0.82X). This figure indicates that regardless of when the patients acquired the HCV infection, they developed HCC at approximately the same time of life. Noda et al. examined the relation between the period from blood transfusion to the diagnosis of HCC and the age of the patient at the time of blood transfusion in 85 HCC patients.[27] In a retrospective and preliminary study, patients were collected from 11 hospitals for the purpose of investigating whether habitual alcohol intake and the age at HCV infection were factors for the progression of chronic hepatitis C to cirrhosis and HCC in patients with history of blood transfusion. Our Y intercept was 6 years higher than that reported by Noda et al.[27] The reason for this discrepancy may be that the current study was a cohort study and we were able to determine precisely the time from blood transfusion to the diagnosis of HCC and the age of the patient at the time of blood transfusion. The current study data thus demonstrated the natural history of blood transfusion-related chronic hepatitis C.

It is not clear why the majority of patients developed HCC in their 60s, regardless of the duration of the infection. We previously reported that the rate of progression of fibrosis to cirrhosis in patients with chronic hepatitis C was accelerated by aging.[7,22] In addition, Poynard et al. concluded that the progression of liver fibrosis is closely related to patient age at the time of HCV infection. They found that the rate of progression of fibrosis was proportional to patient age at the time of HCV infection, which is the main factor associated with progression to fibrosis.[28] Similarly, the rate of progression of fibrosis in the current study was proportional to patient age at the time of HCV infection (data not shown). The histologic deterioration that promotes HCC development may take place rapidly in the seventh decade of life.

Based the results of the current study, it would be natural to speculate that host-dependent factors associated with age may play a pivotal role in hepatocarcinogenesis in patients with posttransfusion hepatitis C. Aging may be associated with progressive loss of various stress tolerances due to a decline in the functional reserve of multiple organ systems.[29] It has been shown that aging is associated with aberrant cytokine function and decreased capacity for DNA repair,[30] which may facilitate the development of HCC. However, we are unable to explain the precise relation between patient age and HCC development. Further assessment of the role of aging in the progression of HCV is needed.

Posttransfusion chronic hepatitis C is a disease with considerable mortality and morbidity. Age, fibrosis, duration of HCV infection, and alcohol consumption appear to be associated independently with HCC development. However, the majority of HCC patients in the current study developed HCC when they were age > 60 years regardless of the duration of HCV infection. The current study may be useful in establishing an adequate follow-up policy for patients with posttransfusion chronic hepatitis C.

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The Des-γ-Carboxy Prothrombin Index Is a New Prognostic Indicator for Hepatocellular Carcinoma

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BACKGROUND. Des-γ-carboxy prothrombin (DCP) has been reported to be an important prognostic factor in patients with hepatocellular carcinoma (HCC). Recently, a monoclonal antibody, 19B7, which recognizes the Gla domain of DCP, has been identified. The 19B7 antibody recognizes an epitope different from that recognized by MU-3, which is another antibody against DCP. In this study, the authors investigated the measurement of DCP using the antibodies MU-3 and 19B7, respectively, as a prognostic factor for patients with HCC who had solitary, small tumors and or Child Stage A HCC.

METHODS. One hundred four patients with HCC who had solitary, small tumors or Child Stage A tumors were enrolled in the study between 1991 and 2001. All patients were treated and were followed for a mean of 3.2 years. The authors analyzed the correlation between the DCP Index (DCP measured by MU-3 and DCP measured by 19B7) and patient prognosis. The patients were classified into 3 groups based on their DCP Index: 1) DCP negative (DCP < 40 milli arbitrary unit ([mAU]/mL); 2) low DCP Index (DCP ≥ 40 mAU/mL; MU-3:19B7 ratio, < 3.0; and 3) high DCP Index (DCP > 40 mAU/mL; MU-3:19B7 ratio, > 3.0).

RESULTS. The survival rate for patients in the high DCP Index group was lower compared with the survival rate for patients in the DCP-negative group and was significantly lower compared with the survival rate for patients in the low DCP Index group. In a univariate Cox proportional hazards model, the positive factors were high DCP Index and low DCP Index. Among the positive predictive factors that were analyzed using a multivariate Cox proportional hazards model were age (hazard ratio, 3.27; $P = 0.006$), low DCP Index (hazard ratio, 2.87; $P = 0.012$), and high DCP Index (hazard ratio, 12.3; $P < 0.0001$).

CONCLUSIONS. The prognosis of patients who had a high DCP Index score was poorer compared with patients who had a low DCP Index score and patients who were classified as DCP negative. The authors concluded that the DCP Index is a prognostic indicator for patients with HCC. *Cancer* 2003;98:2671–7.
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KEYWORDS: hepatocellular carcinoma, des-γ-carboxy prothrombin, prognosis, index.

Des-γ-carboxy prothrombin (DCP) is useful as a marker of hepatocellular carcinoma (HCC). However, because of its low sensitivity, DCP is not suitable for the initial detection of small HCC tumors.[1,2] Recently, a sensitive method for serum DCP determination has been developed and reportedly is positive in 30–40% of patients with small and solitary HCC.[3,4] Furthermore, it has been reported that a positive DCP determination at the time of diagnosis of HCC indicates a poor prognosis.[5] Hence, measurement of DCP has been suggested not only for the diagnosis of HCC but also as a prognostic indicator after treatment.[6,7]

A new monoclonal antibody (19B7) against DCP produced from a human HCC cell line (PLC/PRF/5), has been used in the development of an enzyme immunoassay (EIA).[8] The 19B7 kit for detection of DCP due to vitamin K deficiency is superior to the MU-3 kit.[9,10] It is reported that 19B7 can be used as a tumor marker and can distinguish the DCP subtype produced in HCC from that produced in various other diseases. Nakao et al. reported that the elevation of the DCP level measured by 19B7 is higher compared with the level measured by MU-3 in pancreatobiliary malignancies and that the 19B7-related DCP level is significantly high in alcoholic liver disease.[11] The ratio of DCP determined by MU-3:19B7 (henceforth referred to as the MU-3:19B7 ratio or the DCP Index) has been measured in patients with liver disease, chronic hepatitis, cirrhosis, and HCC. The ratio was about 1.0–1.5 in patients with chronic hepatitis and cirrhosis and > 2.0 in patients with HCC.[12] However, to the best of our knowledge, the relation between this ratio and the prognosis of patients with HCC is not known. In the current study, we analyzed the correlation between the ratio of plasma DCP concentrations and prognosis in patients with small and solitary HCC.

MATERIALS AND METHODS

Patients and Diagnosis

Between June 1991 and March 2001, 539 patients with HCC were admitted to the Liver Center at National Nagasaki Medical Center (Nagasaki, Japan) for tumor therapy. The current cohort study included the following criteria for enrollment: 1) a solitary HCC lesion measuring ≤ 3 cm in greatest dimension; 2) no evidence of extrahepatic or intrahepatic metastasis; 3) Child-Pugh risk grouping, Child Stage A HCC, ranked according to the criteria of Pugh et al.;[13] and 4) collection of serial serum samples (Fig. 1). Written informed consent was obtained from all patients, and an institutional review board approved the study.

The diagnosis of HCC was made by a combination of ultrasonography, computed tomography, magnetic resonance imaging, and digital subtraction angiography (DSA). Most patients underwent percutaneous fine-needle aspiration liver biopsy under ultrasonography guidance to confirm the diagnosis.

The degree of tumor differentiation was determined histologically according to a modified Edmondson and Steiner classification. On the basis of nuclear overcrowding, increased cytoplasmic basophilia, and microacinar formation, tumors were defined as well differentiated, moderately differentiated (Grade 3), or poorly differentiated (Grade 4).

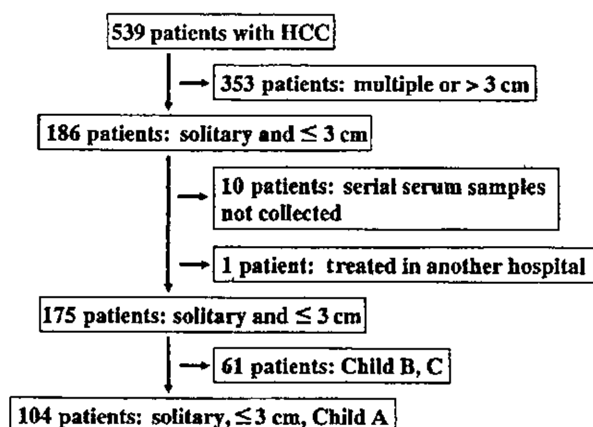

FIGURE 1. Summary of the enrollment of the study population. HCC: hepatocellular carcinoma.

Treatment and Follow-Up

The following treatment criteria were used in the study: 1) Percutaneous ethanol injection therapy (PEIT) was performed to allow ultrasonography detection of a solitary HCC lesion. 2) Transcatheter arterial embolization (TAE) was performed for a lesion that could not be detected by ultrasonography to allow detection by DSA. 3) An operation was performed for a lesion that could not be treated using PEIT or TAE.

After initial treatment, the condition of each patient was followed carefully. Serum α-fetoprotein (AFP) and DCP concentrations were measured monthly. Ultrasonography and computer tomography were performed every 3 months until 6 months posttreatment and every 6 months thereafter until 30 months posttreatment. Recurrences of HCC were confirmed by tumor enlargement or by the appearances of new lesions on imaging studies. When a recurrence was suspected, an angiography or a percutaneous fine-needle aspiration liver biopsy was performed under ultrasonography guidance. Subsequent treatments for recurrent HCC were selected according to the number of tumors and liver function. The therapy for patients with recurrent tumors included the following: 1) PEIT usually was selected for patients with recurrent HCC tumors that measured ≤ 3 cm with ≤ 3 nodules. 2) TAE or transcatheter arterial chemoembolization was selected for patients with single nodules measuring > 3 cm or with multiple nodules that produced unequivocal tumor stains.

The closing date of the current study was March 2002 or the time of a patien's death. The possible causes of death were defined as follows: 1) liver-related disease (tumor progression, liver failure, or bleeding from esophageal-gastric varices) and 2) others (all other disease). If a patient had not been mon-

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 (%)[a]
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.
[a] 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.

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 (× 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.

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.

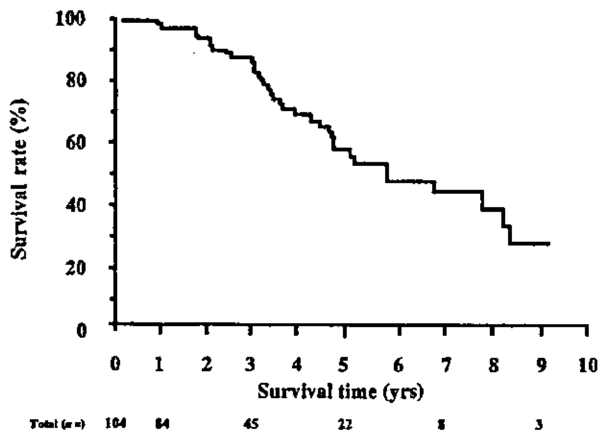

FIGURE 2. Overall survival rates for 104 patients who underwent initial treatment for hepatocellular carcinoma that measured ≤ 3 cm in greatest dimension.

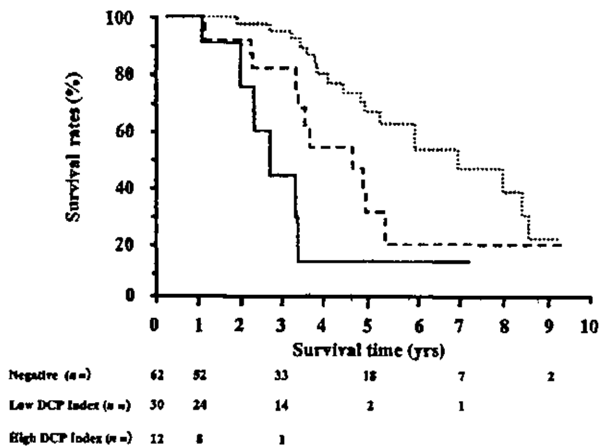

FIGURE 3. Survival rates prepared according to the Kaplan–Meier method are shown for the groups defined by the des-γ-carboxy prothrombin (DCP) Index. The survival rate of the high DCP Index group (solid line; $n = 12$) was significantly lower compared with the negative group (dotted line; $n = 62$) and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

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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

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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)

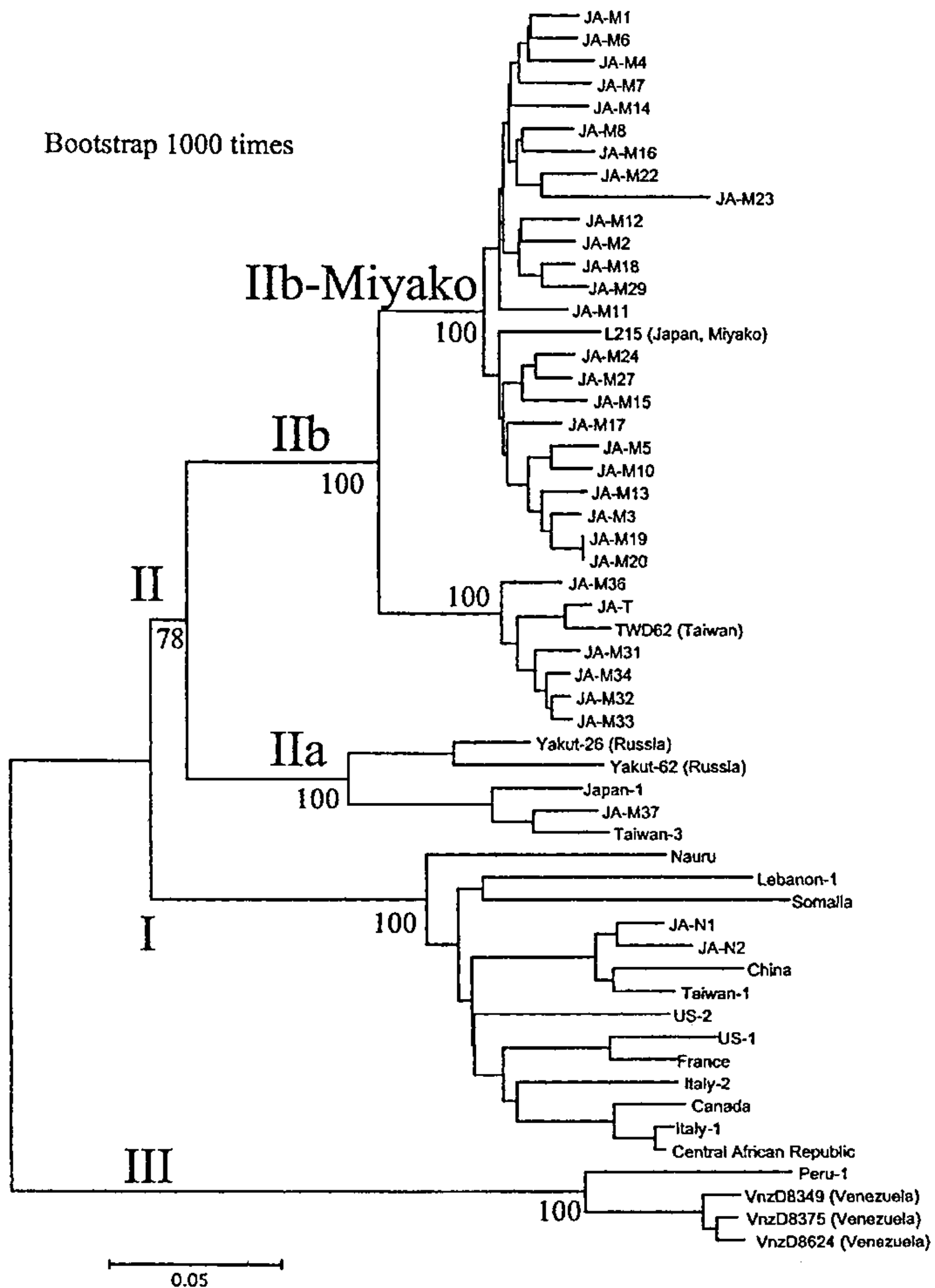

Fig. 1. For legend see page 3278.

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 [$<1\cdot0\times10^6$ 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 ($P=0\cdot0009$ 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).

```
              nt. 1
IIb-M(JA-M1)  GATGGGCCACAGTG-CCGACGAAGAGGCCGCAGGTGGGAGGATCAG-CCA--CCGGAGAGGGACGCGATCGT--AGAGTGGAGGAAACTTCGGAAGCGGA
IIb-M (L215)  .......T....-.......G...T....A...........-..--.....................C-A....................G..AA.
IIb    (JA-T) .......G....TC.......G...C................-..--.....-...........CCAA......G.....TC...CG....A.
IIb  (TWD62)  .......G....TC.......G...C................-..--.....-...........CCAA......G.....TC...CG....A.
IIa (JA-M37)  ........G..--AC.G.G...G.G..G..A..TG....A.GGG.TAT.---...AG...-..TC.TCAAC.AA............TC......A.CTT
IIa (Japan-1) ....A...G..-..C.G.G..GGAG..A..AG.TC...G.A.GGG..A..--...AG...-..TCTCCA.C.AA............TC......A.CTT
IIa (Yakut-26) ........G..-.AC.GAG.....GATA.CGGTAA...G...GG..GAAT-....AG.A.--.TCGCCCAC.AA.....A......TTC......A.CTC
I    (JA-N1)  C.......--.TCTC.GAG........A...GCCG-.A........CT.---...AG-....GTAA..C...AA....CATT....-CGC..AG..T.AG
I    (China)  C.......--.TCTC.GAG.....G.AA...GCTG.A...-....TCT.---...AG-....GATGA.C...AA....CATT...-TCA...A.G.TAAG
I  (Italy-1)  CT..A....-...TC.GAG...G...A-...G..G-..........CT.---...AGAG....T.TC.C...AA....CATT....C..CG.AG.AACT.
I    (Nauru)  C...A...--.A.TC.GA........AG...GA.G..A........CT.---...AGAG..--A.TCG:...AA...A...G...........G..CTC
III (Peru-1)  -A......G.--.T..G-G.-...G..GTC.GAAATC.....CGG.G.AGAA.TCCC...A.TT.G.AGAAGAA....G.CGAA..TTC.....C..ATC

              96
IIb-M(JA-M1)  T--CCCAAGAGGATCACTCGAGATTCAAGAGGTGAGGAGGGATCCCCGAGACGCTGGAGGAACGCCGGGAAAAAGA-AAAGAA-GGCAAGAGATTGGTAG
IIb-M (L215)  .--.........G....C...........C............C...............T...G.C............A......-......G........
IIb    (JA-T) AT-.......A.G..GAGAA--TC.......AC....G....C...............ACG.C.G..A.--C.AGG...C.G-AA....GT.-C.....
IIb  (TWD62)  AT-.......A.G..GAGAA-.TC.....CTAC....G....C...............ACG.C.G..A.--C.AGG...T.G-AA....GT.-C.....
IIa (JA-M37)  CT-.......A..A.TGGGAC.C.CTC.AGAAG....-A.TT.T.....-...T.....GAT.CGG.-A.TTTCATTC.---.GAAG...GT.GC..-..
IIa (Japan-1) CC-.......A..A.TGGGAC.C.CTC.AGAAG....-A.AT.T.....-.A.......ACT...G.-A.TTTCATTC.---.GAAG...GT.AC..-..
IIa (Yakut-26) .--.......A..A.CA..A-TCCCTC.AGA.A.AG.A.AATC...ATG-........A..GACTTCC.GGT..CC..GTC..G.AG...GA.-C.....
I    (JA-N1)  .CT.......A..AA.AAA....AAG.......C..AC.ATT.....ATA..........AC.-T..T....--G.GG.....GG.AAGGTG..A----..
I    (China)  .C-.......A.GAA.AAA....AAG......AC..AC.ACC....TAT.G....A...AC.-T..T....--G.GG.....GG.AAGGTG..A----.A
I  (Italy-1)  CT-.......A.CAA.--GAGAGG..TCAG.AA-GC.GAC..GAT..CC-..AAC.CC...GAAT.TCT-GG...GGG...G.G.AAGGTG..A---G.A
I    (Nauru)  .--.......A.G...AGAG.AGCAAGGA.ACG..A.CATTTC...AT.-.........A..CTT..T....--T.GGG.GA..GA.GG.TG..A----..
III (Peru-1)  C--......CTCCCA..AGAG..AGAG.ACAAGATA....AGC....AC......A.CAA.C..T..C.C.CTG.GAG.G..GT..GCG..AGA---...

              186
IIb-M(JA-M1)  AAAAGAGCG-AGCCTCCCGATACGAGTTTGCCA-GGACCTATCAAGTTTGGAGTCATCCG-GCCCGTAGGGGAGAATAGAACACCGGG--GGGTGATCCA
IIb-M (L215)  .........C.......................-..........................-.G...........................--..........
IIb    (JA-T) .........-.......................-..........................-.G..CC....TG.....T...........--..........
IIb  (TWD62)  .........-.......................-..........................-.G..C..A..T..........C........--.........
IIa (JA-M37)  ...G.....-G..........C.....GGT..GC..-.G.....................-.-A..A.A..TT..GAT.-..C.ACA.TC.........
IIa (Japan-1) ...G.....-G..........C.....GGT...C..-.G.....................-.-...A...TT..GAT.-..C.ACA.TC.........
IIa (Yakut-26) .........-......T........AAGG...GC.AG......G...................-.G...A....TT..GAC.C..C.ACA.AC...A.CCT--
I    (JA-N1)  G..G.G...-G..........C....GGGC...A----.ATC.G..........AGCA.TCC.G...A....-TCG.-G.T..C.A.AAG.A..A....--
I    (China)  G..G.G...-G..........C....GGGC...A----..GC............AGCA..CC.G...A....TC..G--.T..C..A.AAG.A.GA...--
I  (Italy-1)  ...G.G...-G..........C....GGGC...A----.CTC..GA.C.....AGCA.TCC.G..CG.....TTG.--.T.GCA.CCAGA...A.GAATC
I    (Nauru)  ...G.....-....CT.....C....GGGC...A----.C.C.G.A.......AGCACTTCCGG.CG.AT..TCG.--GT.GCA.TCAGA...A..AA-T
III (Peru-1)  ...C.GAGACCC.GGT....ATGCCAA.C....GC----A.A.TCC.C.......C....-.G...A.A..AGG..--ACTAC.G.C.GA..........

              279
IIb-M(JA-M1)  CCAGGAGAAGTAG-CGGAGAACCCACCTCCAGAGGACCCCTTCTGCGAACAGAAAAGCTCTTCCCCCTCGGGAGT-AGGGCCGTAGCGATGGGAGGGGAT
IIb-M (L215)  ..........G..-.............................A..........G....C...T.TG.......-.................G..A...
IIb    (JA-T) .........-TG.AA.......AT...................A..........G.TACTCC..T.GATA....C-.A....A.........G..A...
IIb  (TWD62)  .........-TG.A-....A..AT...................A..........G..ACTCC..T.GATA....C-.A.A..A.........G..A...
IIa (JA-M37)  .......GGTGGA--....GGTTA..T................A......G...G....TCCT..TTCG.A...A-.A.A..A.........G.-A...
IIa (Japan-1) .......GGTGGA--.....GA.....................A..........G.....CCT..T.CG.A...AG.A.A..A......A..G..A...
IIa (Yakut-26) .......GGT.T.-A.......T....................A.........GG..TCTC.A..T.GGA....A-.A.A.............G.-A...
I    (JA-N1)  -TCACG..GAA.AG.A..C..AT....................A.........-G......-GA.G.G..A.GAGT.A.C..A..........GA.A...
I    (China)  -TCTCG..GAA.AGGA..C..AT....................A.........-G.A....-GA.G.G..A.GACG.A.C..A.........A.A.A...
I  (Italy-1)  .ACTCGG.GATG.G.A.....AT....................A.......AG.G-..G....GAG.GGTA.GAGT.A.A..A.......A.....A...
I    (Nauru)  ...CAC.G..A.AG.A..C..AT....................A..........G......CGGAG.G-TA...C-.A.A.............G..A...
III (Peru -1) ..C....T-TG.A-....C..G....A................G..........G.C.CTGGTA..GG-.A.G.AATA.C..A...TACAA..G.-A...

              378
IIb-M(JA-M1)  GCTAGGAGTTGGGAG-AAACCGAACCGAGGAGGAAACCAAAGAAAGCAACCGGGCTACCGAGTGGATGTTCCTCTCCCCG-A-AGCTCCCGAGTGAGGCT
IIb-M (L215)  ...............-.................................................................-.-...............
IIb    (JA-T) .........C..AG.-.G...............................................G.C.....-.GG..G..-..........
IIb  (TWD62)  .........C..AG.-.G...............................................G.C.....-.GG..G..-..........
IIa (JA-M37)  .........A..CG.-CG..........G......T..............................G.C.....-GCG..G..-..........
IIa (Japan-1) .........A..AG.-CG..........G......T..............................A.C....A-GCG.TG..-.........C
IIa (Yakut-26) .........A..CG.-CG.................C...............................G.C..AA.--GG.AG..-..........
I    (JA-N1)  .........A.AG.-.GG...............................CG....G......A.CT....-.G...GGA..........
I    (China)  .........A.AG.-.G...AG.A...........T..............TCG.....G......G.CT....-.G...GGA..........
I  (Italy-1)  .........A..AG.-.G.................T..............CG.....G......G.C.....-.G...GGA..........
I    (Nauru)  .........A...C.G.G...............................CG-.CAG......G.C......-.G...GGA..........
III (Peru -1) .........C..AG.-..G..AG.A...CTCA..........G...............C.CC..G......ATC.ATG.G.TC...G............

              476
IIb-M(JA-M1)  TATCCCGGGGA--TCGGCC---TCCGCTCCTCCATGGTGGA-TCCAGGACCCCC---GAAAGGAGGGGGGTGGGCCTTGGACCTCC--GGGAA-CCATCG
IIb-M (L215)  ...........--......--TC.GC....C.........CC........................--...........................C..A-..GT-......
IIb    (JA-T) ...........--.....G.---GT...C..CA...A.CA...CC..........TTCC...C..........AC..T......A..TC...AG...GC-......
IIb  (TWD62)  ...........--.....G----T...C..CA...A.CA...CC..-..GT....TTCCG..C.........AC..T...........-AG...GC-......
IIa (JA-M37)  ...........--.....G--AAT..TC..GAG....G.ACTC.G-A..G....TTCCG...T.AC.....G...GTCACTAGGAA.ACG..GG....GT.
IIa (Japan-1) ...........--.....TG--AAT..TC..GGA....-...C...GA.GG....TTCCG...TG.C.....G...GTCACTAGGAA.ACT..GGT...GT.
IIa (Yakut-26) ...........AC....TA--A.T...C..GAA....AG.ACTC.GG.G-T....TTCCG.....AC..A.G...GTGACTAGGAA--TCA.G.T...GT.
I    (JA-N1)  ...........AC.....GCGAAT..TC..CA...A.CA.CCC..-...G.....TTCC....ATGACC.A.G...CTGGCTAGGAA.GC...GG...AGT.
I    (China)  ...........AC.....G--AAT..TC..CA...A.CA.CTC..-...G.....TTCA....AT..CC.A.G...GTGGCTAGGAA.GC...GG...GGT.
I  (Italy-1)  ...........AC...A.--TTAT..TC..CATC.A.C..GAC...C........TTC.....TGACC..AGG...-.GCTGGGAA.ACC..GG.C..GT.
I    (Nauru)  ...........ACG..C.G--AAT..TC..CATC.A.CA.CTC..-.........TTCCA..ATGACCAA.A.A.GGTG.CTTGGAGCGCG..GGA.CGT.
III (Peru -1) ..........GTGA..C.T----.G..C.T...T.A.CATCGGAATCCCGGG..CCCTCCCA.GAAT...AACAGGGGGAGATCGACC...GCC.GCA..
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Fig. 2. For legend see page 3282.

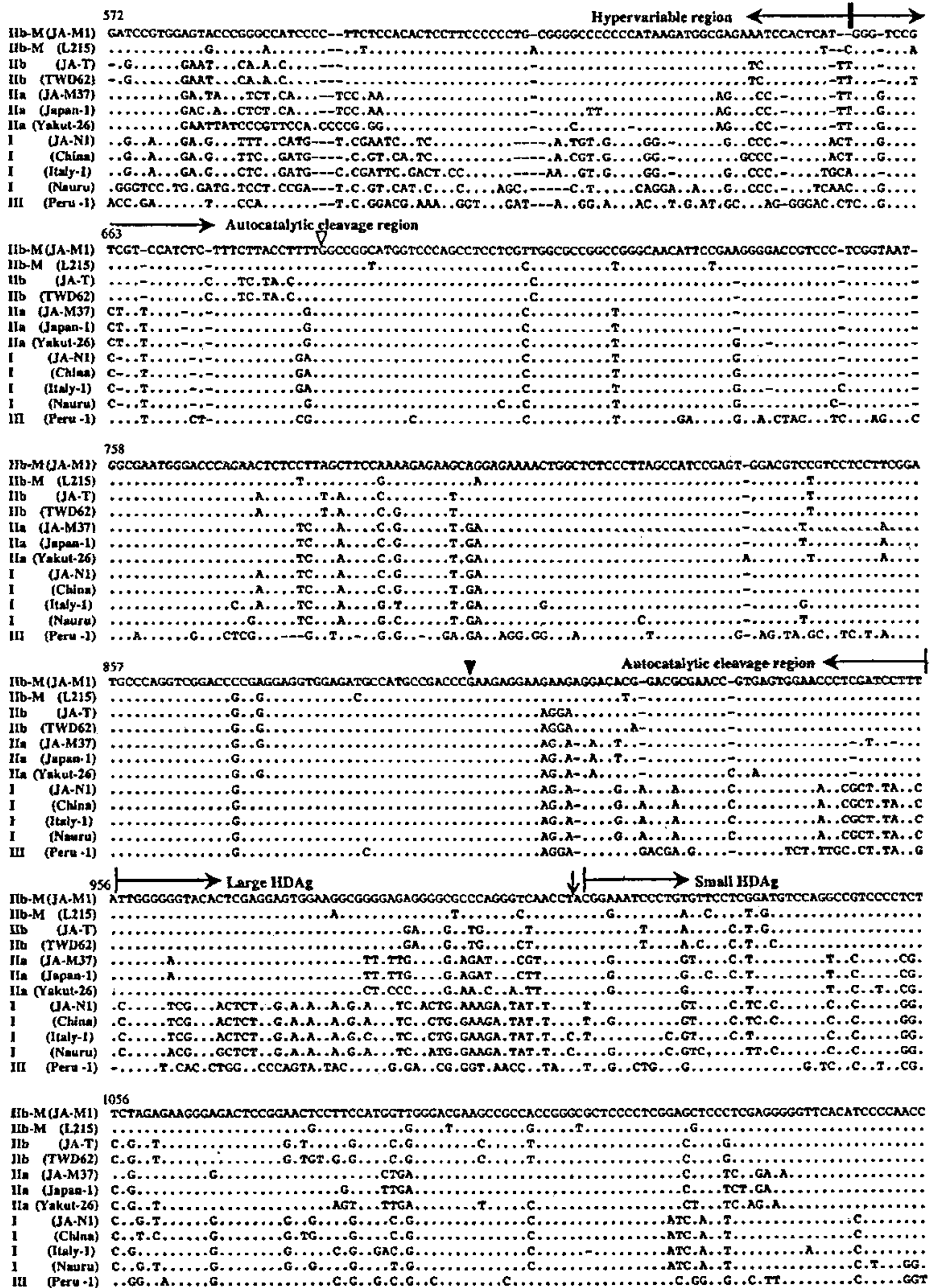

Fig. 2. (*cont.*) For legend see page 3282.

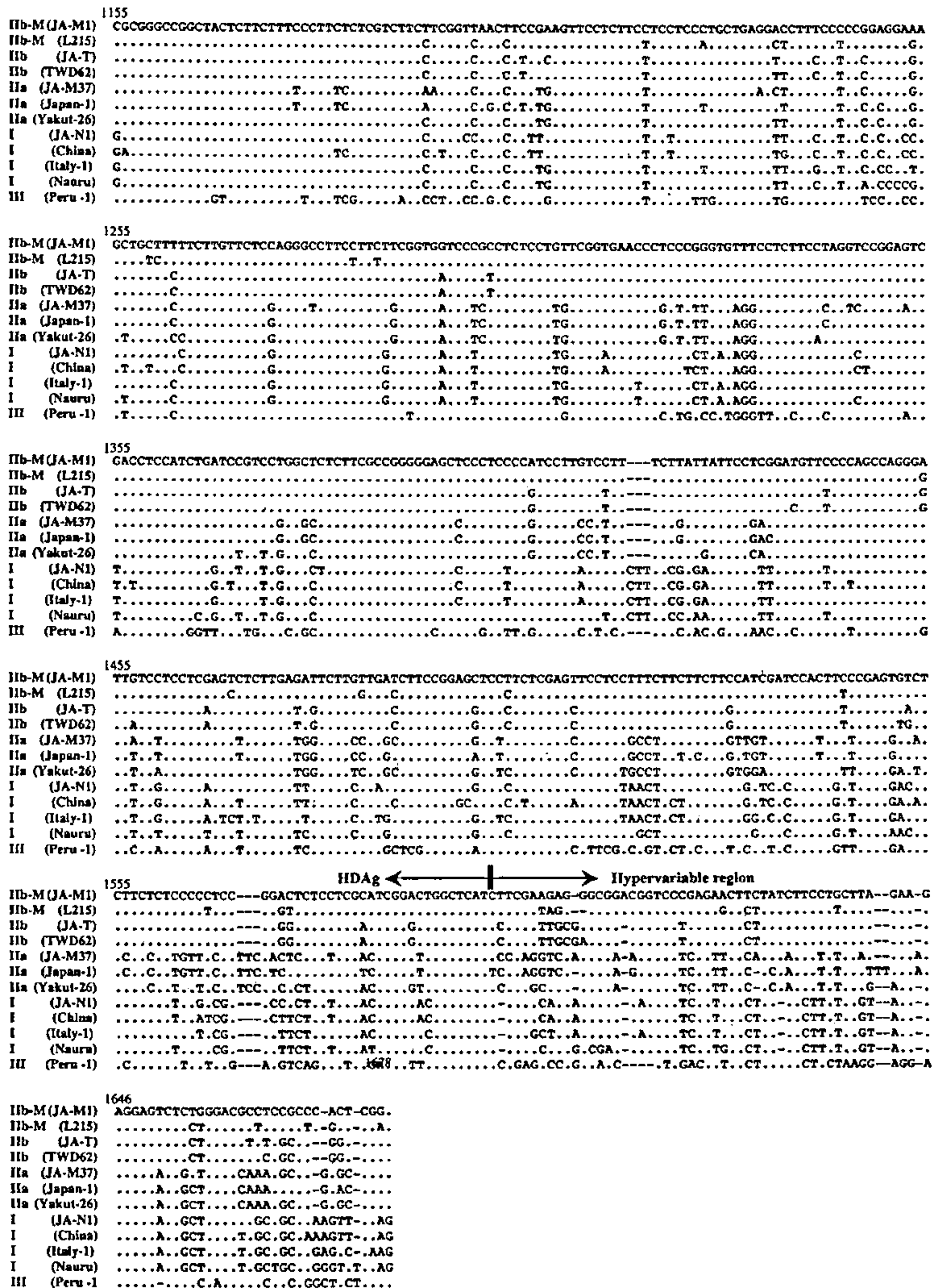

Fig. 2. (*cont.*) For legend see page 3282.

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.

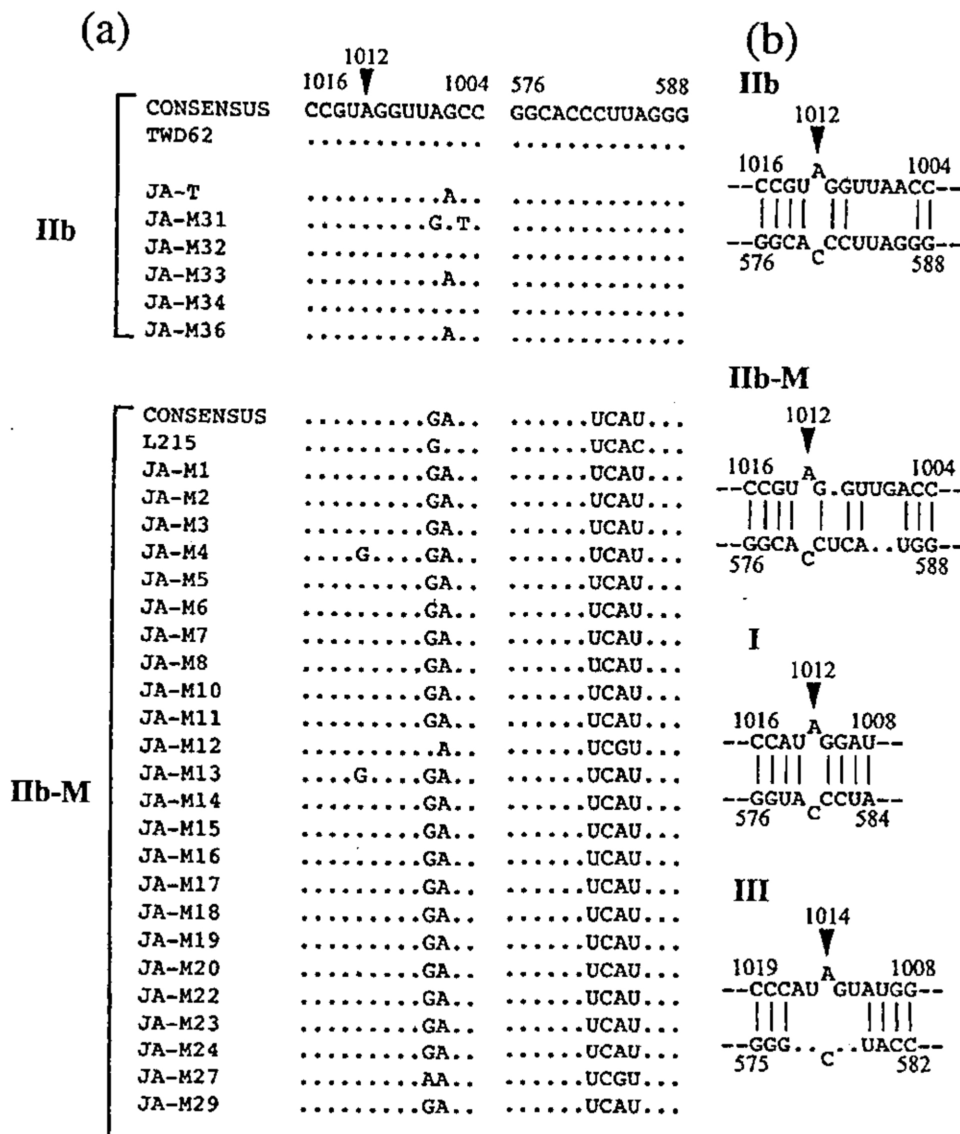

Fig. 3. RNA editing site of HDV genotype IIb and IIb-M. (a) Nucleotide sequences of the RNA editing site of HDV genotypes IIb and IIb-M, which is formed between anti-genome RNA surrounding the edited A residue (nt 1012) and nucleotide sequences of the opposite site (nt 580) of the unbranched rod structure 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

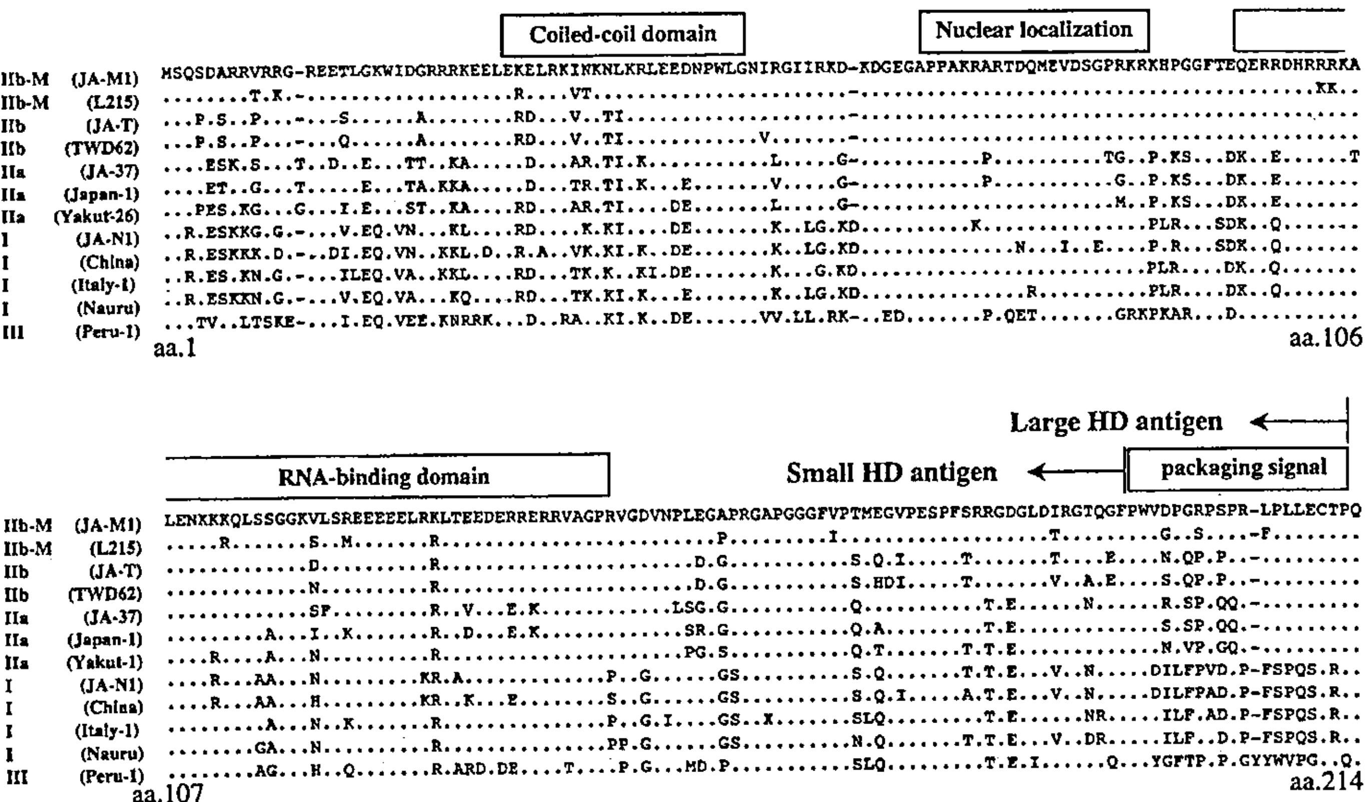

Fig. 4. HDAg amino acid alignment of HDV isolates. The amino acids are numbered according to Wang *et al.* (1986). Dots indicate conserved amino acids and dashes indicate missing amino acids. Sources of isolate sequences are as follows: L215, AB088679; TWD62, AF18077; Japan-1, X60193; Yakut-26, AJ309879; 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

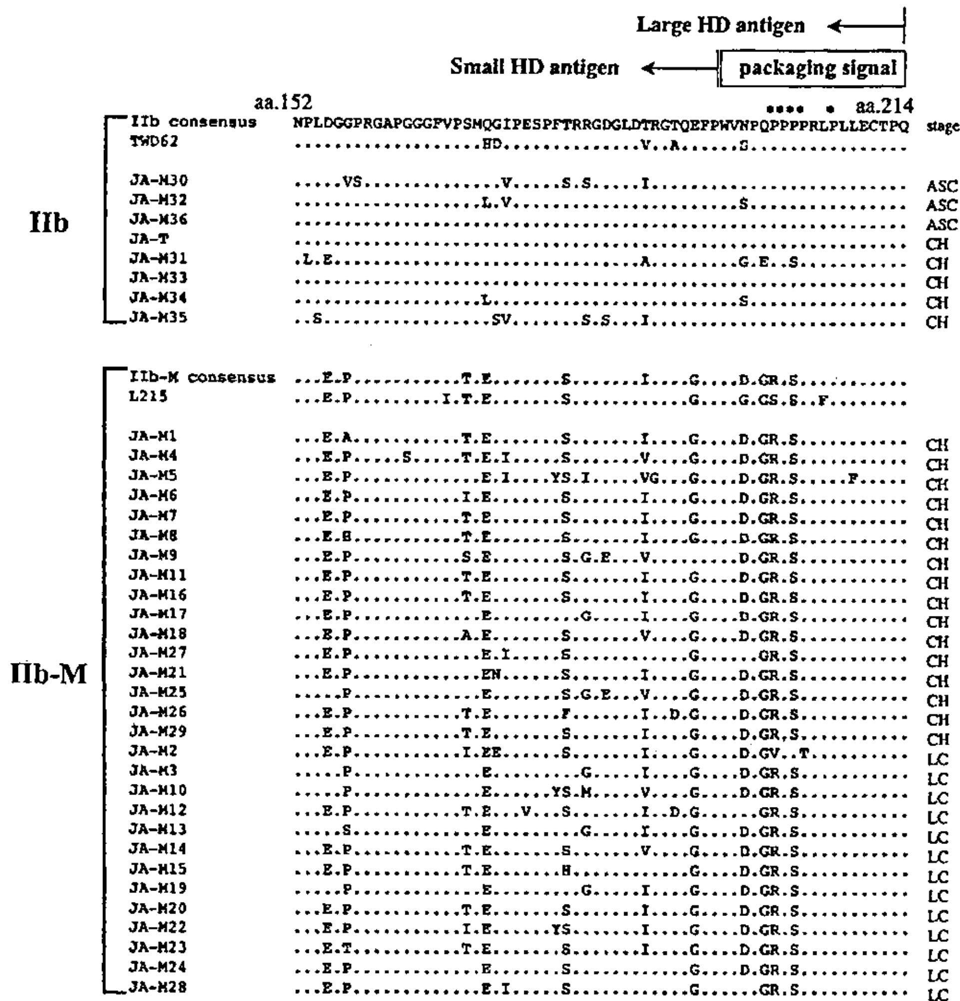

Fig. 5. C-terminal end of HDAg of HDV genotypes IIb and IIb-M. The C terminus of the HDAg from all the IIb ($n=8$) and IIb-M ($n=29$) genotypes were aligned. Dots indicate the same amino acids as the consensus sequence of genotype IIb. The stage (ASC, CH or LC) is described at the right side of the sequences. 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.