itored in our hospital or in a related private hepatology clinic for more than 1 year, then the patient was considered lost to follow-up.

DCP Determinations

Serial serum samples had been collected over many years and maintained at −20 °C. The concentration of the serum DCP was determined by the newly developed, revised EIA kit for DCP in a retrospective examination of the serum samples. Measurement of plasma DCP concentrations were performed with a high-sensitivity EIA kit using the MU-3 monoclonal antibody (ED036 kit; Eisai Company Ltd., Tokyo, Japan) and a new EIA kit using the newly developed 19B7 monoclonal antibody, which is specific for DCP, produced by the human HCC cell line PLC/PRF/5. The cutoff value was set at 40 mAU/mL for the MU-3 measurement.[3] The DCP values in preserved serum and fresh serum were compared to determine the influence of preservation at −20 °C using 10 samples. Because the standard deviation was less than 10% (data not shown), we concluded that serum preservation at −20 °C did not affect measured values. Patients were classified into 3 groups based on their DCP Index scores—1) DCP negative: serum DCP concentration (measured by MU-3) < 40 mAU/mL; 2) low DCP Index: DCP concentration ≥ 40 mAU/mL and DCP ratio (MU-3:19B7) < 3; and 3) high DCP Index: DCP concentration ≥ 40 mAU/mL and DCP ratio (MU-3:19B7) ≥ 3.

Statistics

Survival rates were determined using the Kaplan–Meier method, and differences in the survival rates between the groups were compared using the log-rank test. An analysis of multiple covariates among the prognostic factors from the patient's background was performed with a Cox proportional hazards model. The chi-square test and the Kruskall–Wallis rank test were used for comparisons of discrete variables. Statistical significance was defined as a P value < 0.05.

The eight factors examined were age at diagnosis with HCC, gender, positivity for hepatitis B surface antigen (HBsAg), positivity for antibodies to hepatitis C virus (anti-HCV), positivity for AFP, positivity for DCP, DCP Index, and initial treatment. Initial treatment was classified into two groups: 1) PEIT and 2) hepatectomy and TAE. The cutoff value for the AFP level was set at 200 ng/mL.

RESULTS

Patient characteristics

One hundred four patients were enrolled in the study, and the prognostic factor of a solitary HCC lesion measuring ≤ 3 cm in greatest dimension was analyzed prospectively by follow-up for a mean of 3.24 years (median follow-up, 2.65 years; range, 0.15–9.4 years). Patient characteristics are listed in Table 1. The ages of the patients ranged from 38.1 years to 85.2 years (median age, 64.7 years), and there were 73 males and 31 females. Of these patients, 15 (14.4%) were positive for HbsAg but negative for anti-HCV. Seventy-six patients (73.1%) were positive for anti-HCV but negative for HBsAg. Eight patients (7.7%) were negative for both HBsAg and anti-HCV, whereas 5 patients (4.8%) were positive for both HBsAg and anti-HCV. Of the 104 total patients with HCC, 42 patients (40.3%) had DCP serum concentrations ≥ 40 mAU/mL at the time of diagnosis, and 10 patients (9.6%) had serum concentrations of AFP ≥ 200 ng/mL.

A percutaneous fine-needle aspiration liver biopsy was performed under ultrasonography guidance for confirmation of the diagnosis in 68 of 104 patients. In 68 patients (65.3%), HCC had been diagnosed by pathology. The remaining 36 patients (34.7%) exhibited clinical features of HCC in imaging studies, and all patients were treated for HCC. Seventy-eight patients received PEIT, 23 patients underwent TAE, and 3 patients underwent hepatectomy.

TABLE 1
Patient Characteristics (n = 104)

Variable	No. of patients (%)*
Median age (yrs)	64.7
Gender (male/female)	73/31
Positive for HBsAG and negative for anti-HCV	15 (14.4)
Positive for anti-HCV and negative for HBsAG	76 (73.2)
Positive for both HBsAG and anti-HCV	5 (4.8)
Negative for both HBsAG and anti-HCV	8 (7.7)
Albumin (mg/dL)	3.9
Total bilirubin (mg/dL)	0.8
Serum aspartate aminotransferase (IU/mL)	56.5
Serum alanine aminotransferase (IU/mL)	59.0
Prothrombin time (%)	79.8
Platelet count (× 10^4)	10.5
AFP (ng/mL)	14.5
DCP (mAU/mL)	24.5
Positive for AFP (> 200 ng/mL)	10 (9.6)
Positive for DCP (≥ 40 mAU/mL)	42 (40.3)
Histology (well-differentiated/moderately-to-poorly differentiated	62/6
Initial treatment (hepatectomy/PEIT/TAE)	3/78/23
Average interval (yrs)	3.24

HBsAG: hepatitis B surface antigen; HCV: hepatitis C virus; IU: International Units; mAU: milli Arbitrary Units; AFP: α-fetoprotein; DCP: des-γ-carboxy prothrombin (MU-3); PEIT: percutaneous ethanol injection therapy; TAE: transcatheter arterial embolization.
* Data are expressed as median values.

TABLE 2
Summary of Patient Outcomes

Outcome	No. of patients (%)
Survival	56 (53.8)
Death	
Hepatic disease	34 (32.7)
Other	2 (2.0)
Lost during follow-up	12 (11.5)

Long-Term Outcomes

Follow-up data were obtained on 92 patients (88.5%) (Table 2), and 12 patients (11.5%) were lost during follow-up. Thirty-four patients (32.7%) died of hepatic disease (33 died of tumor progression and hepatic failure, and 1 patient died of gastrointestinal bleeding). Two patients died of other diseases (one due to a hemorrhagic stroke and one due to renal failure).

Characteristics of the Three Groups Classified According to the DCP Index

Patients with HCC were divided into three groups. Twelve patients had a high DCP Index score at the time they were diagnosed with HCC, and 30 patients had a low DCP Index score. Sixty-two patients were classified as negative for DCP. Table 3 shows that the patients in these three groups were identical with regard to age, etiology of cirrhosis, degree of liver function, and AFP levels. The DCP levels measured by MU-3 and 19B7 differed significantly among the high DCP Index group, the low DCP Index group, and the DCP-negative group. The other variables were not significantly different across the three groups.

Survival Rates

Figure 2 shows the survival rates of 104 patients who were classified with Child Stage A disease at the time they were diagnosed with a solitary HCC lesion that measured ≤ 3 cm in greatest dimension. The 3-year survival rate of the 104 patients was 80.3%, and their 5-year survival rate was 52.1%.

The survival rate of patients in the high DCP Index group was significantly lower compared with the survival rate of patients classified as DCP negative ($P < 0.01$; log-rank test). The survival rate of patients with in the high DCP Index group also was significantly lower compared with the survival rate of patients in the low DCP Index group ($P < 0.05$; log-rank test) (Fig. 3).

Univariate and Multivariate Analyses

The independent predictors of survival are summarized in Table 4. A Cox proportional hazards regression analysis was performed to determine the factors that affected survival. According to the univariate analysis, the DCP concentration has a significant effect. However, other factors (age, gender, positive HBsAg status, positive anti-HCV status, AFP ≥ 200 ng/mL, initial treatment) did not affect survival. Multivariate Cox regression analyses were performed on the six variables in the model due to the possibility that the variables were correlated mutually. Of these six variables, two factors, age and DCP Index score at entry, were associated independently with survival. The risk of death in the group of patients age ≥ 65 years at entry was increased 3.27-fold compared with the group of patients age < 65 years. The death rate in the low DCP Index group was 2.87-fold higher compared with the DCP-negative group, and the death rate in the high DCP Index group was 12.3-fold higher compared with the DCP-negative group.

TABLE 3
Comparison of Characteristics in Patients with Positive Des-γ-Carboxy Prothrombin Status and Negative Des-γ-Carboxy Prothrombin Status[a]

Variable	High DCP Index (n = 12)	Low DCP Index (n = 30)	DCP negative (n = 62)
Median age (yrs)	63.1	67.1	64.2
Gender (male/female)	10/2	24/6	39/23
Positive for HBsAG and negative for anti-HCV (%)	3 (25.0)	3 (10.0)	9 (14.5)
Positive for anti-HCV and negative for HBsAG (%)	7 (58.3)	24 (80.0)	45 (72.6)
Positive for both HBsAG and anti-HCV (%)	0 (0)	2 (6.7)	3 (4.8)
Negative for both HBsAG and anti-HCV (%)	2 (16.7)	1 (3.3)	5 (8.1)
Albumin (mg/dL)	4.0	3.9	3.9
Total bilirubin (mg/dL)	0.75	0.8	0.8
Serum aspartate aminotransferase (IU/mL)	37.5	64.0	57.5
Serum alanine aminotransferase (IU/mL)	42.5	55.0	66.0
Prothrombin time (%)	88.3	73.9	82.0
Platelet count ($\times 10^4$)	11.9	10.8	10.8
AFP (ng/mL)	16.5	14.5	13.0
DCP (MU-3) (mAU/mL)[b]	904.0	103.0	16.0
DCP (19B7) (mAU/mL)[c]	149.0	89.0	11.5
Histology (well-differentiated/moderately-to-poorly differentiated	8/2	13/2	41/2
Initial treatment (hepatectomy/PEIT/TAE)	1/10/1	1/19/10	1/49/12

DCP: des-γ-carboxy prothrombin; HBsAG: hepatitis B surface antigen; HCV: hepatitis C virus; IU: International Units; mAU: milli Arbitrary Units; AFP: α-fetoprotein; PEIT: percutaneous ethanol injection therapy; TAE: transcatheter arterial embolization.

[a] Data are expressed as median values.

[b] $P < 0.05$ for high DCP Index versus low DCP Index and for low DCP Index versus DCP negative.

[c] $P < 0.05$ for high DCP Index versus DCP negative and for low DCP Index versus DCP negative.

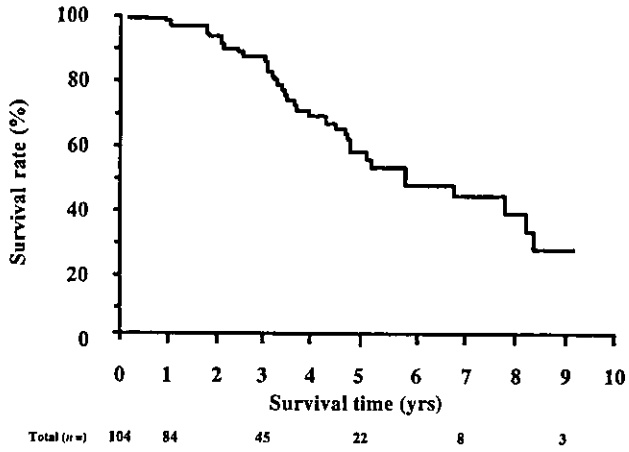

FIGURE 2. Overall survival rates for 104 patients who underwent initial treatment for hepatocellular carcinoma that measured ≤ 3 cm in greatest dimension.

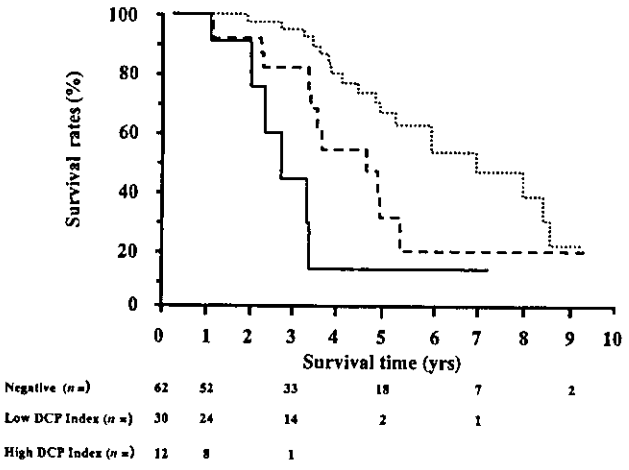

FIGURE 3. Survival rates prepared according to the Kaplan–Meier method are shown for the groups defined by the des-γ-carboxy prothrombin (DCP) Index. The survival rate of the high DCP Index group (solid line; $n = 12$) was significantly lower compared with the negative group (dotted line; $n = 62$) and the low DCP Index group (dashed line; $n = 30$). $P < 0.05$ for negative versus low DCP Index and for low DCP Index versus high DCP Index. $P < 0.01$ for negative versus high DCP Index.

DISCUSSION

Serum DCP levels have been found to be significantly correlated with tumor size, intrahepatic metastasis, vascular invasion, and morphologic development of HCC.[5,6,14] Moreover, DCP has been suggested as a measure of tumor progression. Indeed, serum DCP levels are correlated significantly with the development of portal venous invasion[15] and early intrahepatic recurrence after PEIT.[16] Shimada at el. reported that the DCP level in noncancerous liver tissue with multicentric occurrence of HCC was significantly greater compared with the DCP level in tissue without multicentric occurrence of HCC.[17]

The lens culinaris agglutinin–reactive fraction of AFT (AFP L3%) is a described marker of HCC. Serum AFP L3% levels have been found to be significantly correlated with the histologic grade of malignancy and the prognosis after treatment in patients with HCC.[18–20] AFP L3% levels are considered superior in specificity and sensitivity compared with total serum AFP levels.[21] In the same manner that the AFP L3% level measures a fraction of the total AFP, the DCP Index, the ratio of the two DCP measurements (MU-3:19B7), reflects the amount of DCP produced by HCC cells as a percentage of the total DCP. The serum DCP produced by patients with HCC has greater reactivity with the MU-3 antibody compared with the 19B7 antibody. We consider that the MU-3 measurement reflects the amount of HCC, and the DCP Index reflects the malignant potential of HCC. An increased DCP Index score suggests either premalignancy in noncancerous parts of the liver or tumor progression that was more advanced than suggested by imaging studies. The DCP Index is not a diagnostic marker but is a marker of a poor prognosis and tumor progression.

In the current study, we analyzed prognostic factors in patients with HCC who had solitary, small tumors and a Child A classification. We chose this category of tumors for several reasons: first, it is clear that liver function tests and tumor stage were prognostic factors. Second, we wanted to know the malignant potential of the tumor, and we believed that malignant potential should be determined at the same tumor stage and Child grade. The study showed that the survival rate of patients in the high DCP Index group was significantly lower compared with the survival rate of patients in the low DCP Index group (Fig. 3). Multivariate Cox regression analyses demonstrated that the serum DCP concentration at the time of diagnosis of HCC is the most important factor determining the prognosis in patients with HCC. The finding in the multivariate analysis that DCP is an independent prognostic factor supports this idea. Six of 7 patients who were in the high DCP Index group died of tumor progression within 3 years. There were almost no significant differences between the high DCP Index group and the low DCP Index group. Only the DCP measured using MU-3 was significantly high in the high DCP Index group (Table 4).

The reasons for deciding on a cutoff value of 3.0 for the DCP ratio as a new prognostic indicator in this study are described below. In the current study, univariate analyses and multivariate Cox regression analyses were performed using many cut-off values for the DCP ratio (MU-3:19B7 ratio: 2.0, 2.5, 3.0, 3.5, and 4.0).

TABLE 4
Univariate and Multivariate Analyses of Survival in Patients with Solitary Hepatocellular Carcinoma

Variable	No. of patients	Univariate analysis			Multivariate analysis		
		RR	95% CI	*P* value	RR	95% CI	*P* value
Age ≥ 65 yrs	51	1.51	0.79–2.89	0.21	3.27	1.38–7.71	0.006
Male gender	73	1.32	0.66–2.64	0.42	1.69	0.76–3.78	0.19
HBV positive	15	1.32	0.44–3.99	0.61	2.56	0.78–8.37	0.11
HCV positive	76	0.62	0.23–1.65	0.34	0.71	0.25–2.00	0.71
AFP ≥ 200 ng/mL	10	1.98	0.82–4.79	0.12	2.38	0.91–6.23	0.076
DCP							
High DCP Index	12	6.71	2.69–16.6	< 0.0001	12.3	4.07–37.7	< 0.0001
Low DCP Index	30	2.18	1.04–4.56	0.001	2.87	1.27–6.47	0.012
Initial treatment: TAE	23	0.87	0.38–2.01	0.76	1.06	0.41–2.69	0.90

RR: relative risk; 95% CI: 95% confidence interval; HBV: hepatitis B virus; HCV: hepatitis C virus; AFP: α-fetoprotein; DCP: des-γ-carboxy prothrombin; TAE: transcatheter arterial embolization.

With the cutoff value of the DCP ratio set at 2.5 in univariate analyses, the survival rates of patients in the high DCP Index group and patients in the low DCP Index group did not differ significantly. Using each cutoff value (MU-3:19B7 ratio: 2.0, 2.5, 3.0, 3.5, and 4.0) in multivariate analyses, the DCP Index at entry was found to be independently associated with survival. The results from using each cutoff value were as follows: at an MU-3:19B7 ratio of 2.0, patients in the low DCP Index group had a hazard ratio of 2.99 ($P = 0.009$), and patients in the high DCP Index group had a hazard ratio of 7.41 ($P = 0.0001$); at an MU-3:19B7 ratio of 2.5, patients in the low DCP Index group had a hazard ratio of 3.02 ($P = 0.012$), and patients in the high DCP Index group had a hazard ratio of 8.36 ($P = 0.0001$); at an MU-3:19B7 ratio of 3.5, patients in the low DCP Index group had a hazard ratio of 2.87 ($P = 0.019$), and patients in the high DCP Index group had a hazard ratio of 12.4 ($P < 0.0001$); and at an MU-3:19B7 ratio of 4.0, patients in the low DCP Index group had a hazard ratio of 3.01 ($P = 0.006$), and patients in the high DCP Index group had a hazard ratio of 11.0 ($P < 0.0001$). For these reasons, we chose a cutoff value of 3.0 for the DCP (MU-3:19B7) ratio.

The current study had certain shortcomings. The therapeutic effects of the second and third series of treatments for HCC were not evaluated as prognostic factors. Many patients developed recurrent disease during long-term follow-up, and the average number of courses of the different combinations of treatment was 4.73 courses. It is difficult to evaluate all of the therapeutic effects as prognostic factors. At a minimum, the initial therapy did not affect the prognosis of patients with HCC in univariate and multivariate Cox regression analyses. The patients in the current study who did not undergo surgical resection were treated mainly by PEIT. Recently, PEIT has become a common treatment for small HCC and reportedly is as beneficial as surgery.[22] Several investigators have discussed the prognosis of patients with small HCC, and the results of our study were similar to other reports (Fig. 2).[22,23] In addition, we performed a needle biopsy in 68 of patients (65.3%) with HCC, they had been diagnosed by pathology. We did not analyze the pathologic diagnosis in all patients, and there are risks that sampling errors may have occurred. The most atypical portions of the nodules may not always have been obtained by needle biopsy. Sixty-two patients were diagnosed with well differentiated HCC, but the correlation between the histologic grade of the malignancy and the DCP Index could not be analyzed.

It has been reported that the vitamin K levels in HCC tissue are significantly lower than in the surrounding tissue, although this remains controversial.[17,24] The mechanism of DCP production is not known to date. Many recent findings point to a role for vitamin K in controlling cell growth, and vitamin K analogues cause cell growth inhibition in hepatoma cell lines.[25,26] Wang et al. reported that vitamin K is a potent inducer of apoptosis in rat hepatocytes.[27] Administration of vitamin K to patients with HCC may alter the metabolism of hepatoma cells and also may have a growth-inhibitory effect. Sekiya et al. administered vitamin K to four patients with DCP-positive HCC and examined the changes in the 19B7 and MU-3 assay DCP levels.[12] The DCP level was decreased in both assays, but changes in the MU-3:19B7 ratio and in the prognosis of the patients were not reported. Liver transplantation is an effective treatment for patients with small, unresectable HCC,[28] but the indication for liver transplantation in patients with HCC still is considered controversial.[29] Considering that almost all patients with a high DCP Index score died from tumor progression within 3 years, we should consider

the indication for liver transplantation in such patients.

In conclusion, it is apparent that the DCP Index reflects the exact state of the tumor. The current study appears to show that a high DCP Index score is a new prognostic factor in patients with HCC who have small, solitary tumors and a Child A classification at the time of diagnosis.

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Chronic hepatitis delta virus infection with genotype IIb variant is correlated with progressive liver disease

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We determined the sequence of the hepatitis delta virus (HDV) genome in 40 Japanese patients, most of whom were from the Miyako Islands, Okinawa, Japan. Consensus sequences from 33 HDV full genomes out of a total of 40 patients were determined by directly sequencing four partially overlapping PCR products. Phylogenetic tree analysis classified these 33 complete HDV genomes as HDV genotype I (two patients), genotype IIa (one patient) and genotype IIb (30 patients). Among the 30 genotype IIb patients, there were two clusters of genetic variants. One group consisted of six isolates showing significant homology with genotype IIb, previously reported from Taiwan. The other group consisted of 24 isolates, whose sequences formed a new genetic subgroup (genotype IIb-Miyako; IIb-M). When the genetic structures were compared in detail between IIb and IIb-M, characteristic variations were found in the C-terminal sequence of the large delta antigen-conferring packaging signal as well as the RNA editing site. Determination of subclasses of genotype IIb in a total of 37 patients, including seven HDV patients whose partial HDV sequence was determined, revealed eight patients with IIb and 29 patients with IIb-M. Although there was no significant difference in the clinical background or virological state of hepatitis B virus between these two groups, patients with genotype IIb-M showed greater progression of chronic hepatitis and cirrhosis than those with genotype IIb ($P=0{\cdot}0009$). These data indicate the existence of a genetic subgroup of HDV genotype IIb, which is associated with different clinical characteristics and which could be related to genetic variations in functionally important parts of the HDV genome.

INTRODUCTION

Hepatitis delta virus (HDV) is a defective virus that requires hepatitis B virus (HBV) surface antigen for virion assembly (Rizzetto *et al.*, 1980) and infection and contains a negative single-stranded circular RNA genome of 1·7 kb (Wang *et al.*, 1986; Makino *et al.*, 1987). HDV is classified into three genotypes (I, II and III) based on genetic sequence analysis (Casey *et al.*, 1993). HDV genotypes correlate with the clinical outcome of HDV infection. HDV genotype I, which is found worldwide, often causes aggressive hepatitis and is more frequently associated with liver cirrhosis (LC) and hepatocellular carcinoma (HCC) than genotype II, which is mainly isolated from East Asia and is generally related to milder diseases (Wu *et al.*, 1995a). On the other hand, acute infection with HDV genotype III, which is isolated from the northern part of South America, is closely associated with fulminant hepatitis (Casey *et al.*, 1993). However, information about the precise relationship between the genetic structure of HDV and the clinical characteristics within each

genotype is somewhat limited. Comparative analysis, in which genetic variations are correlated with clinical presentations in a population with homogeneous clinical backgrounds or virological states of HBV, can provide valuable information about HDV genetic structures that determine the severity of liver disease.

In Japan, chronic HDV infection is relatively rare (Tamura *et al.*, 1993) but is endemic in the Miyako Islands of Okinawa, where the HDV genotype II is prevalent (Sakugawa *et al.*, 1999). Although the route by which HDV is spread on this island is unclear, our previous studies demonstrated that the severity of liver disease was heterogeneous within this population, despite relatively uniform clinical backgrounds. Thus, a detailed analysis in which the HDV genomes of these patients are correlated with clinical profiles could provide a unique opportunity to define the critical genetic features of HDV that determine liver injury.

To delineate the features of HDV isolates in this area, we determined the sequence of the full-length HDV genome from a large group of patients with chronic HDV infection, the majority of whom were from the Miyako Islands. As a result, we identified a new genetic variant of HDV genotype IIb that was associated with more progressive disease. Subsequently, specific genetic differences among these HDV genotype IIb isolates were correlated with the clinical features in order to reveal the variations in the HDV genome responsible for the progression of liver disease.

METHODS

Patients. A total of 40 patients were enrolled in the study: 16 males and 24 females ranging in age from 23 to 83 years old. Of the 40 patients, 37 were from the Miyako Islands of Okinawa, where HDV infection is endemic, two were from Nagasaki and one was from Tokyo, Japan. All patients showed positive serum anti-HD and -HBsAg, including three asymptomatic carriers (ASC) who had consistently normal alanine aminotransferase (ALT) levels at least bimonthly for more than 2 years, 23 patients with chronic hepatitis (CH) with abnormal ALT levels and 13 patients with LC. The diagnosis of LC was based on clinical findings, such as oesophageal varices or ascites, with histological and/or radiological findings consistent with LC. The abdominal ultrasound findings for all ASC patients appeared normal without any evidence of liver disease. All subjects were hepatitis B e antigen (HBeAg) negative. To exclude other factors contributing to ALT elevation, the following subjects were excluded from the present study: subjects who were positive for antibody to hepatitis C virus (anti-HCV) or antinuclear antibodies, those with fatty liver on ultrasound examinations and those with a history of excess alcohol intake or hepatotoxic drugs. Written informed consent was obtained from patients in this study. The HBV DNA levels in the sera were quantified with a commercial kit (DNA probe Chugai-HBV; Chugai Diagnostics, Tokyo, Japan) using a transcription-mediated amplification assay (Kamisango *et al.*, 1999). The detection range of this assay was from $5{\cdot}0\times10^3$ to $5{\cdot}0\times10^8$ copies ml^{-1}. HBV genotype was determined using the PCR-RFLP method (Mizokami *et al.*, 1999).

Sequencing of HDV. The full-length HDV genome was sequenced in 33 patients. In the other seven patients, the partial genetic sequence enoding the delta antigen (HDAg) was determined. Extraction of RNA from 150 µl of serum by the acid guanidinium thiocyanate/phenol/chloroform method (Chomczynski & Sacchi, 1987) using ISOGEN (Wako, Osaka, Japan) and RT-PCR were performed as described previously (Enomoto *et al.*, 1994). Four partially overlapping fragments were amplified by nested PCR using the primers shown in Table 1. These primers were designed and numbered based on HDV genotype II sequences in GenBank. PCR was initially performed with primers designed for HDV genotype II. If HDV cDNA was not amplified with these primers, PCR was performed with primers for HDV genotype I (primer sequences are available on request). Both strands of the PCR products were directly cycle sequenced with the PRISM dye termination kit (Applied Biosystems) and nested PCR primers.

Sequence analysis. The 33 HDV full genome sequences determined in this study were aligned with 22 complete nucleotide sequences of HDV retrieved from the international DNA databases (DDBJ/EMBL/GenBank). An initial alignment was made using the Clustal X 1.81 program (Thompson *et al.*, 1997), followed by manual correction. Based on the alignment, a phylogenetic tree was constructed using the neighbour-joining method (Saitou & Nei, 1987), with genetic distances calculated using the Kimura two-parameter method using MEGA version 2.1 (http://www.megasoftware.net/). A maximum-likelihood tree was also constructed using PAUP 4.0 (D. L. Swofford, Sinauer Associates). To confirm the reliability of the phylogenetic trees, bootstrap resampling and reconstruction were carried out 1000 times for neighbour-joining trees and 100 times for maximum-likelihood trees. The nucleotide and amino acid identities were calculated using MEGA version 2.1. Phylogenetic

Table 1. The primer sets for the HDV genome sequence

The nucleotides are numbered according to Wang *et al.* (1986).

		Outer primer	Inner primer
Fragment 1	5′	CGACGAGGAGCCCGCAGGTGGGAGG (nt 16–39)	GCCAAAGAGTGGGGGAAATCTCG (nt 64–86)
	3′	AAGGGAGGTCTGGGGATTCCCACG (nt 577–600)	ATGGGCCCCCTGAGGTCCAAGGACC (nt 545–570)
Fragment 2	5′	TGTTCCGCCCCCCGAGGGGGGCCGA (nt 443–467)	TGAGGCTTATCCCGGGGATCG (nt 469–491)
	3′	TTCCACTCCTCGAGTGATCCCCCA (nt 958–982)	CAGGGTTCCACTCACGGTTCGCGTC (nt 922–948)
Fragment 3	5′	GATGCCCAGGTCGGACCGCGAGGAG (nt 855–879)	GGAGATGCCATGCCGACCCGAAGAG (nt 882–906)
	3′	AAAAGGGAAGGACGGGGAGGGGGCT (nt 1394–1421)	GGCGAAGAGGCCCCGGACGGATCAG (nt 1364–1388)
Fragment 4	5′	GAGATCCTCCCTCTCCTTGTCGGTG (nt 1292–1316)	GTGAGGCCTCTTCCCAGGTCCGGA (nt 1328–1351)
	3′	TCTTGAATGAAATTCCGGGAGTCTC (nt 146–173)	CAGCGTTCGGGAAATCTCCTC (nt 125–145)

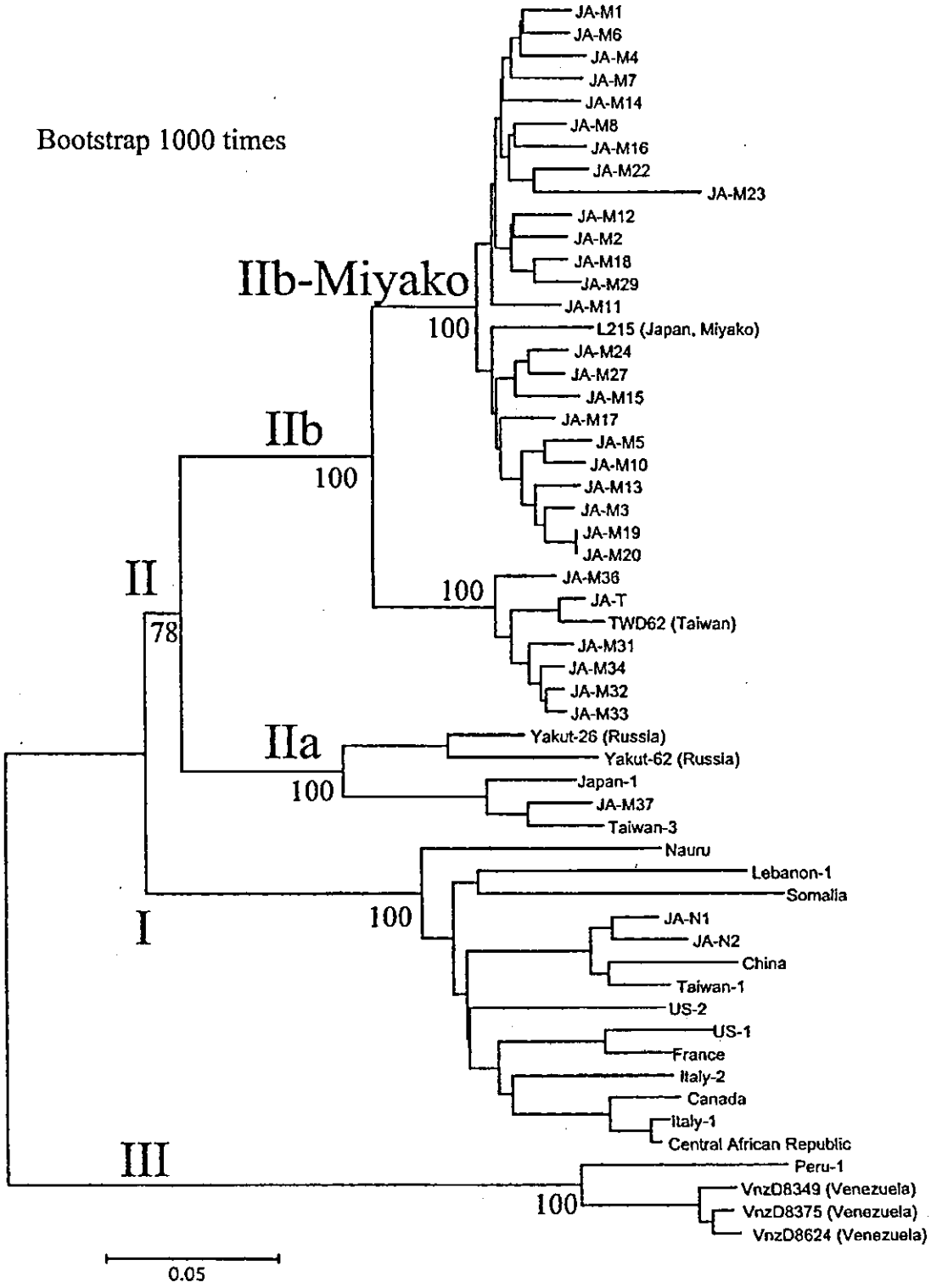

Fig. 1. For legend see page 3278.

analysis of the partial HDV genome encompassing the HDAg region, including seven additional partial sequences determined in this study, was also performed.

Statistical analysis. Categorical data were compared by chi-square or Fisher's exact test. Distributions of continuous variables were analysed by the Mann–Whitney *U*-test or Student's *t*-test using Statview 5.0 software (Abacus Concepts). All tests of significance were two-tailed and *P* values of less than 0·05 were considered as statistically significant.

RESULTS

Homology and phylogenetic tree analysis of the HDV genome

Fig. 1 shows the neighbour-joining phylogenetic tree based on the complete sequences of 55 HDV isolates, including the 33 newly sequenced HDV isolated from the present study and another 22 available full-length genome sequences. Of the 33 new HDV isolates, two were located in the genotype I cluster and one in the genotype IIa. The two patients with genotype I were from Nagasaki and the patient with genotype IIa was from Miyako Islands, Okinawa. The other 30 strains clustered with the reported complete sequences of genotype IIb TWD62 or L215 (Wu *et al.*, 1998; Ma *et al.*, 2003). Six strains clustered with the prototype genotype IIb isolate TWD62, originally reported from Taiwan, whereas the other 24 isolates formed a unique cluster with the IIb strain L215, a recently reported full genome sequence from the Miyako Islands (Ma *et al.*, 2003). The two clusters were divided distinctly with high bootstrap values of 100 %. Five of the six strains clustered with the prototype genotype IIb were from the Miyako Islands, while the other patient with prototype genotype IIb was from Tokyo. In contrast, the strains from the cluster including L215 were all from the Miyako Islands. These results indicate the existence of at least three lineages of HDV variant, IIa, prototype IIb and Maiyako Islands-specific variant genotype IIb-Miyako (IIb-M) in the Miyako Islands. A maximum-likelihood tree constructed from the same sequences also showed distinct clusters of prototype IIb and IIb-M with high bootstrap values (data not shown).

The typical genomic sequences of genotype IIb-M with representative isolates of other HDV genotypes (I, IIa, IIb, III) are shown in Fig. 2. The length of the complete HDV sequence of genotype IIb-M was 1676 nt and the overall identities in the HDV genomic sequences between genotype IIb-M and genotype I (Wang *et al.*, 1986), IIa (Imazeki *et al.*, 1991), IIb (Wu *et al.*, 1998), IIb-L215 (Ma *et al.*, 2003) and III (Casey *et al.*, 1993) isolates were 72·7, 78·1, 87·3, 93·8 and 64·6 %, respectively, showing that IIb-M is most closely related to IIb-L215, the recently reported HDV isolate from the Miyako Islands.

The lengths of the complete HDV sequences of prototype IIb and of IIb-M genotypes determined in this study were 1677–1679 nt and 1675–1685 nt, respectively. The nucleotide identities among isolates within prototype IIb and within genotype IIb-M were 94–97 % similar, whereas those between prototype IIb and IIb-M had similarities of 88–90 %. There were marked variations in the degree of genetic divergence among different regions of the HDV genome when comparing IIb-M (JA-M1) and the other genotypes (Modahl & Lai, 2000). As shown in Table 2, the greatest divergence was in the hypervariable region (54·7–92·5 %), followed by the HDAg coding sequence (70·7–94·4 %), and the least divergence was in the autocleavage region (74·8–95·6 %).

Clinical characteristics of patients with HDV genotype IIb-M

A phylogenetic tree analysis of the partial genome sequences encompassing the HDAg coding region, including additional sequences isolated from seven patients from the Miyako Islands, showed similar clustering (data not shown). Five of them clustered with genotype IIb-M and the remaining two strains clustered with prototype IIb. Finally, the clinical pictures between eight patients infected with prototype IIb and 29 patients with IIb-M were compared. As shown in Table 3, age, sex, HBV status [all patients were HBeAg negative and anti-HBe positive with low [$< 1{\cdot}0 \times 10^6$ copies (ml serum)$^{-1}$] HBV DNA levels] did not differ between these two groups. However, the severity of liver disease was significantly higher in patients with genotype IIb-M than in those with prototype IIb. In prototype IIb patients, three were ASC, five were CH and none were LC. In contrast, there were 16 CH and 13 LC in genotype IIb-M. Thus, patients with HDV IIb-M showed significantly greater disease progression compared with patients with HDV prototype IIb ($P = 0{\cdot}0009$ by the Kruskal Wallis test).

Genetic features of HDV genotype IIb-M

The secondary structure of the antigenomic sequence corresponding to the 3′ end of the small HDAg gene containing the RNA editing site (Casey *et al.*, 1992) showed that IIb-M has a particular structure located at nt 1012, the amber/tryptophan site (Fig. 3). All known HDV genotypes I

Fig. 1. Phylogenetic tree analysis of HDV isolates. Sources of isolate sequences are as follows: TWD62, AF018077; Taiwan-3, U19598; Taiwan-1, M92448; Yakut-26, AJ309879; Yakut-62, AJ309880; Japan-1, X60193; Nauru, M58629; Lebanon-1, M84917; Somalia, U81988; China, X77627; US-1 (M28267; US-2, L22066; France, D01075; Italy-1, X04451; Italy-2, X85253; Canada, AF098261; Central African Republic, AJ000558; Peru-1, L22063; VnzD8349, AB037948; VnzD8375, AB037947; VnzD8624, AB037949. JA-M1 to JA-M37 (from Miyako), JA-N1 and JA-N2 (from Nagasaki) and JA-T (from Tokyo) were sequenced in this study. These sequences have been deposited in the GenBank database (AF309420 and AB118818–AB118849).

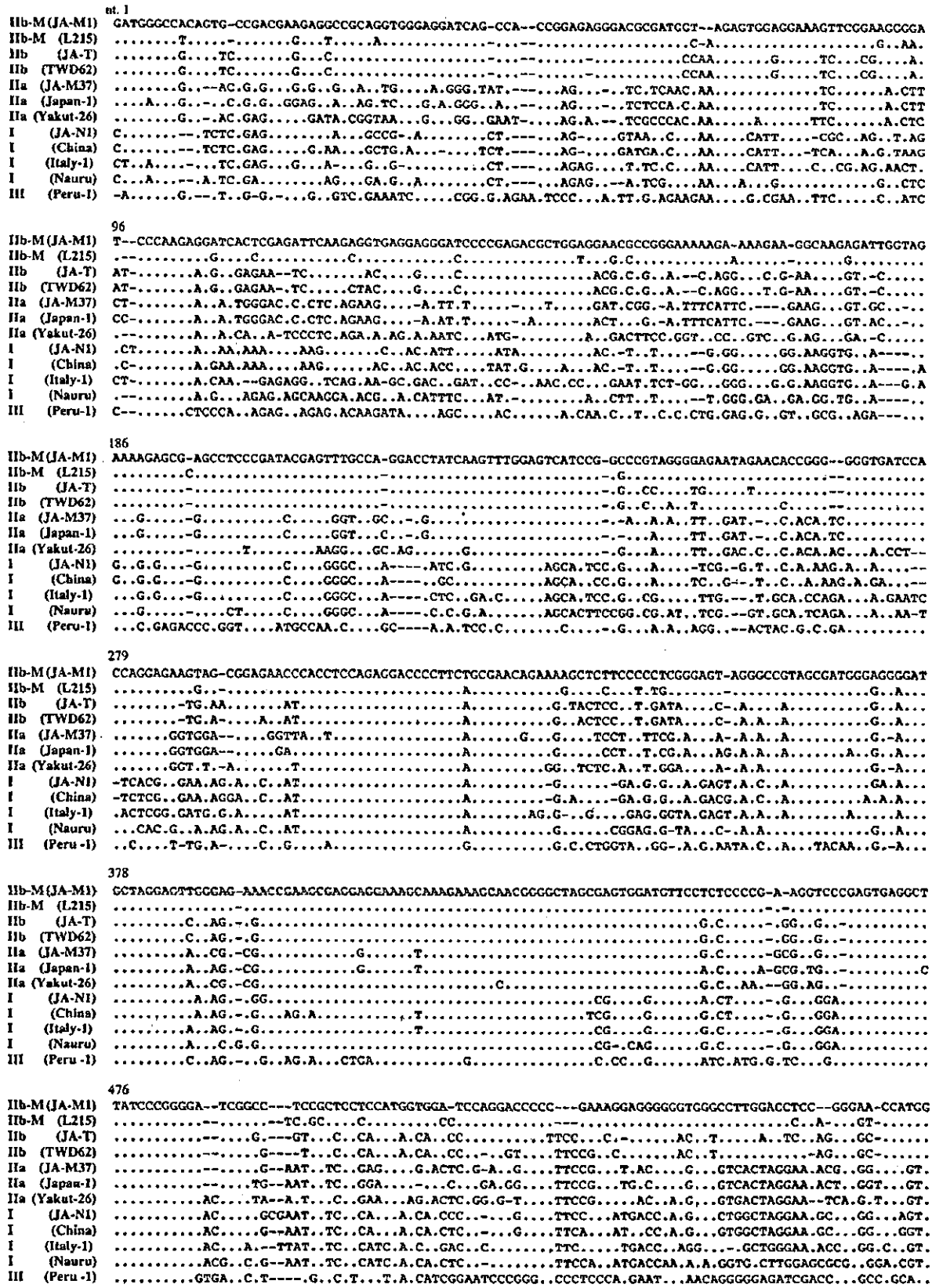

Fig. 2. For legend see page 3282.

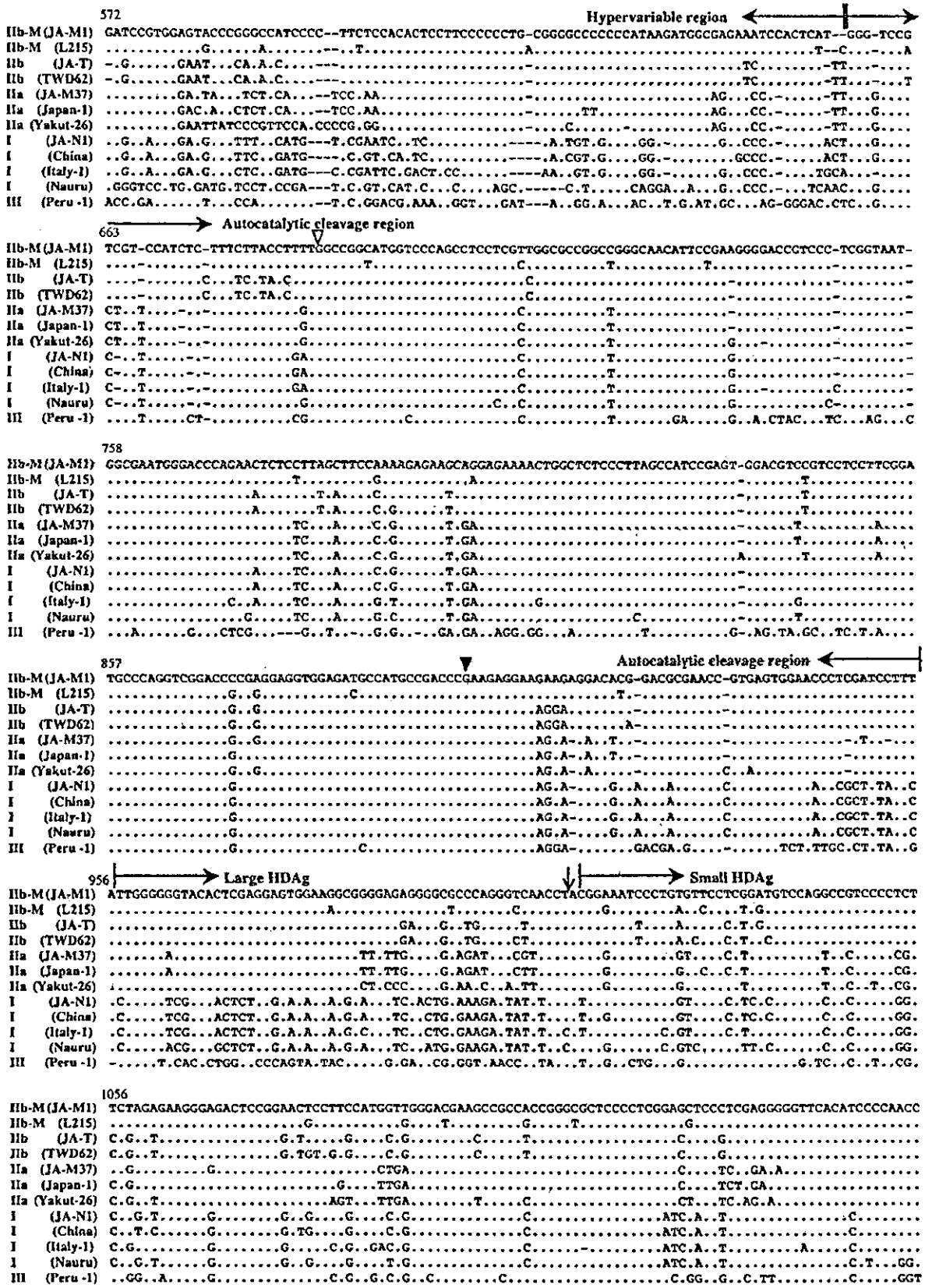

Fig. 2. (*cont.*) For legend see page 3282.

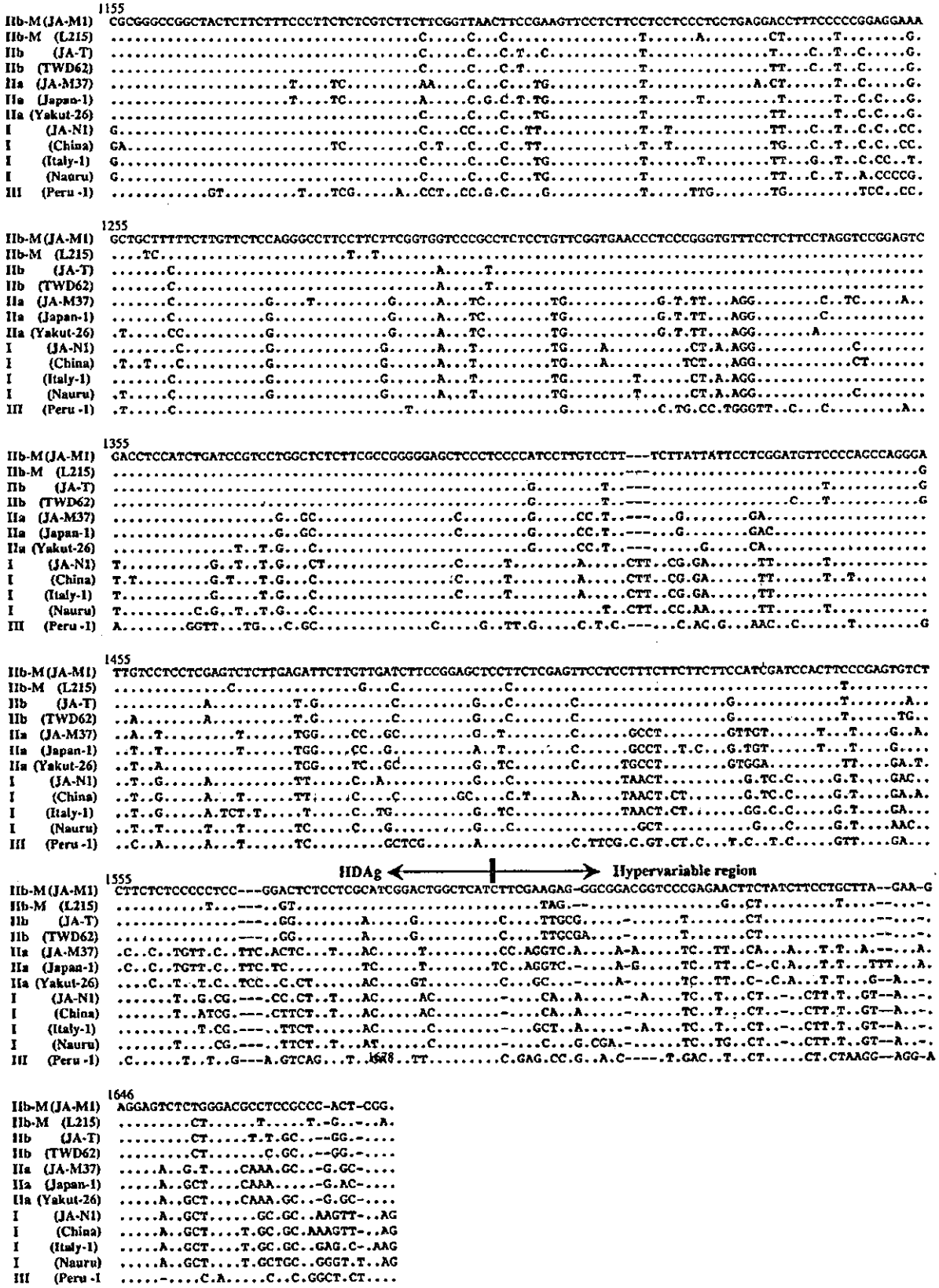

Fig. 2. (*cont.*) For legend see page 3282.

Table 2. Nucleotide identities between IIb-Miyako and other genotypes

Numbers given are the nucleotide identities (%) between the isolate JA-M1 (AF309420) and each isolate listed below. Sources of the isolates: L215, AB088679; TWD62, AF018077; Japan-1, X60193; Italy-1, X04451; Peru-1, L22063.

	Genotype				
	IIb-M (L215)	IIb (TWD62)	IIa (Japan-1)	I (Italy-1)	III (Peru-1)
Complete sequence (%)	93·8	87·3	78·1	72·7	64·6
Autocleavage region (nt 658–956)	95·6	93·0	92·9	89·2	74·8
Delta antigen (nt 957–1597)	94·4	90·1	81·5	77·9	70·7
Hypervariable region (nt 1598–657)	92·5	82·5	69·0	61·1	54·7

Table 3. Clinical and virological features according to HDV genotype

HBeAg was negative in all the patients.

	Genotype				
	I (n=2)	IIa (n=1)	IIb (n=8)	IIb-Miyako (n=29)	P
Sex (male:female)	0:2	1:0	4:4	18:11	NS
Age (years)	34±16	39	59±14	61±12	NS
ALT (IU l^{-1})	57±7	63	52±27	94±182	NS
HBV genotype	A 1/B 1	B 1	B 8	B 29	NS
HBV DNA level*	3·7(<3·7)†	<3·7†	<3·7 (<3·7–4·1)†	<3·7 (<3·7–5·5)†	NS
Stage (ASC:CH:LC)	0:1:1	0:1:0	3:5:0	0:16:13	0·0009‡
HCC	0	0	0	4	NS
Distribution	Nagasaki 2	Miyako 1	Miyako 7 Tokyo 1	Miyako 29	NS

*$\log_{10}$ HBV DNA (copies ml^{-1}).
†Median range.
‡Between IIb and IIb-Miyako.

and II, including the prototype genotype IIb, have a 2–4 bp structure on both sides of the RNA editing site, located on the opposite strand at around nt 580, and this structure is required for efficient RNA editing and HDV replication (Nakano *et al.*, 2001; Ma *et al.*, 2003; Ivaniushina *et al.*, 2001). The RNA editing site of IIb-M showed a unique structure, where this base-paired structure was disrupted one nucleotide upstream adjacent to the RNA editing site. Furthermore, these structures around the RNA editing site and the opposite site were well conserved among genotype IIb and among genotype IIb-M, despite being different between genotypes IIb and IIb-M.

Deduced HDAg sequence of HDV genotype IIb in Okinawa

Fig. 4 shows the predicted HDAg amino acid sequence of the representative isolate of HDV genotype IIb-M aligned with HDV isolates of other genotypes. The identities in the amino acid sequences of the large HDAg between IIb-M and I (Wang *et al.*, 1986), IIa (Imazeki *et al.*, 1991), IIb (Wu *et al.*, 1998), IIb-M (L215) (Ma *et al.*, 2003) and III (Casey *et al.*, 1993) were 70, 79, 87, 92 and 61 %, respectively (Table 4). There was considerable variation among genotypes within several domains: the RNA-binding domain was the most

Fig. 2. Nucleotide alignment of whole genomic sequences of HDV isolates. The nucleotides are numbered according to Wang *et al.* (1986) The genomic autocatalytic cleavage site (nt 685/686) is indicated by the white arrowhead. The site (nt 900/901) corresponding to the cleavage site of the antigenomic RNA is indicated by the black arrowhead. The RNA editing site (nt 1012) is indicated by an arrow. Sources of isolate sequences are as follows: L215, AB088679; TWD62, AF018077; Japan-1, X60193; Yakut-26, AJ309879; China, X77627; Italy-1, X04451; Nauru, M58629; Peru-1, L22063. JA-M1 and JA-M37 (from Miyako), JA-N1 (from Nagasaki) and JA-T (from Tokyo) were sequenced in this study.

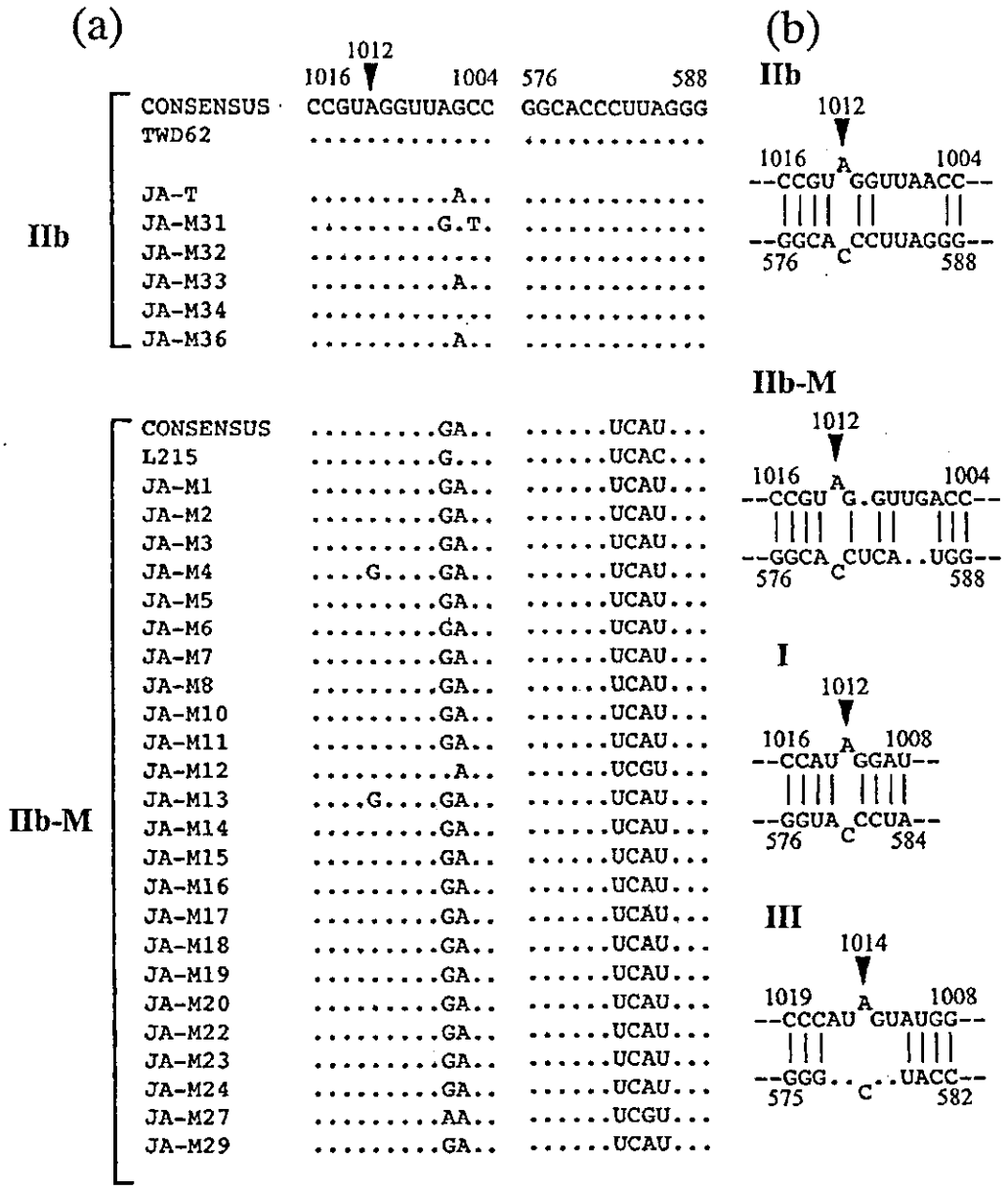

Fig. 3. RNA editing site of HDV genotype IIb and IIb-M. (a) Nucleotide sequences of the RNA editing site of HDV genotypes IIb and IIb-M, which is formed between anti-genome RNA surrounding the edited A residue (nt 1012) and nucleotide sequences of the opposite site (nt 580) of the unbranched rod structure of HDV. Sources of isolate sequences are as follows: L215, AB088679; TWD62, AF18077. JA-M1 to JA-M36 (from Miyako) and JA-T (from Tokyo) were sequenced in this study. (b) The base-paired structure formed by genotype IIb, IIb-M, I and III for RNA editing.

conserved region, followed by the nuclear localization signal (NLS) region, the coiled-coil sequence containing the leucine zipper motif and the N terminus, and the C-terminal packaging sequences of 19 amino acids (Modahl & Lai, 2000). The cysteine residue four amino acids from the C terminus, the site of prenylation required for binding with HBsAg (Glenn *et al.*, 1992), was well conserved in every isolate. Comparing IIb and IIb-M, the NLS and RNA binding domains were well conserved, whereas the coiled-coil domain and packaging sequences showed significant differences. In the coiled-coil domain, the substituted amino acid residues had similar properties (D → E, T → N and I → L) and the leucine zipper motif was completely conserved (Chen *et al.*, 1992; Wang & Lemon, 1993), while the packaging sequence of most of the IIb-M isolates showed the characteristic four amino acid substitutions in the proline

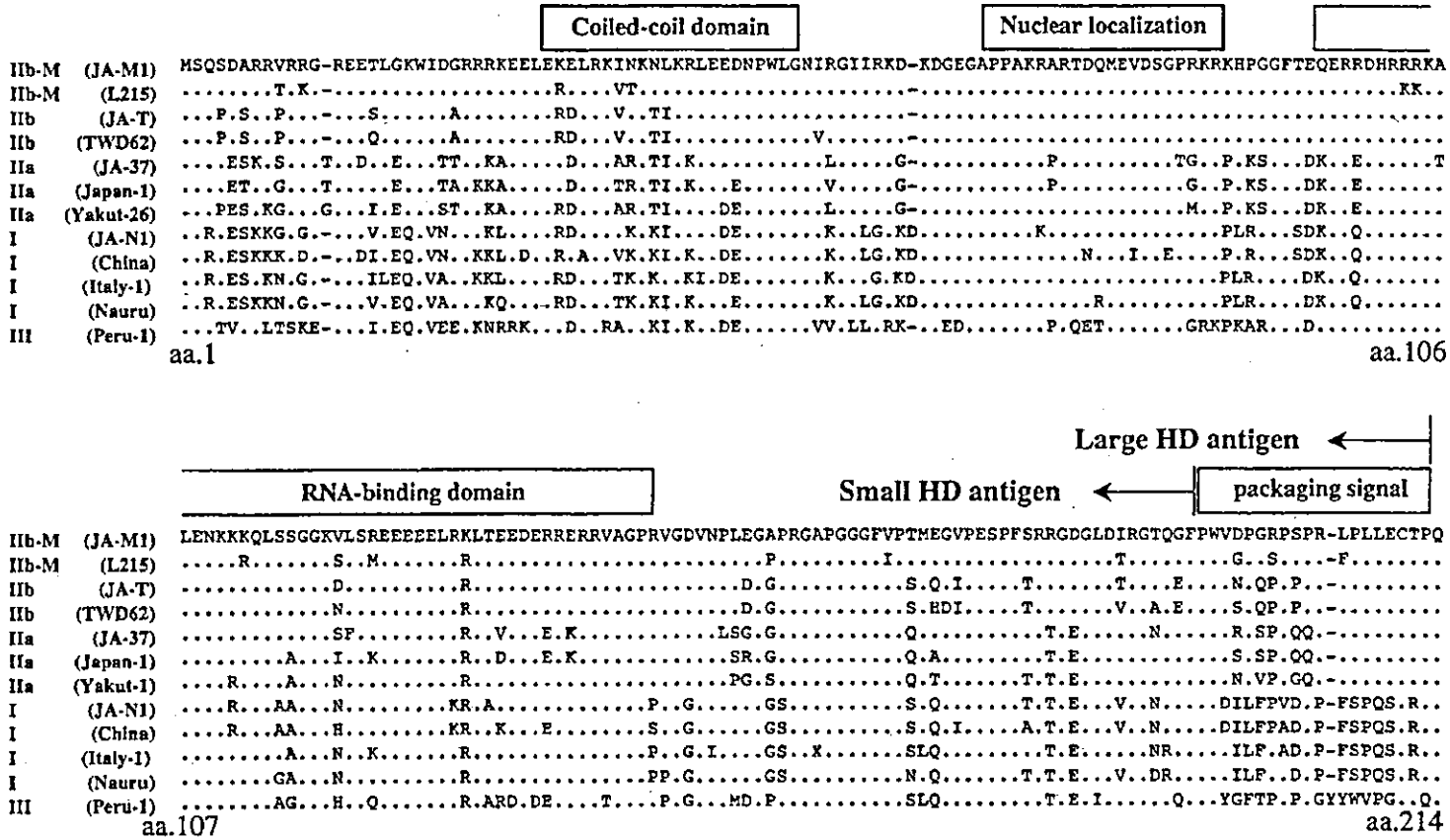

Fig. 4. HDAg amino acid alignment of HDV isolates. The amino acids are numbered according to Wang *et al.* (1986). Dots indicate conserved amino acids and dashes indicate missing amino acids. Sources of isolate sequences are as follows: L215, AB088679; TWD62, AF18077; Japan-1, X60193; Yakut-26, AJ309879; China, X77627; Italy-1, X04451; Nauru, M58629; Peru-1, L22063. JA-M1 and JA-M37 (from Miyako), JA-N1 (from Nagasaki) and JA-T (from Tokyo) were sequenced in this study.

Table 4. Amino acid identities of HDAg between IIb-Miyako and other genotypes

Numbers given are the amino acid identities of HDAg (%) between the isolate JA-M1 (AF309420) and each isolate listed below. Sources of the isolates: L215, AB088679; TWD62, AF018077; Japan-1, X60193; Italy-1, X04451; Peru-1, L22063.

	Genotype				
	IIb-M (L215)	IIb (TWD62)	IIa (Japan-1)	I (Italy-1)	III (Peru-1)
Complete sequence (%)	92	87	79	70	61
Coiled-coil domain (aa 31–52)	86	77	68	59	64
Nuclear localization domain (aa 68–88)	100	100	86	95	62
RNA-binding domain (aa 97–146)	88	96	84	90	78
Packaging signal domain (aa 195–214)	84	79	74	26	21

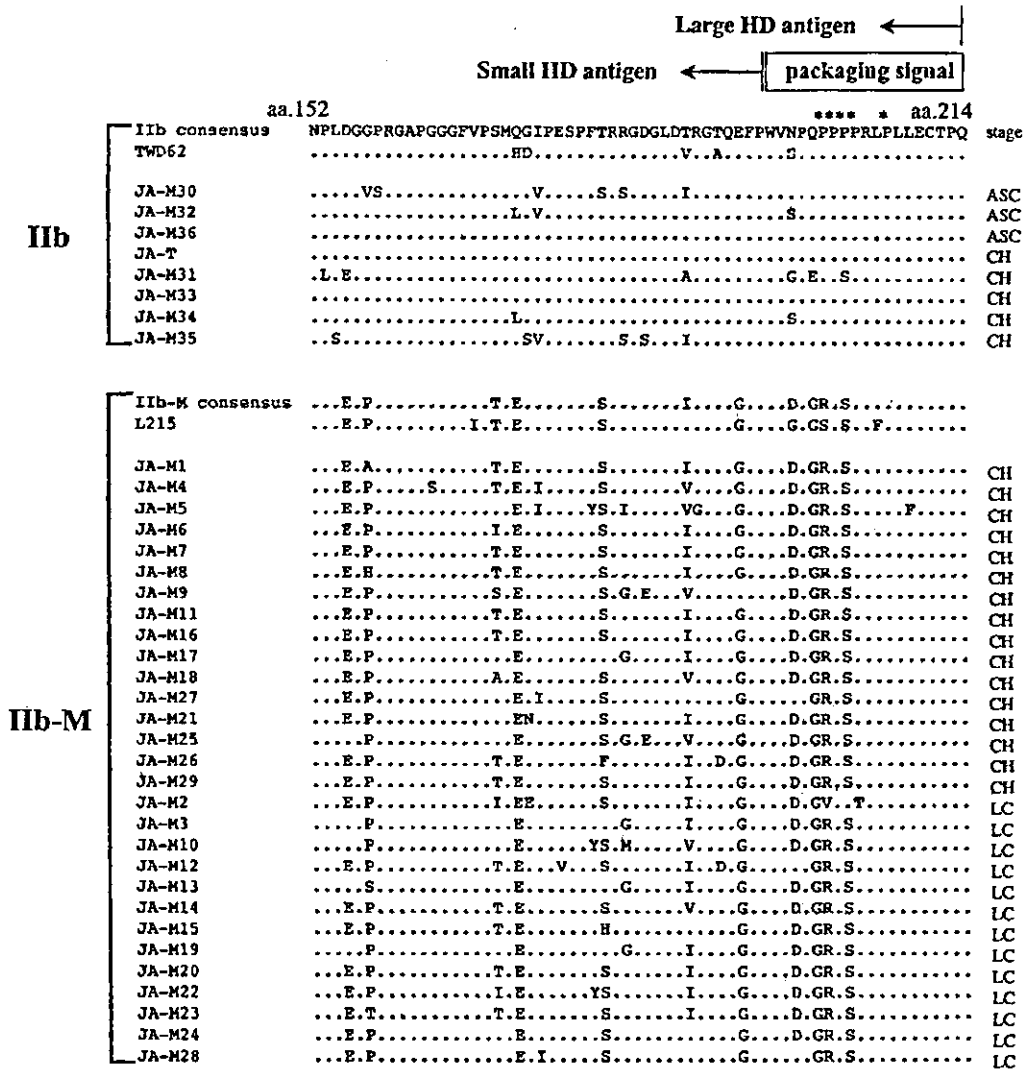

Fig. 5. C-terminal end of HDAg of HDV genotypes IIb and IIb-M. The C terminus of the HDAg from all the IIb ($n=8$) and IIb-M ($n=29$) genotypes were aligned. Dots indicate the same amino acids as the consensus sequence of genotype IIb. The stage (ASC, CH or LC) is described at the right side of the sequences. An asterisk indicates a proline residue in the packaging signal. Sources of isolates are as follows: L215, AB088679; TWD62, AF18077. JA-M1 to JA-M36 (from Miyako) and JA-T (from Tokyo) were sequenced in this study.

stretch that regulates extranuclear export of HDAg (Lee *et al.*, 2001) (Fig. 5).

DISCUSSION

In the present study, we initially sequenced the full-length HDV genome from 33 patients in Japan with chronic HDV infection and demonstrated a new HDV genotype IIb variant in the Miyako Islands, Okinawa, the Japanese islands nearest to Taiwan. Patients infected with genotype IIb-M showed greater progression to CH or liver cirrhosis than genotype IIb-infected patients with similar clinical backgrounds. HDV genotype IIb-M has specific genetic structures in the RNA editing site and the packaging signal sequence of HDAg, which could potentially influence the efficiency of HDV replication (Casey, 2002; Hsu *et al.*, 2002; Lee *et al.*, 1995, 2001; Wu *et al.*, 1995b; Yang *et al.*, 1995). The observed correlation between HDV genetic structure and clinical characteristics suggests a critical role for variations in the RNA editing site and packaging signal of the HDAg gene in determining the diversity of clinical outcome, even among patients infected with the same genotype of HDV.

We identified the new HDV genotype IIb variant by phylogenetic analysis of the complete genomes of 33 HDV isolates. Among them, 30 isolates, mostly from the Miyako Islands, were classified as genotype IIb (Wu *et al.*, 1998) or its variant, IIb-M. In previous studies including our own, HDV genotypes in the Miyako Islands have been considered as genotype IIb (Sakugawa *et al.*, 1999; Ma *et al.*, 2003; Arakawa *et al.*, 2000). However, the present detailed phylogenetic analysis using the full genome successfully identified a cluster distinct from the prototype IIb cluster. In fact, the nucleotide homologies between genotype IIb and IIb-M and among genotype IIb-M were clearly different, i.e. 88–90 % and 94–97 %, respectively. HDV genotype II is divided into two types in Taiwan (i.e. IIa and IIb), with 77 % nucleotide homology between the complete sequences of genotype IIa and IIb (Wu *et al.*, 1998). Although the criteria for defining identical genotype by homology analysis were not determined, the difference between IIb and IIb-M seems to be less than that between IIa and IIb, as shown by phylogenetic tree analysis. In fact, a IIa variant was recently reported in Siberia (IIa-Yakutia), which in comparison with IIa showed a similar degree of genetic differences (Ivaniushina *et al.*, 2001). Based on these results, we conclude that IIb-M should be considered as a genetically relevant IIb variant.

Genotype II is confined to East Asia (mainly Siberia, Japan and Taiwan), in contrast to the ubiquitous global distribution of genotype I (Gerin *et al.*, 2002). Genotype IIb was first identified in Taiwan (Wu *et al.*, 1998) and we subsequently reported it among patients from the Miyako Islands (Sakugawa *et al.*, 1999). However, the origin of clusters of IIb-M cannot be precisely determined. If in the future the precise evolution rate of the HDV genome can be determined, then the temporal estimation of the spread of HDV using a molecular clock might be possible.

One of the most important findings in the present study is that the clinical pictures differ between genotype IIb and IIb-M. Our previous studies demonstrated that HDV genotype II is predominant in this area and that these patients show heterogeneous clinical pictures ranging from ASC to HCC (Sakugawa *et al.*, 1999; Nakasone *et al.*, 1998); however, the reason for this diversity could not be explained based on the known clinical and virological factors of HBV. In the present study, all of the patients with chronic HDV genotype IIb infection were ASC or CH and none were at the LC or HCC stage. In contrast, 55 % and 45 % of patients with genotype IIb-M were in the CH and LC stages, respectively, and none of them was ASC. These findings indicate that patients with genotype IIb-M are more likely to progress to LC and HCC than those with genotype IIb and that differences in HDV genotype could cause the different clinical pictures observed in this population.

The main cause of the difference in liver disease between patients with IIb and IIb-M seems to be the diversity of HDV itself. Although the severity of liver disease in hepatitis D can be influenced by a variety of host factors including genetic backgrounds as well as HBV status, no apparent differences were found between patients with genotype IIb and IIb-M. In particular, in most patients, serum HBV DNA levels were below 10^5 copies ml^{-1} with negative HBeAg, which were too low to cause HBV-related liver injury (Sakugawa *et al.*, 2001; Lok *et al.*, 2001). Similarly, the HBV genotype, which is also known to cause diversity of liver disease (Kao *et al.*, 2000; Orito *et al.*, 2001), was genotype B in all of the patients from the Miyako Islands. Differences in HDV genotype are known to affect the pathogenesis and diverse clinical pictures of HDV infection (Casey *et al.*, 1993; Wu *et al.*, 1995a; Ivaniushina *et al.*, 2001). Genotype III, exclusively found in the northern part of South America, is associated with fulminant hepatitis (Casey *et al.*, 1993). On the other hand, genotype II in Taiwan is generally associated with a more favourable outcome than genotype I, which causes liver disease with diverse clinical presentation from asymptomatic carrier to rapidly progressive CH (Wu *et al.*, 1995a). A IIa variant recently reported in Yakutia, Siberia, Russia, also causes a severe hepatitis comparable with genotype I in this cohort (Ivaniushina *et al.*, 2001). These findings strongly suggest that the genetic structure of HDV can profoundly influence the pathogenesis of liver injury in HDV infection. However, the genetic structure responsible for such clinical features could not be readily determined because the genetic differences between the different genotypes are too diverse, as seen in Fig. 2. In contrast, despite the different clinical pictures between IIb and IIb-M, the genetic differences are small enough to enable the definition of the genetic features of HDV pathogenesis and replication *in vivo*.

By comparative analysis between the genotype IIb and IIb-M genomes, the highest difference was found in the

hypervariable region (nt 1598–657) and moderately high in HDAg (nt 957–1597), whereas the autocatalytic regions encoding ribozyme activity were well conserved (Wu & Lai, 1989). The hypervariable region was markedly variable even within the same genotype, supporting the notion that this region cannot confer any relevant biological function aside from the formation of the rod structure of HDV RNA required for RNA synthesis by RNA polymerase II (Modahl & Lai, 2000). On the other hand, the requirement for strict secondary or tertiary structure of the autocatalytic domain seems to be so crucial for full activity of the ribozyme needed for the rolling-circle mechanism of HDV replication that divergence of this region could not exist among isolates. Therefore, HDV genetic regions other than the hypervariable region or the autocatalytic domain, i.e. the HDAg coding region, confer the clinical difference between IIb and IIb-M. In the HDAg coding region, we found that the most prominent differences are in the RNA editing site and the packaging signal in the C terminus of the large HDAg (Modahl & Lai, 2000). Although the coiled-coil domain (Wang & Lemon, 1993) also showed modest differences, the leucine zipper motif (Chen *et al.*, 1992) was preserved, and the nuclear localizing signal (Xia *et al.*, 1992) and RNA binding domain (Lin *et al.*, 1990) were identical in IIb and IIb-M, indicating that these regions are not responsible for liver damage.

In genotype IIb-M, there was particular disruption of the base-pairing structure two bases upstream of the editing site, resulting in a characteristic structure in this region distinct from genotype IIb and IIa (Fig. 3). There is a possibility that the unique structure of the RNA editing site of genotype IIb-M may affect the observed difference in pathogenesis between genotype IIb and IIb-M. RNA editing is a pivotal event during the HDV replication cycle (Casey *et al.*, 1992), where initially in HDV infection, small HDAg transactivates HDV RNA synthesis by RNA polymerase II (Wu *et al.*, 1995b). Large HDAg, which has 19 additional amino acids (the packaging signal sequence) at the C terminus of small HDAg, is produced in the late stage of infection by RNA editing of the umber stop codon (UAG) to a tryptophan codon (UGG) in the small HDAg gene by the host adenosine deaminase (Modahl & Lai, 2000). Large HDAg suppresses HDV RNA replication and promotes virion assembly by extranuclear export of the HDAg–RNA complex and binding to HBsAg. The regulatory mechanism of this RNA editing is not fully understood, but the secondary structure of the antigenomic region corresponding to the 3′ end of the small HDAg gene influences the editing efficiency (Casey *et al.*, 1992; Wu *et al.*, 1995b; Casey, 2002). A recent *in vitro* mutational study clearly demonstrated that the base-pairing structure surrounding the RNA editing site profoundly influences RNA editing efficiency (Hsu *et al.*, 2002). In genotype I, the base pairing surrounding this site is particularly strong (four base pairs on each side), whereas a weaker secondary structure is found within genotype II that is associated with milder liver disease. In addition, the distinct structure of genotype III is thought to be involved in fulminant hepatitis (Casey, 2002). Collectively, the specific differences in the base-paired structure of the RNA editing site might explain to some extent the difference in virulence among HDV genotypes. Therefore, although *in vitro* confirmation is necessary, it appears that the loose structure around the RNA editing site found in genotype IIb-M might influence the editing efficiency in comparison with genotype IIb, leading to the observed clinical differences.

In addition to the difference in the RNA editing site, there are four characteristic amino acid differences (codons 198, 200, 201 and 203) in the packaging signal sequence of the large HDAg between genotype IIb and IIb-M (Fig. 5). This region is almost completely conserved among IIb-M isolates. As mentioned above, addition of this packaging signal reverses the property of HDAg (Modahl & Lai, 2000; Chang *et al.*, 1993). The exact molecular mechanism of this phenomenon is not completely understood, but, as shown in Fig. 4, a sequence of 19 amino acids was highly genotype specific. *In vitro* analysis demonstrated that swapping the packaging signal sequence of genotype IIa with that of genotype I HDAg decreases the virus replication of genotype I, while the replication of genotype II was intensified, indicating that this region directly regulates HDV RNA replication (Hsu *et al.*, 2002). Thus, the structural characteristics of this region in IIb-M can profoundly influence virus replication. In particular, two of the four amino acid differences found in IIb-M were located in the proline residues, which are implicated in the assembly process by extranuclear export of the HDAg–RNA complex. In fact, in a recent study with cultured cells, mutation of the proline residue in this region attenuated the extranuclear export of large HDAg (Lee *et al.*, 2001). However, these data did not directly prove that the C-terminal domain structure of HDAg influences the pathogenesis. In the future, *in vitro* mutational studies should be performed to verify the hypothesis that differences in the packaging signal sequence in genotype IIb-M can modulate HDV replication and lead to progressive disease.

In conclusion, we have identified a new genetic subclass of HDV genotype IIb in the Miyako Islands, Okinawa, Japan. This HDV variant is associated with more aggressive liver disease and has specific genetic changes in the C-terminal packaging signal of large HDAg as well as the RNA editing sequence. These findings should prompt further investigation into the relationship between HDV genetic structures and their function and pathogenesis. This study provides valuable information for molecular epidemiology and diagnosis, contributes to a better understanding of HDV biology and offers the potential for new therapies for HDV – a disease for which no effective therapy has yet been established.

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