

- [16] McHutchison J, Blatt L, Sedghi-Vaziri A, Russell J, Schmid P, Conrad A. Is there an optimal time to measure quantitative HCV RNA to predict non-response following interferon treatment for chronic HCV infection? *J Hepatol* 1998;29:362–8.
- [17] McHutchison JG, Shad JA, Gordon SC, et al. Predicting response to initial therapy with interferon plus ribavirin in chronic hepatitis C using serum HCV RNA results during therapy. *J Viral Hepat* 2001;8:414–20.
- [18] Ferenci P, Schiffman M, Freid MW, et al. Early prediction of response to 40-kDa peg-interferon alfa-2a (Pegasys) plus ribavirin (RBV) in patients with chronic hepatitis C (CHC). *Hepatology* 2001;34:351 [abstract 716].
- [19] Okanoue T, Itoh Y, Minami M, et al. Interferon therapy lowers the rate of progression to hepatocellular carcinoma in chronic hepatitis C but not significantly in an advanced stage: a retrospective study in 1148 patients. *J Hepatol* 1999;30:653–9.
- [20] Kasahara A, Hayashi N, Mochizuki K, et al. Risk factors for hepatocellular carcinoma and its incidence after interferon treatment in patients with chronic hepatitis C. *Hepatology* 1998;27:1394–402.
- [21] Davis GL, Esteban-Mur R, Rustgi V, et al. Interferon alfa-2b alone or in combination with ribavirin for the treatment of relapse of chronic hepatitis C. *N Engl J Med* 1998;339:1493–9.
- [22] Poynard T, Marcellin P, Lee SS, et al. Randomized trial of interferon α 2b plus ribavirin for 48 weeks or for 24 weeks versus interferon α 2b plus placebo for 48 weeks for treatment of chronic infection with hepatitis C virus. *Lancet* 1998;352:1426–32.
- [23] McHutchison JG, Gordon SC, Schiff ER, et al. Interferon alfa-2b alone or in combination with ribavirin as initial treatment for chronic hepatitis C. *N Engl J Med* 1998;339:1485–92.
- [24] Poynard T, McHutchison J, Goodman Z, Ling MH, Albrecht J. Is an “a la carte” combination interferon alfa-2b plus ribavirin regimen possible for the first line treatment in patients with chronic hepatitis C? *Hepatology* 2000;31:211–8.
- [25] Iino S, Matsushima T, Kumada H, et al. Comparison of ribavirin (SCH18098) and interferon α -2b combination therapy and interferon α -2b monotherapy in chronic hepatitis C patients of genotype 1b and high viral load – a double-blind parallel study to determine dosage and administration. *J Clin Ther Med* 2002;4:565–91 [in Japanese].
- [26] Toyota J, Sainokami S, Yasuda K, et al. Comparison of interferon α -2b and ribavirin (SCH18908) combination therapy and interferon α -2b monotherapy in chronic hepatitis C patients who have not responded or relapsed to previous interferon therapy – a double-blind comparative study to examine concomitant efficacy. *J Clin Ther Med* 2002;4:539–63 [in Japanese].
- [27] Wong JB, Davis GL, McHutchison JG, et al. Clinical implication of testing viral response during ribavirin and peginterferon alfa-2b treatment for chronic hepatitis C. *Hepatology* 2002;36:281A [abstract 472].
- [28] Karino Y, Toyota J, Arakawa T, et al. Advanced age and serum ribavirin level affect the degree of hemolytic anemia during the combination therapy of IFN- α 2b and ribavirin for chronic hepatitis C. *Hepatology* 2003;38(1):743A [abstract 1211].
- [29] Kasahara A, Hayashi N, Hiramatsu N, et al. Ability of prolonged interferon treatment to suppress relapse after cessation of therapy in patients with chronic hepatitis C: a multicenter randomized controlled trial. *Hepatology* 1995;21:291–7.
- [30] Arase Y, Ikeda K, Tsubota A, et al. Randomized trial of prolonged interferon retreatment for chronic hepatitis C patients with HCV-genotype 1b and high virus load. *Hepatol Res* 2003;25:364–70.
- [31] Pawlotsky JM, Dahari H, Neuman AU, et al. Antiviral action of ribavirin in chronic hepatitis C. *Gastroenterology* 2004;126:703–14.
- [32] Brouwer JT, Nevens F, Bekkering FC, et al. Reduction of relapse rates by 18-month treatment in chronic hepatitis C A Benelux randomized trial in 300 patients. *J Hepatol* 2004;40:689–95.

Comparison of Genotypes C and D of the Hepatitis B Virus in Japan: A Clinical and Molecular Biological Study

Tran Nhu Duong,¹ Norio Horiike,¹ Kojiro Michitaka,² Chen Yan,¹ Masashi Mizokami,³ Yasuhito Tanaka,³ Kouji Jyoko,⁴ Kazutoshi Yamamoto,⁵ Hiroaki Miyaoka,⁶ Yoshimasa Yamashita,⁷ Naofumi Ohno,⁸ and Morikazu Onji^{1*}

¹Third Department of Internal Medicine, Ehime University School of Medicine, Ehime, Japan

²Endoscopy Center, Ehime University School of Medicine, Ehime, Japan

³Department of Clinical Molecular Informative Medicine, Nagoya City University Graduate School of Medical Sciences, Nagoya, Japan

⁴Internal Medicine, Matsuyama Red Cross Hospital, Ehime, Japan

⁵Internal Medicine, Matsuyama Shimin Hospital, Ehime, Japan

⁶Internal Medicine, Matsuyama Saiseikai Hospital, Ehime, Japan

⁷Internal Medicine, Uwajima City Hospital, Ehime, Japan

⁸Internal Medicine, Ohno Naika Hospital, Ehime, Japan

The genotype-related differences between genotype C and genotype D of the hepatitis B virus (HBV) remain unknown. The relationship was studied between the HBV genotypes and their clinical features, paying special attention to genotypes C and D. Serum samples from 413 HBV carriers were genotyped using an enzyme immunoassay (EIA) and by restriction fragment length polymorphism. The nucleotide sequences at the basic core promoter (BCP) and precore (PreC) regions were analysed by direct sequencing. The full genome sequences of three HBV genotype D cases were also examined. Almost all carriers with HBV genotype D were asymptomatic carriers (84.2%). Genotype D was not found in patients with liver cirrhosis and hepatocellular carcinoma. In contrast, carriers with genotype C had mainly chronic liver disease (63.2%; $P < 0.001$). The ratio of hepatitis B e antigen (HBeAg)/anti-HBe was significantly higher in genotype C than in genotype D in the young age-matched group ($P < 0.01$). The mutation at BCP (T1762, A1764) was significantly lower in genotype D than in genotype C among HBeAg-negative patients ($P < 0.05$). The HBV full-genome sequences are very similar to certain HBV genotype D sequences from Europe. In conclusion, genotype C was associated with chronic liver disease, whereas genotype D was related to asymptomatic carriers with earlier HBeAg seroconversion. Thus, the outcome of chronic HBV infection may be different in persons infected with HBV genotypes C and D. **J. Med. Virol.** 72:551–557, 2004. © 2004 Wiley-Liss, Inc.

KEY WORDS: hepatitis B virus; HBV genotype; basic core promoter mutation; precore mutation; HBV Japan

INTRODUCTION

Infection by the hepatitis B virus (HBV) may be mild and transient (acute), chronic and progressive (chronic) or even fatal (fulminant). The course of chronic HBV infection is very diversified. Some partials infected chronically with HBV exhibit repeated exacerbation and remission of chronic hepatitis and a considerable number of them ultimately develop more severe complications such as liver cirrhosis and hepatocellular carcinoma. On the contrary, many people chronically infected with HBV do not display any features of liver disease throughout their life-time [Chen, 1993; Lee, 1997]. Extensive studies on viral and host factors could not clarify the underlying mechanism of the diverse clinical course of chronic HBV infection.

Only recently, it has become evident that the serological heterogeneity of the hepatitis B surface antigen (HBsAg) may play a role in this regard. Initially,

*Correspondence to: Morikazu Onji, MD, Third Department of Internal Medicine, Ehime University School of Medicine, Ehime, Japan. E-mail: onjimori@m.ehime-u.ac.jp

Accepted 27 October 2003

DOI 10.1002/jmv.20044

Published online in Wiley InterScience (www.interscience.wiley.com)

HBsAg was classified into nine different subtypes, based on the antigenic determinants of their HBsAg [Nishioka et al., 1975; Magnus and Norder, 1995]. Alternatively, a genetic classification based on the complete nucleotide sequence (inter group divergence of 8% or greater) of HBV was established [Okamoto et al., 1988]. Accordingly, eight genotypes of HBV have been detected and designated from A to H. HBV genotypes have distinct geographical distribution. Overall, HBV genotypes B and C are mostly predominant in Asian countries, including Japan, whereas people infected with HBV genotypes A and D are abundant in European countries and in the USA [Okamoto et al., 1988; Norder et al., 1994; Lindh et al., 1997; Usuda et al., 1999; Ruiz et al., 2002].

Recent studies also support the notion that genotype heterogeneity of HBV may be associated with a diverse clinical course of chronic HBV infection. In general, in areas where HBV genotypes B and C are predominant, genotype C is associated with more severe liver disease than genotype B [Kao et al., 2000; Orito et al., 2001a]. In contrast, in areas where HBV genotypes A and D circulated commonly, an association between genotype A and the chronic outcome of HBV infection was found [Mayerat et al., 1999]. However, in the absence of genotypes C and D in the same geographical region, it has not been possible to compare the clinical implication of these HBV genotypes among the same population.

Several million people in Japan are infected chronically with HBV, and most of them harbour either genotype C or B (corresponding to serotype *adr* or *adw*, respectively). Genotype D with serotype *ayw* is extremely rare throughout the country [Usuda et al., 1999; Orito et al., 2001a]. However, HBV serotype *ayw* has also been detected in a town in the Southwest of Japan with a history of an infantile papular acrodermatitis (Gianotti's disease) outbreak during the 1970s [Ishimaru et al., 1976]. Accordingly, it is postulated that if persons with chronic HBV infection with genotype D are detected in and around this town, it may be possible to compare the clinical features of different HBV genotypes in the same population.

In this study, the distribution of HBV genotypes was studied among persons with chronic HBV infection in Ehime prefecture, situated in the Southwestern part of Japan. A considerable number of the studied subjects

were infected with genotype D in addition to the prevalence of genotype C. This permitted the comparison of the clinical characteristics of people infected with HBV genotypes C and D in the same geographical region. The full-genome sequences of genotype D were also studied to gain insight into the possible origin of this genotype.

PATIENTS AND METHODS

Patients

Serum samples were obtained consecutively from 413 Japanese chronic HBV carriers (positive for HBsAg for more than 6 months) who visited our hospital and other hospitals affiliated with Ehime University in Ehime, Japan from January 1995 to December 2002. As shown in Table I, 179 asymptomatic carriers, 138 with chronic hepatitis, 50 with liver cirrhosis and 46 with hepatocellular carcinoma were enrolled in the study. People who had consistently normal serum alanine aminotransferase (ALT) level for more than 1 year, and had never exhibited any symptom, were considered to be asymptomatic carriers. Chronic hepatitis, liver cirrhosis and hepatocellular carcinoma were diagnosed using the liver function test, serum levels of alpha-fetoprotein, abdominal ultrasonography and computed tomography. A liver biopsy was performed on some patients. Persons with evidence of the hepatitis C virus infection, the hepatitis D virus infection, a history of alcohol abuse and drug addicts were excluded from this study. Serum samples from each subject were stored at -80°C until used. Informed consent was obtained from all subjects.

Serological Markers

The presence of HBsAg was determined using a chemiluminescent immunoassay kit (Architect HBsAg, Dainabot, Tokyo, Japan). The hepatitis B e antigen (HBeAg) and antibody to HBeAg (anti-HBe) were checked by an enzyme immunoassay (EIA) kit (AxSYM HBeAg assay and AxSYM HBeAb assay, Dainabot).

Genotyping of HBV

HBV genotypes were determined using an HBV genotype EIA kit (Institute of Immunology, Tokyo,

TABLE I. Demographic, Clinical Data and Distribution of HBV Genotypes of 413 HBV Carriers With Various Liver Diseases

Diagnosis	No.	Demographic and clinical data				HBV genotypes			
		Sex (M)	Age (years)	ALT	HBeAg (+)	A	B	C	D
Asymptomatic carriers	179	109	36.0 ± 14.9	23.8 ± 10	31 (17.3)	8 (4.5)	10 (5.6)	129 (72.1)	32 (17.8)
Chronic hepatitis	138	92	40.5 ± 12.0	101.3 ± 167	57 (41.3)	3 (2.2)	3 (2.2)	126 (91.3) ^b	6 (4.3) ^a
Liver cirrhosis	50	35	51.7 ± 12.7	92.3 ± 193	20 (40.0)	0	0	50 (100) ^b	0 ^a
Hepatocellular carcinoma	46	36	55.0 ± 10.3	61.7 ± 51	17 (36.9)	0	1 (2.2)	45 (97.8) ^b	0 ^a
Total	413	272	41.6 ± 14.9	62.2 ± 124	125 (30.2)	11 (2.7)	14 (3.4)	350 (84.7)	38 (9.2)

Numbers in parentheses indicate the percentage.

No., number studied; M, male; ALT, alanine aminotransferase.

^a $P < 0.05$ vs. asymptomatic carriers.

^b $P < 0.01$ vs. asymptomatic carriers.

Japan) according to the manufacturer's instructions, as reported previously [Usuda et al., 1999]. The 50 random samples that were genotyped by the EIA method were also reconfirmed by the restriction fragment length polymorphism method on the small-S gene sequence amplified by a PCR with nested primers [Mizokami et al., 1999].

Mutations of Basic Core Promoter (BCP) and Precore (PreC) Regions of HBV

To determine the mutations at the BCP and PreC regions, 35 samples with genotype D and 51 random samples with genotype C were examined by direct sequencing, as described previously [Chen et al., 2003]. The G-to-A substitution at nucleotide 1,896 in PreC (A1896), plus A-to-T at nucleotide 1,762 and G-to-A at nucleotide 1,764 in BCP were considered mutations.

HBV Full-Genome Sequencing

The complete nucleotide sequences of HBV from three samples with genotype D (two patients with chronic hepatitis and one asymptomatic carrier) were done by employing PCR and the full-genome sequence method as described previously [Chen et al., 2003]. In short, the PCR product was isolated and purified from 2% agarose gel by a QIAquick Gel Extraction kit (QIAGEN, MD) and was directly sequenced using the bigdye terminator cycle sequencing method.

Molecular Evolutionary Analysis

The three full-genome sequences of genotype D obtained from this study, together with the full-genome sequences of three other HBV genotype D cases from a previous study [Chen et al., 2003] (one patient with fulminant hepatitis, accession no.: AB078031; one patient with acute hepatitis, accession no.: AB078032; one patient with chronic hepatitis, accession no.: AB078033), were aligned with other known sequences from DNA databases (DDBJ/EMBL/GenBank). The computer program ODN, version 1.1.1 [Ina, 1994] using the six-parameter method [Gojobori et al., 1982], was used to determine the number of nucleotide

substitutions per site (genetic distance) between the isolates. Based on these values, a phylogenetic tree was constructed by the neighbour-joining method [Saitou and Nei, 1987]. The program from DDBJ with the midpoint rooting option was used to plot the tree. To confirm the reliability of the phylogenetic tree, bootstrap re-sampling tests were performed 1,000-times [Felsenstein, 1985].

Statistical Analysis

The data were analysed by using the chi-square test and Fisher's exact test. The Mann-Whitney U-test was used as required. Differences were considered significant for *P*-values of less than 0.05.

RESULTS

The genotype distribution in 413 HBV carriers is shown in Table I. The genotypes determined by the two methods (EIA or PCR-RFLP) were completely identical in all of the 50 subjects. Three hundred and fifty (84.7%) of the total 413 samples were infected with genotype C and a considerable proportion of HBV genotype D (9.2%) co-existed with this genotype. An interesting observation was made when we examined the genotype distribution among asymptomatic carriers and symptomatic carriers. Although more than 90% of the patients with chronic hepatitis (91.3%), liver cirrhosis (100%) and hepatocellular carcinoma (97.8%) were infected with genotype C, only 72.1% of the asymptomatic carriers were harbouring this genotype. Surprisingly, genotype D was detected in 32 of 179 (17.8%) asymptomatic carriers. Genotype D was not detected in any patient with liver cirrhosis or hepatocellular carcinoma. This tendency was more remarkable when the distribution of clinical diagnosis in patients infected with genotypes C and D was analysed (Table II). Thirty-two of the 38 HBV genotype D cases (84.2%) were asymptomatic carriers, while only 129 of the 350 HBV genotype C cases (36.8%) were asymptomatic carriers ($P < 0.001$).

The clinical characteristics of patients with different genotypes are shown in Table III. A male predominance was found in subjects with genotype D ($P < 0.01$). People with genotype D were significantly younger than people

TABLE II. Distribution of Clinical Diagnosis in People Infected With Different HBV Genotypes

Genotypes	No.	Diagnosis			
		ASC	CH	LC	HCC
Genotype A	11	8 (72.7)	3 (27.3)	0	0
Genotype B	14	10 (71.4)	3 (21.4)	0	1 (7.2)
Genotype C	350	129 (36.8)	126 (36.0)	50 (14.3)	45 (12.9)
Genotype D	38	32 (84.2) ^b	6 (15.8) ^a	0 ^a	0 ^a

Numbers in parentheses indicate the percentage.

ASC, asymptomatic carrier; CH, chronic hepatitis; LC, liver cirrhosis; HCC, hepatocellular carcinoma;

No., number studied.

^a $P < 0.05$ vs. genotype C.

^b $P < 0.001$ vs. genotype C.

TABLE III. Clinical Characteristics of Different HBV Genotypes in 413 HBV Carriers

HBV genotypes	No.	Age (years)	Gender (male, %)	ALT (IU/L)	HbeAg (+)
Genotype C	350	43.0 ± 14.6 ^a	223 (64) ^b	67.3 ± 133.4	117 (33.4) ^c
Genotype D	38	28.4 ± 12.1	33 (87)	32.8 ± 20.6	5 (13.2)
Others (A, B)	25	41.8 ± 13.6	16 (64)	35.6 ± 42.0	3 (12.0)
Total	413	41.6 ± 14.9	272 (66)	62.2 ± 124	125 (30.2)

Numbers in parentheses indicate the percentage.
 ALT, alanine aminotransferase; No., number studied.
^a $P < 0.001$ vs. genotype D.
^b $P < 0.01$ vs. genotype D.
^c $P < 0.05$ vs. genotype D.

with genotype C ($P < 0.001$). HBeAg, however, was more prevalent in people with genotype C than in people with genotype D (33.4% vs. 13.2%, $P < 0.05$).

Further analysis of the age factor revealed that the prevalence of HBV genotype D was found mainly among the 20–29 years of age group, which was significantly higher than other age groups (30–39; 40–49; >50 years) ($P < 0.01$, $P < 0.01$, $P < 0.01$, respectively; Fig. 1).

To minimise the impact of age on genotype distribution, the distribution of genotypes C and D among asymptomatic carriers and chronic liver disease patients (chronic hepatitis, liver cirrhosis and hepatocellular carcinoma) in young age-matched people (under 30-years-old with mean age: genotype C: 23.5 ± 4.2 years and genotype D: 24.8 ± 3.3 years; $P = 0.13$) was evaluated. As shown in Table IV, genotype D was again significantly more prevalent than genotype C in young asymptomatic carriers ($P < 0.05$). In contrast, the

proportion of genotype C was significantly higher in people who developed chronic liver disease. The anti-HBe-positive rate was also found to be significantly more frequent in genotype D than in genotype C in this age group (87% vs. 54%, $P < 0.01$) (Table IV).

The distribution of BCP and PreC mutations was analysed according to HBeAg status, BCP mutations being found less frequently in genotype D than in genotype C among HBeAg-negative carriers (50% vs. 82%, $P < 0.05$) (Table V).

The full genome of HBV was analysed in three persons with genotype D, the sequence homology being 98.8–99.3% among these samples (the nucleotide sequence data of the three full-genome isolates reported in this article were deposited in the DDBJ/EMBL/GenBank databases under accession numbers AB090268, AB090269 and AB090270). Their S genes encoded Arg¹²², Thr¹²⁷ and Lys¹⁶⁰, indicating that they

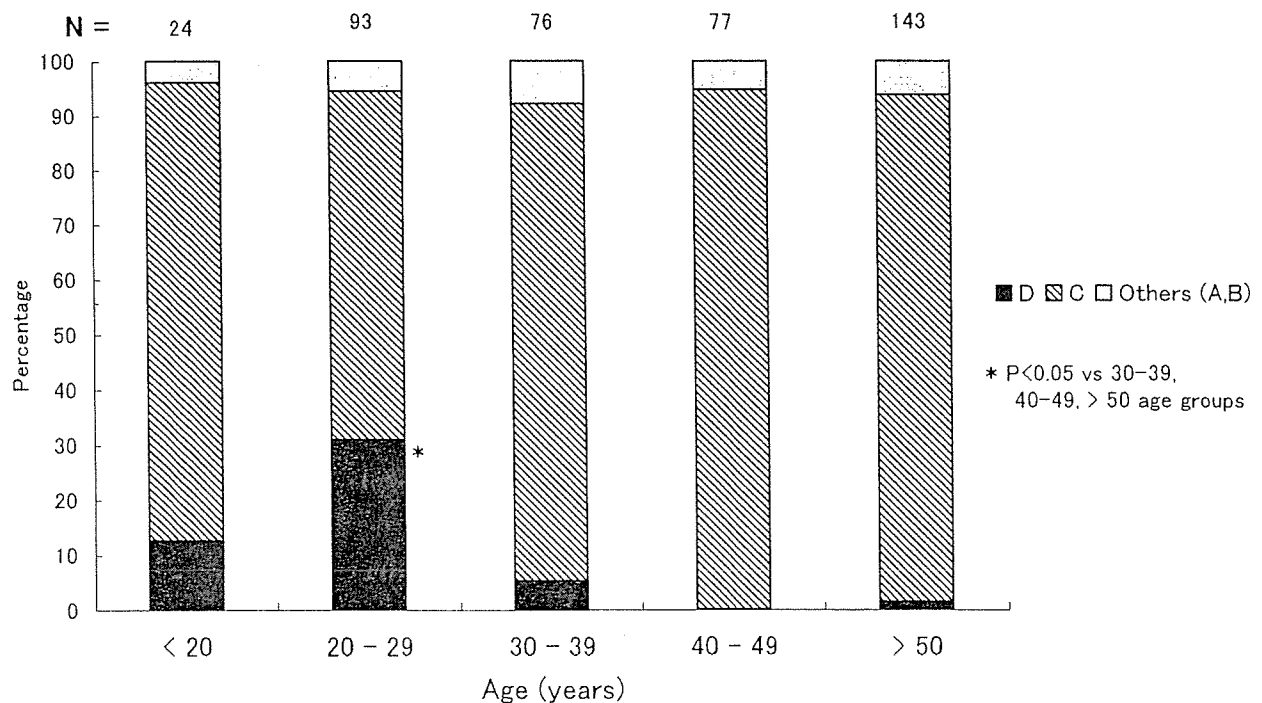


Fig. 1. HBV genotypes of 413 HBV carriers stratified by age. The prevalence of HBV genotype D was mainly found in age group 20–29 years and was significantly higher than other age groups (30–39; 40–49; >50) ($P < 0.01$, $P < 0.01$, $P < 0.01$, respectively).

TABLE IV. Diagnosis and HBeAg Status of HBV Genotypes C and D in HBV Carriers Under 30 Years of Age

Genotype	No.	Diagnosis		HBeAg status	
		ASC	CLD	HBeAg	Anti-Hbe
C	79	50 (63.3)	29 (36.7)	36 (46)	43 (54)
D	32	28 (87.5) ^a	4 (12.5)	4 (13)	28 (87) ^b

Numbers in parentheses indicate the percentage.

ASC, asymptomatic carriers; CLD, chronic liver disease (chronic hepatitis, liver cirrhosis); No., number studied.

^aP < 0.05 vs. genotype C.

^bP < 0.01 vs. genotype C.

all belonged to subtype *ayw3*. The length of the HBV genome in two samples was 3,182 bp, the standard length for HBV genotype D. Another strain was 3,194 bp in length, with a 12-bp insertion ranging from nucleotide 2,858 to 2,870 in the preS1 region. The genetic relationship among our three HBV genomes together with three other HBV genotype D full-genome sequences from our previous study [Chen et al., 2003] and some other complete HBV genomes obtained from databases is shown in Figure 2. The phylogenetic tree confirmed that the HBV full genomes were of genotype D, and the six HBV genomes were very closely related and were clustered as a separate branch. These Japanese sequences are related to some genotype D sequences originating from Europe (Fig. 2).

DISCUSSION

In agreement with previous studies about HBV genotype distribution in Japan [Usuda et al., 1999; Orito et al., 2001a], genotype C was the predominant strain, accounting for 84.7% of our studied samples. But, our results for the first time showed that a considerable proportion of HBV genotype D (9.2%) co-existed with genotype C in the same geographical area in the Southwestern part of Japan. The diagnosis of genotype D was confirmed by analysing the complete HBV genome sequence in some samples. This finding differed from previous studies in showing that HBV genotype D represented a very small proportion in Japan, only 0.4% in both previous studies [Usuda et al., 1999; Orito et al., 2001a]. This discrepancy diversifies further the geographical distribution of the HBV genotype in Japan.

TABLE V. The Incidence of BCP, PreC Mutations in HBV Carriers Infected With Genotypes C and D

Genotype	No.	HBeAg (+)		HBeAg (-)		
		PreC	BCP	No.	PreC	BCP
C	23	4 (17)	11 (48)	28	20 (71)	23 (82) ^a
D	5	0	3 (60)	30	17 (57)	15 (50)

Numbers in parentheses indicate the percentage.

No., number studied; PreC, precore: A1896; BCP, basic core promoter (T1762, A1764).

^aP < 0.05 vs. genotype D.

Recently, the clinical implications of HBV genotypes have been clarified partially. There have been several reports concerning the comparison between genotypes C and B in Asia. It is reported that HBV genotype B is associated with earlier HBeAg seroconversion than genotype C or that genotype C is associated with more severe liver disease than genotype B [Kao et al., 2000; Orito et al., 2001a; Chu et al., 2002; Furusyo et al., 2002; Sumi et al., 2003]. There have also been some reports concerning the comparison between genotypes A and D in Europe. In Switzerland, genotype A was associated with chronic hepatitis more frequently than genotype D [Mayerat et al., 1999]. Nevertheless, observations from Spain indicate that in chronic hepatitis B, the development of beneficial events such as biochemical remission, clearance of HBV DNA and clearance of HBsAg are more frequent in patients infected with genotype A than in those infected with genotype D [Sanchez-Tapias et al., 2002]. However, our present study was the first investigation that made a comparison directly between genotypes C and D in terms of the clinical relevance and virological characteristics in the same race and the same geographical area.

Interestingly, our results showed that the majority of the people with genotype D in our area were asymptomatic carriers (84.2%), but not patients having chronic hepatitis (15.8%), liver cirrhosis (0%) or hepatocellular carcinoma (0%). These findings were further confirmed when the distribution of genotypes D and C was evaluated between asymptomatic carriers and chronic liver disease in young age-matched HBV carriers. The anti-HBe-positive rate was also found more frequently in genotype D (87%) than in genotype C (54%) in young people. In contrast, genotype C was frequent among people who developed chronic liver disease. At this stage, it is unknown why most of the people infected with genotype D remained asymptomatic. It is premature to conclude, however, that infection with HBV genotype D is more likely to take a benign course, since most of the persons infected with it are still young. These patients should be followed-up in order to observe the progression of liver disease in the future. Because almost all chronic HBV infection in Japan occurs perinatally from their mothers at birth, the duration of infection is almost the same as the age of the carriers. In this study, the genotype D carriers are younger than the genotype C carriers. It seems that the epidemiology of genotype D may differ from that of genotype C. HBV genotype D may be spread by routes other than perinatal infection, while those infected with genotype C are mainly infected perinatally. If the route of transmission is horizontal in genotype D, the differences observed between genotypes D and C may be due to differences in duration of carrier-ship being shorter for those infected with genotype D and a higher age at the time of infection may also explain the more rapid seroconversion to anti-HBe for those infected with genotype D. However, we did not suspect any drug addicts among the patients which we studied.

Genotype D prevailed mainly among younger HBV carriers (age 20–29 years) and it appears that HBV

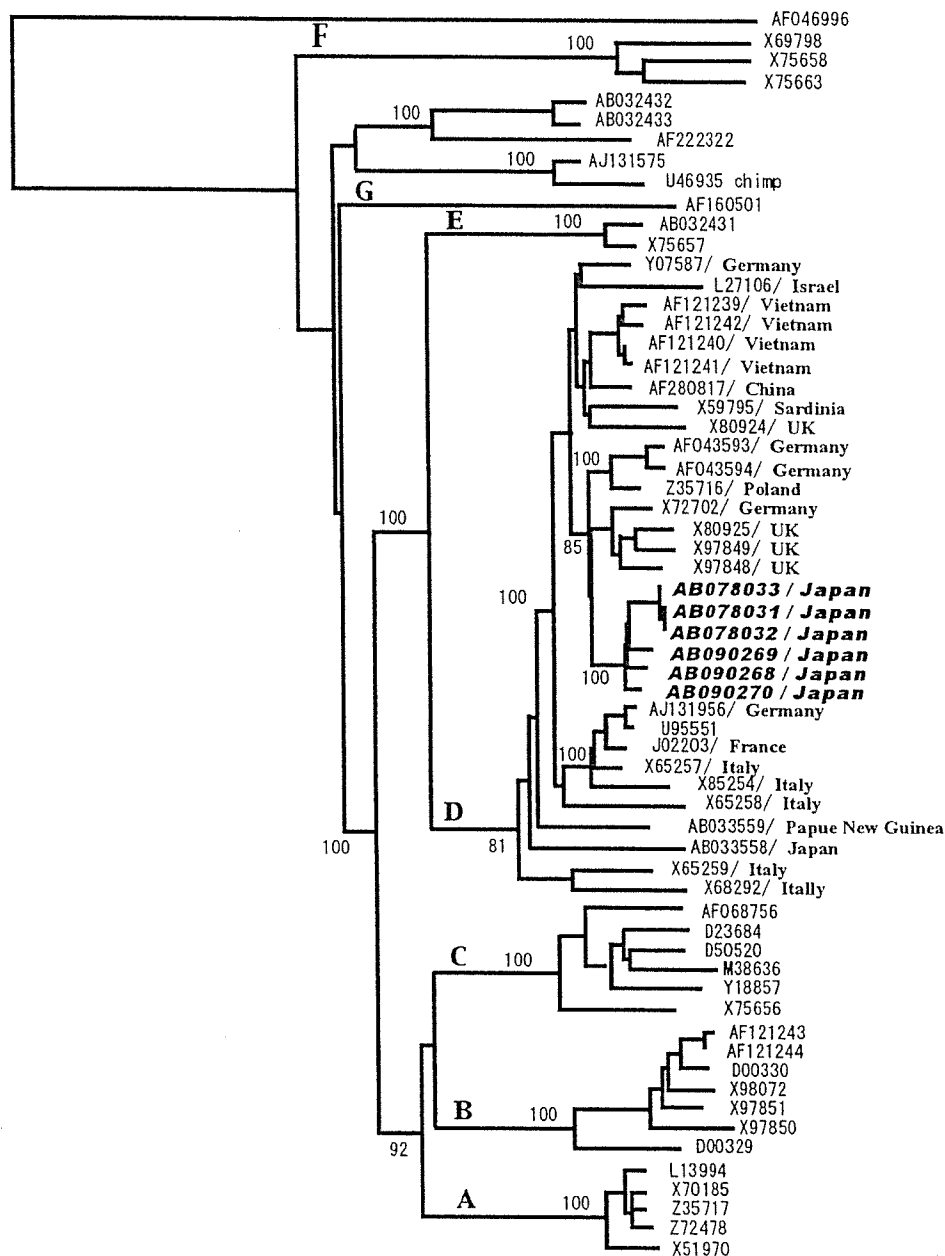


Fig. 2. Full nucleotide sequences of HBV with genotype D from our studied subjects were done and a phylogenetic tree of HBV genotype was constructed. HBV genotype D from our study was related to some HBV genotype D sequences originating from Europe. Bootstrap values are shown along each main branch. Countries of origin are indicated for genotype D. The numbers indicate the accession numbers.

genotype D might have been spread in this community 20–30 years ago. Related to this phenomenon, an epidemic of infantile papular acrodermatitis (Gianotti's disease) was reported in this area 30 years ago in children mainly under 4 years of age. HBsAg subtype *ayw* was detected in 87% of these patients [Ishimaru et al., 1976]. Although HBV serotypes *adr* and *adw* (corresponding to genotypes C and B, respectively) are the most prevalent in Japan [Usuda et al., 1999], patients with Gianotti's disease had a serotype of *ayw*. Because Ehime is a port city, it is possible that HBV

genotype D with serotype *ayw* in the present study and HBV serotype *ayw* in a previous study may have originated from the outside [Ishimaru et al., 1976]. The phylogenetic tree confirmed that genotype D of this area was similar to some sequences from Europe. This suggests that the spread of genotype D from Europe might have occurred, but the infectious source as well as the route of transmission is not clear.

Many studies have shown a strong relationship between HBV genotypes and mutations in the PreC and BCP regions that may abolish or diminish the

production of HBeAg [Carman et al., 1989; Lindh et al., 1999]. The most common PreC mutation, A1896, that creates a premature stop codon at codon 28, is found in association with HBV genotypes B, C and D, but not genotype A [Li et al., 1993]. On the other hand, mutations in the BCP were found more frequently in genotype C than in genotype B in some reports [Orito et al., 2001b; Chan et al., 2002]. BCP mutations are generally related to more severe liver disease and the progression of HBV infection [Lindh et al., 1998; Yotsuyanagi et al., 2002]. In this study, it was found that mutations at BCP (T1762, A1764) were significantly less frequent in genotype D than in genotype C among HBeAg-negative patients. However, the clinical implication of the lower frequency of BCP mutation in genotype D compared to genotype C should be clarified in the future.

In summary, we have found HBV genotype D among asymptomatic carriers in the Southwestern part of Japan. Overall, people with HBV genotype D were younger and were related to earlier HBeAg seroconversion than those with genotype C. These findings further strengthen the possible pathogenic differences among different HBV genotypes. Further long-term longitudinal studies including clinical, virological and host genetic assessment in this population would be required to confirm more precisely these observations in the future.

REFERENCES

- Carman WF, Jacyna MR, Hadziyannis S, Karayiannis P, McGarvey MJ, Makris A, Thomas HC. 1989. Mutation preventing formation of hepatitis B e antigen in patients with chronic hepatitis B infection. *Lancet* 2:588–591.
- Chan HLY, Tsang SWC, Liew CT, Tse CH, Phil M, Wong ML, Ching JYL, Leung NWY, Tam JSL, Sung JYJ. 2002. Viral genotype and hepatitis B virus DNA levels are correlated with histological liver damage in HBeAg-negative chronic hepatitis B virus infection. *Am J Gastroenterol* 97:406–412.
- Chen DS. 1993. From hepatitis to hepatoma: Lesions from type B viral hepatitis. *Science* 262:369–370.
- Chen Y, Michitaka K, Matsubara H, Yamamoto K, Horiike N, Onji M. 2003. Complete genome sequence of hepatitis B virus (HBV) from a patient with fulminant hepatitis without precore and core promoter mutations: Comparison with HBV from a patient with acute hepatitis infected from the same infectious source. *J Hepatol* 38:84–90.
- Chu CJ, Hussain M, Anna SFL. 2002. Hepatitis genotype B is associated with earlier HBeAg seroconversion compared with hepatitis B virus genotype B. *Gastroenterology* 122:1756–1762.
- Felsenstein J. 1985. Confidence limits on phylogenies. *Evolution* 39:783–791.
- Furusyo N, Nakashima H, Kashiwagi K, Kubo N, Hayashida K, Usuda S, Mishiro S, Kashiwagi S, Hayashi J. 2002. Clinical outcome of hepatitis B virus (HBV) genotypes B and C in Japanese patients with chronic HBV infection. *Am J Trop Med Hyg* 67(2):151–157.
- Gojobori T, Ishii K, Nei M. 1982. Estimation of average number of nucleotide substitutions when the rate of substitution varies with nucleotide. *J Mol Evol* 18:414–423.
- Ina Y. 1994. ODEN: A program package for molecular evolutionary analysis and database search of DNA and amino acid sequences. *CABIO* 10:11–12.
- Ishimaru Y, Ishimaru H, Toda G, Baba K, Mayumi M. 1976. An epidemic of infantile papular acrodermatitis (Gianotti's disease) in Japan associated with hepatitis B surface antigen subtype ayw. *Lancet* 1(7962):707–709.
- Kao JH, Chen PJ, Lai MY, Chen DS. 2000. Hepatitis B genotypes correlate with clinical outcomes in patients with chronic hepatitis B. *Gastroenterology* 118:554–559.
- Lee WM. 1997. Hepatitis B virus infection. *N Engl J Med* 337:1733–1745.
- Li JS, Tong SP, Wen YM, Vitvitski L, Zhang Q, Trepo C. 1993. Hepatitis B virus genotype A rarely circulates as an HBe-minus mutant: Possible contribution of a single nucleotide in the precore region. *J Virol* 67:5402–5410.
- Lindh M, Andersson AS, Gusdal A. 1997. Genotypes, nt 1858 variant, and geographic origin of hepatitis B virus—large scale analysis using a new genotyping method. *J Infect Dis* 175:1285–1293.
- Lindh M, Gustavson C, Mardberg K, Norkrans G, Dhillon AP, Horal P. 1998. Mutations of nucleotide 1762 in the core promoter region during hepatitis B e seroconversion and its relation to liver damage in hepatitis B e antigen carriers. *J Med Virol* 55:185–190.
- Lindh M, Hannoun C, Dhillon AP, Norkrans G, Horal P. 1999. Core promoter mutations and genotypes in relation to viral replication and liver damage in East Asia hepatitis B virus carriers. *J Infect Dis* 179:775–782.
- Magnius LO, Norder H. 1995. Subtypes, genotypes and molecular epidemiology of the hepatitis B virus as reflected by sequence variability of the S-gene. *Intervirology* 38:24–34.
- Mayerat C, Mantegani A, Frei PC. 1999. Does hepatitis B virus (HBV) genotype influence the clinical outcome of HBV infection? *J Viral Hepat* 6:299–304.
- Mizokami M, Nakano T, Orito E, Tanaka Y, Sakugawa H, Mukaide M, Roberson BH. 1999. Hepatitis B virus genotype assignment using restriction fragment length polymorphism pattern. *FEBS Lett* 450:66–71.
- Nishioka K, Levin AG, Simons MJ. 1975. Hepatitis B antigen, antigen subtypes, and hepatitis B antibody in normal subjects and patients with liver disease. *Bull World Health Organ* 52:293–300.
- Norder H, Courouce AM, and Magnius LO. 1994. Complete genomes, phylogenetic relatedness, and structural proteins of six strains of hepatitis B virus, four of which represent two new genotypes. *Virology* 198:489–503.
- Okamoto H, Tsuda F, Sakugawa H, Sastrosowignjo RI, Imai M, Miyakawa Y, Mayumi M. 1988. Typing hepatitis B virus by homology in nucleotide sequence: Comparison of surface antigen subtype. *J Gen Virol* 69:2575–2583.
- Orito E, Ichida T, Sakugawa H, Sata M, Horiike N, Hino K, Okita K, Okanoue T, Iino S, Tanaka E, Suzuki K, Watanabe H, Hige S, Mizokami M. 2001a. Geographic distribution of hepatitis B virus (HBV) genotype in patients with chronic HBV infection in Japan. *Hepatology* 34:590–594.
- Orito E, Mizokami M, Sakugawa H, Michitaka K, Ishikawa K, Ichida T, Okanoue T, Yotsuyanagi H, Iino S. 2001b. A case-control study for clinical and molecular biological differences between hepatitis B virus of genotype B and C. *Hepatology* 33:218–223.
- Ruiz PA, Norder H, Robertson BH, Magnius LO. 2002. Genotype H: A new Amerindian genotype of hepatitis B virus revealed in Central America. *J Gen Virol* 83:2059–2073.
- Saitou N, Nei M. 1987. The neighbor-joining method: A new method for reconstructing phylogenetic trees. *Mol Biol Evol* 4(4):406–425.
- Sanchez-Tapias JM, Costa J, Mas A, Bruguera M, Rodes J. 2002. Influence of hepatitis B virus genotype on the long-term outcome of chronic hepatitis B in Western patients. *Gastroenterology* 123:1848–1856.
- Sumi H, Yokosuka O, Seki N, Arai M, Imazeki F, Kurihara T, Kanda T, Fukai K, Kato M, Saisho H. 2003. Influence of hepatitis B genotypes on the progression of chronic type B liver disease. *Hepatal* 37:19–26.
- Usuda S, Okamoto H, Iwanari H, Baba K, Tsuda F, Miyakawa Y, Mayumi M. 1999. Serological detection of hepatitis B genotype by ELISA with monoclonal antibodies to type specific epitopes in the preS2-region product. *J Virol Methods* 80:97–112.
- Yotsuyanagi H, Hino K, Tomita E, Toyoda J, Yasuda K, Iino S. 2002. Precore and core promoter mutations, hepatitis B virus DNA levels and progressive liver injury in chronic hepatitis B. *J Hepatol* 37:355–363.

Gianotti-Crosti Syndrome Caused by Acute Hepatitis B Virus Genotype D Infection

Kojiro MICHITAKA, Norio HORIIKE*, Yan CHEN*, Tran Nhu DUONG*, Ichiro KONISHI*, Toshie MASHIBA*, Yoshio TOKUMOTO*, Yoichi HIASA*, Yasuhito TANAKA**, Masashi MIZOKAMI** and Morikazu ONJI*

Abstract

A 12-year-old girl with Gianotti-Crosti syndrome caused by hepatitis B virus (HBV) infection was admitted due to eruption on her extremities. Laboratory findings revealed elevation of transaminase, positivity for HB surface antigen (HBsAg), and an IgM type anti-HB core. The eruption and level of transaminase improved, and HBsAg became negative within 2 months of onset. Analysis of the virus revealed it to be genotype D with a genomic length of 3,182 bases and the HBsAg serotype was ayw3, which is very rare in Japan. The possible relationship between Gianotti-Crosti syndrome and HBV genotype D infection is discussed. (Internal Medicine 43: 696–699, 2004)

Key words: Gianotti-Crosti syndrome, hepatitis B virus, genotype D

Introduction

Gianotti-Crosti syndrome is characterized by papular eruption, which is mainly observed on the dorsal side of hands and feet of children. The disease has also been designated as infantile papular acrodermatitis, with hepatitis B virus (HBV) infection reported to be the most common cause (1, 2). In the 1970s, an epidemic of Gianotti-Crosti syndrome occurred in a town in the western part of Japan, and the relationship between the disease and HBV with hepatitis B surface antigen (HBsAg) serotype ayw, which is a very rare serotype in Japan, was reported (3). In the present case report, we analyze the genome of an HBV infection in a patient with Gianotti-Crosti syndrome whose HBsAg

serotype was ayw.

Case Report

A 12-year-old Japanese girl was admitted to our hospital on December 25, 1987, because of eruption on the extremities that had emerged at the beginning of December. A papular eruption was observed on all four extremities, especially on the hands and feet (Fig. 1). Laboratory findings revealed an elevation of serum transaminase, and positivity for the hepatitis B surface antigen (HBsAg) and IgM-type anti-hepatitis B core (IgM-anti-HBc) (Table 1). The patient had no past history of liver disease. She was diagnosed with Gianotti-Crosti syndrome due to acute hepatitis B virus (HBV) infection, because of the typical eruption symptoms and serum HBV markers. Following admission, the eruption improved, and completely disappeared on January 4, 1988. Further, alanine transaminase (ALT), which had become elevated to 1,452 IU/l, gradually improved and returned to a normal range at the beginning of February 1988 (Fig. 2). Icterus was not observed during the course of the disease. A liver biopsy showed typical findings of acute hepatitis. HBsAg became negative in March, while anti-HBs later became positive in August. She did not progress to become a chronic HBV carrier. Both parents were negative for HBsAg and the infectious route of HBV was not clarified.

The HBsAg serotype, genotype, and full genome sequence of the HBV were analyzed using serum obtained on the day of admission which had been stored at -70°C . Written informed consent was obtained from the patient and her parents prior to the analysis. The HBsAg serotype was assayed using an enzyme immunoassay, the HBV genotype was determined based on restriction fragment length polymorphism patterns of the S gene sequence amplified by polymerase chain reaction (PCR-RFLP) (4), and sequencing

From the Endoscopy Center, *the Third Department of Internal Medicine, Ehime University School of Medicine, Ehime and **the Department of Internal Medicine and Molecular Science, Nagoya City University Graduate School of Medical Sciences, Nagoya

Received for publication November 28, 2003; Accepted for publication March 10, 2004

Reprint requests should be addressed to Dr. Morikazu Onji, the Third Department of Internal Medicine, Ehime University School of Medicine, Shigenobu-cho, Onsen-gun, Ehime, 791-0295

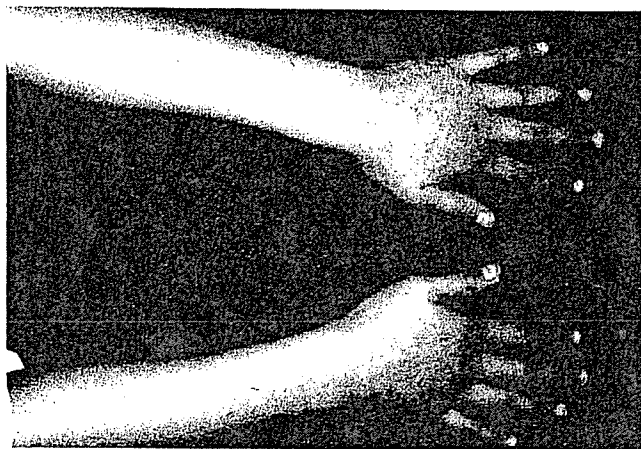


Figure 1. Photograph of an area of skin eruption. Eruption was mainly observed on the dorsal side of the four extremities.

was done by direct sequence, according to the previously reported method (5). Accuracy of the sequences was ensured by identification of the sequence data of the genome obtained using sense and anti-sense sequencing primers. The genotype of the present strain was shown to be D. A full genome sequence analysis revealed the genomic length to be 3182 bases (accession number AB116266), while the HBsAg serotype deduced from the amino acid sequence of the S region was ayw3, because the deduced amino acid sequence at position 122 of the S protein was Arg, which corresponded to antigen y, and that at 127 was Thr, which corresponded to antigen w3 of HBsAg. Core promoter (nt 1762T, nt 1764A) and precore (nt 1896A) mutations were not found.

Discussion

In addition to HBV, other viruses such as Epstein-Barr virus, human herpesvirus 6, and respiratory syncytial virus, have been reported as possible causal agents of Gianotti-Crosti syndrome (6–9), while a combined vaccination for

HBV and measles has also been shown to be involved in its etiology (10). Further, several studies have indicated the participation of immunological mechanisms, such as the formation of immune complex (11, 12).

Several reports have found the HBsAg serotype of HBV in patients with Gianotti-Crosti syndrome, and HBV serotypes ayw, adr and adw have been noted (3, 13–16). However, there have been no reports regarding HBV genotypes in patients with this syndrome. The present patient lived in an area where an epidemic of Gianotti-Crosti syndrome occurred in the 1970s, and a relationship with this disease in that area and HBV with serotype ayw has been reported (3, 17). Stored serum samples from those patients with Gianotti-Crosti syndrome epidemic in the 1970s are not available, and the epidemic has ended and no patients with this syndrome have been admitted to our hospital since 1989. Thus, the present case was the only patient with this syndrome whose serum of acute phase was stored in our laboratory.

In the present patient, the HBsAg serotype was also ayw and the HBV genotype was determined to be D. In Japan, the major HBV genotypes are C and B (18), and the main serotypes of HBsAg are adr and adw (19). Genotype D and serotype ayw are very rare in Japanese. From our results, we suspect that the HBV organisms with serotype ayw in patients with Gianotti-Crosti syndrome in the 1970s epidemic were genotype D. The genomic length of the virus in the present study was 3182 bases, which is typical for genotype D. Further, the sequence of this strain was similar to that of 3 HBV strains from patients living in the same prefecture, which we reported previously (5). Phylogenetic analysis showed that the strain from the present patient had a cluster with the 3 strains described above, which were previously obtained from 3 adult patients (asymptomatic carrier, acute hepatitis and fulminant hepatitis) living in Ehime Prefecture (data not shown). Eruption was not observed in those 3 patients, which may indicate that age at time of infection might be related to the occurrence of this syndrome, as noted by Toda et al (17).

The route for HBV transmission was not clarified in the

Table 1. Laboratory Data on Admission (December 25, 1987)

WBC	7,000/mm ³	T. Bil	1.7 mg/dl	TP	8.0 g/dl	HBsAg	(+) 2*
Seg.	52.6%	D. Bil	1.2 mg/dl	Alb	4.4 g/dl	anti-HBs	(-)
Ly.	40.1%	GOT	528 U//	T. chol	133 mg/dl	HBeAg	0.6
Mo.	4.6%	GPT	1,452 U//	TG	197 mg/dl	anti-HBe	81.3%
Eo.	0.9%	ChE	74 IU//	BUN	6 mg/dl	IgM anti-HBc	(+) 7.8
Ba.	1.8%	LDH	144 IU//	Cr	0.7 mg/dl	anti-HBc ×1	99%
RBC	516×10 ⁴ /mm ³	ALP	700 IU//	Na	140 mEq//	×200	71%
Hb	14.8 g/dl	γ-GTP	52 IU//	K	4.4 mEq//	HBV-DNA-P	48 cpm
Ht	45.2%	LAP	122 IU//	Cl	108 mEq//	IgM-HA	(-)
Plt	35.8×10 ⁴ /mm ³	ZTT	19 U//	FPG	92 mg/dl	Anti-HCV	(-)
		TTT	24 U//	IgG	1,636 mg/dl	ANA	×20
		ICG K	0.184	IgA	201 mg/dl	AMA	<×20
		R15	7%	IgM	156 mg/dl	ASMA	<×20

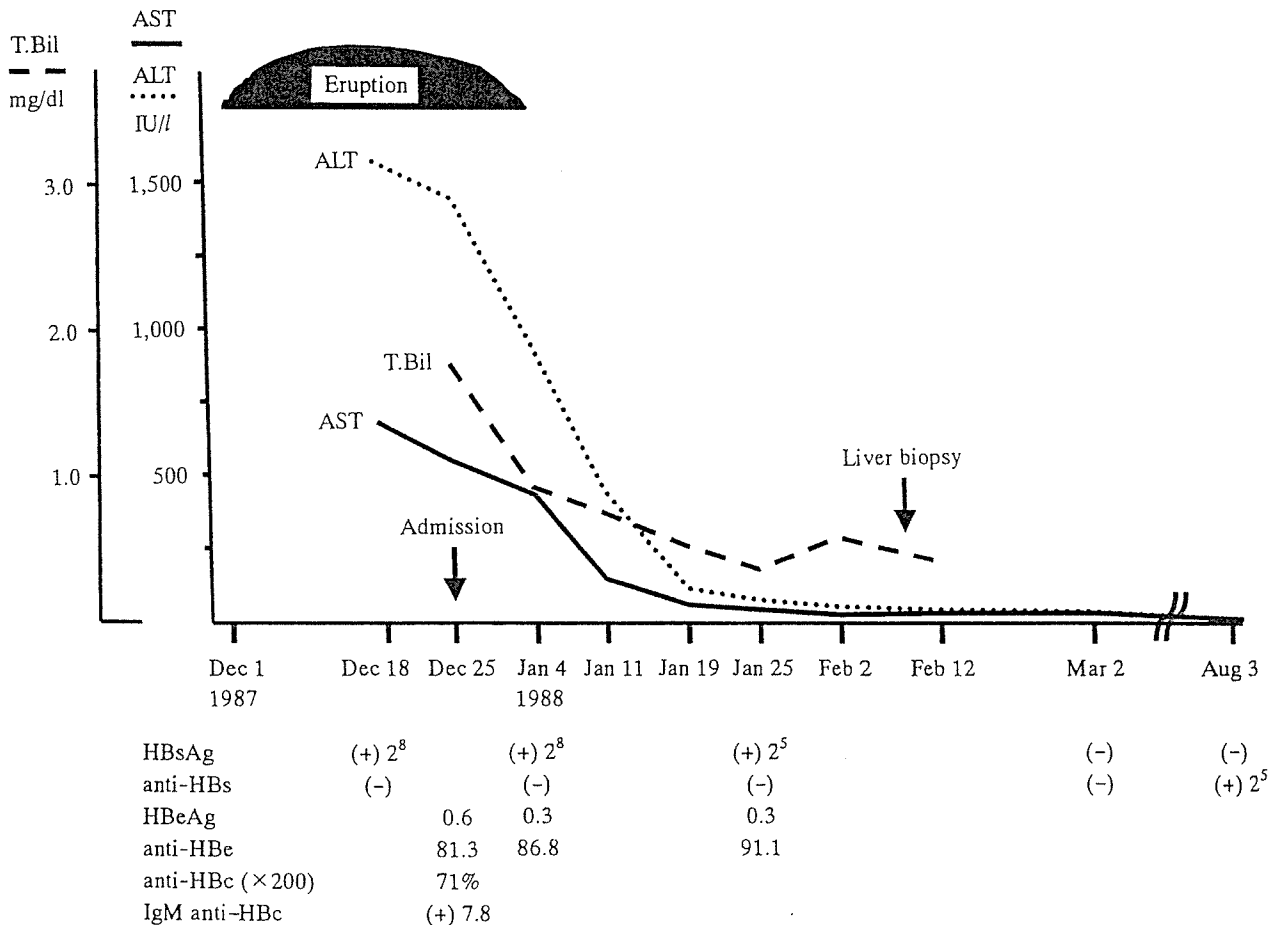


Figure 2. Clinical course. Changes in liver function test results and HBV markers were similar to a typical course of acute hepatitis B. Eruption was observed for approximately 5 weeks after onset.

present case, however, since no family members were found to be positive for HBsAg, horizontal transmission from a non-family member was suspected. For HBV transmission in adulthood, sexual transmission is the most common route in Japan (20), however, this patient had no previous sexual contact or intravenous drug injection. Ishimaru et al reported that the positivity of HBsAg in parents of patients with Gianotti-Crosti syndrome infected with HBV serotype ayw epidemic in western Japan during the 1970s was low (3), and speculated that the main routes for HBV transmission in patients with Gianotti-Crosti syndrome was not vertical but rather horizontal.

Some reports have described the clinical characteristics of HBV genotypes. We found that chronic infection with HBV genotype D was related to lower ALT and earlier seroconversion, as compared with genotype C (21). Additionally, it has been reported that the rate of progression to chronic carrier state from acute infection and that of HBsAg clearance in chronic infected patients were lower in genotype D as compared to genotype A (22, 23). However, it has not

been clarified whether HBV genotype D has a characteristic to induce Gianotti-Crosti syndrome, while the mechanism of the syndrome is also not well known. Genotyping and sequencing of a large number of HBV organisms from patients with Gianotti-Crosti syndrome would help to elucidate the relationship and clarify these issues.

References

- 1) Gianotti F. Papular acrodermatitis of childhood. An Australia antigen disease. *Arch Dis Child* 48: 794-799, 1973.
- 2) De Gaspari G, Bardare M, Costantino D. AU antigen in Crosti-Gianotti acrodermatitis. *Lancet* I: 1116-1117, 1970.
- 3) Ishimaru Y, Ishimaru H, Toda G, Baba K, Mayumi M. An epidemic of infantile papular acrodermatitis (Gianotti's disease) in Japan associated with hepatitis-B surface antigen subtype ayw. *Lancet* I: 707-709, 1976.
- 4) Mizokami M, Nakano T, Orito E, et al. Hepatitis B virus genotype assignment using restriction fragment length polymorphism patterns. *FEBS Lett* 450: 66-71, 1999.
- 5) Chen Y, Michitaka K, Matsubara H, Yamamoto K, Horiike N, Onji M. Complete genome sequence of hepatitis B virus (HBV) from a patient with fulminant hepatitis without precore and core promoter mutations:

Gianotti-Crosti Syndrome by HBV Genotype D

- comparison with HBV from a patient with acute hepatitis infected from the same infectious source. *J Hepatol* **38**: 84–90, 2003.
- 6) Caputo R, Gelmetti C, Ermacora E, Gianni E, Silvestri A. Gianotti-Crosti syndrome: a retrospective analysis of 308 cases. *J Am Acad Dermatol* **26**: 207–210, 1992.
 - 7) Chuh AA, Chan HH, Chiu SS, Ng HY, Peiris JS. A prospective case control study of the association of Gianotti-Crosti syndrome with human herpesvirus 6 and human herpesvirus 7 infections. *Pediatr Dermatol* **19**: 492–497, 2002.
 - 8) Taieb A, Plantin P, Du Pasquier P, Guillet G, Maleville J. Gianotti-Crosti syndrome: a study of 26 cases. *Br J Dermatol* **115**: 49–59, 1986.
 - 9) Draelos ZK, Hansen RC, James WD. Gianotti-Crosti syndrome associated with infections other than hepatitis B. *JAMA* **256**: 2386–2388, 1986.
 - 10) Andiran N, Senturk GB, Bukulmez G. Combined vaccination by measles and hepatitis B vaccines: a new cause of Gianotti-Crosti syndrome. *Dermatology* **204**: 75–76, 2002.
 - 11) Colombo M, Gerber MA, Vernace SJ, Gianotti F, Paronetto F. Immune response to hepatitis B virus in children with papular acrodermatitis. *Gastroenterology* **73**: 1103–1106, 1977.
 - 12) Kacprzak-Bergman I, Halasa J. Frequency of complement third component (C3) and properdin factor (BF) phenotypes in patients with various clinical manifestations of HBV infection. *Eur J Immunogenet* **23**: 1–6, 1996.
 - 13) Lee S, Kim KY, Hahn CS, Lee MG, Cho CK. Gianotti-Crosti syndrome associated with hepatitis B surface antigen (subtype adr). *J Am Acad Dermatol* **12**: 629–633, 1985.
 - 14) Schneider JA, Poley JR, Millunchick EW, Orcutt MA, de Triquet JM. Papular acrodermatitis (Gianotti-Crosti syndrome) in a child with anicteric hepatitis B, virus subtype adw. *J Pediatr* **101**: 219–222, 1982.
 - 15) Kanzaki S, Kanda S, Terada K, et al. Detection of hepatitis B surface antigen subtype adr in an epidemic of papular acrodermatitis of childhood (Gianotti's disease). *Acta Med Okayama* **35**: 407–410, 1981.
 - 16) Ono E. Natural history of infantile papular acrodermatitis (Gianotti's disease) with HBsAg subtype adw. *Kurume Med J* **35**: 147–157, 1988.
 - 17) Toda G, Ishimaru Y, Mayumi M, Oda T. Infantile papular acrodermatitis (Gianotti's disease) and intrafamilial occurrence of acute hepatitis B with jaundice: age dependency of clinical manifestations of hepatitis B virus infection. *J Infect Dis* **138**: 211–216, 1978.
 - 18) Orito E, Ichida T, Sakugawa H, et al. Geographic distribution of hepatitis B virus (HBV) genotype in patients with chronic HBV infection in Japan. *Hepatology* **34**: 590–594, 2001.
 - 19) Usuda S, Okamoto H, Iwanari H, et al. Serological detection of hepatitis B virus genotypes by ELISA with monoclonal antibodies to type-specific epitopes in the pre-S2-region product. *J Virol Methods* **80**: 97–112, 1999.
 - 20) Arima S, Michitaka K, Horiike N, et al. Change of acute hepatitis B transmission routes in Japan. *J Gastroenterol* **38**: 772–775, 2003.
 - 21) Duong TN, Horiike N, Michitaka K, et al. Comparison of genotypes C and D hepatitis B virus in Japan: a clinical and molecular biological study. *J Med Virol* **72**: 551–557, 2004.
 - 22) Mayerat C, Mantegani A, Frei PC. Does hepatitis B virus (HBV) genotype influence the clinical outcome of HBV infection? *J Viral Hepat* **6**: 299–304, 1999.
 - 23) 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* **123**: 1848–1856, 2002.

Hepatitis B Virus DNA in Liver, Serum, and Peripheral Blood Mononuclear Cells After the Clearance of Serum Hepatitis B Virus Surface Antigen

Yoshiki Murakami,^{1,2*} Masahito Minami,¹ Yukiko Daimon,¹ and Takeshi Okanoue¹

¹Department of Internal Medicine, National Fukui Hospital, Sakuragaoka, Tsuruga, Fukui, Japan

²Third Department of Internal Medicine, Kyoto Prefectural University of Medicine, Kyoto, Japan

The integration of hepatitis B virus (HBV) DNA in the liver of chronic HBV carriers has been documented extensively. However, the status of the viral genome during acute infection has not been assessed conclusively. While HBV DNA sequences are detected often in serum, liver, and peripheral blood mononuclear cells (PBMCs) after the clearance of serum the hepatitis B virus surface antigen (HBsAg), the precise status of the viral genome, and in particular the possible persistence of integrated genomes in PBMCs, has not been established. A highly sensitive PCR-derived assay (Alu-PCR) was employed to re-examine liver and PBMC specimens obtained from patients with acute (n = 19) and chronic (n = 22) hepatitis in whom serum HBsAg was present (n = 12) (HBV-related chronic active hepatitis) or absent with anti-HCV (n = 10) (HCV-related chronic active hepatitis). Viral integration was demonstrated in 3 out of 19 liver specimens from patients with acute hepatitis and 12 out of 12 specimens from patients with chronic hepatitis. Viral integration was also observed in 4 out of 7 PBMC samples from HBV-related chronic active hepatitis patients and 2 out of 10 liver and PBMC samples from HCV-related chronic active hepatitis patients. In one liver specimen from an acute hepatitis patient, HBV DNA was found integrated in the intronic sequence of the tumour necrosis factor (TNF)-induced protein gene; viral integration into cellular sequences was also found in the PBMCs of four HBV-related chronic active hepatitis and two HCV-related chronic active hepatitis. The results demonstrate the early integration of HBV genome during acute viral infections and the persistence of the viral genome in an integrated form in PBMCs. *J. Med. Virol.* 72:203–214, 2004. © 2004 Wiley-Liss, Inc.

KEY WORDS: HBV integration; viral persistence; HBV/HCV co-infection

INTRODUCTION

Hepatitis B virus (HBV) is a double-stranded DNA virus responsible for acute and chronic liver diseases. Unlike retroviruses, HBV does not need to form a provirus once it has completed its replication cycle [Miller and Robinson, 1986; Seeger et al., 1986]. However, some HBV DNA molecules enter the nuclei and are diverted into an integration pathway, and integrated HBV DNA has been cloned from chronically infected liver specimens, including specimens from hepatocellular carcinoma (HCC) patients. HBV DNA integration into host chromosomal DNA has been reported at various stages of chronic HBV infection [Takada et al., 1990; Hino et al., 1994], with a very high integration rate of HBV DNA into the cellular DNA of HCC type B patients [Bréchet et al., 2000] and an integration rate of more than 80% in HBV-related chronic active hepatitis patients [Matsubara and Tokino, 1990]. However, whether HBV integration occurs in transient HBV infection, such as in acute hepatitis or fulminant hepatitis has not been determined. Attempts to demonstrate the integration of the viral genome in ducks acutely infected with duck hepatitis B virus (DHBV) have been successful [Yang and Summers, 1995], and a few studies using Southern blots have shown HBV DNA patterns consistent with the integration of the viral genome, but integration in

*Correspondence to: Yoshiki Murakami, MD, Department of Internal Medicine, National Fukui Hospital, Sakuragaoka 33-1, Tsuruga, Fukui 914-0195, Japan. E-mail: cim87490@rio.odn.ne.jp

Accepted 22 July 2003

DOI 10.1002/jmv.10547

Published online in Wiley InterScience
(www.interscience.wiley.com)

transient infections has not been established conclusively [Lugassy et al., 1987; Marconi et al., 1988; Yoffe et al., 1990]. This issue is of importance in clarifying the natural course of viral infection and the sequence of events favouring clonal liver cell expansion.

The existence of HBV DNA observed in extrahepatic tissues are still a matter of debate [Laskus et al., 1999], and the infection of PBMCs by HBV, in particular, remains controversial despite the detection of HBV DNA by Southern blotting and *in situ* hybridisation [Yoffe et al., 1986; Catterall et al., 1994]. The possible persistence of viral sequences in an integrated form in such cell types remains an important question. Finally, several studies have now conclusively shown that HBV DNA is present in the serum of patients who have recovered from an HBV infection [Michalak et al., 1994; Yotsuyanagi et al., 1998; Marusawa et al., 2000]. HBV DNA is detected frequently in the lymphocytes of acute hepatitis patients after clearance of serum HBsAg [Penna et al., 1996; Rehmann et al., 1996]. Serum HBV persists for many years in the form of an immune-complex after seroconversion to an anti-HBs antibody (anti-HBs) [Yotsuyanagi et al., 1998], and the presence of infectious viral particles in these sera has been established by transmission experiments in chimpanzees as well as by the analysis of well-characterized post-transfusion hepatitis and recurrent hepatitis in liver grafts after liver transplantation [Sureau et al., 1988; Chazouilleres et al., 1994].

Reports of persistent HBV infections (occult HBV infection) of clinical relevance have also been made. In chronic hepatitis C infection, the presence of occult hepatitis B co-infection has been associated with a poor interferon alpha response [Cacciola et al., 1999; Chemin et al., 2001; Sagnelli et al., 2001]. Epidemiological studies have suggested that the hepatocarcinogenesis is closely associated with HBV/hepatitis C virus (HCV) co-infection [Paterlini et al., 1993; Brechot et al., 1998]. Follow-up studies have shown that alanine aminotransferase (ALT) flares are associated with the detection of HBV DNA in the serum of patients with HBV/HCV co-infection [Feitelson et al., 1995; Zignego et al., 1997]. However, the precise HBV DNA status of such patients has not been established, given the small proportion of HBV-DNA-positive cells.

The aim of this study was to clarify the precise status of HBV DNA infection, especially the pattern of integrated HBV DNA, in specimens from patients with acute or occult HBV infections using a highly sensitive PCR-based method (Alu-PCR) [Minami et al., 1995]. We also investigated whether HBV DNA persists in an integrated form in PBMCs. The clonal expansion was examined of HBV-integrated cells during early HBV infection and HBV/HCV co-infections.

MATERIALS AND METHODS

Study Population

The study population consisted of 31 HBV-infected patients, 10 HCV-infected patients, and 3 normal con-

trols (23 males and 21 females) ranging in age from 3 months to 70 years. Among the 31 patients with HBV infections, 8 had acute hepatitis, 8 had fulminant hepatitis, 3 had subacute fulminant hepatitis, and 12 had chronic active hepatitis (Table I). Ten patients had anti-HCV-positive chronic active hepatitis; their serum was negative for HBsAg and positive for an antibody to the HBV core (anti-HBc). Samples from three healthy blood donors with no history of liver disease were used as controls. All patients or their guardians provided their written informed consent, and the Ethics Committee of the Kyoto Prefectural University of Medicine approved all aspects of the study.

Sample Preparation

Peripheral blood mononuclear cells (PBMCs) were prepared from heparinized whole blood using Ficoll-Hypaque (Amersham-Pharmacia, Buckinghamshire, UK) gradient centrifugation, according to the manufacturer's instructions. DNA from liver tissue and PBMCs was extracted using a "G'NOME DNA isolation kit" (BIO 101, Joshua Way, CA), according to the manufacturer's instructions. All samples were carefully handled and stored to avoid contamination by other nucleic acids, and the cells and tissues were stored at -80°C until use. The genomic DNA extracted from Huh2 was kind gift from Dr. Koike [Koike et al., 1983].

Detection of HBV DNA

Genomic DNA was amplified using three independent primer sets targeting the HBV core, S, and X regions (Table II). HBV DNA was amplified from 100 ng of the extracted DNA in a total volume of 50 μl in the presence of 10 pM of each primer and 2.5 U of recombinant Taq (rTaq) polymerase (Toyobo). PCR was performed in a DNA thermal cycler (Perkin Elmer Cetus, Norwalk, CT) with 35 cycles of denaturation at 94°C for 30 sec, annealing at 55°C for 30 sec, extension at 72°C for 1 min and an initial denaturation at 94°C for 2 min, and a final extension at 72°C for 10 min. Sample preparation and mixing were carried out in a different room from the one in which the amplified samples were handled, and a filter pipette was used for all steps. Results were considered as valid only if the same results were obtained in at least two separate experiments.

Detection of Viral-Host Junctions

A PCR-based technique (Alu-PCR) was employed using specific primers for a human Alu sequence and the HBV sequence to detect effectively the viral-host junction, as described previously [Minami et al., 1995]. The strategy of this PCR method is illustrated in Figure 1. The first ten cycles of amplification were undertaken in a thermal cycler in a final volume of 50 μl , containing 100 ng of genomic DNA as a template, 10 pM of Alu primer, 100 pM of HBV primer, and 2.6 U of Taq DNA-Two DNA polymerase mixed with an ExpandTM High Fidelity assay kit (Roche, Mannheim, Germany). The reaction was carried out as a "hot start" PCR using the Taq start antibody (Clontech, Palo Alto, CA). The

TABLE I. Baseline Characteristics of the Patients

Sex	Age	Diagnosis	HBsAg	Anti-HBs	HBeAg	Anti-HBe	Anti-HBc	IgM-HBc	Anti-HCV	Anti-HIV
1	F	49	Fulminant hepatitis	+	-	-	+	NT	+	-
2	F	9		+	-	-	+	+	-	-
3	F	3m		+	-	-	+	+	-	-
4	M	54		+	-	-	+	NT	+	-
5	M	44		+	-	-	+	+	-	-
6	F	48		+	-	-	+	+	-	-
7	F	51		+	-	+	-	+	-	-
8	F	18		+	-	-	+	+	-	-
1	M	46	Subacute fulminant hepatitis	-	-	-	+	+	+	-
2	M	19		-	-	-	+	+	NT	-
3	F	51		-	-	NT	NT	+	+	-
1	M	29	Acute hepatitis	+	-	-	+	+	NT	-
2	M	37		+	NT	NT	NT	NT	NT	-
3	F	52		+	+	-	-	+	+	-
4	M	70		+	NT	+	-	+	+	-
5	M	28		+	-	-	-	+	+	-
6	M	59		+	-	-	+	+	NT	-
7	M	37		+	-	-	+	+	+	-
8	M	46		+	+	-	-	-	+	-
1	M	36	HBV-related chronic active hepatitis	+	NT	NT	NT	NT	NT	-
2	M	29		+	NT	-	-	NT	NT	-
3	M	31		+	NT	-	+	NT	NT	-
4	F	33		+	NT	NT	NT	NT	NT	-
5	M	51		+	NT	-	+	NT	NT	-
6	F	44		+	NT	+	+	NT	NT	-
7	F	15		+	NT	NT	NT	NT	NT	-
8	M	46		+	-	-	+	-	-	-
9	M	31		+	-	-	+	NT	-	-
10	M	35		+	-	-	+	+	NT	-
11	F	20		+	NT	-	-	NT	NT	-
12	M	45		+	-	+	-	+	NT	-
1	F	56	HCV-related chronic active hepatitis	-	+	NT	NT	+	NT	+
2	M	38		-	-	NT	NT	+	NT	+
3	M	63		-	+	NT	NT	-	NT	+
4	F	52		-	+	NT	NT	+	NT	+
5	F	57		-	+	NT	NT	+	NT	+
6	F	62		-	+	-	+	+	NT	+
7	F	37		-	-	NT	NT	+	NT	+
8	M	40		-	+	NT	NT	+	NT	+
9	F	67		-	-	NT	NT	+	NT	+
10	F	62		-	+	NT	NT	+	NT	+
1	M	34	Healthy volunteer	-	-	-	-	-	-	NT
2	F	24		-	-	-	-	-	-	NT
3	F	22		-	-	-	-	-	-	NT

NT, not tested; M, male; F, female.

cycling conditions consisted of denaturation for 30 sec at 94°C, annealing for 30 sec at 59°C, and extension for 3 min at 70°C, with an initial denaturation period of 1 min at 94°C. One unit of uracil DNA glycosylase (UDG) (GIBCO/BRL, Paisley, UK) was then added to each of the tubes, and the tubes were incubated for 30 min at 37°C. After heating for 10 min at 94°C to break the DNA strands at apurinic dUTP sites, 10 pM of each primer were added for the next amplification.

The "touchdown" PCR technique was employed for this amplification [Don et al., 1991]. Denaturation was carried out at 94°C for 30 sec and extension at 70°C for 3 min. The annealing step was started at 65°C for 30 sec; the temperature was then reduced by 1°C every second cycle until a temperature of 55°C was reached, at which point 20 cycles has been carried out. The final extension was carried out for 8 min at 72°C. Thus, a total of 40 cycles were made, and 1 µl of the product was subjected

TABLE II. Sequence of Primers

Code	Primer sequence	Position (HBV)	Note
MM6	5'-AGGTATGTTGCCCGTTTGTCT-3'	459-480	HBV S
MM7	5'-CAGCAAAGCCCAAAGACCCAC-3'	1001-980	HBV S
MM37	5'-TGCCAAGTGTGCTGACGC-3'	1174-1193	HBV X
MM26	5'-GTTACGGTGGTCTCCATG-3'	1627-1609	HBV X
HW1	5'-CCGAAAAGCTT GAGCTCTTCTTTTTCACCTCTGCCTAAT-3'	1818-1839	HBV core
MM31	5'-AGTGCGAATCCACACTC-3'	2288-2269	HBV core
MM24	5'-TGCCAAGTGGATCCTTCGCGGGACGTCCTT-3'	1392-1423	ccc Ditection (1st)
MM25	5' GGAAGGAAAGAAGTCAGAAGG-3'	1978-1960	ccc Ditection (2nd)
HW2	5'-CCGAAAAGCTT GAGCTCTTCAAAAAGTTGCATGGTGCTG-3'	1826-1807	ccc Ditection
UP5	5'-CAGUGCCAAGUGUUUGCUGACGCCAAAGUGCUGGGAUUA-3'		Alu-sense
T3-515	5'-AUUAACCCUCACUAAAAGCCUCGAUAGAUYRYRCCAYUGCAC-3'		Alu-antisense
UP6	5'-CAAGTGTTGCTGACGCCAAAG-3'		Alu-sense (tag)
midT3	5'-ATTAACCTCACTAAAGCCTCG-3'		Alu-antisense (tag)
pUTP	5'-ACAUGAACCUUUACCCCGUUGC-3'	1131-1152	HB1 (HBV X)
MM37			HB2 (HBV X)
MM60	5'-CTGCCGATCCATACTGCGGAAC-3'	1258-1279	HB3 (HBV X)
uPre 31	5'-GAGUUCUUCUUCUAGGGGACCUG-3'	2350-2328	HB1 (HBV core)
MM31			HB2 (HBV core)
MM25			HB3 (HBV core)

Numbering of nucleotides is according to Ono et al. [1983].

Underlined bases represent added restriction enzyme sites. U = dUTP; Y = (C,T); R = (A,G).

to hemi-nested PCR with the initial primers to obtain discrete bands.

Amplified PCR products were analysed by electrophoresis on 1.0% agarose gel and transferred to a Hybond-N⁺ nylon membrane (Amersham-Pharmacia,

Buckinghamshire, UK). To prepare a total HBV probe, the total HBV genome was amplified according to the method of Günther et al. [1995], and the HBV-specific bands were detected by hybridisation with a DIG-labelled probe (Roche).

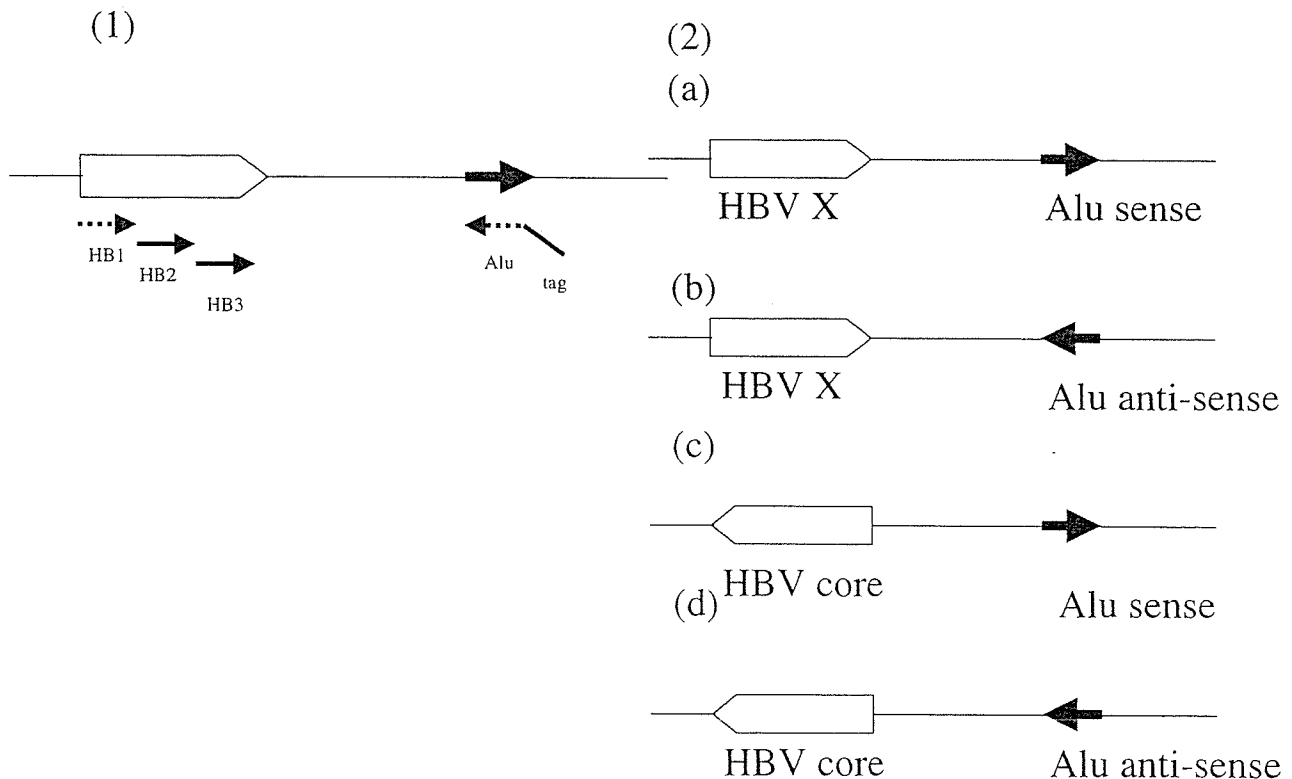


Fig. 1. 1: Technical protocol for the detection of the viral-host junction. The small broken arrows and small intact arrows represent the primer that synthesizes dUTP instead of dTTP and the usual primer, respectively. 2: Four Alu-PCR primer pairs. The locations of the primers are as follows: HBV X region and Alu-sense, HBV X region and Alu-antisense, HBV core region and Alu-sense, and HBV core region and Alu-antisense. The open boxes, lines, and large arrows represent the integrated HBV sequence, genomic sequence, and Alu sequence, respectively.

Direct sequencing. The sequences of the viral host junction were determined from the PCR products using the dideoxy chain termination method. After removing excess primer and deoxyribonucleotides using an Easy trap kit (Takara, Otsu, Japan), the DNA was subjected to a sequencing reaction using the Prism Taq DyeDeoxy Terminator cycle sequencing kit (Applied Biosystems, Inc., Foster City, CA), according to the manufacturer's instructions. The sequencing products were precipitated with ethanol and then subjected to electrophoretic separation and analysis with a 377 Prism DNA sequencer (Applied Biosystems, Inc.).

Detection of Covalently Closed Circular (ccc) HBV DNA

The amplification method used in this study was similar to that employed to identify ccc duck hepatitis B virus (DHBV) DNA [Mason et al., 1998]. A 100 ng sample of genomic DNA was amplified in a 50 μ l reaction mixture containing 10 pM of each primer (MM24 and MM25) and 2.5 U of rTaq DNA polymerase (Toyobo). Thirty-five cycles of amplification were performed (94°C for 30 sec, 53°C for 30 sec, and 72°C for 2 min), with an initial denaturation at 94°C for 1 min and a final extension at 72°C for 10 min in a thermal cycler. One microliter of the first PCR product was amplified with 10 pM of hemi-nested primer (MM24 and HW2).

RESULTS

Sensitivity of PCR and Alu-PCR

To assess the sensitivity of the PCR technique, cloned HBV DNA was diluted serially with double distilled water. A mixture of HBV DNA diluted serially and 100 ng of human genomic DNA from the peripheral blood mononuclear cells (PBMCs) of control subjects without any history of liver disease was used as the template. The fifth dilution (10⁻² pg/100 ng of genomic DNA) was detected using each primer pair (HBV-core, S, and X regions) (data not shown). Alu-PCR sensitivity was determined using a mixture of the DNA from the hepatoma cell line Huh2, containing one copy of integrated HBV DNA per cell [Koike et al., 1983] and human genomic DNA. A 10-fold serial dilution of Huh2 DNA was also prepared; the third dilution (10² cells) was detected. Thus, the method can be used to detect 100 copies of integrated HBV DNA sequence in a reaction mixture (data not shown).

Detection and Sequence Analysis of Integrated HBV DNA in the Liver Tissues During Acute Infection

HBV DNA integration was detected by Alu-PCR in the liver cells from two of the eight patients with acute hepatitis (patients 4, 8; Fig. 2). The structures of the viral-host junctions and the cellular flanking sequence were examined using the BLAST search system. In case 8, HBV DNA was integrated into the intronic sequence of the tumour necrosis factor (TNF)-induced protein gene (between the second and third

exon) (Accession no.: XM090935.1), while in case 4, the HBV DNA was integrated into a repetitive sequence. HBV DNA integration was detected in one of the three patients with subacute fulminant hepatitis (case 1); this patient had two viral-host junctions. In one, HBV DNA was integrated into the intronic sequence of a hypothetical protein gene (LOC169443) (between fifth and sixth exon); in the other, it was integrated into a repetitive sequence (Fig. 3a). HBV DNA integration was not detected in any of the other 8 patients with fulminant hepatitis (Table III).

Detection of HBV DNA Integration in the Liver Tissue of Patients With HBV-Related Chronic Active Hepatitis and HCV-Related Chronic Active Hepatitis

HBV DNA integration was demonstrated in the liver of all 12 patients with HBV-related chronic active hepatitis using Alu-PCR (Table III). Several hybridised bands of polyclonal integration were observed as a smear on autoradiography (Fig. 2). Two of the 10 patients with HCV-related chronic active hepatitis (cases 3 and 6) also exhibited HBV DNA integration (Table III).

Detection of HBV DNA in PBMCs

HBV DNA integration in PBMCs was investigated in 17 patients (7 with HBV-related chronic active hepatitis and 10 with HCV-related chronic active hepatitis). HBV DNA integration was detected in the PBMCs from four of the HBV-related chronic active hepatitis patients (cases 1, 3, 6, and 7) (Table IV and Fig. 3) and from two of the HCV-related chronic active hepatitis patients (cases 4 and 6). The structure of the viral-host junctions was examined using the BLAST search system. In HBV-related chronic active hepatitis (case 1), the HBV DNA was integrated 73 kb downstream of the inositol 1,4,5-triphosphate 3-kinase B (ITPKB) gene (Accession no.: NM002221.1). In HBV-related chronic active hepatitis (case 6a), the viral genome was integrated into the intronic sequence of the gene for an unnamed protein (LOC144362) between eighth and ninth exon (Accession no.: XM038659.3). In HBV-related chronic active hepatitis (case 7), the HBV DNA was integrated 4 kb downstream of a member of the RAS oncogene family (RAB30) (Accession no.: XM006025.4). In HCV-related chronic active hepatitis (case 6), the HBV DNA was integrated into the intronic sequence of the gene for a hypothetical protein (FLJ 12303) between the second and third exon (Accession no.: XM052594.1) (Table III). In the remaining cases, the viral DNA was integrated repetitive sequences. One HCV-related chronic active hepatitis patient (case 4) showed a rearranged viral sequence, and the host junction DNA sequence showed no homology to the EMBL/Genbank sequences (Fig. 3b).

Forms of HBV DNA in Serum, Liver, and PBMCs

Three primer pairs (for the core, S, and X regions) were used to detect HBV DNA. In all seven HBV-related

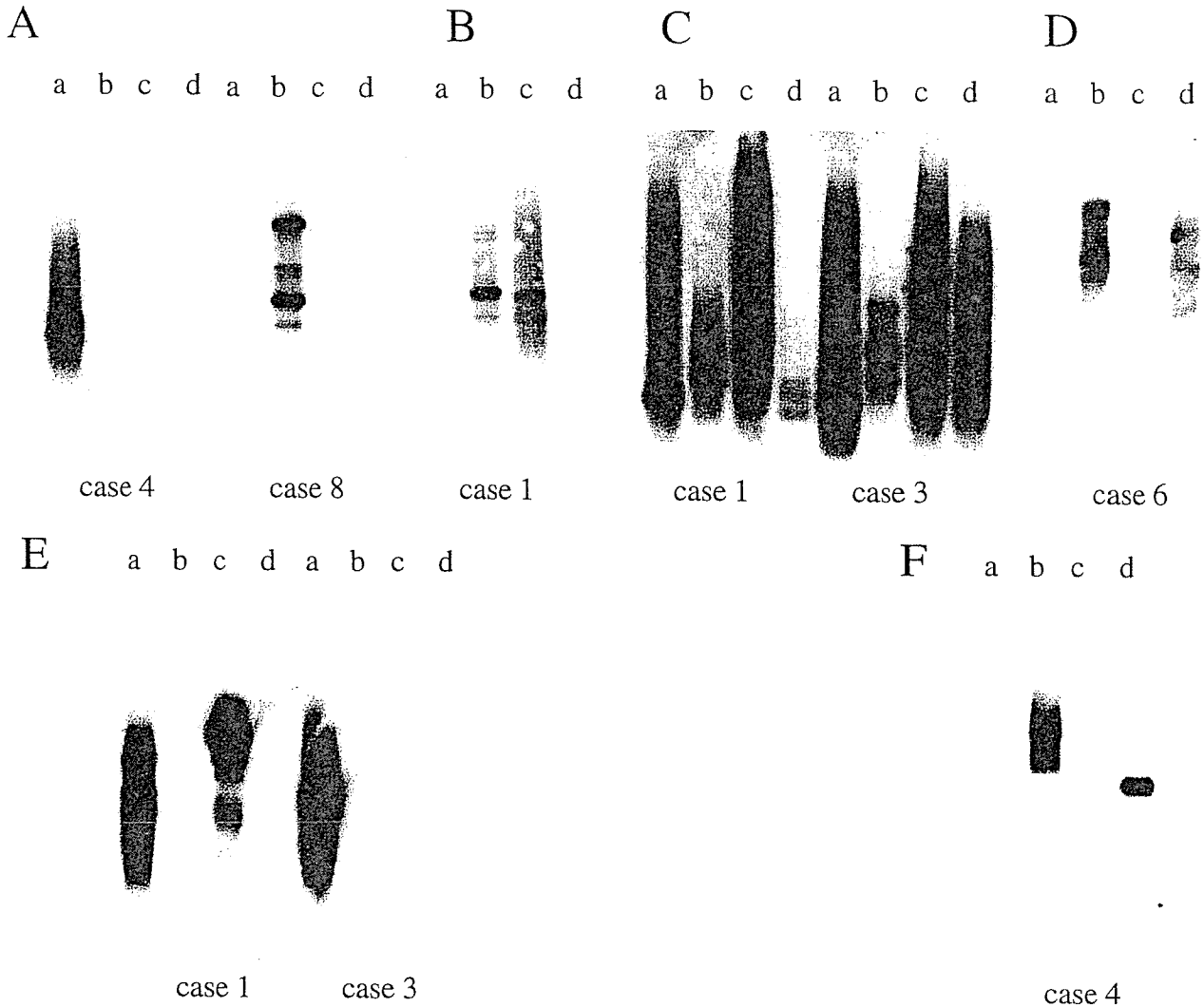


Fig. 2. Detection of viral-host junctions by Alu-PCR. A-D: The HBV hybridised bands in liver specimens from acute hepatitis, subacute fulminant hepatitis, HBV-related chronic active hepatitis, and HCV-related chronic active hepatitis patients, respectively. E and F: The HBV hybridised bands in PBMCs from HBV-related chronic active hepatitis, and HCV-related chronic active hepatitis patients, respectively. Lanes a, b, c, and d represent the primer pairs corresponding to Figure 1.

chronic active hepatitis patients, HBV DNA was detected in the serum using all three-primer sets. HBV DNA was also detected in the PBMCs of all of these patients. However, HBV DNA was only detected using one or two primer sets in four cases of PBMCs (case 2, 3, 5, and 6); the other three cases had detectable levels of HBV DNA using all primer sets. Among the HCV-related chronic active hepatitis patients, HBV DNA was detected using all three primer sets in sera from cases 2 and 7, in liver tissue from cases 1, 2, 5, 7, and 9, and in PBMCs from cases 2 and 9. HBV DNA was only identified using one or two primer sets in the other cases (Table IV).

Finally, the presence of covalently closed circular (ccc) HBV DNA was examined in the serum, liver, and PBMC samples. ccc HBV DNA was detected in the liver tissue of all seven patients with HBV-related chronic active hepatitis and in one patient (case 1) with HCV-related chronic active hepatitis, but ccc HBV DNA was not

detected in the PBMCs from four of the seven patients with HBV-related chronic active hepatitis (cases 2, 3, 4, and 6) or any of the 10 patients with HCV-related chronic active hepatitis, nor was it detected in any of the sera samples (Table IV).

DISCUSSION

Alu-PCR is more sensitive for detecting viral-host junctions than Southern hybridisation and has several other advantages as well. This PCR-based method can effectively amplify the viral-host junction in small samples, such as liver tissue specimens obtained by needle biopsy. This PCR reaction can also be carried out in a single tube and without ligation; as a result, there is less chance of cross-contamination. The Alu-specific and HBV-specific primers provide a fragment containing a hybrid of the viral and cellular sequence, and nonspecific amplification of viral or host sequences can be avoided.

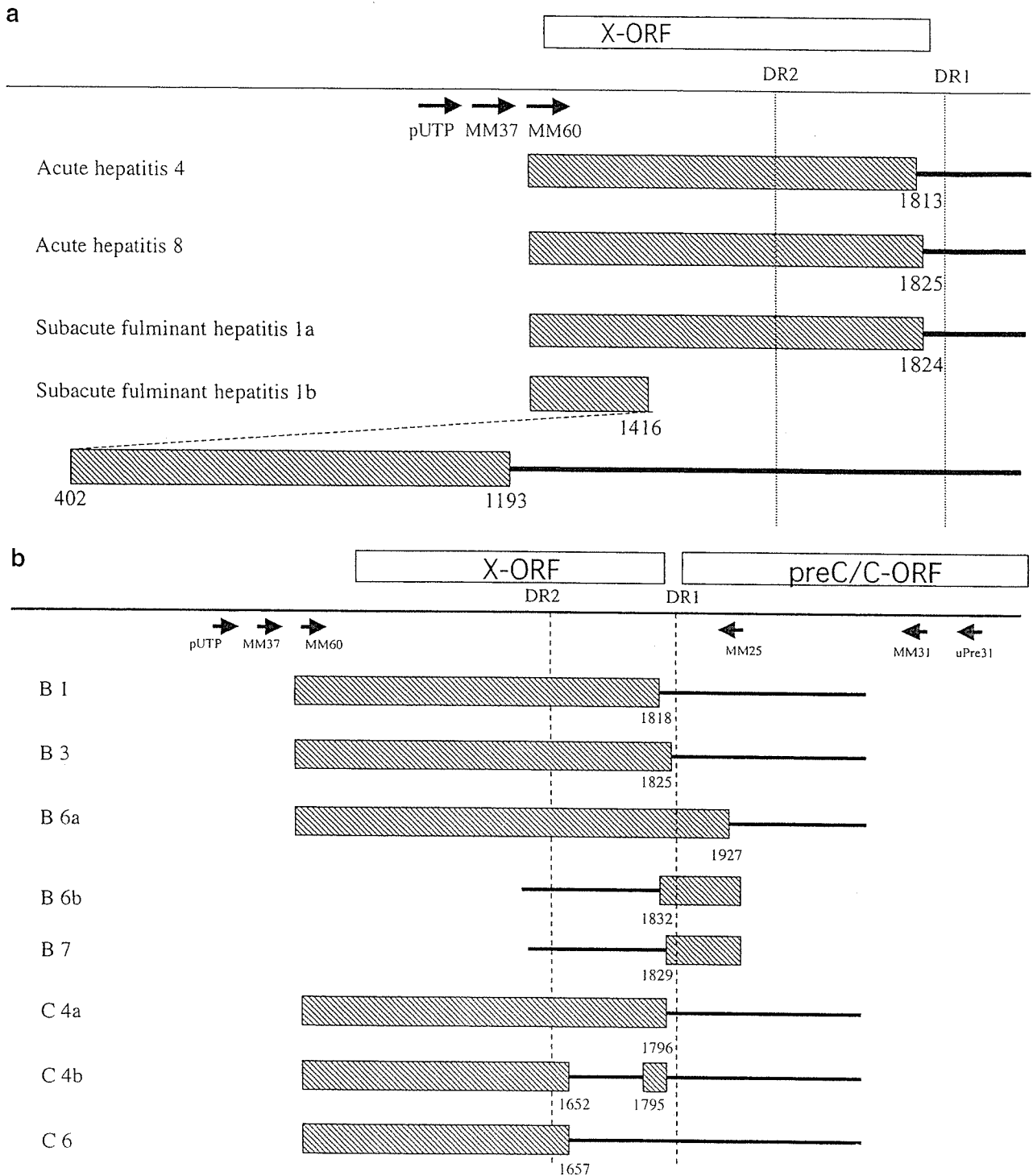


Fig. 3. Structure analysis of the viral-host junctions in liver (a) and PBMCs (b). B and C: HBV-related chronic active hepatitis and HCV-related chronic active hepatitis, respectively. The open boxes represent the HBV open reading frame. X and preC/C correspond to HBV X and precore/core region, respectively. The hatched box, thick

line, and arrow represent the HBV sequence, cellular flanking sequence, and primer, respectively. The dotted lines represent rearrangements of the HBV sequence. The nucleotide numbers of the virus pointing at the junctions are presented under the sequence, numbering from the hypothetical EcoRI site of subtype adr [Ono et al., 1983].

The detection limit of this method is about 100 copies of the same viral-host junction per reaction. Positive results for integrated HBV DNA with this method indicate the presence of more than 100 clonally expanded cells with HBV DNA integration.

The present study provides the first definite demonstration of viral DNA integration during the acute stage of HBV infection, both in liver tissues and peripheral blood mononuclear cells (PBMCs). The rates of viral integration detection in acute hepatitis, HBV-related

TABLE III. Summary of HBV DNA Integration

Tissue	Clinical diagnosis	Case no.	HBV integration	Gene/type of sequence	Accession no.	Supercontig (position)	Orientation	Chromosomal localisation
Liver	Fulminant hepatitis	1	-					
		2	-					
		3	-					
		4	-					
		5	-					
		6	-					
		7	-					
		8	-					
	Subacute fulminant hepatitis	1a	+	Intronic sequence of hypothetical protein (LOC 169443)	XM_095704.1	NT_017568.8 (642470)	Same	9q33
	Fulminant hepatitis	1b	+	Repetitive sequence				
2		-						
3		-						
	Acute hepatitis	1	-					
2		-						
3		+		Repetitive sequence				
4		-						
5		-						
6		-						
7		-						
8		+			Intronic sequence of TNF-induced protein	XM_090935.1	NT_010194.8 (1832076)	Opposite
	HBV-related chronic active hepatitis	1	+					
2		+						
3		+						
4		+						
5		+						
6		+						
7		+						
8		+						
9		+						
10		+						
11		+						
12		+						