Table 2 Annual care cost and health related QOL assigned to each health state

Annual care cost ($)	Outpatient	Inpatient	Total
Combination therapy			
Standard protocol (first year)	23 660*†	6322‡	29 982
Extended protocol (first year)	23 481*†	6322‡	29 803
Extended protocol (second year)	9443*		9443
Post-SVR	697		697
Chronic hepatitis	1597		1597
Compensated cirrhosis	1743		1743
Decompensated cirrhosis	2370§	13 414	15 784
Ascites (76.0%)¶	1976		
Variceal bleeding (18.5%)¶	2472		
Encephalopathy (5.5%)¶	8235		
HCC	3706	18 036	21 742
Liver transplantation			
1st year			9201 [6,22,27]
Subsequent years			12 962 [6,22,27]

Health-related QOL	Value	Reference
Chronic hepatitis	0.94	30
Compensated cirrhosis	0.89	30
Decompensated cirrhosis	0.81	30
HCC	0.81	30
Liver transplantation	0.86	30
Combination therapy	0.90	30
Cure (post-SVR)	1	30

*Dose modification considered.
†Costs affected by each discontinuation rate.
‡Drug cost not included.
§Weighted average according to the rates of three states in decompensated cirrhosis.
¶Percentage in the parenthesis showed the rates in the state of decompensated cirrhosis.
SVR, sustained virologic response; QOL, quality of life; HCC, hepatocellular carcinoma.

probabilities, varied based on each 95% confidence interval. The rate from decompensated cirrhosis to liver transplantation varied between 1 and 5% according to expert opinions. The annual care cost of HCC and liver transplantation and the price of peginterferon α-2a plus ribavirin varied from −50 to +50%. The costs and benefits were discounted at a rate of 3% per year in the base-case analysis, based on the current well-accepted recommendation [31]. They also varied between 0 and 5% in the sensitivity analysis.

The analyses were performed using the TREEAGE PRO 2006 software program (TreeAge Software, Williamstown, MA, USA).

RESULTS

Base-case analysis

The 10-year cumulative risk of the progression to cirrhosis in chronic hepatitis C patients without treatment was estimated in order to assess the robustness of our model. It was calculated to be 45.6% in our model and was not appreciably different from the rate of 47.4% reported by Yano

Table 3 The base-case analysis in the Markov model based on each treatment strategy

	No treatment	Standard protocol	Extended protocol
LEs (years)	14.88	15.99	16.40
QALYs	13.52	14.80	15.35
Lifetime cost ($)	59 640	71 559	69 438

The extended protocol for genotype 1-infected patients with a slow virologic response presented the prolonged LEs and QALYs, and also reduced lifetime cost, in comparison with the standard protocol. The costs and benefits were discounted at 3% per year.
LEs, life expectancies; QALYs, quality-adjusted life years.

et al. [11]. As shown in Table 3, the extended protocol would be superior to the standard protocol in the LEs, the QALYs and the lifetime cost.

Sensitivity analysis

Table 4 demonstrates the results of the sensitivity analysis. In all of the one-way sensitivity analyses, the extended protocol resulted in prolonged QALYs and reduced lifetime costs in comparison with the standard protocol. In the cost-effectiveness analysis, when the new protocol or the strategy of treatment showed superior effectiveness and a reduction in lifetime costs, it was assessed to be dominant. Therefore, the extended protocol would be the dominant strategy in comparison with the standard protocol.

DISCUSSION

It was our finding that the treatment with the extended protocol for the genotype 1-infected patients with a slow virologic response was superior in effectiveness and in lifetime cost in comparison with the standard treatment protocol.

Table 4 Differences of QALY and lifetime cost (extended protocol–standard protocol) in the sensitivity analysis

Parameter	Difference of QALY	Difference of lifetime cost ($)	Cost-effectiveness
Base-case	0.55	−2762	Dominant
SVR rate	0.51 to 0.60	−2133 to −1890	Dominant
Transition probability	0.35 to 0.74	−3737 to −415	Dominant
Ribavirin 1200 mg	0.55	−1110	Dominant
Treatment cost			
Cost of combination therapy	0.55	−2139 to −2101	Dominant
Annual care cost for HCC	0.55	−3840 to −400	Dominant
Cost of LT (both cost of first and subsequent years)	0.55	−8916 to −2027	Dominant
Discount rate			
5%	0.39	−918	Dominant
0%	1.00	−4998	Dominant

The SVR rate and the transition probabilities were varied between their probable ranges as shown in Table 1. Treatment costs were varied between −50 and +50%.
QALY, quality adjusted life year; SVR, sustained virologic response; HCC, hepatocellular carcinoma; LT, liver transplantation.

In our study, ribavirin was assumed to be used at a fixed dose of 800 mg day^{-1}, which was less than the dose used in the recent recommendations. The National Institute of Health Consensus Development Conference and the American Association for the Study of Liver Disease recommended that untreated patients should be treated with peginterferon in combination with ribavirin (1000–1200 mg day^{-1}) for 48 weeks in genotype 1 cases [32,33]. Berg *et al.* justified the dose of 800 mg day^{-1} in their report because more than a few patients treated with 1200 mg day^{-1} required a reduction in the dose and the cumulative doses of ribavirin were similar between 800 mg day^{-1} for 72 weeks and 1200 mg day^{-1} for 48 weeks [7]. As shown in Table 4, the cost-effectiveness of the extended protocol at a ribavirin dose of 1200 mg day^{-1} would not be changed in our analysis. However, we assumed that the rate of SVR could improve by using the higher dose of ribavirin. Regarding treatment effectiveness, Hadziyannis *et al.* reported the effectiveness of a treatment regimen with the higher dose of ribavirin. They reported that treatment with peginterferon α-2a and weight-based ribavirin (1000 or 1200 mg day^{-1}) for genotype 1-infected patients achieved an SVR rate of 52%, which was superior to that of 41% with peginterferon α-2a and low dose rivabirin (800 mg day^{-1}) [34]. As they did not perform the stratified analysis on the viral kinetics, it was not known whether the difference in the ribavirin dose might have affected the rates of SVR for the slow virologic responders. Recently, the randomized clinical trial reported by Pearlman *et al.* compensated for these problems, thus suggesting the effectiveness of the extended treatment with peginterferon α-2b plus weight-based ribavirin (800–1400 mg day^{-1}) for genotype 1-infected patients with slow virologic response [35]. Although their definition of the slow virologic response was different from that in the study by Berg *et al.*, they reported that an SVR rate of 39% in the extended 72 weeks of treatment was superior to that of 18% in the standard 48 weeks of treatment. In their trial, the slow virologic response was defined as less than 2 $\log_{10}$ drop in pretreatment HCV-RNA level but still detectable at week 12 and not at week 24. When we re-evaluated the cost-effectiveness based on the data by Pearlman *et al.* in order to confirm the effect of the weight-based ribavirin, the lifetime cost and the QALYs of the extended treatment with peginterferon α-2b plus weight-based ribavirin were estimated to be $78 305 and 15.99 QALYs, respectively. In contrast, the estimates of the standard 48 weeks of treatment with peginterferon α-2b plus weight-based ribavirin were calculated to be $79 829 in lifetime cost and 14.79 QALYs, respectively. We therefore confirmed that these results could support our findings if weight-based ribavirin was used.

Our findings could therefore be of help for a limited number of patients receiving the combination therapy. More specifically, when 1000 patients start combination therapy, only 218 patients would be predicted to be slow virologic responders based on the findings of Berg *et al.* [7]. However,

the extended protocol could lead to a reduction in the wasted costs, such as the cost for patients who relapsed after the treatment, which were not considered when making the current cost-effectiveness analysis. Davis *et al.* suggested that genotype 1-infected patients who failed to become HCV-RNA negative or to achieve a 2 $\log_{10}$ decrease from the pre-treatment HCV-RNA level at week 12, should discontinue the treatment from an economic perspective [36]. In other words, the treatment costs for those patients without a sufficient virologic response at week 12 would be a waste. In fact, in the previous cost-effectiveness analyses, patients without an early virologic response were excluded from the analyses [5,6]. Conversely, our results indicated that the treatment costs for genotype 1-infected patients who did not achieve an early virologic response, but who became HCV-RNA negative at week 24, would incur justifiable costs if the treatment duration was extended to 72 weeks.

Finally, we defined a slow virologic responder as a patient whose HCV-RNA was detectable at week 12 but undetectable at week 24. But the stratification at week 12 still remains controversial. Sánchez-Tapias *et al.* described the effectiveness of the extended 72-week course of treatment for patients with detectable HCV-RNA at week 4 [37]. The SVR rates among genotype 1-infected patients were reported to be 44% by the extended treatment and 28% by the standard 48-week course of treatment, respectively. Although this result may appear to be superior in the cost-effectiveness analysis, significantly more patients discontinued treatment in the extended course of treatment. The reason why treatment discontinuation was more frequent in the extended treatment course was reported to be not due to adverse events, but because of a refusal of further treatment or a failure of the patient to return for follow-up care [37]. Because of the insufficient compliance in the extended course of treatment, the evaluation for the stratification of treatment duration should therefore be undertaken at week 12.

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Heterogeneity of Hepatitis B Virus Genotype D in Japan

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Key Words
Hepatitis B virus · Genotype D · Hepatitis B surface antigen

Abstract
Objective: Hepatitis B virus (HBV) genotypes B and C are predominant in Japan. Previously, we reported that approximately 9% of HBV carriers in the Ehime area of western Japan were infected with genotype D (HBV/D) and their sequences closely related. Recently, serum samples from 3 patients with chronic HBV/D infections living in Tokyo and the surrounding area became available for testing. The purpose of this study was to determine whether the HBV/D isolates from these different areas of Japan are closely related. ***Methods:*** Of the 3 Tokyo area patients infected with HBV/D, 2 had chronic hepatitis, and 1 had hemophilia with a history of frequent coagulation factor injections. The complete HBV/D genome sequences of each were determined, and compared with those of subjects from the Ehime area. ***Results:*** All 3 HBV/D sequences had a genomic length of 3,182 bases, and the hepatitis B surface antigen subtype was ayw3. Phylogenetic analysis revealed that the 1 of the HBV/D isolates was closely related to the isolates from Ehime Prefecture, while 1 was similar and 1 was clearly distinct. ***Conclusion:*** Our results indicate that HBV/D infections in Japan are heterogeneous.

Introduction

Hepatitis B virus (HBV) is an incomplete double-stranded DNA virus with approximately 3,200 bases in genomic length. More than 350 million people throughout the world are chronically infected with HBV, which had been classified into several serotypes and genotypes according to the antigenicity of the hepatitis B surface antigen (HBsAg) and the entire nucleotide sequence, respectively [1–4]. HBV genotypes are classified as A to H [4–7], and have distinct geographic distributions [8–11], as genotypes B (HBV/B) and C (HBV/C) are found mainly in East Asia, while genotypes A (HBV/A) is mainly distributed in Europe, North America and central Africa, with genotype D (HBV/D) is distributed widely. A number of studies regarding the significance and clinical relevance of HBV genotypes have been reported [12–18].

In Japan, HBV/B and HBV/C are the most prevalent. Although the frequency of HBV/D was reported to comprise only 0.4% of HBV carriers in Japan [8], approximately 9% of the HBV carriers in a small geographic locale in western Japan (Ehime Prefecture) were found to be infected with HBV/D [19]. It was reported previously that Gianotti-Crosti syndrome caused by HBV subtype *ayw* was endemic in the 1970s in the Ehime area and an HBV isolate with subtype *ayw* from a patient with that

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Table 1. Clinical and virological data of the 3 patients

Patient	Sex	Age	Diagnosis	Year of sampling	ALT IU/l	HBeAg	Anti-HBe	HBV-DNA LGE/ml (TMA)*	Isolate	Accession No.	Length bp	Deduced HBsAg subtype
1	M	12	CH, hemophilia A	1990	53	–	+	<3.7	Toshima 1	AB210819	3182	ayw3
2	M	27	ASC	2001	12	–	+	<3.7	Tokyo 1	AB210820	3182	ayw3
3	M	8	CH	2001	501	+	–	8	Kokusai-Iryo 2-OS	AB210822	3182	ayw3

CH = Chronic hepatitis; ASC = asymptomatic carrier (inactive HBsAg carrier state).
* Log genome equivalents/ml, transcription-mediated assay (Chugai Diagnostics Science Co. Ltd., Tokyo, Japan).

syndrome was determined to be HBV/D [20–22]. In our previous report, we showed that HBV/D strains from Ehime Prefecture formed a definite cluster in a phylogenetic tree, and that several isolates from Russia and north Europe were closely related to them [23]. Further, evolutionary analyses revealed that the root of the HBV/D phylogenetic tree in Ehime was approximately 1900. Combined with the history of Japan, we speculated that the origin of HBV/D in Ehime is Russia.

HBV/D is rare in other areas of Japan, however, serum samples from 3 patients in Tokyo and the surrounding area (greater Tokyo area) infected with HBV/D have become available for study. In the present study, we analyzed the genomic sequences of the HBV/D isolates from the Tokyo area and compared them to those from the Ehime area to elucidate whether the strains from these areas are closely related.

Patients and Methods

Patients

Three Japanese patients living in the Tokyo area and confirmed to be infected with HBV/D were included in this study. Patient 1 was a 12-year-old male, with chronic hepatitis and hemophilia, who had a history of frequent injections of coagulation factor VIII, which was considered to be the route of infection. Patient 2 was a 27-year-old male, asymptomatic HBV carrier, with an unknown infectious route, and patient 3 was an 8-year-old male, with chronic hepatitis, and an unknown infectious route. None of the 3 patients had ever lived in the Ehime area, and were not related. Serum samples were stored at –80° prior to genotyping and sequencing.

The purpose and the details of the study were explained, and written informed consent was obtained from the subjects or their parents.

HBV Genotyping

The HBV genotype was determined using the genotype-specific epitopes of the preS2 region of HBV with a commercially available enzyme immunoassay kit (HBV Genotype EIA Kit, Institute of Immunology, Tokyo, Japan) according to the manufacturer's instructions, as reported previously [24].

Complete Genome Sequencing

The complete genome sequences were determined by direct sequencing of the PCR- products, as explained in detail in our previous report [25]. Briefly, DNA was extracted from the serum sample, then to obtain the full-length HBV-DNA sequence, 2 amplicons were obtained by PCR, with 1 fragment shown to be 2,936 bases in length (nt 1994 to nt 1747), and the other 1,080 bases in length (nt 1399 to nt 2478). Sequencing was performed by direct sequencing using a commercially available kit (BigDye Terminator Cycle Sequencing FS Ready Reaction Kit, Applied Biosystems, Alameda, Calif., USA) with suitable sequencing primers. The accuracy of the sequences was ensured by identification of the sequence data of the complete genome obtained by the sense and anti-sense sequencing primers.

Phylogenetic Tree

The 3 complete HBV genomes in the present study were compared with those of HBV/D isolates in Ehime as well as other isolates available in databases. The nucleotide sequences were multiple-aligned using software (GENETYX for Windows version 7.0, Genetyx, Tokyo, Japan). Genetic distances were calculated using the Kimura two-parameter method and phylogenetic tree was constructed by the neighbor-joining method [26]. Phylogenetic and molecular evolutionary analyses were performed using MEGA version 2.1 [27].

Results

Complete Genome

Complete genome sequencing revealed that all 3 isolates were 3,182 bases in length, and the deduced HBs antigen subtype was ayw3 in each (table 1). The homol-

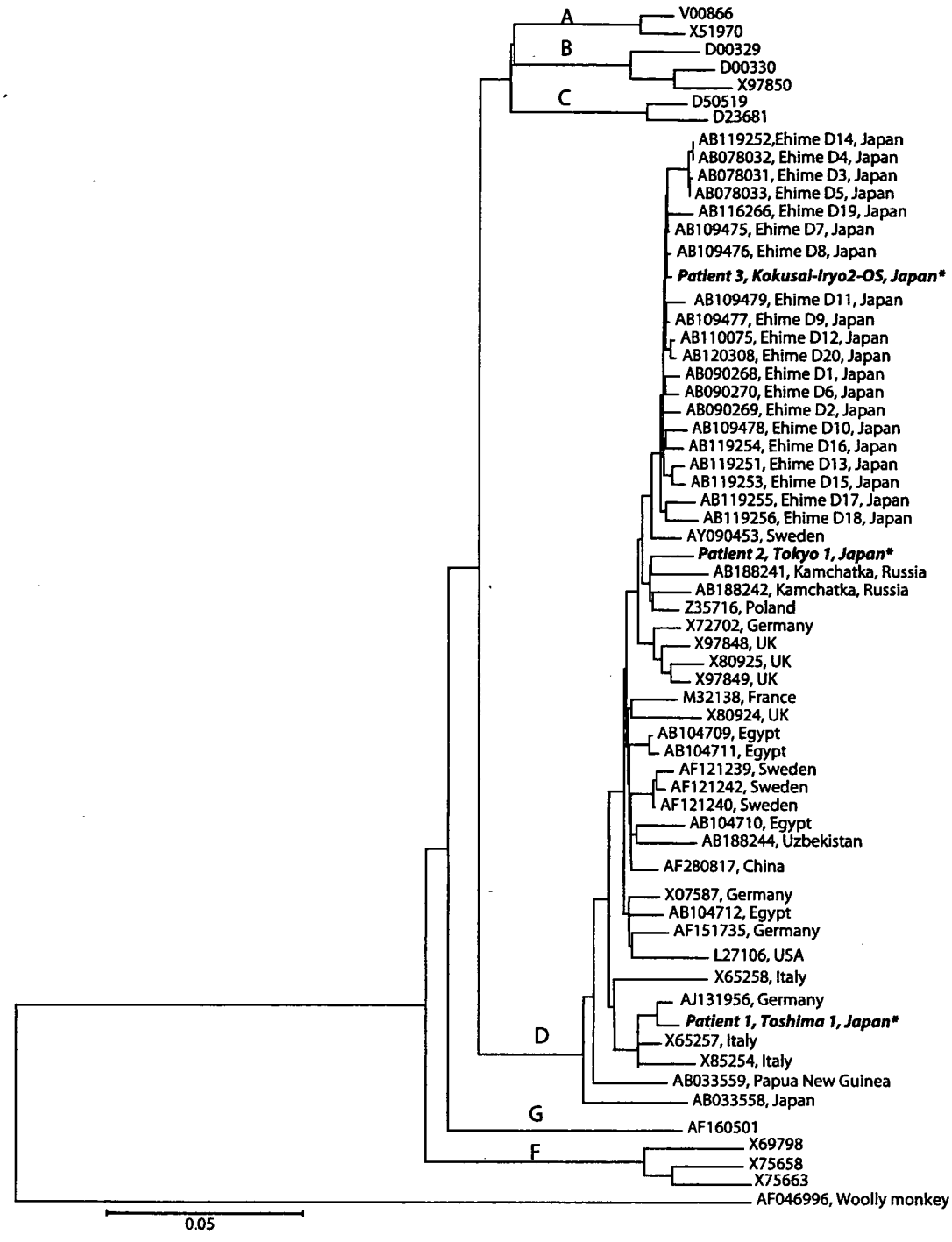

Fig. 1. Phylogenetic tree of HBV isolates. The accession numbers and information regarding the country or area where the isolate was obtained are included. Those from Japanese subjects are shown in italic bold letters and marked with an asterisk.

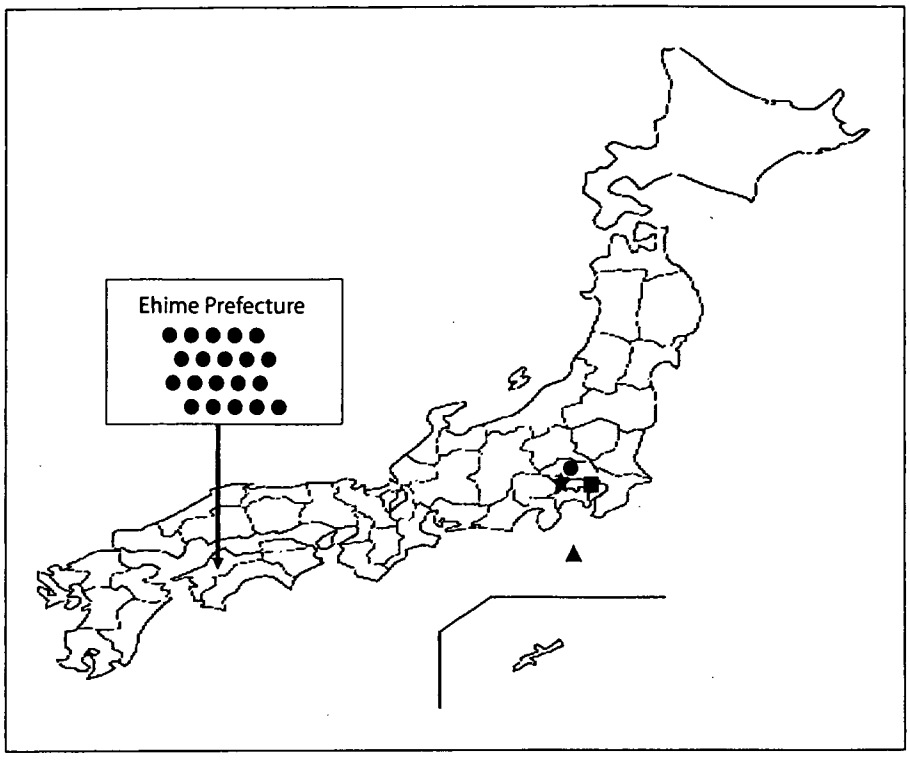

Fig. 2. HBV/D isolates in Japan and residence of subjects who harbored the isolates. ● = Isolates from Ehime [23] and the isolate from patient 3 in the present study that formed the cluster in the phylogenetic tree shown in figure 1. ■ = The isolate from patient 2 that has a close relation with several isolates from Kamchatka in Russia. ★ = The isolate from patient 1 that is distinct from the isolates from Ehime. ▲ = The isolate reported by Okamoto et al. [4] that is distinct from isolates from the Ehime, though residence information for that person is not available.

ogy of the nucleotide sequences (3,182 bases) among the 3 isolates was 96.39% between AB210819 (patient 1) and AB210820 (patient 2), 96.64% between AB210819 (patient 1) and AB210822 (patient 3), and 97.77% between AB210820 (patient 2) and AB210822 (patient 3).

Phylogenetic Analysis

Figure 1 shows a phylogenetic tree constructed from the complete nucleotide sequence of HBV. In addition to the 3 isolates in the present study, the complete genome sequences of 21 isolates of HBV/D isolated from Japanese subjects have been reported (fig. 1), which include 20 isolates from the Ehime area [23], and 1 (AB033558) from a Japanese blood donor reported by Okamoto et al. [4]. The isolate from the present patient 3 (AB210822) was very closely related to the isolates from the Ehime area, while that from patient 2 (AB210820) was closely related to several isolates from Kamchatka in Russia and relatively close to those from Ehime in the tree. In contrast, the isolate from patient 1 (AB210819), who had hemophilia, was clearly distinct from the isolates described above. Based on the present phylogenetic tree, we suspect that 1 of the isolates (AB210822) have the same origin as those in Ehime Prefecture, while the isolate from patient 1 was clearly distinct from the other 2 isolates and the isolates from Ehime area. The isolate reported by Okamoto et al. [4] was also shown to be distinct from the 3 Tokyo area isolates and all of those from Ehime. Therefore, we concluded that HBV/D in Japan is heterogeneous (fig. 2).

The nucleotide homologies between the 3 HBV/D isolates in the present study and the 20 from Ehime shown in figure 2 were calculated. The nucleotide homology between AB210819 (patient 1) and the 20 isolates from Ehime ranged from 95.91 to 96.64%, while that between AB210820 from patient 2 and the 20 isolates ranged from 97.01 to 97.86%, and that between AB210822 from patient 3 and the 20 isolates ranged from 98.68 to 99.81%.

Discussion

In the present study, we found that 1 HBV/D isolates from a patient living in Tokyo area was closely related to the isolates from Ehime, while 1 was close to several isolates from Kamchatka in Russia, and 1 was distinct from all of the isolates from Ehime. In our previous study, we speculated that HBV/D infection in the Ehime area had originated from Russia, and that Japanese-Russo war may relate with the import of HBV/D as approximately 6,000 Russian military personnel were interned in a pris-

on camp in Ehime during the Japanese-Russo war [23]. In addition, during and after that war, approximately 70,000 Russian individuals were interned in several prison camps throughout Japan, including the Tokyo area. Therefore, the 2 isolates from patients 2 and 3 are also suspected to have originated from Russia. From our results, we speculated that HBV/D strains originating from Russia may be the dominant HBV/D strains in Japan.

One of the present isolate from a patient in the Tokyo area (AB210819) was clearly distinct from the other 2 isolates from Tokyo and the 20 isolates from Ehime. That patient has a history of repeated injections with a coagulation factor produced in the 1970s and 1980s, during which time the screening and elimination of contaminated viruses in blood products was not sufficient. The ingredients used in coagulation factor products during those years were imported into Japan mainly from the United States, therefore, the origin of the HBV/D isolate in that patient may be related to imported coagulation factor product ingredients. Thus, imported coagulation factor products is suspected to be another route of HBV/D infection in Japan.

A few studies have reported subgrouping of HBV genotypes [28–30]. Norder et al. [11] classified HBV/D into 4 subgenotypes (D1-D4). According to that classification, the 20 isolates from Ehime area [23] and the isolate from the present patient 3 (AB210822) are D2, whereas the isolate from patient 1 (AB210819) and the isolate reported by Okamoto et al (AB033558) [4] are distinct from D2. These findings support the notion that HBV/D in Japan is heterogeneous.

HBV/B and HBV/C are indigenous to Japan, though it has been reported that HBV/A is increasing not only in Japanese patients with acute hepatitis, but also in chronic carriers [31–33], while other genotypes, such as HBV/E, F, G, H, have been identified from Japanese patients [34–37]. These HBV genotypes, as well as HBV/D, are suspected to have been transmitted horizontally, as they are not indigenous to Japan. A nationwide prophylactic policy for preventing mother to child transmission of HBV has been introduced in Japan, however, horizontal transmission, especially sexual transmission of HBV, remains uncontrolled [38], because a universal vaccination of HBV has not introduced. To prevent the transmission of HBV, especially HBV with non-indigenous genotypes, additional efforts are needed to prevent horizontal transmission routes.

In conclusion, our results show that HBV/D in Japan is heterogeneous. Further, we speculated that the various HBV/D strains in Japan originated from different areas of the world.

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INTERNAL MEDICINE

□ CASE REPORT □

Development of Hepatocellular Carcinoma (HCC) in a Patient 17 Years after Recovery from Chronic Hepatitis B and Seroconversion to Anti-HBs

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Abstract

A 63-year-old man was admitted to hospital in 2003 for treatment of a hepatocellular carcinoma (HCC). He was negative for HBs antigen (HBsAg) and anti-HCV antibody, and positive for anti-HBs. He had a past history of chronic hepatitis B. In 1986, HBsAg had become negative with the development of anti-HBs. In 2003, an HCC was detected and liver resection was carried out. Histological examination revealed moderately differentiated HCC and slightly fibrotic liver. It is suggested that a diagnosis of HCC, combined with negativity for HBsAg and anti-HCV antibody, may include cases of past recovery from chronic hepatitis B, such as this case.

Key words: hepatocellular carcinoma, hepatitis B virus, anti-HBs, liver cirrhosis, long-term follow up

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Introduction

The causes of HCC include hepatitis B virus (HBV) infection, hepatitis C virus (HCV) infection, Budd-Chiari syndrome, and congenital genetic abnormalities, such as hemochromatosis and Wilson's disease. Alcohol intake also is considered to be a risk factor (1). In Japan, approximately 35,000 people die from HCC annually. Among patients with HCC, those who are HBsAg or HCV antibody-positive comprise the majority, accounting for approximately 90% of the total. On the other hand, approximately 5% are patients with HCC of unknown cause, where HBsAg and HCV antibody are both negative and other possible causes are not clear. It is known that among such cases of unknown cause, there are many patients with low titers of HBc antibodies. However, it is not known whether this antibody indicates a past transient HBV infection or recovery from chronic hepatitis B. It is also unclear whether HBV participates in the oncogenesis. We have encountered a case of HCC, positive for anti-HBs, for which the cause initially was thought to be unknown. However, prior treatment for chronic hepatitis B was identified through interview. Therefore, the case of HCC had developed after seroconversion to anti-HBs.

Case Report

A 63-year-old man was admitted in December 2003 for further examination of a space-occupying lesion (SOL) in the liver which had been detected by routine abdominal ultrasonography (US). On admission, the serum albumin was 4.2 g/dl, total bilirubin was 0.7 mg/dl, the disappearing rate of indocyanine green serum was 0.160, and the prothrombin time was 105.8%. The protein induced by vitamin K absence or antagonist II (PIVKA-II) was elevated (112 mAU/ml). HBsAg and HBV DNA (Amplicor) were negative and

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Table 1. Laboratory Data on Admission

⟨Blood chemistry⟩		⟨Tumor markers⟩	
Total protein (g/dL)	7.8	alpha-fetoprotein (ng/ml)	18
Albumin (g/dL)	4.2	PIVKA-II	112
Total bilirubin (mg/dL)	0.7	⟨Serology⟩	
Direct bilirubin (mg/dL)	0.1	HBs antigen	(+)
Aspartate aminotransferase (IU/L)	28	anti-HBs antibody (mIU/mL)	34
Alanine aminotransferase (IU/L)	25	anti-HBc antibody (S/CO)	95
Alkaline phosphatase (IU/L)	248	anti-HBc×200 (S/CO)	0
γ-Glutamyl transpeptidase (IU/L)	30	HBe antigen (COI)	<0.3
Fasting plasma glucose (mg/dl)	87	anti-HBe antibody (S/CO)	±35
Hemoglobin A1c (%)	5.4	HBV-DNA (LGE/mL)	2.6<
ICG K	0.160	anti-HCV antibody (2nd)	(-)
⟨Coagulation⟩		anti-HTLV-1	(-)
Prothrombin time (%)	105.8	anti-nuclear antibody	(-)
Hepaplastin time (%)	59.4	anti-mitochondrial antibody	(-)

ICGK, disappearing rate of indocyanine green. PIVKA-II, protein induced by vitamin K absence or antagonist II. HBV-DNA was detected by the PCR method.

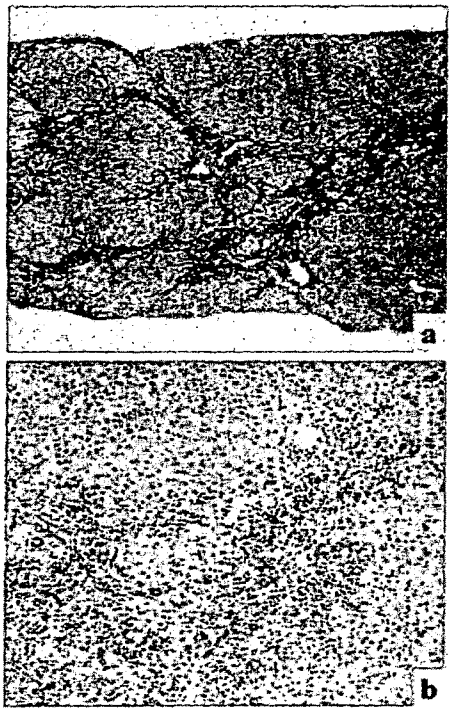

Figure 1. Histological findings of the liver. Silver staining of a liver biopsy in 1970 revealed severe portal fibrosis (a) and hematoxylin-eosin staining showed moderate infiltration of lymphocytes into the portal tract (b).

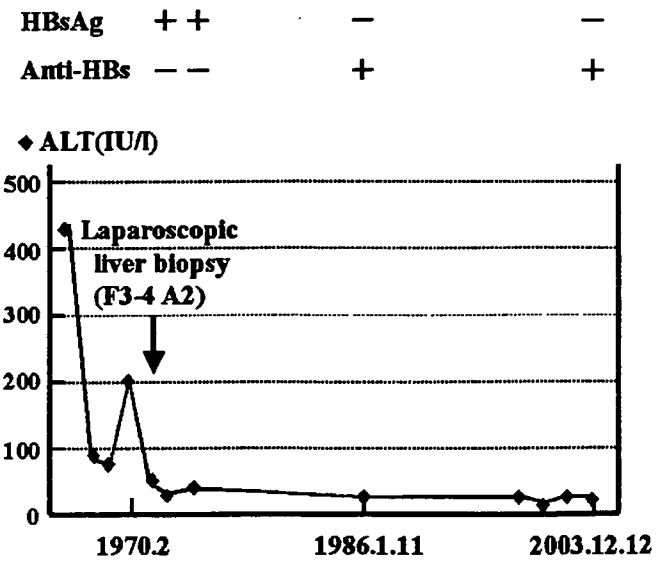

Figure 2. The clinical course of the patient.

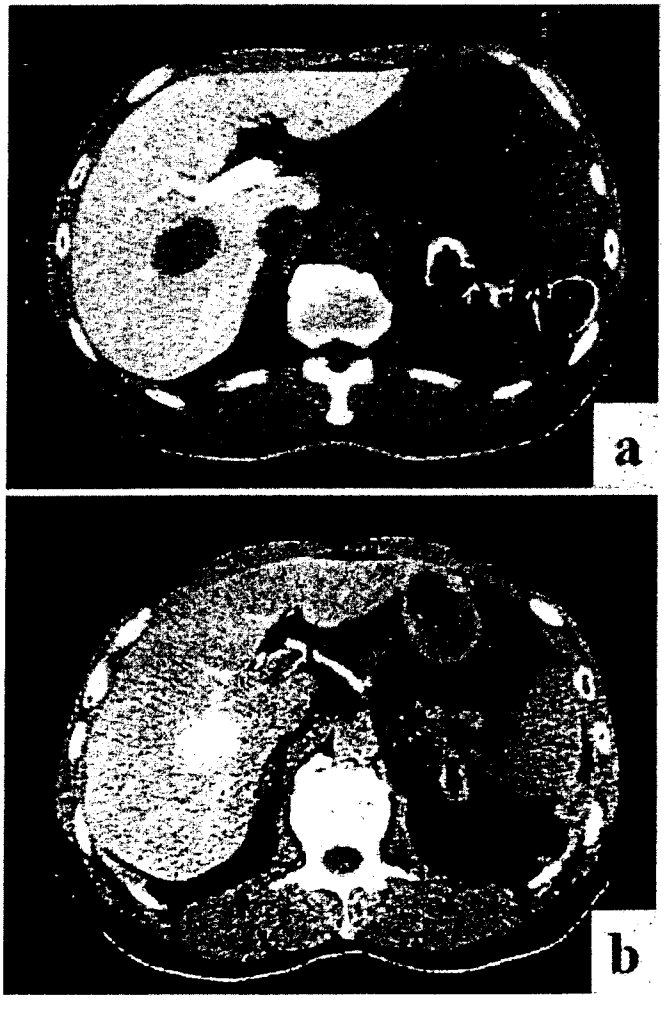

Figure 3. Abdominal CT. A perfusion defect in the S6 region of the liver is shown by the portography (a). This tumor was shown to be hypervascular by arterial angiography (b).

anti-HBs antibody was positive (Table 1). The patient had no history of excessive alcohol intake. His height was 156.5 cm, and his weight was 60.9 kg (BMI is 24.9 kg/m^2). Oral glucose tolerance test (OGTT) revealed a normal pattern. Autoimmune antibodies were negative, and anti-HTLV-1 antibody was also negative. After obtaining a detailed clinical history, he was found to have had an abnormality in a liver function test and he had been admitted to Okayama University hospital in February 1970. We obtained the following data and histopathology upon inquiring at the hospital. The alanine aminotransferase (ALT) value had been 200 IU/L, albumin was 3.3 g/dL, and total bilirubin was 0.7 mg/dL in the serum. HBsAg was positive in the serum. Liver biopsy under laparoscopy revealed severe fibrosis and moderate infiltration of lymphocytes into the portal tract (Fig. 1). He

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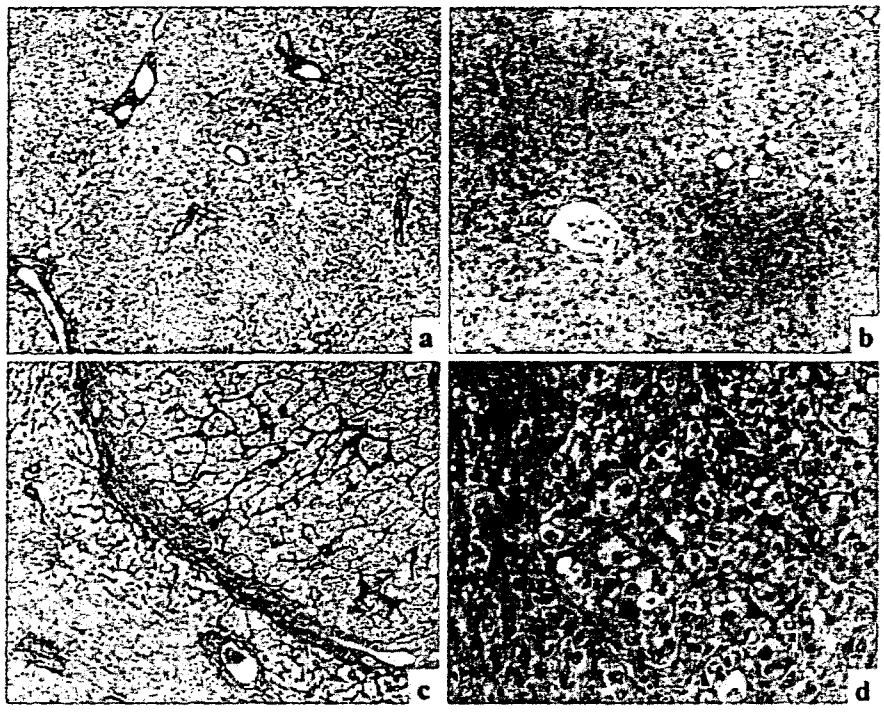

Figure 4. Microscopic appearance of the resected liver specimen. Silver staining of the resected liver specimen revealed a decrease of the portal fibrosis (a) and hematoxylin-eosin staining showed minimal infiltration of lymphocytes into the portal tract of the non-cancerous portion (b). Silver staining (c) and hematoxylin-eosin staining (d) of the tumor obtained at the liver resection revealed a moderately differentiated HCC of the thin trabecular type.

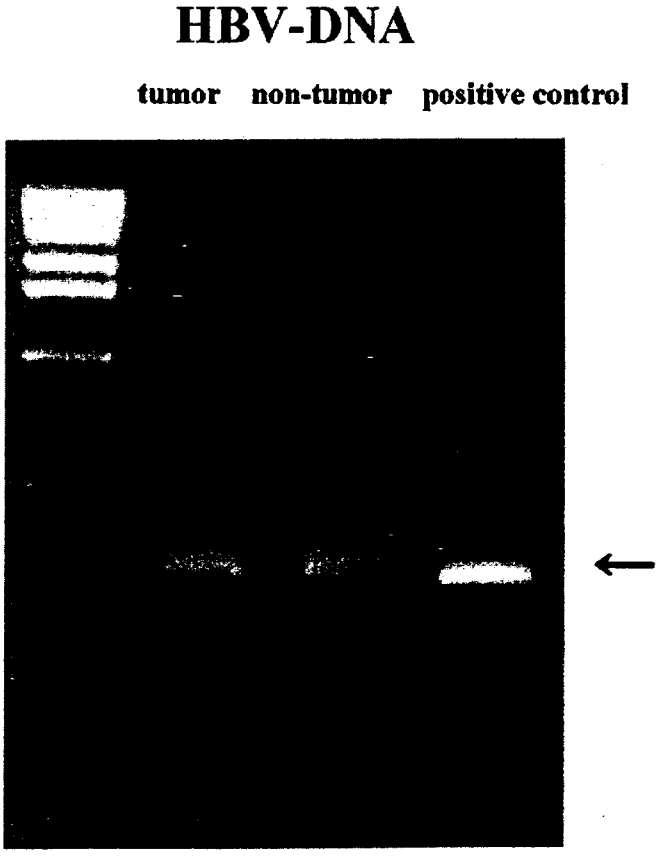

Figure 5. PCR. HBV DNA was detected in the non-cancerous and cancerous portions by PCR.

was diagnosed with chronic hepatitis B. He was treated with steroid therapy and discharged after normalization of the liver function tests. The level of transaminase in the serum had not been elevated since 1971, and anti-HBs was confirmed to be positive in 1986 (Fig. 2).

An abdominal US revealed a SOL with a diameter of 35 mm in the S6 region of the liver, showing a mosaic pattern and accompanied by a halo. Using abdominal CT scanning, hypervascular enhancement at the arterial phase and a perfusion defect at the portal phase was observed (Fig. 3). The SOL was diagnosed as HCC from these images. From these results, the diagnosis of HCC with negativity for HBsAg and anti-HCV antibody was made.

Transcathetel arterial embolization (TAE) was carried out, followed by liver resection on the 32nd hospital day. Silver staining and hematoxylin-eosin staining of the resected liver specimen revealed slight fibrosis and infiltration of lymphocytes into the portal tract of the non-cancerous portion, respectively, showing fibrotic liver. The cancerous portion was a moderately differentiated HCC (Edmondson type 2) of the thin trabecular type (Fig. 4). HBV DNA was detected in both the cancerous and non-cancerous portions by polymerase chain reaction (PCR) with the method we have already reported (2, 3) (Fig. 5), however, HBsAg and HBcAg were not detected by immunohistological staining in both portions. Recurrence of HCC was not observed after 18 months

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of follow-up with normalization of PIVKA-II in the serum.

Discussion

It is known that HBV carriers have a certain rate of clearance of HBsAg (4-8). Among these individuals are those in whom the HBc antibodies are decreased to a low titer and an open question remains as to how frequently HBsAg seroconversion occurs. Unfortunately, there have been only a few sporadic reports and no definitive data is available. The present case clearly indicates that there are cases of HBV carriers in whom HBs antibodies develop after recovery from a long period of chronic hepatitis B. Our case is also valuable in suggesting that such an individual can develop HCC.

HCC usually develops on a background of liver cirrhosis or in individuals with advanced liver fibrosis. However, in contrast to HCV, it is known that HBV-related HCC has a relatively high probability of developing in individuals with less severe fibrosis. A background of liver cirrhosis is found in at least 90% of cases attributable to hepatitis C but in only approximately 70% of those attributable to hepatitis B (9-11). Our case showed slight liver fibrosis at the time of HCC resection but even the biopsy 30 years previously had shown fibrosis at stage F3. In most cases of hepatitis B, when HBe antigen seroconversion occurs and hepatitis subsides, there is an alleviation of liver fibrosis (12).

It is not clear when the HCC developed in the present case. However, it is speculated that when the fibrosis and activity of hepatitis were severe, gene abnormalities in the liver cells accumulated, leading to a predisposition to oncogenesis. Although it was not evaluated in the present case, if clonal integration of HBV DNA is found in the tumor tissue, the possibility of HBV related HCC is high. In the future, it appears feasible to prove the involvement of HBV more directly by detection of HBV DNA integration in the tumor tissue in those cases with HCC of unknown cause (13-16).

In the cases with HCC of unknown cause, there are cases with recovery from the carrier state, such as our case, even if positive for HBs antibodies. The cases found to have chronic liver disease in the past, regardless whether HBV or HCV, should be considered a high risk group for HCC development and screened regularly for HCC (17).

In conclusion, we have encountered a case of past chronic hepatitis B in which the patient had recovered from the carrier state and acquired HBs antibodies. This was followed by the development of HCC. The diagnosis of HCC of unknown cause may include cases with a relevant past history of chronic hepatitis B.

We thank Mr. Kenji Tanimoto for immunohistological staining, and Ms. Yoshie Kondo for PCR assay for HBV-DNA.

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Original Article

Characteristics of geographic distributions and route of infection for hepatitis B virus genotype D in Ehime area in western Japan

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Aims: Hepatitis B virus genotype D (HBV/D) is rare in Japan, and has been shown to circulate in Ehime prefecture in western Japan. HBV/D is suspected to have been transferred into Ehime from Russia as a result of the Japanese–Russian War. This study examined the current geographic spread and infectious route for HBV/D in Ehime.

Methods: HBV genotype was determined for 508 patients with chronic HBV infection and 46 patients with acute HBV infection hepatitis (acute hepatitis, AH), all of whom were living in Ehime. Ehime was divided into three areas and genotypic distributions were studied.

Results: The ratio of genotypes A,B,C and D in chronically infected patients were 1.8%, 4.5%, 87.8% and 5.9%, respectively. Most patients chronically infected with HBV/D lived in the central area. Only two patients lived in the east and southwest areas, and both had lived in the central area in childhood. Patients with AH due to HBV/D were found only in the central area.

Conclusion: HBV/D has not yet spread widely to areas other than central Ehime, although small numbers of infected people have moved to other areas. The major infectious route for patients with AH is sexual transmission, regardless of HBV genotype.

Key words: acute hepatitis, genotype D, hepatitis B virus, infectious route

INTRODUCTION

HEPATITIS B VIRUS (HBV) is one of the major causes of liver disease throughout the world and more than 350 million people are persistently infected. The HBV genome is a double-stranded circular DNA consisting of approximately 3200 nucleotides. Based on nucleotide diversity, HBV has been classified into eight genotypes, designated A–H.[1–3] HBV genotypes reportedly display definite geographic distributions. Genotype A (HBV/A) is common in Europe and North America, whereas genotypes B (HBV/B) and C (HBV/C) are predominant in east Asia.[4–7] Genotype D (HBV/D) is widespread in Europe, the Mediterranean, and western and central Asia.

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Clinical differences among genotypes have been studied by many investigators. The prognosis for chronically infected patients is reportedly worse for HBV/C than for HBV/B, whereas the severity of acute infection is reportedly higher in patients infected with HBV/B compared with HBV/C.[8–10] Comparisons of HBV/A and HBV/D have revealed that the efficacy of antiviral therapy is worse in HBV/D compared with HBV/A, whereas progression from acute hepatitis (AH) to chronic hepatitis is suspected to be more frequent with HBV/A than with HBV/D.[11] Clinical differences have also been reported between infection with HBV/C and HBV/D.[12] Routes of infection also differ among HBV genotypes. Vertical transmission from mother to child is dominant in HBV/C and HBV/B, whereas horizontal transmission is dominant in HBV/A.[5] In this context, examining HBV genotypes in populations from specific countries or areas is important, in addition to examinations of individual patients.

In Japan, HBV/C is predominant, followed by HBV/B, whereas HBV/D is very rare.[6] However, HBV/D has been

found to circulate in the Ehime area of western Japan.[12] Analysis of HBV sequences isolated from the Ehime area revealed that HBV/D in Ehime is closely related to strains from Russia and Europe.[13] Molecular evolutionary analyses suggest that HBV/D was transferred to the Ehime area in approximately 1900. HBV/D in Ehime was suspected to have been transferred from Russia to Ehime as a result of the Japanese–Russian War from 1904 to 1905, as Matsuyama city in central Ehime contained large prisoner-of-war camps and hospitals where approximately 6000 prisoners of war were transferred during and after the war.

In the area of Matsuyama city, endemic Gianotti–Crosti syndrome[14] caused by HBV subtype *ayw* reportedly occurred in 1970s.[15] The HBV causing this syndrome was speculated to be an imported strain, as the majority of HBV subtypes in Japan are *adr* and *adw*, corresponding to HBV/C and HBV/B, respectively.[15–17] We have found that HBV isolates with subtype *ayw* obtained from a patient with Gianotti–Crosti syndrome in this area represented HBV/D on complete genome sequencing.[18] We thus speculated that the spread of HBV/D is closely related to the endemic Gianotti–Crosti syndrome in the 1970s. However, there is a paucity of available information regarding the geographic spread of HBV/D in this area. The present study, therefore, tried to clarify the prevalence of HBV genotypes in chronically and acutely infected patients in areas other than central Ehime, to determine whether HBV/D has spread beyond the area of Matsuyama city and how HBV/D is currently transmitted.

METHODS

Patients

THE 554 PATIENTS infected with HBV enrolled in this study comprised 508 patients with chronic HBV infection who visited our hospitals and affiliate hospitals from 1999 to 2005, and 46 patients with acute HBV infection (acute hepatitis B, AH-B) from 1980 to 2005. Ehime prefecture (approximately 5700 km^2, with a population of 1.5 million) was divided into three areas: the east area; central area; and south-west area. Of the 554 subjects, 127 lived in east Ehime, 284 lived in central Ehime and 143 lived in south-west Ehime.

The 508 cases of chronic HBV infection comprised 18 hepatitis B e antigen (HBeAg)-positive asymptomatic carriers, 222 cases of inactive hepatitis B surface antigen (HBsAg) carrier state and 266 cases of chronic liver disease. Criteria for AH-B comprised positive results for antihepatitis B core antibody (anti-HBc) with low titer (<90% in 200-fold diluted serum) and positive results for IgM-class anti-HBc. IgM-class antihepatitis A virus antibody (IgM-class anti-HA), antihepatitis C virus antibody (anti-HCV) and HCV-RNA were negative in all patients with acute hepatitis B in this study. A past history of Gianotti–Crosti syndrome was present in two patients displaying inactive HBsAg carrier state, and one patient with this syndrome displayed AH. Sera were stored at −80°C until tested. The purpose and details of the study were explained to all patients in this study, and written informed consent was obtained prior to enrollment.

Serological test

Hepatitis B surface antigen (HBsAg) was assayed by chemiluminescent immunoassay (CLIA) (Abbott Japan, Tokyo, Japan). Levels of antibody to hepatitis B core antigen (anti-HBc) and IgM-anti-HA were assayed using enzyme immunoassay (EIA), while anti-HCV levels were measured using CLIA (Abbott Japan, Tokyo, Japan), and HCV-RNA levels were determined by polymerase chain reaction (PCR) methods (Roche Japan, Tokyo, Japan)

Genotyping

HBV genotype was determined by EIA using monoclonal antibodies that detect genotype-specific epitopes on preS2 antigen (Institute of Immunology, Tokyo, Japan), according to the manufacturer's instructions.[19] When the genotype was not determined by this method, sera were assayed using a PCR-restriction fragment length polymorphism method.[20]

Sequencing

DNA was extracted from 200 μL of one serum sample using a QIAmp DNA Mini Kit (Qiagen, Hilden, Germany). Nested PCR was performed to amplify two fragments (A and B). Fragment A was amplified using primer SP3 (sense, 5′-CTCTCTTTTTTGCCTTCTGAC-3′) and primer AP5 (antisense, 5′-CCTCCTAGTACAAA GACCTT-3′) for first PCR, and primer SP4 (sense, 5′-GACACCGCCTCTGCTCTGTAT-3′) and primer AP1 (antisense, 5′-TCCCCCAACTCCTCCCAGTCCTT-3′) for second PCR. Fragment B was amplified using primer B1 (sense, 5′-CTCTCTCGGAAATACACCTC-3′) and primer P6 (antisense, 5′-TTCCCACCTTATGAGTCCAA-3′) for first PCR and primer B3 (sense, 5′-TGGATC CTTCGCGGGACGTCCTT-3′) and P6 for second PCR. Details of the sequencing method have been described previously.[21] A phylogenetic tree was then constructed by the neighbor joining method using MEGA3.1

Table 1 Ratio of HBV genotypes and clinical data of chronically infected patients

	Genotype			
	A	B	C	D
n	9 (1.8%)	23 (4.5%)	446 (87.8%)	30 (5.9%)
Age (years)	46.0 ± 12.3	55.3 ± 16.0	47.6 ± 14.6	34.0 ± 18.4
Sex (male/female)	4/5	13/11	249/197	17/13
ALT (IU/L)	30.7 ± 21.6	70.6 ± 84.7	64.7 ± 129.2	30.9 ± 24.4
HBeAg	1 (11.1%)	2 (8.6%)	135 (30.3%)	6 (20%)
Inactive HBsAg carrier state	5 (55.6%)	10 (43.4%)	183 (41.0%)	24 (80.0%)

ALT, alanine aminotransferase; HBsAg, hepatitis B surface antigen; HBeAg, hepatitis B e antigen.

software (Center for Evolutionary Functional Genomics, AZ, USA).

Statistical analyses

The data were analyzed using the χ^2-test or Fisher's exact test for categorical data. The Mann–Whitney *U*-test was used as required. Values of $P < 0.05$ were regarded as statistically significant.

RESULTS

Genotypes in chronic HBV infection

FREQUENCIES OF GENOTYPES A, B, C and D were 1.8%, 4.5%, 87.8% and 5.9%, respectively. Clinical features are shown in Table 1. The mean age of patients infected with HBV/D was 34.0 years (range, 16–66 years), more than 10 years younger than the mean age for the other genotypes. Inactive HBsAg carrier state was present in 80% of patients infected with HBV/D, the highest rate among the four genotypes.

Genotype distributions among the three areas in Ehime prefecture are shown in Figure 1. The majority of patients infected with HBV/D lived in central Ehime. The rate of HBV/D in the central area (10.9%) was similar to a previous study in which most sera were collected from the central area.[12] However, one patient lived in the east area and one patient lived in the south-west area. In both cases, they had been born and had lived in central Ehime prefecture in childhood, before moving to the other area after growing up. No differences in geographic distribution were found in the other genotypes.

Although sequences of HBV/D from the patient in the south-west area could not be determined because of low HBV-DNA levels, a complete genome sequence was

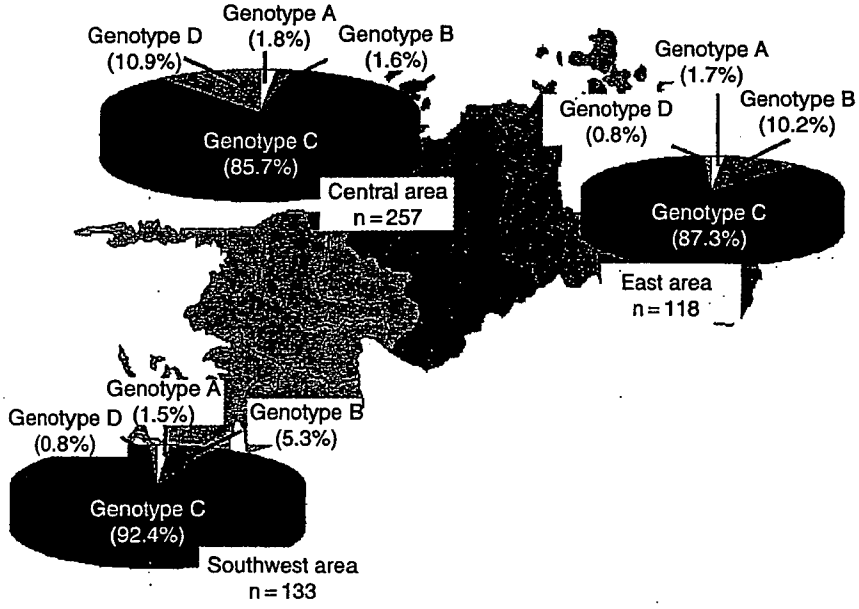

Figure 1 Distribution of HBV/D of chronically infected patients in Ehime. In central Ehime, 28 of 258 chronically infected patients (10.9%) were infected with HBV/D. In east and south-west Ehime, only one of 118 patients (0.8%) and one of 133 patients (0.8%), respectively, were infected with HBV/D.

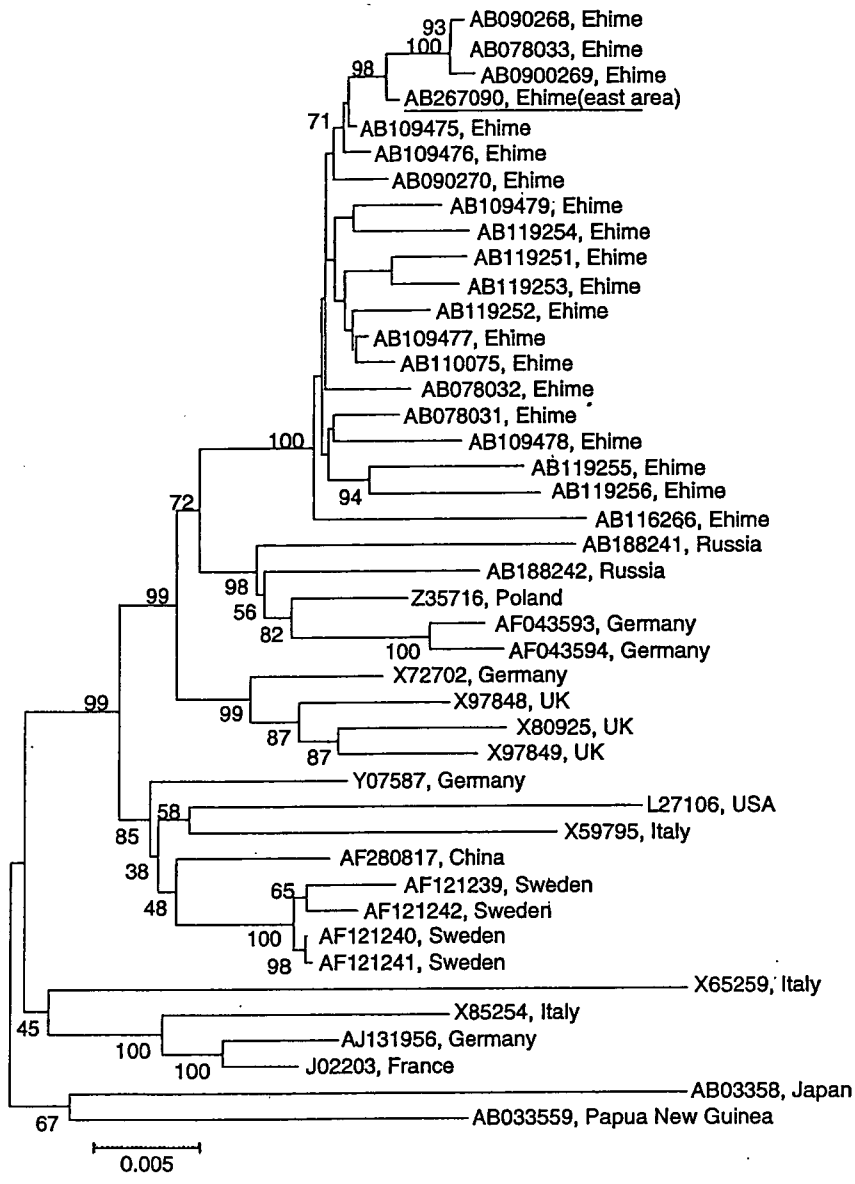

Figure 2 Phylogenetic tree of HBV/D in Ehime and other areas (neighbor-joining method). Bootstrap resampling (100 replicates) was performed. The isolate obtained from east Ehime in the present study and 19 previously reported isolates from Ehime formed a cluster, which was surrounded by a rectangle. All isolates in the rectangle were obtained from patients in Ehime.

available for the patient infected with HBV/D living in east Ehime (DDBJ/GenBank accession number, *AB267090*). Genomic length of the HBV-DNA was 3182 bp and deduced HBsAg subtype was *ayw3*, as with other previously reported HBV/D isolates from Ehime.[12] Nucleotide homology between isolates in the present study and 20 isolates from Ehime reported previously was 98.9 ± 0.3%. All of those 20 isolates were obtained from patients living in the central area of Ehime prefecture. Phylogenetic tree analyses revealed that the isolate from the east area in the present study was closely related to the 19 isolates from the central area, forming a definite cluster (Fig. 2).

Prevalence and genotypes of acute HBV infection

Among the 46 patients with AH-B, rates of genotypes A, B, C and D were 8.7%, 6.5%, 63.0% and 21.7%, respectively (Table 2). Rates of infection with HBV/A and HBV/D were higher in AH-B than in chronically infected patients (1.8% and 5.9%, $P = 0.02$ and $P = 0.002$, respectively). Progression to chronic hepatitis occurred in one of 29 patients with HBV/C and one of 10 patients with HBV/D.

Frequencies of genotypes in each area are also shown in Table 2. All 10 patients with AH-B due to HBV/D lived

Table 2 Clinical data and genotype distributions in three parts of Ehime in patients with acute HBV

	Genotype			
	A	B	C	D
n	4 (8.7%)	3 (6.5%)	29 (63.0%)	10 (21.7%)
Age (years)	28.0 ± 2.6	32.0 ± 20.0	33.5 ± 16.7	32.2 ± 9.3
Sex (male/female)	2/2	2/1	18/11	4/6
T. Bil (mg/dL)	17.4 ± 14.6	9.9 ± 4.6	11.1 ± 10.7	4.3 ± 2.8
ALT (IU/L)	1425.3 ± 630.1	1810.4 ± 683.2	2892.7 ± 2622.3	2236.0 ± 2202.1
PT (%)	81.0 ± 29.4	69.0 ± 32.7	52.9 ± 37.0	93.3 ± 35.3
Fulminant hepatitis	0 (0%)	0 (0%)	4 (13.7%)	1 (10.0%)
Progression to chronic infection	0 (0%)	0 (0%)	1 (3.4%)	1 (10.0%)
Distribution				
East	1 (11.1%)	1 (11.1%)	7 (77.8%)	0 (0%)
Central	3 (11.1%)	1 (3.7%)	13 (48.1%)	10 (37.1%)
South-west	0 (0%)	1 (10.0%)	9 (90.0%)	0 (0%)

ALT, alanine aminotransferase; PT, prothrombin time; T.Bil, total bilirubin.

in the central area, and three of four patients with AH-B due to HBV/A lived in the central area, whereas HBV/B and HBV/C were found in all areas in Ehime prefecture. In the central area, frequencies of genotypes A, B, C and D were 11.1%, 3.7%, 48.1% and 37.1%, respectively.

Chronological changes in the numbers of patients with AH-B and infectious routes in patients with AH-B in relation to HBV genotypes are shown in Figure 3. Chronological changes in genotype frequencies were not obvious. Infectious routes of HBV transmission in patients with AH before and after 1990 were studied, because the screening of donated blood by anti-HBc has begun since 1989, in addition to the screening by HBsAg.[22] Before 1990, blood transfusion and medical routes such as needle stick accidents by medical staff represented important infectious routes. Although a few patients were still transmitted by medical routes, the major route of infection after 1990 was sexual transmission (18 out of 29, heterosexual in all cases in the present study). Sexual transmission was the major route in all genotypes except HBV/B, for which the number of patients was too small to discuss. The four patients with AH-B sexually transmitted with HBV/D comprised three women and one man. All three women were infected from their boyfriends (sexual partners), and the one man was suspected to be infected from a commercial sex worker in central Ehime.

DISCUSSION

IN EAST ASIAN countries, HBV/B and HBV/C represent the predominant HBV genotypes, and the frequencies of HBV genotypes A, B, C and D in Japan have been reported as 1.7%, 12.2%, 84.7% and 0.4%, respectively, in a multicenter cross-sectional study.[6] HBV genotypes other than HBV/B and HBV/C are thus suspected to represent foreign genotypes that were transferred from other countries. We have supposed that HBV/D in the Ehime area was transferred from Russia in the early 20th century and rooted to this area, and the high rate of HBV/D in this area appears to have been caused by a rapid spread of HBV/D in the 1970s.[13]

The present study examined the geographic spread of HBV/D infection. The majority of patients infected with HBV/D were living in central Ehime, in the vicinity of Matsuyama city and the presumed site of initial HBV/D transfer. Only a small number of individuals infected with HBV/D lived in the east or south-west areas, and these were proven to have lived in the central area in childhood and subsequently moved. These patients thus seem likely to have become infected while residing in the central area. Frequencies of HBV/D among HBV carriers in the present study (5.9%) were lower compared with our previous study (9.2%),[12] because the majority of serum samples in the previous study were obtained from hospitals in central Ehime. In the present study, serum samples were collected from HBV carriers in central, east and south-west areas of the prefecture, and the frequency of genotype D in the central area was 10.3%, similar to the previous study. The sequencing result from HBV/D in the patient from east Ehime clarified that HBV in this case was very similar to the 20 isolates from central Ehime. Norder *et al.* subdivided HBV/D into four subtypes, D1–D4.[17] The previously reported isolates from Ehime were all classified as D2.[13]