2.3. Subgroup analysis

Baseline HCV RNA levels were categorized into three groups: 100 to <500; 500 to <850; and ≥850 kcopies/mL as determined by Amplicor HCV monitor assay. Disappearance of virus and relapse were judged by qualitative Amplicor assay. The time of initial HCV RNA negativity was recorded as the measurement time point (4, 12, and 24 weeks after the start of treatment) at which negativity was first observed; the time of initial relapse was the three measurement time point (4, 12, and 24 weeks after the end of treatment) at which HCV RNA was first detected in patients who achieved HCV RNA negativity during the treatment period. Patients who remained HCV RNA-negative for 6 months after the end of treatment were considered to have achieved SVR.

2.4. Statistical analysis

HCV RNA negativity rate, SVR rate, and relapse rate were compared by baseline viral load between the combination treatment and IFN monotherapy groups by Mantel–Haenzel test using modified RIDIT scores after the lack of interactions in efficacy was confirmed by the Breslow–Day test. Logistic regression analysis was used to identify factors contributing to initial HCV RNA negativity and SVR. The degree of risk of relapse was analyzed using the proportional hazards and grouped exponential models. Intergroup differences in patient profiles were tested by Fisher's exact test, Wilcoxon–Mann Whitney test, and Mantel–Haenzel test. $P < 0.05$ was regarded as statistically significant (two-sided). All calculations were performed by SAS program version 6.12 (SAS Institute, Cary, NC).

3. Results

3.1. Patient characteristics

Table 1 shows the main characteristics of the 209 patients in the combination treatment and 125 patients in the IFN monotherapy groups. In the study in nonresponders and relapsers to previous IFN therapy [26] 41 and 40 patients were allocated to the combination treatment and IFN monotherapy groups, respectively; in the study in patients with genotype 1b and high viral titers, the numbers were 168 and 85, respectively (i.e. 2:1 randomization) [25]. A total of 107 patients (51%) in the combination treatment and 68 (54%) in the IFN monotherapy group had HCV RNA levels ≥850 kcopies/mL. About half of patients in both treatment groups were relapsers after previous IFN therapy. Forty-nine patients (23%) in the combination treatment and 24 (19%) in the IFN monotherapy group had not received prior IFN therapy. No imbalance was observed in background variables between the two groups.

Table 1
Baseline patient characteristics

	IFN + ribavirin	IFN	P value
No. of patients	209	125	–
Sex (male/female)	164/45	94/31	0.503[a]
Mean age (years)	48	49	0.539[b]
Viral load (kcopies/mL)			0.792[c]
Low (<500)	23.0% (48)	24.8% (31)	
Moderate (500 to <850)	25.8% (54)	20.8% (26)	
High (≥850)	51.2% (107)	54.4% (68)	
Previous IFN therapy			0.295[a,d]
Treatment-naive	23.4% (49)	19.2% (24)	
Relapsers	50.7% (106)	50.4% (63)	
Nonresponders	22.5% (47)	30.4% (38)	
Unknown	3.3% (7)	0	

[a] Fisher test.
[b] U-test.
[c] Mantel–Haenzel test.
[d] Excluding unknown.

3.2. Response to therapy

The SVR rate was 18% (38/209) with IFN and ribavirin combination therapy and 2% (2/125) with IFN monotherapy. The results of subgroup analysis by baseline viral levels are shown in Table 2. Patients receiving IFN and ribavirin combination therapy had a significantly higher chance for SVR than those receiving IFN monotherapy at any baseline viral level.

3.3. Initial viral negativity

In patients with viral titers of 500 to <850 kcopies/mL, initial viral negativity occurred in 20% by the first 4 weeks, in 46% by 12 weeks, and in 17% by 24 weeks of treatment in the combination therapy group (Fig. 1a). In the IFN monotherapy group the figures were 15%, 31%, and 12%, respectively (Fig. 1b). In patients with viral titers of ≥850 kcopies/mL, the HCV negativity rates at the same time points were 11%, 50%, and 20%, respectively, in the combination therapy group (Fig. 1a) and 1%, 28%, and 15%, respectively, in the monotherapy group (Fig. 1b). The time to initial viral negativity was slightly earlier in patients with viral titers of <500 kcopies/mL (42%, 25%, and 6% at the same time points, respectively) than in those with ≥500 kcopies/mL in the combination therapy group (Fig. 1a). Logistic regression analysis indicated that low HCV RNA levels and high ALT and creatinine levels before treatment are factors related to achieving HCV RNA negativity by week 4 of combination therapy. High baseline creatinine level was associated

Table 2
SVR rate by baseline viral load

Viral load (kcopies/mL)	IFN + ribavirin ($n = 209$)	IFN ($n = 125$)	P value
Low (<500)	29% (14/48)	0 (0/31)	0.001
Moderate (500 to <850)	17% (9/54)	8% (2/26)	0.001
High (≥850)	14% (15/107)	0 (0/68)	0.001

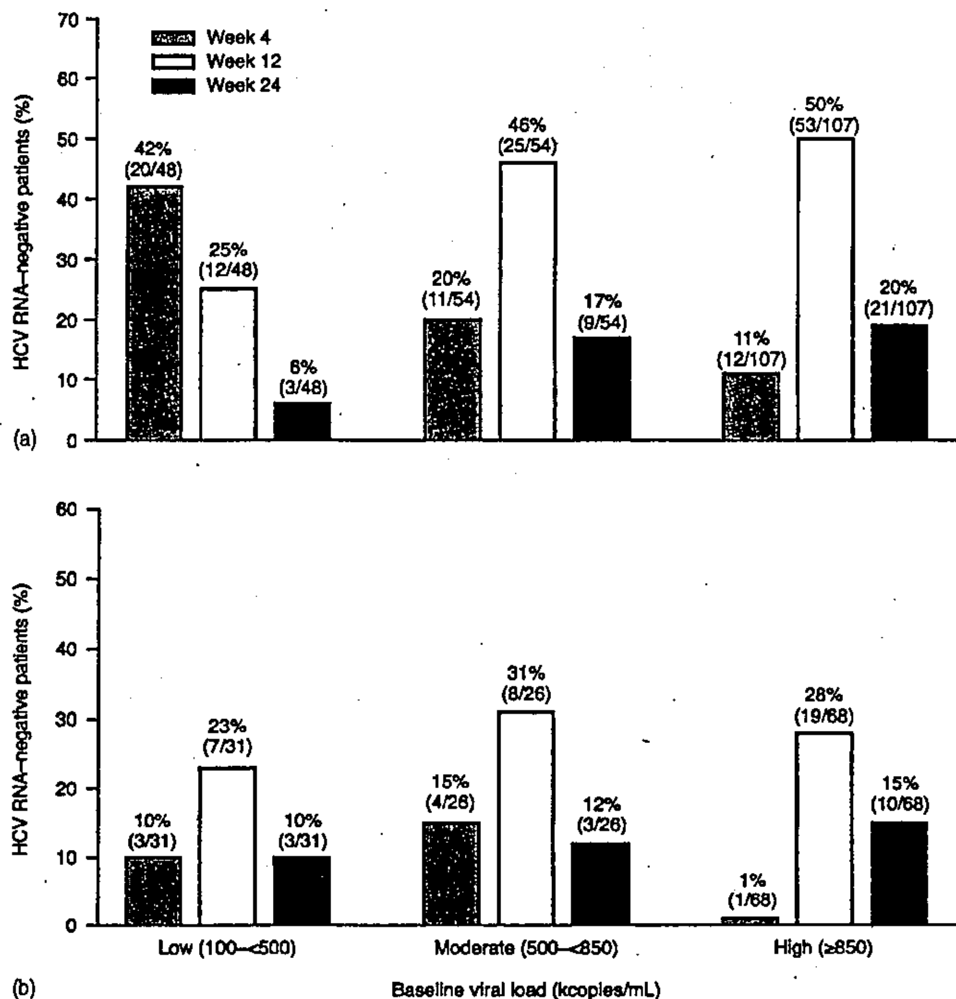

Fig. 1. Percentage of patients testing HCV RNA negative receiving combination therapy (a) and monotherapy (b). Patient numbers are in parentheses.

with achieving HCV RNA negativity by week 12 of treatment

3.4. *SVR by baseline viral level and timing of initial viral negativity*

Fig. 2 shows the SVR rate with respect to the timing of initial viral disappearance for each baseline HCV level. In patients with <500 kcopies/mL, SVR was observed only in those HCV RNA-negative within 4 weeks after the start of combination treatment, with a high SVR rate of 70% (14/20). However, in patients HCV RNA-negative by week 12 or 24, SVR was not observed in either treatment group. In the IFN monotherapy group, three patients were HCV RNA-negative within 4 weeks and the SVR rate was 0% (0/3). Among patients with 500 to <850 kcopies/mL, SVR was observed in 55% (6/11) and 12% (3/25) of patients HCV RNA-negative within 4 and 12 weeks of the start of combination treatment, respectively, and in none of the nine patients HCV RNA-negative within 24 weeks. Among patients treated with IFN alone, SVR was observed only in 50% (2/4) of patients HCV RNA-negative within 4 weeks. In patients with ≥850 kcopies/mL at baseline, SVR was observed in the combination treatment group in 42% (5/12) and 19% (10/53) of patients HCV RNA-negative within 4 weeks and 12 weeks, respectively. However, SVR was not seen in any of the 21 patients HCV RNA-negative within 24 weeks. SVR was not observed in patients with viral levels ≥850 kcopies/mL treated with IFN alone.

In patients HCV RNA-negative within 4 weeks, low baseline viral levels and high body weight were factors contributing to SVR; in those who were HCV RNA-negative within 12 weeks, low baseline viral levels and high baseline platelet count were contributing factors.

3.5. *Relapse rate after end of treatment by baseline viral levels*

In the combination treatment group, the relapse rate was 60% (21/35), 80% (36/45), and 83% (71/86) and in the IFN (-2b alone group 100% (13/13), 86% (13/15), and 100% (30/30) in patients with baseline viral levels <500, 500 to

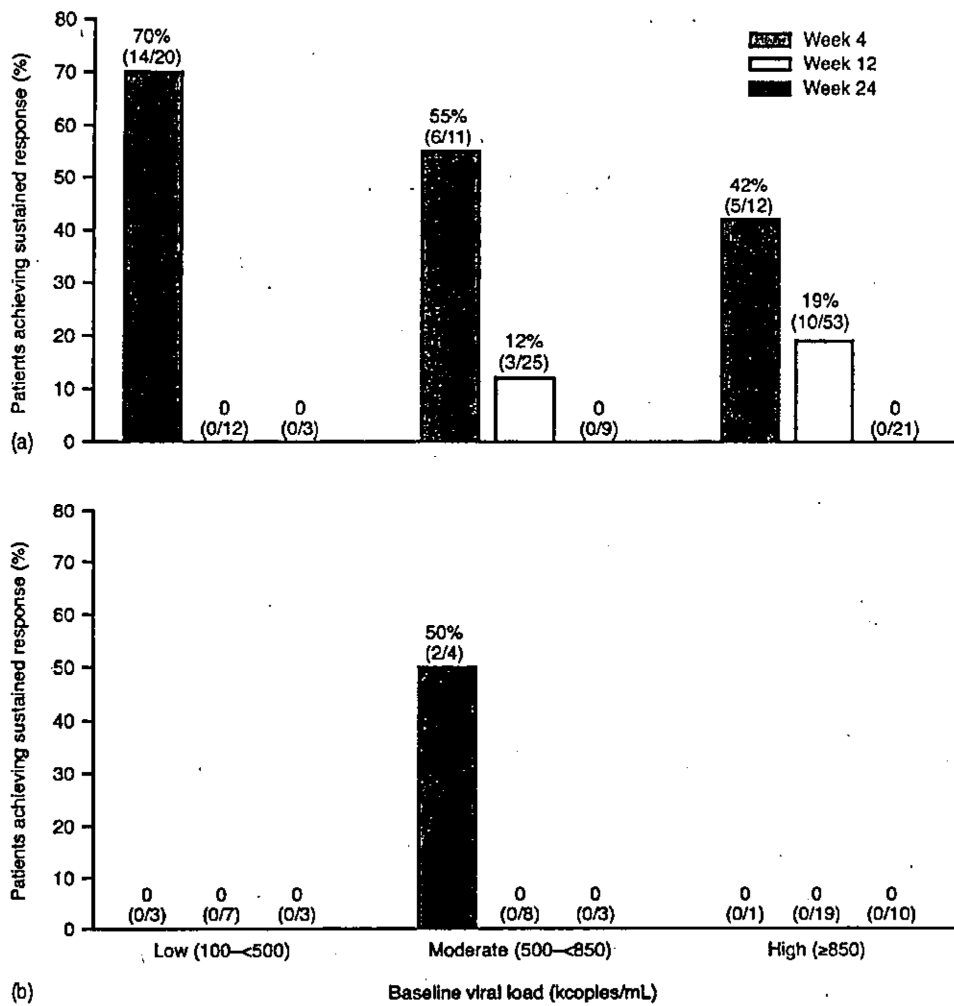

Fig. 2. Sustained response rate in patients receiving combination therapy (a) and monotherapy (b). Patient numbers are in parentheses.

<850, and ≥850 kcopies/mL, respectively (Table 3). Patients in the combination treatment group were 3.7 times (95% CI 1.9–7.3; $P < 0.001$) less likely to relapse than those in the IFN monotherapy group. If the probability of relapse in patients with <500 kcopies/mL is set at 1, then probability was 1.5 (95% CI 0.8–2.9) in patients with 500 to <850 kcopies/mL and 2.0 (95% CI 1.1–3.5) in patients with ≥850 kcopies/mL. However, baseline viral level was not a significant factor ($P = 0.3441$) with regards to risk of relapse. On the other hand, patients HCV RNA-negative within 12 and 24 weeks after the start of treatment were 4.8 (95% CI 2.8–8.1) and 10.3 (95% CI 5.4–19.7) times, respectively, more likely to relapse after the end of treatment than patients HCV RNA-negative within the first 4 weeks ($P < 0.001$).

Table 3
Rate of relapse at 6 months by baseline viral load

Viral load (kcopies/mL)	IFN + ribavirin ($n = 209$)	IFN ($n = 125$)	P value
Low (<500)	60% (21/35)	100% (13/13)	0.001
Moderate (500 to <850)	80% (36/45)	87% (13/15)	0.001
High (≥850)	83% (71/86)	100% (30/30)	0.001

The relative risk of relapse after the end of treatment by initial viral level and time of first viral negativity is shown in Table 4. The risk of relapse in the combination group versus in the IFN monotherapy group was significantly lower by a factor of 0.3 (95% CI 0.1–0.9) and 0.5 (95% CI 0.3–0.8) in patients HCV RNA-negative within 4 weeks ($P = 0.032$) and 12 weeks ($P = 0.011$), respectively. Regarding baseline HCV levels, in patients HCV RNA-negative by 12 weeks, the

Table 4
Relative risk of relapse at 6 months (95% CI)

	First HCV RNA-negative test result		
	4 weeks	12 weeks	24 weeks
Treatment group			
IFN + ribavirin	0.3 (0.1–0.9)*	0.5 (0.4–0.9)*	1.2 (0.6–2.2)
IFN	1	1	1
Baseline viral load (kcopies/mL)			
Low (<500)	1	1	1
Moderate (500 to <850)	0.9 (0.4–2.7)	0.6 (0.4–1.2)	0.8 (0.3–2.3)
High (≥850)	2.1 (0.8–5.6)	0.5 (0.3–0.9)†	0.9 (0.4–2.2)

* $P < 0.05$ vs. monotherapy.
† $P < 0.05$ vs. low baseline viral load.

hazard for relapse was significantly higher (odds ratio: 0.5; $P = 0.021$) in patients with ≥850 kcopies/mL than in those with <500 kcopies/mL. In patients HCV RNA-negative by 24 weeks, no effect on hazard for relapse was observed by treatment group or viral levels.

The relationship between baseline HCV levels and the time of relapse by time of initial HCV negativity is shown in Table 5. The relapse rate in patients HCV RNA-negative within 4 weeks with combination treatment was ≤18% at 4, 12, and 24 weeks after the end of treatment regardless of baseline viral level. In the IFN(-2b alone group, almost all patients relapsed soon after the end of treatment even when HCV RNA-negativity occurred within the first 4 weeks after the start of treatment. The circumstance of relapse in patients HCV RNA-negative within 12 weeks of the start of treatment differed from that in patients HCV RNA-negative within 4 weeks. In those receiving combination treatment, relapse was seen within 4 weeks in 11 (92%), 13 (52%), and 30 (57%) patients whose baseline viral levels were <500, 500 to <850, and ≥850 kcopies/mL, respectively. Relapse within 12 weeks was seen in 1 (8%), 5 (20%), and 13 (26%) patients, respectively. However, even with combination treatment, most patients who first became HCV RNA-negative after 12 weeks from the start of treatment relapsed within 4 weeks after the end of treatment (data not shown)

4. Discussion

In Japan, various IFN regimens for the treatment of CHC have been tried. Under the Japanese health insurance system, the duration of treatment was restricted to 6 months at the time that the present study was conducted. Standard treatment comprises high doses of IFN (6–10 MIU) administered daily in the initial stage of treatment followed by further doses at three times/week for ≤6 months with the aim of eradicating the virus [3]. In 1998, remarkable improvement in efficacy was reported when ribavirin is added to IFN α-2b [21–23], and clinical studies of IFN α-2b plus ribavirin combination therapy were initiated in Japan. Outside Japan, the standard treatment regimen with IFN α-2b was 3 MIU administered three times/week; for combination therapy, ribavirin was added to this standard regimen. The clinical studies in Japan were likewise conducted with ribavirin (600 or 800 mg/day depending on body weight) added to the standard Japanese regimen. When SVR rates by baseline viral levels were compared, combination therapy was superior to monotherapy at all viral levels. A number of reports have been published concerning the timing of first HCV RNA disappearance and its effect on the SVR rate [13–18,27]. To date, however, no study of the SVR rate analyzed in relation to HCV RNA levels has been published. The present study suggests that the timing of first disappearance of HCV RNA is significantly affected by baseline HCV RNA levels. In patients with low baseline viral levels (100 to <500 kcopies/mL), 42% became HCV RNA-negative in comparison with only 11% with high viral levels (≥850 kcopies/mL) following 4 weeks' combination therapy. The study suggests that >4 weeks' treatment is required to achieve HCV RNA negativity in patients with viral levels >500 kcopies/mL. Moreover, with IFN alone the proportion of patients achieving HCV RNA negativity within 4 weeks was especially low among those with HCV genotype 1b and high viral levels; >4 weeks' treatment is required to achieve HCV RNA negativity in this group. Vrolijk et al. [5] reported that when ribavirin was administered in combination with IFNα-2b, HCV RNA negativity was observed by week 4 in nearly half of patients and all patients achieved SVR when treatment was continued for 1.5 years. Tassopoulos et al. [8] reported that when ribavirin was administered in combination with 10 MIU IFNα-2b for 8 weeks, almost half of patients achieved HCV RNA negativity. Treatment was continued thereafter for 48 weeks, and the final SVR rate was roughly 25%. The differences between these studies conducted outside Japan and our results may be explained by the high viral levels in our patients. We also noted that low HCV RNA, high ALT, and high creatinine levels before the start of dosing were factors associated with early disappearance of HCV RNA after treatment was initiated. High serum creatinine levels are related to high serum ribavirin concentrations [28], and this may explain early HCV RNA disappearance.

Kasahara et al. [29] compared the results of 6-month and 1-year treatment and reported that judging from the degree of improvement in ALT, longer duration of treatment with IFN monotherapy may inhibit relapse after the end of treatment. However, no significant difference of SVR rate in CHC patients with genotype 1b and high viral levels between 52 weeks and 78 weeks treatment with IFN monotherapy was reported [30]. Poynard et al. [22] reported that the relapse rate with combination therapy after 48 weeks of treatment in patients not previously treated with IFN and including patients with HCV genotypes other than genotype 1 was significantly lower than after 24 weeks of treatment. McHutchison et al. [23] also reported a similar trend. Portal et al. [6] compared the relapse rate in HCV genotype 1 patients with high viral levels treated with IFN plus ribavirin for 1 year or IFN plus ribavirin for 6 months followed by IFN monotherapy for 6 months, and observed a significantly higher relapse rate in the latter group, indicating the importance of duration of combination treatment in reducing the relapse rate. Although our study of 6-month combination treatment in genotype 1 patients was not adequate to analyze the effects of duration of treatment, our analysis of HCV RNA levels in relation to relapse revealed that relapse is much more likely in patients with high rather than low baseline viral levels. Furthermore, compared with in patients who were HCV RNA negative within 4 weeks, the relative risk for relapse is significantly higher in patients HCV RNA-negative at both 4–12 weeks and 13–24 weeks after the start of treatment. Relative risk of relapse is also reduced by about 0.5 with combination therapy compared with monotherapy. Moderate antiviral effects of ribavirin remaining in the body for long periods after end of

Table 5
Relapse rate by baseline HCV level in patients HCV RNA-negative within 4 weeks and within 12 weeks

	Baseline viral load in patients receiving combination therapy (kcopies/mL)			Baseline viral load in patients receiving monotherapy (kcopies/mL)		
	Low (<500)	Moderate (500 to <850)	High (≥850)	Low (<500)	Moderate (500 to <850)	High (≥850)
(a) HCV RNA-negative within 4 weeks						
Relapse						
4 weeks	15% (3/20)	18% (2/11)	17% (2/12)	67% (2/3)	50% (2/4)	100% (1/1)
12 weeks	5% (1/20)	9% (1/11)	17% (2/12)	0 (0/3)	0 (0/4)	0 (0/1)
24 weeks	10% (2/20)	18% (2/11)	17% (2/12)	0 (0/3)	0 (0/4)	0 (0/1)
Unknown	–	–	8% (1/12)	33% (1/3)	–	–
Total	30% (6/20)	45% (5/11)	55% (7/12)	100% (3/3)	50% (2/4)	100% (0/1)
(b) HCV RNA-negative within 12 weeks						
Relapse						
4 weeks	92% (11/12)	52% (13/25)	57% (30/52)	100% (7/7)	100% (8/8)	84% (16/19)
12 weeks	8% (1/12)	20% (5/25)	26% (13/53)	0 (0/7)	0 (0/8)	5% (1/19)
24 weeks	0 (0/12)	4% (1/25)	0 (0/53)	0 (0/7)	0 (0/8)	5% (1/19)
Unknown	–	12% (3/25)	–	–	–	5% (1/19)
Total	100% (12/12)	88% (22/25)	81% (43/53)	100% (7/7)	100% (8/8)	100% (19/19)

Intergroup within 4 weeks: $P = 0.0319$; time $P = 0.0026$, intergroup within 12 weeks: $P = 0.0109$; time $P = 0.0001$.

treatment might explain the better end-of-treatment response with combination therapy [31]. Although HCV eradication may not be expected in patients HCV positive at 12 weeks, combination therapy should be continued so as to suppress liver inflammation and progression of liver cirrhosis. Moreover, the duration of treatment should be 12 months in patients with genotype 1 and high viral levels.

Many attempts have been made to improve the efficacy of combination therapy. Extending the dosing period from 6 months to 1 year does not affect the HCV RNA negativity rate at the end of treatment [22,23]; improved efficacy with the longer course is attributed to decreases in relapse rate after the end of treatment [32]. Thus it is necessary to conduct a prospective study to determine the optimal duration of combination treatment after HCV RNA becomes negative to improve the efficacy of IFN plus ribavirin combination therapy.

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

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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.

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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,

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HBsAg was classified into nine different subtypes, based on the antigenic determinants of their HBsAg [Nishioka et al., 1975; Magnius 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 chemilumiscent 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

		Demographic and clinical data				HBV genotypes			
Diagnosis	No.	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 ODEN, 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 mid-point 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 [Felsentein, 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

		Diagnosis			
Genotypes	No.	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 Arg[122], Thr[127] and Lys[160], indicating that they

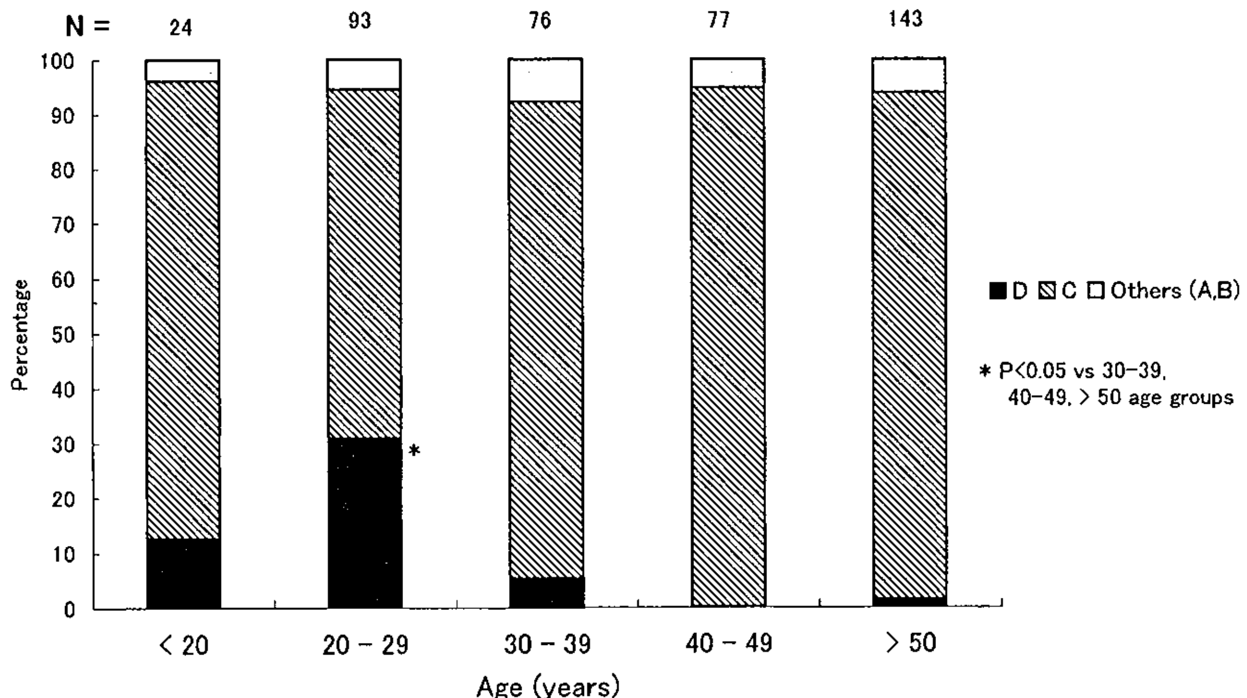

Fig. 1. HBV genotypes of 413 HBV carriers stratified by age. The prevalence of HBV genotype D was mainly found in age group 20–29 years and was significantly higher than other age groups (30–39; 40–49; >50) ($P < 0.01$, $P < 0.01$, $P < 0.01$, respectively).

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

		Diagnosis		HBeAg status	
Genotype	No.	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.
[a]$P < 0.05$ vs. genotype C.
[b]$P < 0.01$ 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 phylogenic 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

	HBeAg (+)			HBeAg (−)		
Genotype	No.	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).
[a]$P < 0.05$ 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 carriership 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

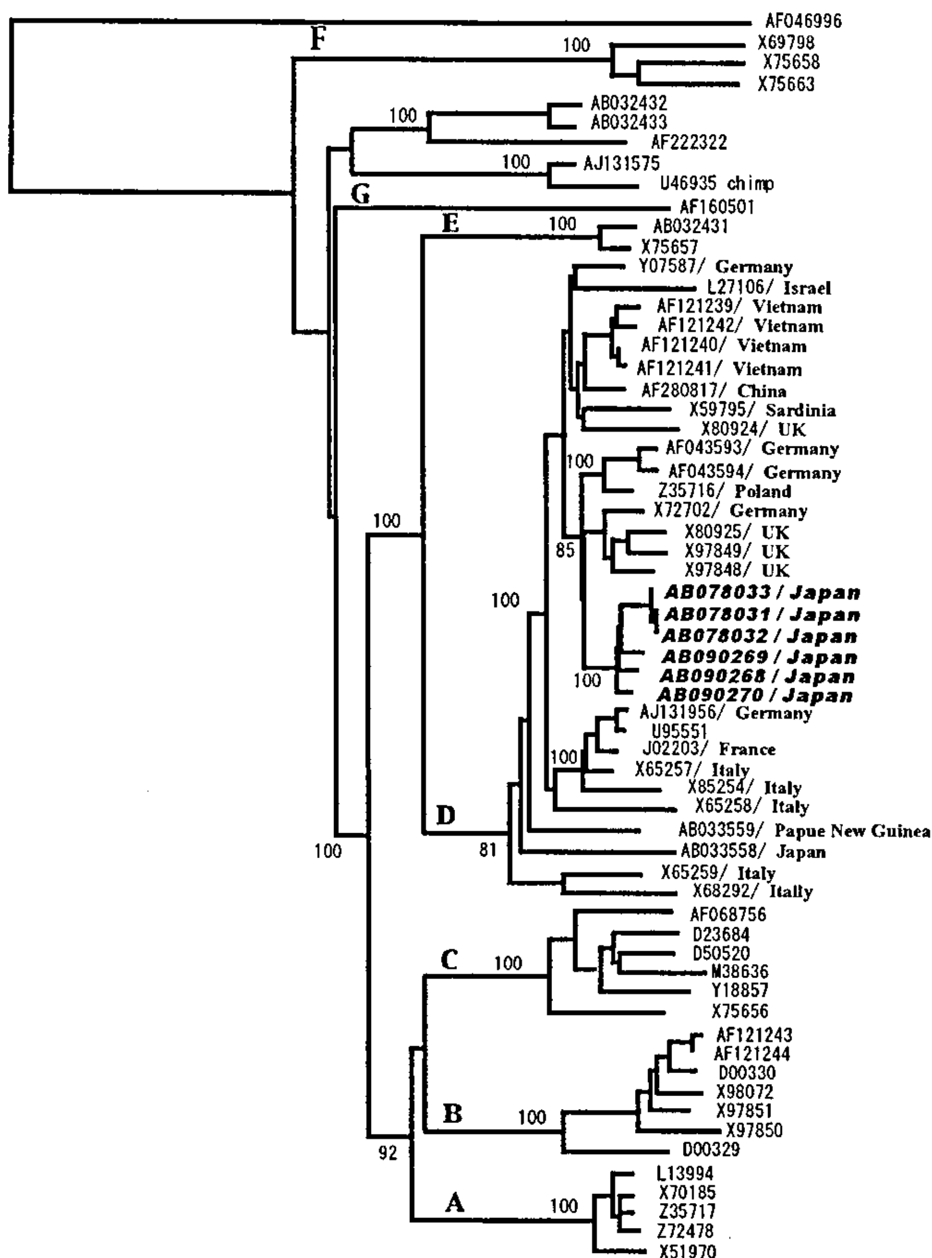

Fig. 2. Full nucleotide sequences of HBV with genotype D from our studied subjects were done and a phylogenetic tree of HBV genotype was constructed. HBV genotype D from our study was related to some HBV genotype D sequences originating from Europe. Bootstrap values are shown along each main branch.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

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☐ CASE REPORT ☐

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

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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

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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/mm³	T. Bil	1.7 mg/dl	TP	8.0 g/dl	HBsAg	(+) 2^8
Seg.	52.6%	D. Bil	1.2 mg/dl	Alb	4.4 g/dl	anti-HBs	(–)
Ly.	40.1%	GOT	528 U/*l*	T. chol	133 mg/dl	HBeAg	0.6
Mo.	4.6%	GPT	1,452 U/*l*	TG	197 mg/dl	anti-HBe	81.3%
Eo.	0.9%	ChE	74 IU/*l*	BUN	6 mg/dl	IgM anti-HBc	(+) 7.8
Ba.	1.8%	LDH	144 IU/*l*	Cr	0.7 mg/dl	anti-HBc ×1	99%
RBC	516×10⁴/mm³	ALP	700 IU/*l*	Na	140 mEq/*l*	×200	71%
Hb	14.8 g/dl	γ-GTP	52 IU/*l*	K	4.4 mEq/*l*	HBV-DNA-P	48 cpm
Ht	45.2%	LAP	122 IU/*l*	Cl	108 mEq/*l*	IgM-HA	(–)
Plt	35.8×10⁴/mm³	ZTT	19 U/*l*	FPG	92 mg/dl	Anti-HCV	(–)
		TTT	24 U/*l*	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

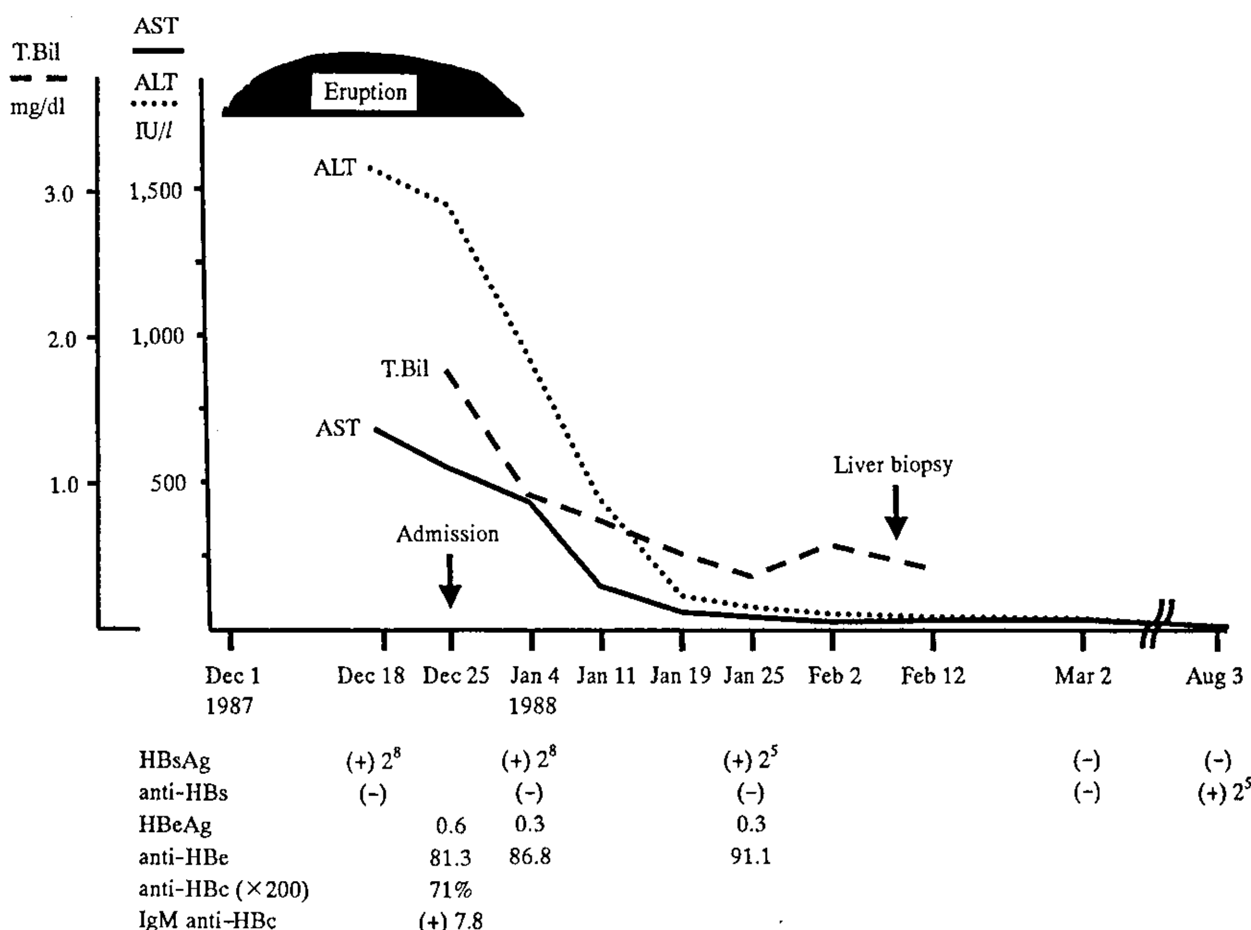

Figure 2. Clinical course. Changes in liver function test results and HBV markers were similar to a typical course of acute hepatitis B. Eruption was observed for approximately 5 weeks after onset.

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.

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

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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.

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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 [Bréchot 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

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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; Rehermann 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 controls (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 Expand^{TM} 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		+	+	–	–	+	NT	–	–
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