virus; Neg, antibody to hepatitis B surface antigen (anti-HBs) titer of <10 IU/mL; OHB, occult hepatitis B; Plt, platelet concentrate; RC, red cell concentrate; UD, samples with HBV DNA detectable by nucleic acid tests but not quantifiable by the real-time polymerase chain reaction assays, either with total absence of signal or below the lower limit of detection.

[a] Recipients suspected of having exposure/reexposure to HBV after receiving the blood products from donors with OHB as suggested by a high anti-HBs titer (>200 IU/mL).

4 cases. One of the 3 recipients (Rx42) was likely to have experienced HBV reactivation after receiving chemotherapy for non-Hodgkin lymphoma. For the fourth recipient (Rx96), who was negative for HBsAg, with 95% homology in the highly variable pre-S genomic between the donor and recipient HBV (Figure 2), it is probable that this immunocompetent recipient acquired the HBV infection through the blood transfusion from a donor with OHB who had undetectable anti-HBs titer in the index sample and low titer (32.7 IU/L) in the sample at the time of further investigations. Discounting the 3 recipients who were probably infected with HBV before transfusion, transmission of HBV resulting in chronic HBV disease was possible but uncommon (1 [2.2%] of 46 recipients). This may be an underestimation, because 13 of the recipients were positive for anti-HBs but negative for anti-HBc, possibly because of previous HBV vaccination. The chronic HBV disease rate for the remaining 33 recipients who were susceptible to HBV infection would therefore be 3.0% (1 of 33 recipients).

Moderately high anti-HBs levels (≥200 IU/mL) were found in 8 recipients (Table 2). One 12-year-old recipient (Rx66) with an anti-HBs titer of 412 IU/mL might have the high titer because of comparatively recent HBV vaccination during childhood. The high anti-HBs levels in the remaining 7 adult recipients (age, >45 years) in samples collected 20–52 months after transfusion with blood components from 4 donors was suggestive of a relatively recent anti-HBs immune response secondary to exposure or re-exposure to HBV. (Of the 4 donors, 2 had undetectable anti-HBs in the index samples; all 4 were positive for anti-HBs at the time of additional investigations for the present study). The recent anti-HBs immune response is compatible with the transfused blood products carrying either circulating viral particles or very low level of the otherwise undetectable free HBsAg. Although not directly indicative of infectivity, it constitutes supportive evidence of potential infectivity of HBV DNA–positive blood products even in the presence of anti-HBs. Future studies correlating the detection of HBV DNA over time with respect to the timing of increase of anti-HBs levels in recipients are required to verify this hypothesis.

Nevertheless, the relative rarity of HBV transmission from donors with OHB held true for different scenarios. These included recipients who were children or elderly (4 recipients were aged <7 years, 1 was aged 12 years, and 10 were aged >65 years in the present study) (Table 2). Of the 15 immunocompromised recipients, none acquired HBV from transfusion (except 1 who had probably OHB before transfusion, with reappearance of HBsAg after chemotherapy, suggesting reactivation) [21].

This low infectivity may only be applicable to donors with OHB who are positive for anti-HBs (75% of all 44 donors [Table 1] and 90% of the 10 donors in the donor-recipient pairs [Table 2] were positive for anti-HBs). According to another study [22], more than one-half (57.8%) of subjects with OHB were anti-HBs positive. It has been suggested that the risk of HBV transmission is low for blood products from anti-HBs-positive donors with OHB [23, 24]. A recent study reported a case of HBV transmission from an anti-HBs positive donor to two recipients when the low level of anti-HBs was insufficient to neutralize an increased level of HBV DNA [25].

The present study has 2 limitations. First, 147 recipients had died, making a complete recipient-tracing not possible. However, according to data on the causes of death, it appeared unlikely that the deaths were related to the transfusions. Second, clinical information with regard to the disease status, including liver function tests and liver histology, was not addressed in the present study. However, those were studied in detail in a previous study of ours: subjects with OHB subjects have normal liver function test results and nearly normal liver histology findings [26].

In conclusion, blood taken from donors with OHB was shown to be potentially infectious in our animal and human studies. However, the risk of chronic hepatitis B transmission through transfusion of blood donated by anti-HBs–positive donors with OHB in human remained low.

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References

1. Allain JP, Candotti D. Diagnostic algorithm for HBV safe transfusion. Blood Transfus **2009**; 7:174–82.
2. Liu CJ, Lo SC, Kao JH, et al. Transmission of occult hepatitis B virus by transfusion to adult and pediatric recipients in Taiwan. J Hepatol **2006**; 44:39–46.
3. Song EY, Yun YM, Park MH, Seo DH. Prevalence of occult hepatitis B virus infection in a general adult population in Korea. Intervirology **2009**; 52:57–62.
4. Bhatti FA, Ullah Z, Salamat N, Ayub M, Ghani E. Anti-hepatitis B core antigen testing, viral markers, and occult hepatitis B virus infection in Pakistani blood donors: implications for transfusion practice. Transfusion **2007**; 47:74–9.
5. Yuen MF, Wong DK, Fung J, et al. HBsAg seroclearance in chronic hepatitis B in Asian patients: replicative level and risk of hepatocellular carcinoma. Gastroenterology **2008**; 135:1192–9.
6. Werle-Lapostolle B, Bowden S, Locarnini S, et al. Persistence of cccDNA during the natural history of chronic hepatitis B and decline during adefovir dipivoxil therapy. Gastroenterology **2004**; 126:1750–8.
7. Allain JP, Candotti D, Soldan K, et al. The risk of hepatitis B virus infection by transfusion in Kumasi, Ghana. Blood **2003**; 101: 2419–25.

8. Tateno C, Yoshizane Y, Saito N, et al. Near completely humanized liver in mice shows human-type metabolic responses to drugs. Am J Pathol **2004**; 165:901–12.
9. Abe A, Inoue K, Tanaka T, et al. Quantitation of hepatitis B virus genomic DNA by real-time detection PCR. J Clin Microbiol **1999**; 37:2899–03.
10. Mason AL, Xu L, Guo L, Kuhns M, Perrillo RP. Molecular basis for persistent hepatitis B virus infection in the liver after clearance of serum hepatitis B surface antigen. Hepatology **1998**; 27:1736–42.
11. Lin HJ, Lai CL, Lau JY, Chung HT, Lauder IJ, Fong MW. Evidence for intrafamilial transmission of hepatitis B virus from sequence analysis of mutant HBV DNAs in two Chinese families. Lancet **1990**; 336:208–12.
12. Thompson JD, Gibson TJ, Plewniak F, et al. The CLUSTAL_X windows interface: flexible strategies for multiple sequence alignment added by quality analysis tools. Nucleic Acids Res **1997**; 25:4876–82.
13. Tamura K, Dudley J, Nei M, Kumar S. MEGA4 Evolutionary Genetics Analysis (MEGA) software version 4.0. Mol Biol Evol **2007**; 24:1596–9.
14. Lin CL, Kao JH, Chen BF, Chen PJ, Lai MY, Chen DS. Application of hepatitis B virus genotyping and phylogenetic analysis in intrafamilial transmission of hepatitis B virus. Clin Infect Dis **2005**; 41:1576–81.
15. Tajiri H, Tanaka Y, Kagimoto S, Murakami J, Tokuhara D, Mizokami M. Molecular evidence of father-to-child transmission of hepatitis B virus. J Med Virol **2007**; 79:922–26.
16. Hoofnagle JH, Seeff LB, Bales ZB, Barker LF. Type B hepatitis after transfusion with blood containing antibody to hepatitis B core antigen. N Engl J Med **1978**; 298:1379–83.
17. Lander JJ, Gitnick GL, Gelb LH, Aach RD. Anticore antibody screening of transfused blood. ox Sang **1978**; 34:77–80.
18. Koziol DE, Holland PV, Alling DW. Antibody to hepatitis B core antigen as a paradoxical marker for non-A, non-B hepatitis agents in donated blood. Ann Intern Med **1986**; 104:488–95.
19. Sugg U, Schenzle D, Hess G. Antibodies to hepatitis B core antigen in blood donors screened for alanine aminotransferase level and hepatitis non-A, non-B in recipients. Transfusion **1988**; 28:386–8.
20. Allain JP. Occult hepatitis B virus infection: implications in transfusion. ox Sang **2004**; 86:83–91.
21. Wands JR, Chura CM, Roll FJ, Maddrey WC. Serial studies of hepatitis-associated antigen and antibody in patients receiving antitumor chemotherapy for myeloproliferative and lymphoproliferative disorders. Gastroenterology **1975**; 68:105–12.
22. Velati C, Romanò L, Fomiatti L, Baruffi L, Zanetti AR; SIMTI Research Group. Impact of nucleic acid testing for hepatitis B virus, hepatitis C virus, and human immunodeficiency virus on the safety of blood supply in Italy: a 6-year survey. Transfusion **2008**; 48:2205–13.
23. Mosley JW, Stevens CE, Aach RD, et al. Donor screening for antibody to hepatitis B core antigen and hepatitis B virus infection in transfusion recipients. Transfusion **1995**; 35:5–12.
24. Satake M, Taira R, Yugi H, et al. Infectivity of blood components with low hepatitis B virus DNA levels identified in a lookback program. Transfusion **2007**; 47:1197–205.
25. Levicnik-Stezinar S, Rahne-Potokar U, Candotti D, Lelie N, Allain JP. Anti-HBs positive occult hepatitis B virus carrier blood infectious in two transfusion recipients. J Hepatol **2008**; 48:1022–5.
26. Yuen MF, Lee CK, Wong DK, et al. Prevalence of occult hepatitis B infection in a highly endemic area for chronic hepatitis B: a study of a large blood donor population. Gut **2010**; 59:1389–93.

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ORIGINAL ARTICLE

Reactivation of hepatitis B virus in HBsAg-negative patients with multiple myeloma: two case reports

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Abstract It was recently reported that hepatitis B virus (HBV) reactivation had occurred in HBsAg-negative lymphoma patients who received rituximab plus steroid combination chemotherapy. HBV reactivation in myeloma patients have not been reported extensively. We describe here two cases of HBV reactivation in HBsAg-negative myeloma patients receiving systemic chemotherapy: one from the medical records of 40 patients and another from 61 patients with prospective HBV-DNA monitoring. In the first case positive for anti-HBs, HBV reactivation was diagnosed when hepatitis developed during conventional chemotherapy such as MP and MCP regimen in a relapsed patient after autologous stem cell transplantation (APBSCT); in the second case positive for anti-HBc and anti-HBs, elevation of HBV-DNA was recognized by serial HBV-DNA monitoring performed prospectively following APBSCT. Interestingly, these two cases had the reduction of the titer of anti-HBs during the treatment, followed by HBV reactivation. These clinical data suggest that the HBV-DNA monitoring is necessary for not only HBsAg-positive but also HBsAg-negative myeloma patients with anti-HBc-positive and/or anti-HBs-positive following transplantation and after conventional chemotherapy in the salvage setting. Establishment of a standard strategy to prevent HBV reactivation is important for myeloma patients receiving systemic chemotherapy.

Keywords Reactivation · HBV · Myeloma · Transplantation

Abbreviations

HBV	Hepatitis B virus
HBsAg	Hepatitis B surface antigen
Anti-HBc	Hepatitis B core antibody
Anti-HBs	Hepatitis B surface antibody
AST	Aspartate transaminase
ALT	Alanine aminotransferase
RTD-PCR	Real-time detection polymerase chain reaction
APBSCT	Autologous peripheral blood stem cell transplantation
VAD	Vincristine, doxorubicin, dexamethasone
MP	Melphalan, prednisolone
MCP	Ranimustine, cyclophosphamide, prednisolone
MMCP	Melphalan, ranimustine, cyclophosphamide, prednisolone
BD	Bortezomib, dexamethasone
TD	Thalidomide, dexamethasone
CHOP	Cyclophosphamide, doxorubicin, vincristine, prednisolone
R-CHOP	Rituximab, cyclophosphamide, doxorubicin, vincristine, prednisolone

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1 Introduction

Most cases of hepatitis B virus (HBV) reactivation have been reported in hepatitis B surface antigen (HBsAg)-positive cancer patients receiving systemic chemotherapy [1]. It was recently reported, however, that HBV reactivation also occurred in HBsAg-negative lymphoma patients who received rituximab plus steroid combination chemotherapy [2–5].

The clinical data on HBV reactivation in myeloma patients have not been reported extensively; therefore, we have performed retrospective and prospective analyses of HBV reactivation in 101 myeloma patients who received systemic chemotherapy at Nagoya City University Hospital. Based on these analyses, we report here two cases of HBV reactivation in HBsAg-negative myeloma patients.

2 Patients and methods

Between January 2001 and July 2009, 101 patients were diagnosed as multiple myeloma at Nagoya City University Hospital. We retrospectively analyzed the medical records of 40 patients for the development of hepatitis B who were diagnosed as multiple myeloma between January 2001 and December 2005. In 2006, we instituted the strategy described below to prevent HBV reactivation, and carried it out prospectively in 61 patients between January 2006 and July 2009. The serological markers for HBsAg, hepatitis B core antibody (anti-HBc) and hepatitis B surface antibody (anti-HBs) were tested to establish HBV infection status before the initial chemotherapy. HBsAg and anti-HBs were determined by enzyme immunoassay (EIA) (AxSYM; Abbott Japan, Tokyo, Japan) or chemiluminescence enzyme immunoassay (CLEIA) (Fujirebio, Tokyo, Japan). Anti-HBc of IgG classes was determined by radioimmunoassay (Abbot Japan) or CLEIA (Fujirebio). If the patient was positive for any of the serological markers, plasma HBV-DNA was measured by real-time detection polymerase chain reaction (RTD-PCR). If the patient was HBsAg-positive and/or had HBV-DNA before chemotherapy, prophylactic therapy with an antiviral drug was administered during and for at least 6 months after the chemotherapy. On the other hand, if the patient was HBsAg-negative, but seropositive for anti-HBc and/or anti-HBs (defined as resolved HBV infection), a serial monitoring of HBV-DNA was performed monthly by RTD-PCR during and for at least 1 year after the chemotherapy. If plasma HBV-DNA levels became detectable, antiviral therapy was started as soon as possible.

In this prospective HBV-DNA monitoring, each case of plasma HBV-DNA was measured at SRL Inc, using methods with the highest sensitivity available at the time in clinical practice; the assays included the following: TaqMan PCR assay (Roche Molecular Systems Inc, between April 2008 and July 2009), or Amplicor-PCR assay (Roche Molecular Systems Inc, between January 2006 and March 2008). The cutoff values of the TaqMan PCR assay and Amplicor-PCR assay were set at 1.8 log copies and 2.6 log copies/mL, respectively. In this retrospective analysis, serum HBV-DNA was measured at our laboratory of Nagoya City University using preserved specimen, and HBV-DNA sequences spanning the S gene were amplified by RTD-PCR in accordance with the previously described protocol with a slight modification; it has a detection limit of 2.0 log copies/mL [6].

The two patients with HBV reactivation provided written informed consent to the publication of this report.

3 Treatment for multiple myeloma

In patients younger than 65 years, autologous peripheral blood stem cell transplantation (APBSCT) was performed using high-dose melphalan (200 mg/m^2) following three courses of a VAD (vincristine, doxorubicin, and dexamethasone) regimen as induction therapy and a high-dose cyclophosphamide regimen as stem cell harvest therapy.

In patients who did not choose the transplantation treatment option, or from whom we could not collect enough hematopoietic stem cells, the initial treatment for symptomatic multiple myeloma was MP (melphalan plus prednisolone) or MMCP (melphalan, ranimustine, cyclophosphamide, prednisolone combination chemotherapy).

Patients over 65 years of age were not candidates for transplantation. The initial treatment regimens for these patients were MP, VAD, MMCP or VAD following MP. In relapsed and refractory patients, BD (bortezomib, dexamethasone: after December 2006), or TD (thalidomide, dexamethasone: after December 2008) or other regimens (MP, VAD, etc.) were administered as salvage treatments for all patients.

4 Results

4.1 A case with HBV reactivation on the basis of the medical records

Based on the retrospective analyses, only one patient developed HBV reactivation among 40 multiple myeloma patients diagnosed from January 2001 to December 2005. The clinical course is shown in Fig. 1. A 59-year-old woman diagnosed as symptomatic multiple myeloma (BJP-κ type) received APBSCT as initial treatment. Before APBSCT, she was seronegative for HBsAg, but no

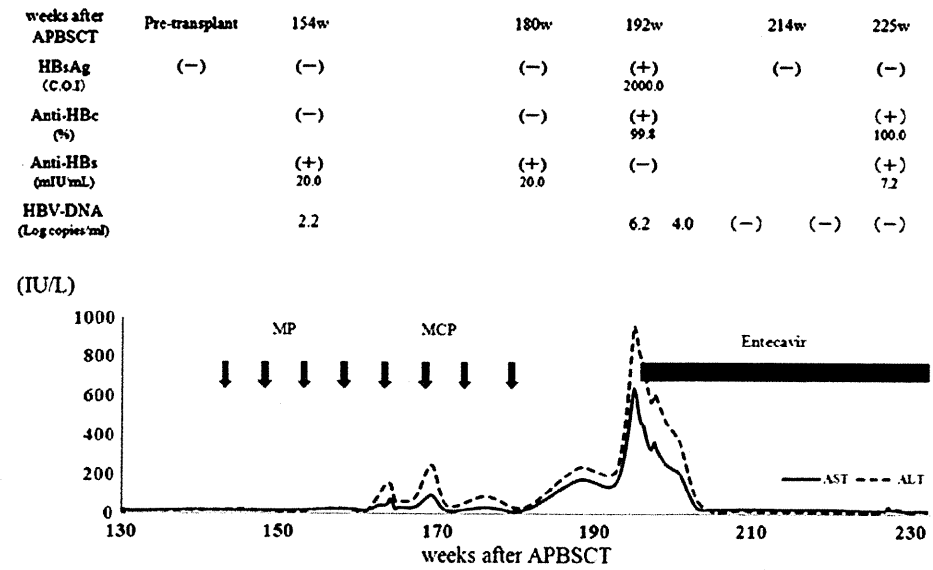

Fig. 1 Clinical course of Case 1. *AST* aspartate transaminase, *ALT* alanine aminotransferase

screening tests for anti-HBc or anti-HBs were performed. Multiple myeloma recurred about 3 years after APBSCT, and MP was administered as salvage treatment. When MP therapy was started, she was seronegative for both HBsAg and anti-HBc, but seropositive for anti-HBs. Because MP could not control the disease, MCP (ranimustine, cyclophosphamide, prednisolone) therapy was administered as the next salvage regimen. Liver damage occurred 32 weeks after the initial salvage chemotherapy was started, and at that time HBsAg changed from negative to positive, and serum HBV-DNA was detectable at 6.2 log copies/mL, so we concluded that the liver damage was caused by hepatitis B virus.

Analyses of specimens preserved during and after salvage therapy showed that serum HBV-DNA was detectable at 2.2 log copies/mL at base line when MP therapy was started, as shown in Fig. 1. In other words, the patient had an occult HBV infection (defined as HBsAg-negative, but HBV-DNA detectable) before salvage chemotherapy.

Furthermore, the HBV gene sequences before and after salvage chemotherapy were confirmed identical in Case 1, so we judged that the liver damage was caused by HBV reactivation. HBV reactivation was reduced after entecavir (0.5 mg, once daily) was administered as an anti-HBV nucleotide analog, and HBV-DNA levels decreased to below the limit of detection.

4.2 HBV-DNA monitoring to prevent HBV reactivation (Fig. 2)

Among 61 patients with symptomatic multiple myeloma diagnosed between January 2006 and July 2009, 1 patient was seropositive for HBsAg, 15 patients were seropositive for anti-HBc and/or anti-HBs (indicating resolved HBV infection), and the remaining 45 patients were seronegative for either anti-HBc or anti-HBs according to the screening tests shown in Fig. 2. One HBsAg-positive patient was given an antiviral drug for prophylaxis before initial treatment. On the other hand, we prospectively performed serial HBV-DNA monitoring during and after myeloma treatment in the 15 patients with resolved HBV infection who had no occult infection.

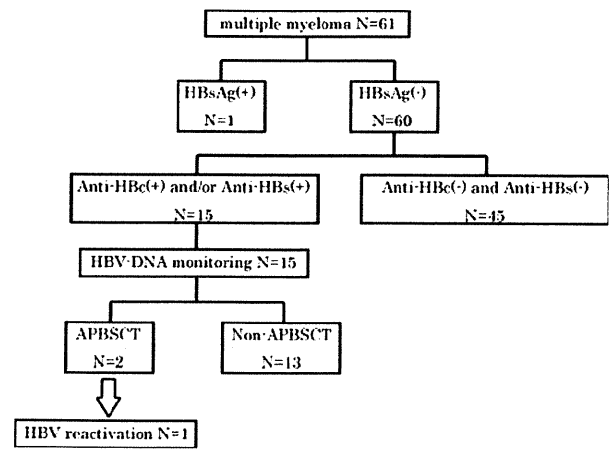

Fig. 2 Screening tests for serological markers of HBV infection and the strategy to prevent HBV reactivation in 61 myeloma patients diagnosed between January 2006 and July 2009 at Nagoya City University Hospital. One patient was seropositive for HBsAg, 15 patients were seropositive for anti-HBc and/or anti-HBs (defined as resolved HBV infection), the remaining 45 patients were seronegative for either anti-HBc or anti-HBs. We prospectively performed serial HBV-DNA monitoring during and after myeloma treatment in 15 patients with resolved HBV infection: following APBSCT, 1 of 15 patients developed HBV reactivation without hepatitis and prior to liver damage because elevation of HBV-DNA was confirmed at an early stage

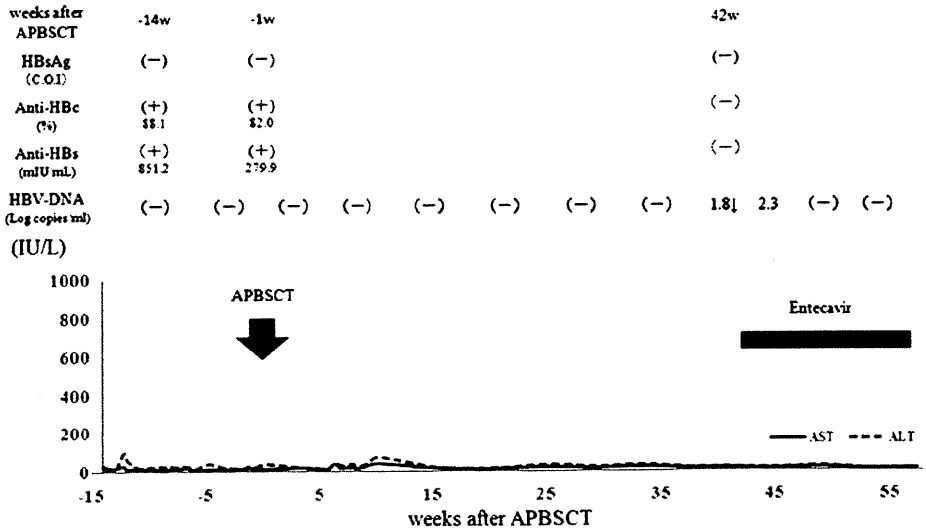

Fig. 3 Clinical course of Case 2

In 8 of these 15 patients, the initial treatment was MP. Following VAD therapy, three patients received MP. Two patients underwent APBSCT. Each of the remaining two patients received MMCP therapy but no further treatment. For salvage treatment, 4 of the 15 patients received BD and/or TD.

One of the 15 patients developed HBV reactivation without hepatitis following APBSCT, after elevation of HBV-DNA was confirmed at an early stage prior to liver damage (Fig. 3). A 61-year-old woman was diagnosed as symptomatic multiple myeloma (BJP-λ type). In screening tests before treatment, HBsAg was negative, both anti-HBc and anti-HBs were positive, and plasma HBV-DNA was below the limit of detection. Therefore, the patient's HBV status was confirmed as a resolved infection. Prospective serial HBV-DNA monitoring was performed monthly, but the testing was sometimes postponed up to 3 months on account of the patient.

Forty-two weeks (about 10 months) after APBSCT, the plasma HBV-DNA level was less than 1.8 log copies/mL but an amplification signal was detectable by the TaqMan PCR assay, and during the following month the HBV-DNA level became detectable with up to 2.3 log copies/mL, as shown in Fig. 2. HBV reactivation was diagnosed at that time, and entecavir (0.5 mg, once daily) was administered immediately as an anti-HBV nucleotide analogue. The plasma HBV-DNA decreased to an undetectable level without liver damage. At the time of HBV reactivation, all HBV serological markers (HBsAg, anti-HBc and anti-HBs) were negative, which suggested that the antibody titers may be reduced by the myeloma treatment. We performed a retrospective search of blood transfusions (red cells and platelets) received by this patient during the previous chemotherapy using the stored specimens from all the blood donors. As a result, it was concluded that the possibility of HBV infection through blood transfusion was extremely low.

5 Discussion

We reported two cases of HBV reactivation in myeloma patients who were seronegative for HBsAg before treatment. HBV was reactivated in a patient with occult infection and definitely diagnosed by a retrospective analysis of preserved specimens when the onset of liver damage occurred after salvage treatment 3 years after APBSCT. In another patient with resolved HBV infection, HBV reactivation at an early stage was detected by the serial HBV-DNA monitoring performed prospectively, and an antiviral drug was administered before liver damage had occurred.

Some HBsAg-negative patients have recently been reported to develop fatal hepatitis by HBV reactivation in the rituximab, cyclophosphamide, doxorubicin, vincristine, prednisolone (R-CHOP) or R-CHOP-like regimens, which combine rituximab and steroid for treatment of CD20-positive malignant lymphoma [2–5]. In 2006, Hui et al. [3] reported that 8 of 244 HBsAg-negative lymphoma patients receiving systemic chemotherapy developed hepatitis by HBV reactivation, and these eight patients were seropositive for either anti-HBc or anti-HBs. It was shown that rituximab plus steroid combination chemotherapy was a risk factor by multivariate analysis. Most recently, Yeo et al. [4] reported that 5 of 80 HBsAg-negative patients diagnosed as diffuse large B cell lymphoma and receiving R-CHOP or CHOP-like regimens had reactivated HBV. All five had received R-CHOP and all were positive for anti-HBc and negative for anti-HBs.

The HBV reactivation following APBSCT in patients with multiple myeloma has been reported sporadically.

Endo et al. [7] reported that 3 of 24 HBsAg-negative patients with resolved HBV infection developed new-onset hepatitis B following APBSCT, and all three patients were multiple myeloma patients. Uhm et al. [8] performed a retrospective analysis of the change of HBV serologic markers following APBSCT. Seven of 129 HBsAg-negative patients became HBsAg-positive after transplantation. All seven patients were seropositive for both anti-HBs and anti-HBc before treatment, and six of the seven patients had multiple myeloma. Furthermore, at reactivation after transplantation, it was shown that the titers of anti-HBs decreased in six of the seven patients. This phenomenon was also shown in our both cases. These data suggested that the decreased titer of anti-HBs may be associated with HBV reactivation, and that the pathophysiology of reactivation may be affected by the dysfunction of humoral immunity in multiple myeloma.

It is necessary to pay attention to the onset of HBV reactivation during salvage treatment; thus, if immunologic inhibition is strong over a longer period, the risk of HBV reactivation may be increased more in patients on second-line or third-line chemotherapy than in those undergoing chemotherapy for the first time [9]. As mentioned above, APBSCT may be one of the important risk factors for HBV reactivation in myeloma patients; however, the reactivation may occur even if the salvage treatment was performed with a mild myelosuppressive regimen such as MP and MCP after the autologous transplantation shown in Case 1. New molecular target drugs such as bortezomib, thalidomide, and lenalidomide improve the survival of myeloma patients remarkably [10–12], so the number of patients who will receive the immunosuppressive therapy for longer periods may increase in the future. Therefore, a standard strategy to prevent HBV reactivation may also become more important in myeloma treatment.

HBV reactivation may lead to fatal fulminant hepatitis, so we hematologists and oncologists should identify high-risk groups in advance before chemotherapy. The latest CDC and Japanese guidelines recommend that patients receiving cytotoxic or immunosuppressive therapy should be tested for serologic markers of HBV infection (i.e., HBsAg, anti-HBc, anti-HBs) [13, 14]. HBV infection status should be established before any chemotherapy or immunosuppressive therapy is initiated (when there is no immunologic inhibition), because antibody titers may be reduced by the treatment, as shown in Case 2. For patients positive for any of the HBV serological markers, the presence of HBV-DNA should be confirmed by RTD-PCR [5, 14].

Prophylaxis with antiviral drugs is essential for HBsAg-positive patients undergoing systemic chemotherapy as recommended by the latest American and Japanese guidelines [14, 15]. Because patients with serum HBV-DNA have more potential risk factors for HBV reactivation, they should be given antiviral drugs as well [5, 14].

If a patient is seropositive for anti-HBc and/or anti-HBs, no standard strategy to prevent HBV reactivation has been established, but making an early diagnosis of HBV reactivation is critical to enable early initiation of active antiviral therapy. Preemptive therapy by serial HBV-DNA monitoring is a reasonable strategy recommended by the latest Japanese guidelines [14]. If HBV-DNA levels become detectable, antiviral therapy should be started as soon as possible.

Only a few studies have reported on the optimal frequency and duration of HBV-DNA monitoring. Hui et al. [3] reported on malignant lymphoma patients that the median time from the elevation of serum HBV-DNA to hepatitis onset was 18.5 weeks (range 12–28 weeks). Most recently, Fukushima et al. [16] conducted a prospective study to monitor HBsAg monthly and HBV-DNA every 3 months during and after systemic chemotherapy in HBsAg-negative but anti-HBc-positive patients with malignant lymphoma; they found that 1 of 24 patients developed HBV reactivation, which was diagnosed by elevation of HBV-DNA level, while their HBsAg was still negative. In fact, as shown in Case 2, we were able to diagnose HBV reactivation at an early stage by the monthly HBV-DNA monitoring and avoid liver damage and decrease plasma HBV-DNA to below the limit of detection by starting the antiviral drug administration.

It is also necessary to make a differential diagnosis in order to distinguish transmission of HBV by blood transfusion from HBV reactivation, because blood transfusion may be received during systemic chemotherapy. In Case 2, we performed a retrospective search of blood transfusion (red cells and platelets) received during previous chemotherapy, using the stored specimens of all blood donors. It was concluded that the possibility of HBV infection through blood transfusion was extremely low.

In conclusion, these clinical data suggest that the HBV-DNA monitoring is necessary for not only HBsAg-positive but also HBsAg-negative myeloma patients with anti-HBc-positive and/or anti-HBs-positive following transplantation and after conventional chemotherapy in the salvage setting. Preemptive therapy by serial HBV-DNA monitoring may be a useful and cost-effective option for preventing HBV reactivation in patients with resolved HBV infection. Establishment of a standard strategy to prevent HBV reactivation is important for myeloma patients receiving systemic chemotherapy.

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References

1. 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.
2. 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.
3. Hui CK, Cheung WW, Zhang HY, Au WY, Yueng YH, Leung AY, et al. Kinetics and risk of de novo hepatitis B infection in HBsAg-negative patients undergoing cytotoxic chemotherapy. Gastroenterology. 2006;131:59–68.
4. Yeo W, Chan TC, Leung NW, Lam WY, Mo FK, Chu MT, 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.
5. Kusumoto S, Tanaka Y, Mizokami M, Ueda R. Reactivation of hepatitis B virus following systemic chemotherapy for malignant lymphoma. Int J Hematol. 2009;90:13–23.
6. Sugiyama M, Tanaka Y, Kurbanov F, Maruyama I, Shimada T, Takahashi S, et al. Direct cytopathic effects of particular hepatitis B virus genotypes in severe combined immunodeficiency transgenic with urokinase-type plasminogen activator mouse with human hepatocytes. Gastroenterology. 2009;136:652–62.e3.
7. Endo T, Sawada K, Fujimoto K, Yamamoto S, Takashima H, Haseyama Y, et al. Reactivation of hepatitis B virus after autologous peripheral blood stem cell transplantation in patients with positive hepatitis B surface antibodies. Rinsho Ketsueki. 2000;41:322–8.
8. Uhm JE, Kim K, Lim TK, Park BB, Park S, Hong YS, et al. Changes in serologic markers of hepatitis B following autologous hematopoietic stem cell transplantation. Biol Blood Marrow Transplant. 2007;13:463–8.
9. Kumagai K, Takagi T, Nakamura S, Sawada U, Kura Y, Kodama F, et al. Hepatitis B virus carriers in the treatment of malignant lymphoma: an epidemiological study in Japan. Ann Oncol. 1997;8(Suppl 1):107–9.
10. San Miguel JF, Schlag R, Khuageva NK, Dimopoulos MA, Shpilberg O, Kropff M, et al. Bortezomib plus melphalan and prednisone for initial treatment of multiple myeloma. N Engl J Med. 2008;359:906–17.
11. Facon T, Mary JY, Hulin C, Benboubker L, Attal M, Pegourie B, et al. Melphalan and prednisone plus thalidomide versus melphalan and prednisone alone or reduced-intensity autologous stem cell transplantation in elderly patients with multiple myeloma (IFM 99–06): a randomised trial. Lancet. 2007;370:1209–18.
12. Dimopoulos M, Spencer A, Attal M, Prince HM, Harousseau JL, Dmoszynska A, et al. Lenalidomide plus dexamethasone for relapsed or refractory multiple myeloma. N Engl J Med. 2007;357:2123–32.
13. Weinbaum CM, Williams I, Mast EE, Wang SA, Finelli L, Wasley A, et al. Recommendations for identification and public health management of persons with chronic hepatitis B virus infection. MMWR Recomm Rep. 2008;57:1–20.
14. Tanaka E, Umemura T. History and prevention of de novo hepatitis B virus-related hepatitis in Japan and the world. Clin J Gastroenterol. 2008;1:83–6.
15. Lok AS, McMahon BJ. Chronic hepatitis B: update 2009. Hepatology. 2009;50:661–2.
16. Fukushima N, Mizuta T, Tanaka M, Yokoo M, Ide M, Hisatomi T, et al. Retrospective and prospective studies of hepatitis B virus reactivation in malignant lymphoma with occult HBV carrier. Ann Oncol. 2009;20:2013–7.

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Review Article

Geographical and genetic diversity of the human hepatitis B virus

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Hepatitis B virus (HBV) is one of the most widely distributed viruses that infect humankind. Distinct clinical and virological characteristics of the HBV-infection have been reported in different geographical parts of the world and are increasingly associated with genetic diversity of the infecting virus. HBV is classified into genotypes and subgenotypes that are associated with ethnicity and geography. The genetic diversity of HBV in its various aspects has been the subject of extensive investigations during the last few decades. Since molecular epidemiology research tools have become widely available, the number of new publications in this field has grown exponentially. This review summarises the recent publications on the geographical distribution of genetic variants of HBV, and proposes updated criteria for the identification of new genotypes and subgenotypes of the virus.

Key words: genotypes, hepatitis B virus, molecular epidemiology, recombination

INTRODUCTION

FOUR DECADES AGO, in 1965, the "Australian antigen" (now reffered to as the hepatitis B surface antigen, HBsAg) was detected in hemophilia patients[1] and was soon specifically associated with hepatits B virus (HBV).[2,3] Three decades ago, the HBV strains were divided into nine major serotypes based on antigenic determinants of HBsAg.[4] Two decades ago, the classification of the HBV by genome nucleotide sequence divergence was proposed.[5] A decade ago, a "unique phylogenetic cluster within genotype A strains was described, triggering consecutive investigations on HBV subgenotypes.[6]

Outlined are the most important key-events in a chain of findings that accumulated in the current image of the HBV diversity. The chain was tortuous before powerful tools such as PCR and nucleotide sequencing became available to researchers. These tools enabled the annual number of publications in this field to grow exponentially. The current review will discuss the most recently published observations on HBV diversity, particularly their geographical distribution, and taxonomical aspects.

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CURRENTLY KNOWN HBV GENOTYPES

A TOTAL OF 18 complete genome sequences were available for comparison when the first four genotypes of HBV (designated A to D, consecutively) were originally proposed and divergence exceeding 8% of the complete genome was indicated as a criterion for genotype identification.[5] Almost at the same time, genotyping based on the phylogenetic clustering was proposed.[7] Consecutively, by sequencing the HBsAg coding region, four new strains were designated as novel genotypes E and F based on both, percent evaluation of nucleotide divergence and phylogenetic analysis. This added new criteria for genotyping; 4% of nucleotide divergence in HBsAg coding sequence.[8] Shortly after, the genotype F was confirmed by an analysis of the full genome sequence.[9] Relatively recent reports identified the last two of the 8 currently known genotypes, genotype G[10] and H.[11]

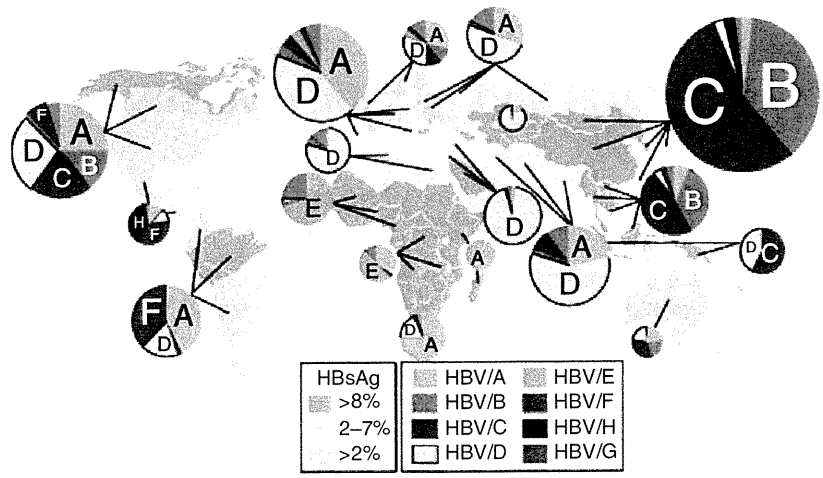

Figure 1 Hepatitis B virus (HBV) infection endemicity is based on the 2005 estimation of hepatitis B surface antigen (HBsAg) seroprevalence (Centers for Disease Control and Prevention Travelers' Health: Yellow Book Chapter 4 – Prevention of Specific Infectious Diseases: Hepatitis, Viral, Type B URL: http://wwwn.cdc.gov/travel/yellowbook/ch4/hep-b.aspx). Percentile distribution of genotypes is indicated for each geographic region.

DISTRIBUTION OF GENOTYPES IN THE WORLD

EPIDEMIOLOGICAL GEOGRAPHICAL DISTRIBUTION of HBV genotypes is being continuously investigated in different parts of the world. For this review we summarised epidemiological studies published within the last decade. A total of 256 papers were analysed. The results of the geographical distribution of genotypes are graphically summarised in Figure 1A. The detailed summary presented in Table 1 contains the number of examined HBV carriers in each particular subregion, and the corresponding references.

The total number of HBV-infected individuals genotyped during the last 10 years consist of approximately 45 000, with more than half of that number in Eastern Asia. From the data accumulated, it can be seen that a single genotype can only be predominately found in

Table 1 Prevalence of hepatitis B virus genotypes in different geographical regions

Geographical subregion	*n*	A	B	C	D	E	F	H	G	Mixed	UT	References
Eastern Africa	43	93.0			2.3						4.7	12–14
South Africa	404	74.3	0.7	1.5	19.3	1.2					3.0	15–17
Central Africa	126	31.0		3.2	3.2	49.2			1.6	11.9		18–20
Western Africa	759	11.3	0.3		1.6	59.2				2.9	24.8	21–29
Northern Africa	331	0.3	5.7	0.9	79.2					9.4	4.5	30–34
Western Asia	1652	0.9	0.2	0.5	94.8		0.1				3.5	35–58
Central Asia	118	11.0		0.8	88.1							59–61
Southern Asia	3023	21.5	0.9	8.9	58.7					3.9	6.1	25,62–79
East Europe	1674	30.5	0.9	0.7	50.4					6.0	11.5	25,80–86
European Union	4968	38.5	3.3	4.3	42.6	3.4	1.4	0.2	0.7	2.0	3.7	10,20,25,81,87–116
North Europe	442	28.3	10.9	10.6	30.8	5.0	1.4	0.2	0.2	2.0	10.6	117–122
North America	3412	25.1	14.3	20.8	27.7	0.2	7.3	0.1	0.9		3.6	10,25,108,123–131
Central America	225	11.6		0.4	11.6		36.0	35.1	3.6	1.3	0.4	132–135
South America	1393	42.6	0.5	1.9	17.4	0.1	35.9		0.1	0.6	0.9	25,136–157
Atlantic Island	84	54.8		1.2	23.8		2.4			17.9		110
Southeastern Asia	2024	6.7	35.2	47.3	4.1	0.7	0.4		0.9	3.4	1.2	20,25,108,158–170
Eastern Asia	23577	2.0	36.9	55.0	2.2					1.9	1.9	25,108,171–255
Pacific Islands	274			57.7	42.3							225,239,256,257
Australia	132	22.7	22.7	31.8	21.2				0.8		0.8	258–260
TOTAL	44661	13.1	22.9	34.5	19.9	1.6	2.1	0.2	0.2	2.1	3.3	

UT, untypeable

Eastern Africa and Western Asia (A and D, respectively) where the prevalence of other genotypes was less than 5%. On the other hand, in countries with high levels of immigration, a variety of genotypes are being reported; all of the known genotypes can be found in the Europe and North America. In Australia, genotypes A, B, C and D were reported in equal prevalence. Two genotypes, A and D are prevalent in European Union (except for the Mediterranean where D predominates), and in Central/Southern Asia. Genotypes B and C are the major variants in South and South East Asia and the Pacific region, while genotypes A and F are the most common in South America or E and A in Central Africa. Genotype E is restricted to Central and West Africa however, its prevalence tends to increase in Europe. Genotype F is subdivided into 4 subgenotypes, and is prevalent in Central and South America and Alaska. In recent reports the subgenotypes of genotype F were further subdivided into clades (Table 2).

A recent study from Peru described a full genome analysis for three strains from Peru that belonged to subtype F1 and suggested they should be considered as clade 1c within subgenotype F1.[281] Genotype G was found in Europe and the United States. A few cases of genotype G infection have been reported from Asia,[227,283] and more recently from Brazil.[284] Despite the geographical dispersion of the reported G strains, they show a very low genetic diversity. Genotype H is frequent in and restricted to Central America where it was also reported in co-infection with genotype G.[133] The pattern of genotype distribution changes according to the pattern of global migration.

EVOLUTIONARY HISTORY OF THE GENOTYPES

THE FIRST ATTEMPT to date the evolutionary history of HBV was carried out by the phylogenetic analysis based on synonymous substitutions in the polymerase coding gene of hepadnavirus family strains isolated from the human, chimpanzee, woodchuck, ground squirrel and duck.[7] The substitution rate estimated in the study was 4.57×10^{-5} substitutions/site/year. This study concluded that the duck strain was the most divergent and shared the most recent common ancestor with other strains approximately 30 000 years ago, whereas different human HBV genotypes emerged about 3000 years ago.[7] However, the overlapping composition of Open Reading Frames (ORFs) in the HBV genome complicates an estimation of the synonymous substitutions, as the same mutation considered synonymous in one of the ORF may cause an aminoacid change in overlapping ORF.[285] The mutation rate of HBV estimated in the serial specimens collected at distant periods of time from genotype B infected carriers, was 7.9×10^{-5} substitutions/site/year.[271] Another study carried on genotype D strains representing localised epidemic in Western Japan, have set the mutation rate to 5.4×10^{-5}.[277] A study aiming to estimate the substitution rate using two independent data sets of non-overlapping ORF coding core protein, concluded that a reliable molecular clock does not exist.[286] Phylogenetic topology of the genotypes heavily depends on the genomic region and substitution model used in analysis, thus hinder any attempt to reconstruct the past spread of this virus.[286] In addition to the complex overlapping structure of the genome, a recombination of HBV severely hampers an assessment of its evolution.[287] New methodological approach is required to explore rules of the HBV evolution.

HBV RECOMBINATION

ONE OF THE most comprehensive analyses of occurrence and composition of HBV intergenotype recombinants indicated the existence of 24 phylogenetically independent recombinant forms of HBV involving all human genotypes as well as both chimpanzee and gibbon variants.[288] Further reports are constantly extending this number.[18,118,283,289] It has been shown that 60% of the intergenotype recombinants have the breakpoints within nucleotides 1640–1900.[289] It was also concluded that recombination sites often localise to gene boundaries.[288,289] Further, using a newly developed approach ("TreeOrder Scan") the authors could demonstrate that analysed in different parts of the HBV genome, genotypes are interchangeably shifting the relative phylogenetic topology. This consists with changes in the overall phylogenetic topology of the HBV genotypes that can be observed in trees reconstructed from different parts of the genome. Genotype G strains in particular demonstrate evidence of recombination with genotype A in the Small S fragment (nucleotides: 250–350) as well as genotype E with genotype D in the core gene (nucleotides: 1950–2500) and genotype H with genotype F within the *Small S* gene (nucleotides: 350–500).[288] It was hypothesised that some of the genotypes that are conventionally regarded as "nonrecombinant," demonstrate evidence of recombination, that is, during evolution in some cases, one or other of the ancestral HBV variants that might have been involved in recombination are virtually replaced by a more viable

Table 2 Hepatitis B virus (HBV) subgenotypes

Genotype	Subgenotype	*n*	Complete genome Nucleotides diversity (complete genome)			Geography	Ref
			Clustering	Intra-subgenotype mean + SD (max)	Next closest negbour mean + SD (min)		
HBV/A							
	A1/Aa	78	yes	2.6 + 0.8 (5.5)	4.4 + 0.4 (3.3) for A4	Africa, Asia, South America	15,261,262
	A2/Ae	94	yes	1.7 + 0.9 (5.5)	4.7 + 0.7 (3.6) for A4	Europe, North America	15,261,262
	A3/Ac	8	yes	3.0 + 0.9 (4.1)	4.7 + 0.4 (3.8) for A1	Western Africa	19,21,22
	A4	3	no	2.9 + 0.9 (3.5)	3.8 + 0.2 (3.4) for A3	Western Africa	21,263
	A5	0	?	?	?	Western Africa	21
HBV/B							
	B1/Bj	38	yes	2.4 + 0.6 (4.1)	4.6 + 0.5 (3.6) for B2	Japan	264–266
	B2	173	yes	1.7 + 0.8 (4.0)	4.4 + 0.5 (2.9) for B4	China, Taiwan	190,200,264–268
	B3	5	yes	1.6 + 0.6 (2.7)	3.6 + 0.5 (2.9) for B5	Indonesia	269
	B4	21	yes	2.7 + 0.6 (4.4)	5.0 + 0.5 (4.3) for B3	Vietnam, Cambodia	269
	B5	7	yes	2.8 + 1.5 (4.5)	5.2 + 0.6 (4.0) for B2	the Philippines	166,167
	B6	27	yes	2.7 + 0.7 (4.2)	5.7 + 0.6 (4.6) for B3	Native populations in Arctic	270,271
	B7	2	no			Indonesia	161
HBV/C							
	C1/Cs	97	yes	2.4 + 0.7 (5.1)	4.4 + 0.5 (3.1) for C2	South and South East Asia	272–274
	C2/Ce	295	yes	2.5 + 0.6 (4.7)	4.9 + 0.5 (3.8) for C3	Eastern Asia (Korea, Japan) and North China	
	C3	3	yes	4.2 + 1.2 (5.2)	5.8 + 0.6 (4.6) for C1	Pacific	269
	C4	2	yes	0.9	6.6 + 0.6 (6.0) for C3	Australia	256
	C5	8	yes	2.0 + 1.0 (3.4)	6.2 + 0.5 (5.0) for C1	Philippines, Vietnam	167
HBV/D							
	D1	88	yes	2.3 + 0.8 (5.2)	3.1 + 0.6 (1.7) for D2	North Africa, Europe, Central Asia	84,269,275
	D2	53	yes	3.0 + 0.8 (5.8)	4.2 + 0.6 (2.6) for D3	North Europe, Russia, Japan (Ehime)	269,276–278
	D3	66	yes	2.9 + 1.1 (5.9)	4.1 + 0.7 (2.3) for D1	South Africa, Europe	
	D4	7	yes	2.6 + 1.2 (4.9)	4.6 + 0.6 (3.5) for D1	Australia	256
	D5	2	yes	2.4	5.2 + 0.5 (4.9) for D4	Eastern India	73
HBV/F							
	F1a	4		1.1 + 0.2 (1.4)	2.0 + 0.2 (1.6) for 1b	Central America: Costa Rica	279,280
	F1b	7		0.4 + 0.1 (0.6)	1.9 + 0.3 (1.5) for 1d	Venezuela, Argentina, Alaska	154,279,281,282
	F1d	2		2.2	2.8 + 0.3 (2.4) for 1a	Japan	279,281
	F2a	9		1.1 + 0.3 (1.4)	3.2 + 0.2 (2.8) for 2b	Brasil, Venezuella, Nicaragua	24,154
	F2b			0.5 + 0.1 (0.6)	4.1 + 0.9 (2.8) for F4		
	F3	23	yes	1.1 + 0.9 (4.2)	4.5 + 0.3 (3.9) for F2	Venezuella	
	F4	6		1.9 + 0.9 (3.7)	4.6 + 0.6 (3.8) for F3	Argentina, Bolivia	142

product of the recombination. Discovery of "pure" genotypes E, G or H strains would confirm this hypothesis. Most of the studies that have found a high prevalence of both D and E were reported in Europe, in particular France.[92–95,103] The only country less affected by recent migration is Cameroon, where these two variants might have been endemic for a longer period of time. However, sequencing of a number of Cameroonian HBV/E strains to date did not reveal any evidence of the presence of a "pure" genotype E that is not "sharing" its core gene sequence with genotype D.[21,22] Genotypes G and H have the highest prevalence in Mexico,[133,134] a country where genotypes A and F are also prevalent.[132,290] Hence further molecular epidemiological studies in Cameroon and Mexico may reveal traces of "pure" non-recombinant ancestors of currently known genotypes.

GENOTYPES COINFECTION

AS MORE THAN one genotype is predominant in most of the geographic regions, coinfection between the predominating genotypes is not a rare finding; especially for B and C,[169,176,179,202,223,251,291] or A and D.[19,21,27,63,69,70,77,79,105,110,138,139] Co-infections with different genotypes of HBV are being reported with various frequencies. The frequency, however, seems to have a stronger association with the genotyping method rather than a geographic region or genotype endemic in a studied population. Most of the reported cases of co-infection with different genotypes were detected by using multiplex PCR or hybridisation assays and are rarely confirmed by conventional cloning and sequencing.[20,81,95,206] However, genotyping based on PCR with specific primers, probes, and/or restriction enzymes may produce misleading non-specific results due to single nucleotide polymorphisms. This is particularly important in case the PCR-based genotyping assays are applied when studying populations with only a few representing HBV sequences in the database, which means that the local variability of HBV strains was not considered when the assay was designed. In some reports, coinfections detected between genotypes not endemic in the studied population. A cross-sectional international population study using PCR-RFLP genotyping, reported 10/47 cases of genotype C in African cohorts and 6 of the 10 cases were found in coinfection (mainly with genotype G.)[20] The same study detected genotypes E to be more frequent in Asian cohorts compared to European and African ones.[20] However these findings are discrepant with previous reports on the geographical distribution of the genotypes therefore the result obtained by PCR-based genotyping assay requires confirmation by cloning and sequencing.

SUBGENOTYPES

GENETIC VARIABILITY WITHIN the genotypes is being extensively investigated since the concept of subgenotyping has evolved from studies on genotype A[15,261] and B.[266] All genotypes except for E, G and H are currently subdivided into subgenotypes (Table 2). Analysis of accumulated sequence data of HBV indicated that nucleotide sequence divergence exceeding 4% but less than 7.5% in the entire genome sequence should be used as criterion for identifying subgenotypes, whereas divisions within the subgenotypes showing less than 4% divergence should be referred to as "clades."[262] In this view, the recently proposed subgenotype A4[21] with a mean and minimal nucleotide divergence from subgenotype A3; 3.8% and 3.4%, respectively, is a clade rather than a subgenotype (Table 2). This can be further supported by a phylogenetic tree constructed on the complete genome of the strains, showing the "A4" strains to group along with the A3 strains (Fig. 2). Similar geographic distribution of the strains (West Africa) concurs that "A4" and A3 strains represent the same subgenotype. The small genetic distance and similar endemicity of the recently proposed subgenotypes B3, B5 and B7 can also suggest that these can be considered as clades representing the same subgenotype of genotype B (Table 2).

EVOLVING OF GENOTYPING CRITERIA

AN EXTENSIVE ANALYSIS of accumulated HBV genome sequence data indicated that the nucleotide diversity of genotype H strains is less than 8% from its closest neighbour; genotype F.[279] It was further proposed to use 7.5% of nucleotide divergence in the complete genome as a cut off for designation of new genotypes.[262] A new genotype "I" was recently reported to be circulating in Vietnam.[294] However, the conclusions of the paper on the new genotype and on the complex intergenotypic recombination did not correspond with existing genotyping criteria.[295] First of all, the complete genome genetic diversity of the strain was lower than 7.5% from the closest neighbour; genotype C (7.0%). Second, the genetic recombination with other genotypes was evident.[288,295,296] And finally, the epidemiological significance of the aberrant variant in terms of a new genotype was questionable as only three strains

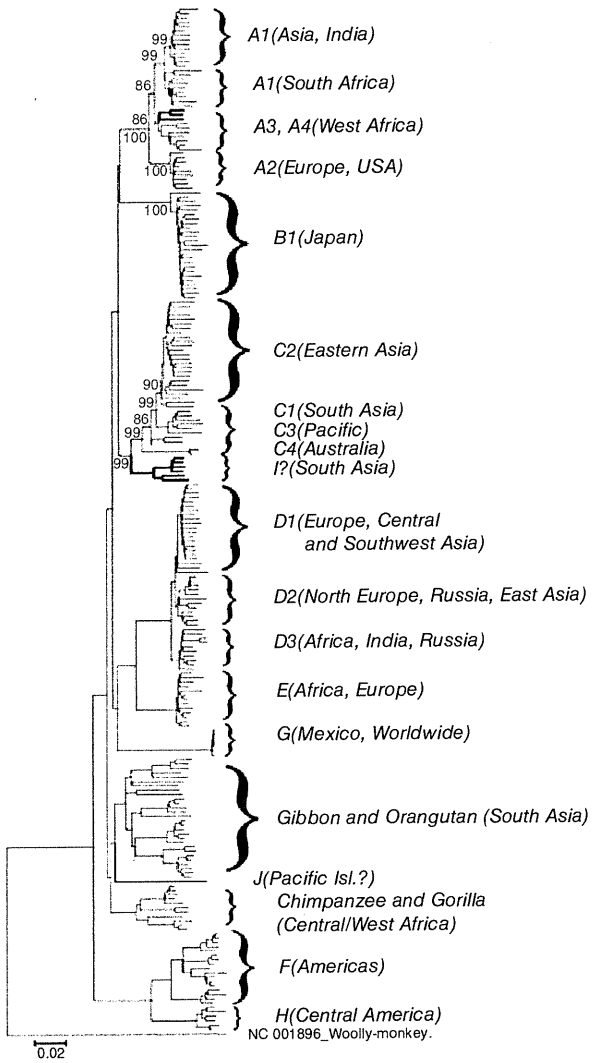

Figure 2 Phylogenetic tree constructed on selected complete genome strain references, which had no evidence of recombination. The neighbour joining tree was constructed using online aligning and tree drawing tools.[292,293]

have been reported during the 8 years since the first report describing this variant.[296] More recent study carried in Laos have revealed more strains that are genetically similar to the three Vietnamese isolates, providing evidence for the epidemiological value of the specific designation of the variant.[297] However, further studies are required to justify the classification of the variant into a separate genotype (HBV-I) or to consider it as a subgenotype of the existing genotype (HBV-C) (Fig. 2.) Another recent study from Japan, based on a strain isolated from an hepatocellular carcinoma patient who had a history of travelling to Borneo, revealed a novel genetic variant of HBV phylogenetically positioned between clusters of human and primate isolates.[298] The tentative genotype J strain show no evidence of recombination with any of known genotypes, and it is phylogenetically close to strains previously isolated from Gibbons and Orangutan (Fig. 2.) Epidemiological, virologic and clinical features of both provisional genotypes I and J require further studies to justify their classification.

Alternative approaches for the genotyping of HBV were recently proposed, suggesting that known variants of HBV can be grouped into 4 groups[299] or 3 groups,[300] however, the relevance of these classifications still needs to be substantiated from epidemiological and clinical points of view.

GENOTYPING CRITERIA

INTENSIVE INVESTIGATIONS HAVE indicated an uneven geographical distribution and epidemiology of distinct HBV genotypes and subgenotypes, however, many questions remain unanswered in terms of their virologic and clinical features. Further investigations in this field require standardised criteria for genotyping and subgenotyping, and these criteria need to be updated regularly in the context of new findings. Currently we propose the following check list of minimal requirements for defining genotypes and subgenotypes:

1 A complete genome sequence analysis is required to identify a new genotype or subgenotype.
2 Nucleotide divergence in a complete genome should exceed 7.5% to distinguish a genotype or 4% to distinguish a subgenotype. Variability below 4% confirmed by specific phylogenetic clustering can be used as a criterion to identify clades within subgenotypes.
3 Genotypes and subgenotypes should be identified by robust independent clustering on molecular evolutionary analysis based on complete HBV genomes.
4 Evidence of recombination with other known or unknown genotypes should be used as a criterion for identifying a subgenotype or clade of genotype involved in recombination rather than a new independent genotype.
5 Identification of a new genotype should be substantiated by its epidemiological, virological or clinical characteristics.

In conclusion, HBV demonstrates significant genetic and geographical divergence. Further studies are required to investigate genetic characteristics of the virus

in less studied developing countries, especially those with a high endemicity. Updated unified criteria are required to resolve future issues in genotype assignment.

REFERENCES

1 Blumberg BS, Alter HJ, Visnich S. A "new" antigen in leukemia sera. *JAMA* 1965; **191**: 541–6.
2 Okochi K, Murakami S. Observations on Australia antigen in Japanese. *Vox Sang* 1968; **15**: 374–85.
3 Prince AM. An antigen detected in the blood during the incubation period of serum hepatitis. *Proc Natl Acad Sci USA* 1968; **60**: 814–21.
4 Courouce-Pauty AM, Lemaire JM, Roux JF. New hepatitis B surface antigen subtypes inside the ad category. *Vox Sang* 1978; **35**: 304–8.
5 Okamoto H, Tsuda F, Sakugawa H *et al.* Typing hepatitis B virus by homology in nucleotide sequence: comparison of surface antigen subtypes. *J Gen Virol* 1988; **69** (Pt 10): 2575–83.
6 Bowyer SM, van Staden L, Kew MC, Sim JG. A unique segment of the hepatitis B virus group A genotype identified in isolates from South Africa. *J Gen Virol* 1997; **78** (Pt 7): 1719–29.
7 Orito E, Mizokami M, Ina Y *et al.* Host-independent evolution and a genetic classification of the hepadnavirus family based on nucleotide sequences. *Proc Natl Acad Sci USA* 1989; **86**: 7059–62.
8 Norder H, Hammas B, Lofdahl S, Courouce AM, Magnius LO. Comparison of the amino acid sequences of nine different serotypes of hepatitis B surface antigen and genomic classification of the corresponding hepatitis B virus strains. *J Gen Virol* 1992; **73**: 1201–8.
9 Naumann H, Schaefer S, Yoshida CF, Gaspar AM, Repp R, Gerlich WH. Identification of a new hepatitis B virus (HBV) genotype from Brazil that expresses HBV surface antigen subtype adw4. *J Gen Virol* 1993; **74**: 1627–32.
10 Stuyver L, De Gendt S, Van Geyt C *et al.* A new genotype of hepatitis B virus: complete genome and phylogenetic relatedness. *J Gen Virol* 2000; **81**: 67–74.
11 Arauz-Ruiz P, Norder H, Robertson BH, Magnius LO. Genotype H: a new Amerindian genotype of hepatitis B virus revealed in Central America. *J Gen Virol* 2002; **83**: 2059–73.
12 Kramvis A, Restorp K, Norder H, Botha JF, Magnius LO, Kew MC. Full genome analysis of hepatitis B virus genotype E strains from South-Western Africa and Madagascar reveals low genetic variability. *J Med Virol* 2005; **77**: 47–52.
13 Sugauchi F, Orito E, Kato H *et al.* Genotype, serotype, and phylogenetic characterization of the complete genome sequence of hepatitis B virus isolates from Malawian chronic carriers of the virus. *J Med Virol* 2003; **69**: 33–40.
14 Hasegawa I, Tanaka Y, Kurbanov F *et al.* Molecular epidemiology of hepatitis B virus in the United Republic of Tanzania. *J Med Virol* 2006; **78**: 1035–42.
15 Kimbi GC, Kramvis A, Kew MC. Distinctive sequence characteristics of subgenotype A1 isolates of hepatitis B virus from South Africa. *J Gen Virol* 2004; **85**: 1211–20.
16 Kew MC, Kramvis A, Yu MC, Arakawa K, Hodkinson J. Increased hepatocarcinogenic potential of hepatitis B virus genotype A in Bantu-speaking sub-saharan Africans. *J Med Virol* 2005; **75**: 513–21.
17 Hardie DR, Williamson C. Analysis of the preS1 gene of hepatitis B virus (HBV) to define epidemiologically linked and un-linked infections in South Africa. *Arch Virol* 1997; **142**: 1829–41.
18 Bekondi C, Olinger CM, Boua N *et al.* Central African Republic is part of the West-African hepatitis B virus genotype E crescent. *J Clin Virol* 2007; **40**: 31–7.
19 Makuwa M, Souquiere S, Telfer P *et al.* Identification of hepatitis B virus subgenotype A3 in rural Gabon. *J Med Virol* 2006; **78**: 1175–84.
20 Toan NL, Song H, Kremsner PG *et al.* Impact of the hepatitis B virus genotype and genotype mixtures on the course of liver disease in Vietnam. *Hepatology* 2006; **43**: 1375–84.
21 Olinger CM, Venard V, Njayou M *et al.* Phylogenetic analysis of the precore/core gene of hepatitis B virus genotypes E and A in West Africa: new subtypes, mixed infections and recombinations. *J Gen Virol* 2006; **87**: 1163–73.
22 Kurbanov F, Tanaka Y, Fujiwara K *et al.* A new subtype (subgenotype) Ac (A3) of hepatitis B virus and recombination between genotypes A and E in Cameroon. *J Gen Virol* 2005; **86**: 2047–56.
23 Suzuki S, Sugauchi F, Orito E *et al.* Distribution of hepatitis B virus (HBV) genotypes among HBV carriers in the Cote d'Ivoire: complete genome sequence and phylogenetic relatedness of HBV genotype E. *J Med Virol* 2003; **69**: 459–65.
24 Huy TT, Ishikawa K, Ampofo W *et al.* Characteristics of hepatitis B virus in Ghana: full length genome sequences indicate the endemicity of genotype E in West Africa. *J Med Virol* 2006; **78**: 178–84.
25 Huy TT, Ushijima H, Win KM *et al.* High prevalence of hepatitis B virus pre-s mutant in countries where it is endemic and its relationship with genotype and chronicity. *J Clin Microbiol* 2003; **41**: 5449–55.
26 Candotti D, Danso K, Allain JP. Maternofetal transmission of hepatitis B virus genotype E in Ghana, West Africa. *J Gen Virol* 2007; **88**: 2686–95.
27 Candotti D, Opare-Sem O, Rezvan H, Sarkodie F, Allain JP. Molecular and serological characterization of hepatitis B virus in deferred Ghanaian blood donors with and without elevated alanine aminotransferase. *J Viral Hepat* 2006; **13**: 715–24.
28 Vray M, Debonne JM, Sire JM *et al.* Molecular epidemiology of hepatitis B virus in Dakar, Senegal. *J Med Virol* 2006; **78**: 329–34.

29 Mulders MN, Venard V, Njayou M *et al.* Low genetic diversity despite hyperendemicity of hepatitis B virus genotype E throughout West Africa. *J Infect Dis* 2004; **190**: 400–8.
30 Ayed K, Gorgi Y, Ayed-Jendoubi S *et al.* Hepatitis B virus genotypes and precore/core-promoter mutations in Tunisian patients with chronic hepatitis B virus infection. *J Infect* 2007; **54**: 291–7.
31 Borchani-Chabchoub I, Gargouri A, Mokdad-Gargouri R. Genotyping of Tunisian hepatitis B virus isolates based on the sequencing of preS2 and S regions. *Microbes Infect* 2000; **2**: 607–12.
32 Saudy N, Sugauchi F, Tanaka Y *et al.* Genotypes and phylogenetic characterization of hepatitis B and delta viruses in Egypt. *J Med Virol* 2003; **70**: 529–36.
33 Zekri AR, Hafez MM, Mohamed NI *et al.* Hepatitis B virus (HBV) genotypes in Egyptian pediatric cancer patients with acute and chronic active HBV infection. *Virol J* 2007; **4**: 74.
34 Ezzikouri S, Chemin I, Chafik A *et al.* Genotype determination in Moroccan hepatitis B chronic carriers. *Infect Genet Evol* 2008; **8**: 306–12.
35 Amini-Bavil-Olyaee S, Sarrami-Forooshani R, Adeli A *et al.* Complete genomic sequence and phylogenetic relatedness of hepatitis B virus isolates from Iran. *J Med Virol* 2005; **76**: 318–26.
36 Amini-Bavil-Olyaee S, Sarrami-Forooshani R, Mahboudi F *et al.* Genotype characterization and phylogenetic analysis of hepatitis B virus isolates from Iranian patients. *J Med Virol* 2005; **75**: 227–34.
37 Sendi H, Mehrab-Mohseni M, Zali MR, Norder H, Magnius LO. T1764G1766 core promoter double mutants are restricted to Hepatitis B virus strains with an A1757 and are common in genotype D. *J Gen Virol* 2005; **86**: 2451–8.
38 Mojiri A, Behzad-Behbahani A, Saberifirozi M *et al.* Hepatitis B virus genotypes in southwest Iran: molecular, serological and clinical outcomes. *World J Gastroenterol* 2008; **14**: 1510–13.
39 Alavian SM, Keyvani H, Rezai M, Ashayeri N, Sadeghi HM. Preliminary report of hepatitis B virus genotype prevalence in Iran. *World J Gastroenterol* 2006; **12**: 5211–13.
40 Moradi A, Kazeminejhad V, Roshandel G, Kalavi K, Ghaemi EO, Semnani S. Hepatitis B virus genotypes in Iran. *Indian J Med Sci* 2008; **62**: 204–5.
41 Ben-Ari Z, Daudi N, Klein A *et al.* Genotypic and phenotypic resistance: longitudinal and sequential analysis of hepatitis B virus polymerase mutations in patients with lamivudine resistance after liver transplantation. *Am J Gastroenterol* 2003; **98**: 151–9.
42 Ben-Ari Z, Ashur Y, Daudi N *et al.* Genotype prevalence, viral load and outcome of hepatitis B virus precore mutant infection in stable patients and in patients after liver transplantation. *Clin Transplant* 2004; **18**: 415–22.
43 El-Zaatari M, Kazma H, Naboulsi-Majzoub M *et al.* Hepatitis B virus DNA in serum of "anti-HBc only"-positive healthy Lebanese blood donors: significance and possible implications. *J Hosp Infect* 2007; **66**: 278–82.
44 Sharara AI, Ramia S, Ramlawi F *et al.* Prevalence of restriction fragment length polymorphism patterns of hepatitis B virus compatible with genotype D in Lebanon. *Eur J Clin Microbiol Infect Dis* 2004; **23**: 861–3.
45 Hajeer AH, Al Knawy B, Alhaj-Hussein BT, Al-Rubiaan SD. Hepatitis B virus: a study of genotypes in an infected Saudi cohort. *Br J Biomed Sci* 2007; **64**: 93–4.
46 Bozdayi G, Turkyilmaz AR, Idilman R *et al.* Complete genome sequence and phylogenetic analysis of hepatitis B virus isolated from Turkish patients with chronic HBV infection. *J Med Virol* 2005; **76**: 476–81.
47 Pinarbasi B, Onel D, Cosan F *et al.* Prevalence and virological features of occult hepatitis B virus infection in female sex workers who work uncontrolled in Turkey. *Liver Int* 2008; **29**: 227–30.
48 Senturker Guldas N, Abacioglu YH. S-gene sequences and genotype-related restriction sites in hepatitis B virus carriers in Turkey. *Infection* 2004; **32**: 344–9.
49 Ozaslan M, Ozaslan E, Barsgan A, Koruk M. Mutations in the S gene region of hepatitis B virus genotype D in Turkish patients. *J Genet* 2007; **86**: 195–201.
50 Serin MS, Akkiz H, Abayli B, Oksuz M, Aslan G, Emekdas G. Genotyping of hepatitis B virus isolated from chronic hepatitis B patients in the south of Turkey by DNA cycle-sequencing method. *Diagn Microbiol Infect Dis* 2005; **53**: 57–60.
51 Ozdemir FT, Duman D, Ertem D *et al.* Determination of hepatitis B genotypes in patients with chronic hepatitis B virus infection in Turkey. *Turk J Gastroenterol* 2005; **16**: 183–7.
52 Bozdayi AM, Bozkaya H, Turkyilmaz AR *et al.* Nucleotide divergences in the core promoter and precore region of genotype D hepatitis B virus in patients with persistently elevated or normal ALT levels. *J Clin Virol* 2001; **21**: 91–101.
53 Sayiner AA, Ozcan A, Sengonul A. Naturally occurring MHR variants in Turkish patients infected with hepatitis B virus. *J Med Virol* 2008; **80**: 405–10.
54 Sunbul M, Leblebicioglu H. Distribution of hepatitis B virus genotypes in patients with chronic hepatitis B in Turkey. *World J Gastroenterol* 2005; **11**: 1976–80.
55 Kaya S, Cetin ES, Aridogan BC, Onal S, Demirci M. Distribution of hepatitis B virus (HBV) genotypes among HBV carriers in Isparta. *Iran Biomed J* 2007; **11**: 59–63.
56 Aksoy A, Ozdarendeli A. [Genotyping of hepatitis B virus by restriction enzyme analysis]. *Mikrobiyol Bul* 2006; **40**: 215–23.
57 Leblebicioglu H, Eroglu C. Acute hepatitis B virus infection in Turkey: epidemiology and genotype distribution. *Clin Microbiol Infect* 2004; **10**: 537–41.

58 Sallam TA, William Tong CY. African links and hepatitis B virus genotypes in the Republic of Yemen. *J Med Virol* 2004; **73**: 23–8.
59 Khan A, Kurbanov F, Tanaka Y *et al.* Epidemiological and clinical evaluation of hepatitis B, hepatitis C, and delta hepatitis viruses in Tajikistan. *J Med Virol* 2008; **80**: 268–76.
60 Kato H, Ruzibakiev R, Yuldasheva N *et al.* Hepatitis B virus genotypes in Uzbekistan and validity of two different systems for genotyping. *J Med Virol* 2002; **67**: 477–83.
61 Avazova D, Kurbanov F, Tanaka Y *et al.* Hepatitis B virus transmission pattern and vaccination efficiency in Uzbekistan. *J Med Virol* 2008; **80**: 217–24.
62 Amini-Bavil-Olyaee S, Alavian SM, Adeli A *et al.* Hepatitis B virus genotyping, core promoter, and precore/core mutations among Afghan patients infected with hepatitis B: a preliminary report. *J Med Virol* 2006; **78**: 358–64.
63 Kumar M, Sarin SK, Hissar S *et al.* Virologic and histologic features of chronic hepatitis B virus-infected asymptomatic patients with persistently normal ALT. *Gastroenterology* 2008; **134**: 1376–84.
64 Borkakoty BJ, Mahanta J, Biswas D. Circulating genotypes of hepatitis B virus in Arunachal Pradesh. *Indian J Med Res* 2008; **127**: 65–70.
65 Gandhe SS, Chadha MS, Arankalle VA. Hepatitis B virus genotypes and serotypes in western India: lack of clinical significance. *J Med Virol* 2003; **69**: 324–30.
66 Chattopadhyay S, Das BC, Hussain Z, Kar P. Hepatitis B virus genotypes in acute and fulminant hepatitis patients from north India using two different molecular genotyping approaches. *Hepatol Res* 2006; **35**: 79–82.
67 Chandra PK, Banerjee A, Datta S, Chakravarty R. G1862T mutation among hepatitis B virus-infected individuals: association with viral genotypes and disease outcome in Kolkata, Eastern India. *Intervirology* 2007; **50**: 173–80.
68 Biswas A, Chandra PK, Datta S *et al.* Frequency and distribution of hepatitis B virus genotypes among eastern Indian voluntary blood donors: association with precore and basal core promoter mutations. *Hepatol Res* 2009; **39**: 53–9.
69 Chattopadhyay S, Das BC, Kar P. Hepatitis B virus genotypes in chronic liver disease patients from New Delhi, India. *World J Gastroenterol* 2006; **12**: 6702–6.
70 Vivekanandan P, Abraham P, Sridharan G *et al.* Distribution of hepatitis B virus genotypes in blood donors and chronically infected patients in a tertiary care hospital in southern India. *Clin Infect Dis* 2004; **38**: e81–6.
71 Chowdhury A, Santra A, Chakravorty R *et al.* 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* 2005; **20**: 1712–20.
72 Banerjee A, Datta S, Chandra PK, Roychowdhury S, Panda CK, Chakravarty R. Distribution of hepatitis B virus genotypes: phylogenetic analysis and virological characteristics of genotype C circulating among HBV carriers in Kolkata, Eastern India. *World J Gastroenterol* 2006; **12**: 5964–71.
73 Banerjee A, Kurbanov F, Datta S *et al.* Phylogenetic relatedness and genetic diversity of hepatitis B virus isolates in Eastern India. *J Med Virol* 2006; **78**: 1164–74.
74 Murhekar MV, Chakravarty R, Murhekar KM, Banerjee A, Sehgal SC. Hepatitis B virus genotypes among the Jarawas: a primitive Negrito tribe of Andaman and Nicobar Islands, India. *Arch Virol* 2006; **151**: 1499–510.
75 Datta S, Chandra PK, Banerjee A, Chakravarty R, Murhekar KM, Murhekar MV. Predominance of hepatitis B virus genotype C among Karens, the "old settlers" of Andaman and Nicobar Islands, India. *Arch Virol* 2007; **152**: 1223–8.
76 Arankalle VA, Murhekar KM, Gandhe SS *et al.* Hepatitis B virus: predominance of genotype D in primitive tribes of the Andaman and Nicobar islands, India (1989–1999). *J Gen Virol* 2003; **84**: 1915–20.
77 Alam MM, Zaidi SZ, Shaukat S *et al.* Common genotypes of Hepatitis B virus prevalent in injecting drug abusers (addicts) of North West Frontier Province of Pakistan. *Virol J* 2007; **4**: 63.
78 Alam MM, Zaidi SZ, Malik SA *et al.* Molecular epidemiology of Hepatitis B virus genotypes in Pakistan. *BMC Infect Dis* 2007; **7**: 115.
79 Abbas Z, Muzaffar R, Siddiqui A, Naqvi SA, Rizvi SA. Genetic variability in the precore and core promoter regions of hepatitis B virus strains in Karachi. *BMC Gastroenterol* 2006; **6**: 20.
80 Olinger CM, Lazouskaya NV, Eremin VF, Muller CP. Multiple genotypes and subtypes of hepatitis B and C viruses in Belarus: similarities with Russia and western European influences. *Clin Microbiol Infect* 2008; **14**: 575–81.
81 Deterding K, Constantinescu I, Nedelcu FD *et al.* Prevalence of HBV genotypes in Central and Eastern Europe. *J Med Virol* 2008; **80**: 1707–11.
82 Krekulova L, Rehak V, da Silva Filho HP, Zavoral M, Riley LW. Genotypic distribution of hepatitis B virus in the Czech Republic: a possible association with modes of transmission and clinical outcome. *Eur J Gastroenterol Hepatol* 2003; **15**: 1183–8.
83 Nemecek V, Strunecky O. [Hepatitis B virus (HBV) genotypes in the Czech Republic]. *Epidemiol Mikrobiol Imunol* 2004; **53**: 55–61.
84 Tallo T, Norder H, Tefanova V *et al.* Hepatitis B virus genotype D strains from Estonia share sequence similarity with strains from Siberia and may specify ayw4. *J Med Virol* 2004; **74**: 221–7.
85 Szomor KN, Dencs A, Toth G *et al.* Variability of the PreS1/PreS2/S regions of hepatitis B virus in Hungary. *Arch Virol* 2007; **152**: 697–704.
86 Manuilov VA, Netesova IG, Osipova LP *et al.* [Genetic variability of hepatitis B virus isolates among population of Shuryskarsky area of Yamal-Nenets autonomous region]. *Mol Gen Mikrobiol Virusol* 2005; **4**: 30–4.

87 Micalessi MI, De Cock L, Vranckx R. Hepatitis B virus (HBV) genotyping in Belgian patients with chronic HBV infection. *Clin Microbiol Infect* 2005; **11**: 499–501.
88 Tran N, Berne R, Chann R *et al.* European multicenter evaluation of high-density DNA probe arrays for detection of hepatitis B virus resistance mutations and identification of genotypes. *J Clin Microbiol* 2006; **44**: 2792–800.
89 Wintermeyer P, Gerner P, Gehring S, Karimi A, Wirth S. Prevalence of hepatitis B virus precore stop codon mutations in chronically infected children. *World J Gastroenterol* 2006; **12**: 2235–8.
90 Oommen PT, Wirth S, Wintermeyer P, Gerner P. Relationship between viral load and genotypes of hepatitis B virus in children with chronic hepatitis B. *J Pediatr Gastroenterol Nutr* 2006; **43**: 342–7.
91 Hannoun C, Krogsgaard K, Horal P, Lindh M. Genotype mixtures of hepatitis B virus in patients treated with interferon. *J Infect Dis* 2002; **186**: 752–9.
92 Soussan P, Pol J, Garreau F *et al.* Expression of defective hepatitis B virus particles derived from singly spliced RNA is related to liver disease. *J Infect Dis* 2008; **198**: 218–25.
93 Ganne-Carrie N, Williams V, Kaddouri H *et al.* Significance of hepatitis B virus genotypes A to E in a cohort of patients with chronic hepatitis B in the Seine Saint Denis District of Paris (France). *J Med Virol* 2006; **78**: 335–40.
94 Trimoulet P, Boutonnet M, Winnock M *et al.* Hepatitis B virus genotypes: a retrospective survey in Southwestern France, 1999–2004. *Gastroenterol Clin Biol* 2007; **31**: 1088–94.
95 Halfon P, Bourliere M, Pol S *et al.* Multicentre study of hepatitis B virus genotypes in France: correlation with liver fibrosis and hepatitis B e antigen status. *J Viral Hepat* 2006; **13**: 329–35.
96 Pas SD, Tran N, de Man RA, Burghoorn-Maas C, Vernet G, Niesters HG. Comparison of reverse hybridization, microarray, and sequence analysis for genotyping hepatitis B virus. *J Clin Microbiol* 2008; **46**: 1268–73.
97 Protzer-Knolle U, Naumann U, Bartenschlager R *et al.* Hepatitis B virus with antigenically altered hepatitis B surface antigen is selected by high-dose hepatitis B immune globulin after liver transplantation. *Hepatology* 1998; **27**: 254–63.
98 Zacharakis G, Koskinas J, Kotsiou S *et al.* Natural history of chronic hepatitis B virus infection in children of different ethnic origins: a cohort study with up to 12 years' follow-up in northern Greece. *J Pediatr Gastroenterol Nutr* 2007; **44**: 84–91.
99 Lok AS, Hussain M, Cursano C *et al.* Evolution of hepatitis B virus polymerase gene mutations in hepatitis B e antigen-negative patients receiving lamivudine therapy. *Hepatology* 2000; **32**: 1145–53.
100 De Maddalena C, Giambelli C, Tanzi E *et al.* High level of genetic heterogeneity in S and P genes of genotype D hepatitis B virus. *Virology* 2007; **365**: 113–24.
101 Dal Molin G, Poli A, Croce LS *et al.* Hepatitis B virus genotypes, core promoter variants, and precore stop codon variants in patients infected chronically in North-Eastern Italy. *J Med Virol* 2006; **78**: 734–40.
102 Koppelman MH, Zaaijer HL. Diversity and origin of hepatitis B virus in Dutch blood donors. *J Med Virol* 2004; **73**: 29–32.
103 Toy M, Veldhuijzen IK, Mostert MC, de Man RA, Richardus JH. Transmission routes of hepatitis B virus infection in chronic hepatitis B patients in the Netherlands. *J Med Virol* 2008; **80**: 399–404.
104 Bielawski KP, Charmuszko U, Dybikowska A, Stalke P, Podhajska AJ. Genetic variability of hepatitis B virus isolates in Poland. *Virus Genes* 2006; **33**: 77–86.
105 Zalewska M, Domagala M, Simon K, Gladysz A. [Hepatitis B virus genotypes and the response to lamivudine therapy]. *Pol Arch Med Wewn* 2005; **114**: 1190–9.
106 Dzierzanowska-Fangrat K, Woynarowski M, Szczygielska I *et al.* Hepatitis B virus genotypes in children with chronic hepatitis B in Poland. *Eur J Gastroenterol Hepatol* 2006; **18**: 655–8.
107 Basaras M, Arrese E, Blanco S, Sota M, de las Heras B, Cisterna R. Characterization of hepatitis B virus genotypes in chronically infected patients. *Rev Esp Quimioter* 2007; **20**: 442–5.
108 Ding X, Park YN, Taltavull TC *et al.* Geographic characterization of hepatitis virus infections, genotyping of hepatitis B virus, and p53 mutation in hepatocellular carcinoma analyzed by in situ detection of viral genomes from carcinoma tissues: comparison among six different countries. *Jpn J Infect Dis* 2003; **56**: 12–18.
109 Ramos B, Nunez M, Martin-Carbonero L *et al.* Hepatitis B virus genotypes and lamivudine resistance mutations in HIV/hepatitis B virus-coinfected patients. *J Acquir Immune Defic Syndr* 2007; **44**: 557–61.
110 Pena-Lopez MJ, Suarez-Bordon P, Garcia-Bardeci D, Rodriguez-San Roman JL, Perez-Gonzalez MC, Lafarga-Capuz B. [Hepatitis B virus genotypes in chronic carriers on the island of Gran Canaria. Clinical and epidemiological characteristics]. *Enferm Infecc Microbiol Clin* 2005; **23**: 415–18.
111 Jardi R, Rodriguez F, Buti M *et al.* Mutations in the basic core promoter region of hepatitis B virus. Relationship with precore variants and HBV genotypes in a Spanish population of HBV carriers. *J Hepatol* 2004; **40**: 507–14.
112 Sanchez-Tapias JM, Costa J, Mas A, Bruguera M, Rodes J. Influence of hepatitis B virus genotype on the long-term outcome of chronic hepatitis B in western patients. *Gastroenterology* 2002; **123**: 1848–56.
113 Avellon A, Echevarria JM. Frequency of hepatitis B virus "a" determinant variants in unselected Spanish chronic carriers. *J Med Virol* 2006; **78**: 24–36.
114 Echevarria JM, Avellon A, Magnius LO. Molecular epidemiology of hepatitis B virus in Spain: identification of