Table 1. Clinical characteristics of HBV+chronic, HCV+chronic and HCV+cleared patients in diabetes study and insulin resistance substudy

				P value		
	HBV+ chronic	HCV+ chronic	HCV+ cleared	HBV+ chronic vs HCV+ chronic	HCV+ chronic vs HCV+ cleared	HBV+ chronic vs HCV+ cleared
Diabetes study						
Number	286	544	122			
Age	45.1 ± 13.6	58.4 ± 13.0	53.2 ± 13.0	< 0.0001	< 0.0001	< 0.0001
Gender male (%)	164 (57.3)	257 (47.2)	82 (67.2)	< 0.01	< 0.0001	NS
Clinical stage (asymptomatic carrier/chronic hepatitis/ cirrhosis)	100/161/25	78/353/113	−/−/−	< 0.0001		
Insulin resistance substudy						
Number	135	232	56			
Age	44.5 ± 13.0	59.6 ± 13.1	53.5 ± 12.0	< 0.0001	< 0.005	< 0.005
Gender male (%)	77 (57.0%)	93 (40.1%)	38 (67.9%)	< 0.005	< 0.0005	NS
Hypertension	5 (3.7%)	49 (21.1%)	5 (9.1%)	< 0.0001	NS	NS
Hyperlipidaemia	32 (23.9%)	33 (14.2%)	17 (30.4%)	< 0.05	< 0.01	NS
Obesity	19 (17.4%)	40 (19.1%)	19 (38.8%)	NS	< 0.01	< 0.01
BMI (kg/m^2)	22.7 ± 3.3	22.8 ± 3.1	23.8 ± 3.3	NS	< 0.05	NS
Clinical stage (ASC/CH/cirrhosis)	40/77/18	37/140/55	−/−/−	< 0.005		
FPG (mg/dl)	96 ± 8	99 ± 10	100 ± 9	< 0.005	NS	< 0.01
IRI (μU/L)	8.7 ± 5.6	9.5 ± 5.3	7.9 ± 5.0	NS	< 0.05	NS
HOMA-IR	2.1 ± 1.4	2.4 ± 1.4	2.0 ± 1.3	NS	NS	NS
AST (IU/L)	42 ± 64	49 ± 27	23 ± 7	NS	< 0.0001	< 0.05
ALT (IU/L)	53 ± 121	49 ± 34	19 ± 9	NS	< 0.0001	< 0.05
γ-GTP (IU/L)	38 ± 68	40 ± 44	30 ± 22	NS	NS	NS
Platelet (× 10^9/L)	201 ± 62	168 ± 69	213 ± 61	< 0.0001	< 0.0001	NS
Total cholesterol (mg/dl)	192 ± 32	176 ± 34	197 ± 32	< 0.0001	< 0.0001	NS
Triglyceride (mg/dl)	92 ± 59	91 ± 46	103 ± 46	NS	NS	NS
HCV-RNA genotype 1/2	–	142/46	22/24		< 0.001	

ALT, alanine aminotransferase; ASC, asymptomatic carrier; AST, aspartate aminotransferase; BMI, body mass index; CH, chronic hepatitis; FPG, fasting plasma glucose; γ-GTP, γ-glutamyl transpeptidase; HBV, hepatitis B virus; HCV, hepatitis C virus; HCV-RNA, hepatitis C virus ribonucleic acid; HOMA-IR, homeostasis model of insulin resistance; IRI, immunoreactive insulin; NS, not significant.

patients with DM (19–21). Most previous studies have provided evidence of a positive association between them, with a few exceptions (22–24).

In this cross-sectional study, we investigated the prevalence of DM and IR in patients with chronic hepatitis C, and compared it with that in patients chronically infected with HBV and those who cleared HCV after interferon treatment as control.

Materials and methods

Diabetes study

Among the 1163 outpatients recruited from patients seropositive for the hepatitis B surface antigen (HBsAg) or the anti-hepatitis C virus antibody (HCV-Ab) who visited the First Department of Medicine, Chiba University Hospital, between January 2003 and December 2004, 87 patients were excluded because 60 patients had HCC and 27 were HCV ribonucleic acid (HCV-RNA) seronegative without previous interferon treatment. Among the remaining 1076 patients, the plasma glucose level and/or the HBA1C level were investigated retrospectively in 952 patients (88.5%), consisting of 544 patients chronically infected with HCV (HCV+chronic), 122 whose HCV had cleared after interferon treatment (HCV+cleared) and 286 chronically infected with HBV (HBV+chronic) (Table 1) (Fig. 1). One hundred and twenty-four patients whose plasma glucose level was not available were excluded: 50 patients chronically infected with HCV, 22 whose HCV had cleared after interferon treatment and 52 chronically infected with HBV. They were younger (45.0 ± 16.5 vs 53.8 ± 14.5, $P < 0.01$) with ASC more prevalent (42.9 vs 21.4%, $P < 0.05$) and cirrhosis less prevalent (3.8 vs 16.6%, $P < 0.05$), and the proportion of males was

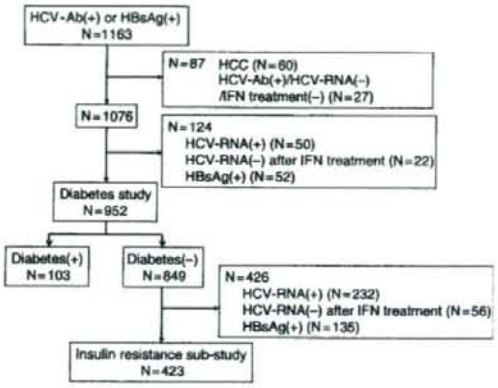

Fig. 1. Flow chart of patients analysed in diabetes study and insulin resistance substudy. IFN, interferon.

almost similar (44.4 vs 52.8%, $P=0.09$) compared with the 952 patients (Fig. 1). More in-depth data including body mass index (BMI), a key confounder, were not available in the diabetes study because it was a retrospective one.

Patients in the HCV+cleared group had completed interferon therapy at least more than 6 months before entry. Patients in the HCV+chronic group were seropositive for HCV-RNA and HCV-Ab, those in the HCV+cleared group were seropositive for HCV-Ab but seronegative for HCV-RNA, and those in the HBV+chronic group were seropositive for HBsAg. Patients seropositive for HBsAg and HCV-RNA were not included and those with autoimmune hepatitis, primary biliary cirrhosis, haemochromatosis, Wilson's disease, excessive alcohol intake of more than 50 g/day, HCC assessed by imaging examinations such as ultrasonography and computed tomography, and with a history of pancreatitis or pancreatic tumours were also excluded from this study. The definition of an asymptomatic carrier depends on normal alanine aminotransferase (ALT) levels in blood examinations at least two times per year for more than 3 years. The diagnosis of cirrhosis was based on histological findings by liver biopsy in 66 of 138 patients (48%) or on clinical features such as the presence of oesophageal varices, platelet counts $< 100 \times 10^9$/L because of hypersplenism and abdominal ultrasonographical findings (25) in the remaining patients. All cirrhotic patients were of Child-Pugh classification A (86%) or B (13%), except for one patient with HCV.

This study received ethics committee approval according to the 1975 Declaration of Helsinki, and informed consent was obtained from each patient.

Insulin resistance substudy

Four hundred and twenty-three patients were enrolled, selected randomly from 849 patients diagnosed as non-diabetic among the above 952(Fig. 1). Among them, 232 patients were chronically infected with HCV (HCV+chronic), 56 patients had HCV cleared after interferon treatment (HCV+cleared) and 135 patients were chronically infected with HBV (HBV+chronic) (Table 1). The remaining 426 patients not recruited into this study, including 238 patients chronically infected with HCV, 55 patients whose HCV had cleared and 133 patients chronically infected with HBV, showed similar distribution in terms of male gender (52.3 vs 49.2%, $P=0.37$) and no difference in age distribution (52.3 ± 15.0 vs 54.0 ± 14.6, $P=0.10$), with cirrhosis being less prevalent (10.0 vs 19.9%, $P < 0.05$).

Presence of diabetes mellitus, hypertension, hyperlipidaemia and obesity and definition of insulin resistance

Patients were considered to have diabetes if they used insulin or hypoglycaemic drugs at the time of the survey or had a fasting plasma glucose level of 126 mg/dl or more, a non-fasting plasma glucose level of 200 mg/dl or more or a haemoglobin A1C level of 6.5% or more. IR was evaluated by the homoeostasis model of IR (HOMA-IR, fasting plasma glucose (mg/dl) $\times$ insulin (μU/ml) $\div$ 405) (26) in patients without overt diabetes and was defined as a HOMA-IR of 2.0 or more. The presence of hypertension was ascertained based on medication history or systolic blood pressure above 140 mmHg or diastolic blood pressure above 90 mmHg. The diagnosis of hyperlipidaemia was made on the basis of the medication history and a total cholesterol level above 220 mg/dl or a triglyceride level above 150 mg/dl, and that of obesity was by a BMI (kg/m^2) of more than 25.0. The use of medications to establish diabetes, hyperlipidaemia and hypertension was not only based on patient self-report but was also confirmed by a medical record review.

Laboratory examination

Anti-hepatitis C virus antibody and HCV-RNA were determined by a second-generation enzyme-linked immunosorbent assay (Ortho Diagnostics, Tokyo, Japan) and the reverse-transcriptional polymerase chain reaction method (Amplicore HCV monitor assay version 2.0; Roche, Tokyo, Japan; lower detection limit, 500 copies/ml) (27). The HCV genotype was determined by serological grouping of serum antibody

(28), assuming that genotypes 1a and 1b correspond to group 1 and genotypes 2a and 2b correspond to group 2. Serum blood chemistries including haematological variables were obtained by a standard method using an autoanalyser. HBsAg was measured by an enzyme-linked immunosorbent assay (Abbott Laboratory, North Chicago, IL, USA).

Statistical analysis

Student's *t*-test and Fisher's exact test were used to analyse quantitative and qualitative data respectively. Multivariate logistic regression analysis was used to determine the adjusted odds ratios (ORs) of type 2 diabetes or IR with respect to HCV infection. Variables considered to be potential confounders in multivariate analysis were age, gender and clinical stage of liver disease for the development of diabetes, and age, gender, clinical stage of liver disease, hypertension, BMI, aspartate aminotransaminase (AST), ALT, γ-glutamyl transpeptidase and triglyceride for the development of IR. A *P*-value of < 0.05 was considered to indicate statistical significance.

Results

Diabetes study: prevalence of diabetes mellitus

Patient characteristics

The characteristics of the patients enrolled in this study are shown in Table 1. The mean age was statistically different among the three groups of patients with HBV infection (HBV+chronic), those with HCV infection (HCV+chronic) and those whose HCV had cleared after interferon treatment (HCV+cleared), and the proportion of male gender was also statistically different among the HCV+chronic and HBV+chronic, and HCV+chronic and HCV+cleared groups (Table 1). The clinical stages differed between the HBV+chronic and HCV+chronic groups, with more asymptomatic carriers in the HBV+chronic group and more cirrhotic patients in the HCV+chronic group (Table 1).

Prevalence of diabetes mellitus with various clinical backgrounds

The prevalence of DM was 18/286 (6.3%) in the HBV+chronic group, 74/544 (13.6%) in the HCV+chronic group and 11/122 (9.0%) in the HCV+cleared group, with the prevalence in the HCV+chronic group being significantly greater than that in the HBV+chronic group ($P < 0.005$) (Table 2). This result was also applicable when considering males and females separately. According to stratified age, the prevalence of DM was higher in the HCV+chronic group than in the HBV+chronic group without statistical significance. The prevalence of DM increased according to the progression of liver disease, but the prevalence was similar in asymptomatic carrier patients between the HBV+chronic and HCV+chronic groups (Table 2).

Table 2. Prevalence of diabetes mellitus with various clinical backgrounds in diabetes study

	HBV+chronic (Group B1)	HCV+chronic (Group C1)	HCV+cleared (Group CC1)
Number	286	544	122
Diabetes mellitus	18 (6.3%)***	74 (13.6%)***	11 (9.0%)
Gender			
Male	14 (8.5%)**	48 (18.7%)**	10 (12.2%)
Female	4 (3.3%)*	26 (9.1%)*	1 (2.5%)
Age			
≤ 49	4 (2.4%)	6 (4.8%)	3 (6.8%)
50–59	10 (11.8%)	23 (18.0%)	4 (12.1%)
≥60	4 (10.8%)	45 (15.4%)	4 (8.9%)
Clinical stage			
ASC	6 (6.0%)	5 (6.4%)	–
CH	9 (5.6%)*	44 (12.5%)*	–
Cirrhosis	3 (12%)	25 (22.1%)	–

*$P < 0.05$.
**$P < 0.01$.
***$P < 0.005$.
ASC, asymptomatic carrier; CH, chronic hepatitis; HBV, hepatitis B virus; HCV, hepatitis C virus.

Clinical factors associated with the development of diabetes mellitus in the HBV+chronic and HCV+chronic groups

Multivariate logistic regression analysis revealed male gender, older age and the presence of cirrhosis as independent risk factors for the development of DM, but not HCV infection (Table 3).

Clinical backgrounds and the prevalence of diabetes mellitus in asymptomatic carrier patients in the HCV+chronic and HCV+cleared groups

The prevalence of DM was 5/78 (6.4%) patients who were HCV-infected asymptomatic carriers and 11/122 (9.0%) patients with eradicated HCV after interferon treatment, showing no difference between them ($P = 0.6$). Multivariate logistic regression analysis with gender, age and HCV infection as confounding factors did not show ongoing HCV infection as an independent risk factor for developing DM (data not shown).

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Insulin resistance substudy: prevalence of insulin resistance in non-diabetic patients

Patient characteristics

The characteristics of the patients enrolled in the IR substudy are shown in Table 1. Age distribution, proportion of male gender and proportion of clinical stage in the HBV+chronic, HCV+chronic and HCV+cleared groups were similar in the diabetes study and the IR substudy (Table 1). Hypertension was most prevalent in the HCV+chronic group, hyperlipidaemia was less prevalent in the HCV+chronic group owing to a lower total cholesterol level, and obesity was most prevalent in the HCV+cleared group.

Table 3. Clinical factors associated with development of diabetes mellitus in HBV+ or HCV+chronic patients using multivariate logistic regression analysis in diabetes study

Variables	Odds ratio	95% confidence interval	*P*-value
Male (vs female)	2.404	1.497–3.861	0.0003
Age (vs ≤ 49)			
50–59	4.484	2.119–9.434	< 0.0001
≥60	3.774	1.792–7.937	0.0005
Clinical stage (vs asymptomatic carrier)			
Chronic hepatitis	1.408	0.698–2.841	0.3393
Cirrhosis	2.273	1.048–4.926	0.0376
HCV (vs HBV)	1.669	0.917–3.040	0.0936

HBV, hepatitis B virus; HCV, hepatitis C virus.

Prevalence of insulin resistance with various clinical backgrounds

The prevalence of IR was 49/135 (36.3%) in the HBV+chronic group, 126/232 (54.3%) in the HCV+chronic group and 20/56 (35.7%) in the HCV+cleared group, with the prevalence in the HCV+chronic group being significantly higher than that in the HBV+chronic group ($P < 0.005$) (Table 4). This result also applied when considering males and females separately. According to stratified age, the prevalence of IR was higher above the age of 50 years in the HCV+chronic group compared with the HBV+chronic group. According to clinical stage, IR in chronic hepatitis was more prevalent in the HCV+chronic group than in the HBV+chronic group ($P < 0.05$), but the prevalence was similar in asymptomatic carrier patients in these two groups (Table 4).

Table 4. Prevalence of insulin resistance with various clinical backgrounds in insulin resistance substudy

				P-value		
	HBV+ chronic	HCV+ chronic	HCV+ cleared	HBV+chronic vs HCV+chronic	HCV+chronic vs HCV+cleared	HBV+chronic vs HCV+cleared
Number	135	232	56			
HOMA-IR≥2.0	49 (36.3%)	126 (54.3%)	20 (35.7%)	< 0.005	< 0.05	NS
Gender						
Male	30 (39.0%)	51 (54.8%)	15 (39.5%)	< 0.05	NS	NS
Female	19 (32.8%)	75 (54.0%)	5 (27.8%)	< 0.01	< 0.05	NS
Age						
≤ 49	34 (43.0%)	18 (40.9%)	6 (30.0%)	NS	NS	NS
50–59	10 (25.6%)	30 (57.7%)	4 (26.7%)	< 0.005	< 0.05	NS
≥60	5 (29.4%)	78 (57.4%)	10 (47.6%)	< 0.05	NS	NS
Clinical stage						
ASC	10 (25.0%)	12 (32.4%)	–	NS		
CH	32 (41.6%)	80 (57.1%)	–	< 0.05		
LC	7 (38.9%)	34 (61.8%)	–	NS		
Hypertension						
(+)	0 (0%)	32 (65.3%)	2 (40%)	< 0.01	NS	NS
(−)	49 (38.0%)	94 (51.4%)	18 (36.0%)	< 0.05	< 0.05	NS
Hyperlipidaemia						
(+)	18 (56.3%)	15 (45.5%)	5 (29.4%)	NS	NS	NS
(−)	31 (30.4%)	111 (55.8%)	15 (38.5%)	< 0.0001	< 0.05	NS
Obesity						
(+)	15 (78.9%)	34 (85.0%)	10 (50%)	NS	*P*< 0.01	NS
(−)	25 (27.8%)	81 (47.9%)	8 (26.7%)	*P*< 0.005	*P*< 0.05	NS

ASC, asymptomatic carrier; CH, chronic hepatitis; HBV, hepatitis B virus; HCV, hepatitis C virus; HOMA-IR, homeostasis model of insulin resistance; LC, cirrhosis; NS, not significant.

Table 5. Clinical factors associated with insulin resistance in HBV+chronic or HCV+chronic patients using multivariate logistic regression analysis in insulin resistance substudy

Variables	Odds ratio	95% confidence interval	*P*-value
Age (vs ≤ 49)			
50–59	1.133	0.534–2.404	0.7444
≥60	1.642	0.769–3.509	0.2003
Body mass index (vs < 25)			
≥25	5.765	2.563–12.967	< 0.001
Clinical stage (vs asymptomatic carrier)			
Chronic hepatitis	1.764	0.792–3.922	0.1652
Cirrhosis	2.183	0.820–5.814	0.1180
HCV (vs HBV)	1.531	0.781–3.003	0.2154
AST≥40 (vs < 40)	0.980	0.475–2.021	0.9567
ALT≥40 (vs < 40)	2.595	1.279–5.265	0.0082
γ-glutamyl transpeptidase (vs < 40 IU/L)			
≥40	2.100	1.108–3.981	0.0229
Triglyceride (vs < 100 mg/dl)			
≥100	1.966	1.077–3.588	0.0276

ALT, alanine aminotransferase; AST, aspartate aminotransferase; HBV, hepatitis B virus; HCV, hepatitis C virus.

Clinical factors associated with insulin resistance in the HBV+chronic and HCV+chronic groups

One hundred and seventy-five of 367 patients had a HOMA-IR >2.0, were older (55.9 ± 14.1 vs 52.4 ± 15.6, $P < 0.05$), had a higher proportion of HCV infection (72.0 vs 55.2%, $P < 0.01$), had more advanced liver disease (asymptomatic carrier/chronic hepatitis/cirrhosis; 22/112/41 vs 55/105/32, $P < 0.001$) and had higher BMI (24.2 ± 3.0 vs 21.4 ± 2.7, $P < 0.0001$), fasting plasma glucose (101 ± 10 vs 96 ± 8, $P < 0.0001$), immunoreactive insulin (13.3 ± 5.2 vs 5.5 ± 1.7, $P < 0.0001$), AST (56 ± 58 vs 37 ± 23, $P < 0.0001$), ALT (66 ± 107 vs 36 ± 30, $P < 0.0001$), γ-glutamyl transpeptidase (50 ± 69 vs 30 ± 32, $P < 0.0001$) and triglyceride levels (99 ± 46 vs 84 ± 54, $P < 0.0001$) than those with a HOMA-IR < 2.0.

Multivariate logistic regression analysis showed BMI ≥25, ALT ≥40, γ-glutamyl transpeptidase ≥40 and triglyceride ≥100 as independent risk factors, with ORs of 5.765, 2.595, 2.100 and 1.966, respectively, but HCV infection compared with HBV infection was not found to be a statistically significant variable (Table 7).

Clinical factors associated with insulin resistance in the HCV+chronic and HCV+cleared groups

One hundred and forty-six patients showed a HOMA-IR >2.0 among 288 patients in the HCV+chronic and HCV+cleared groups. They were older (60.3 ± 11.3 vs 56.5 ± 14.5, $P < 0.05$), had a higher proportion of HCV infection (86.3 vs 74.6%, $P < 0.05$) and had a higher BMI (24.1 ± 2.9 vs 21.8 ± 3.0, $P < < 0.0001$), fasting plasma glucose (102 ± 11 vs 97 ± 8, $P < 0.0001$), immunoreactive insulin (12.9 ± 4.9 vs 5.4 ± 1.7, $P < 0.0001$), AST (49 ± 27 vs 38 ± 24, $P < 0.0005$), ALT (51 ± 35 vs 35 ± 29, $P < 0.0001$) and γ-glutamyl transpeptidase levels (43 ± 43 vs 34 ± 38, $P < 0.05$) than those with a HOMA-IR < 2.0.

Multivariate logistic regression analysis showed age over 60, BMI ≥25 and ALT ≥40 as independent risk factors for IR, with ORs of 2.392, 4.749 and 4.634 respectively. However, ongoing HCV infection compared with HCV eradication was not found to be a statistically significant variable (Table 6).

Clinical factors associated with insulin resistance in asymptomatic carrier patients in the HCV+chronic and HCV+cleared groups

Thirteen variables, including HOMA-IR were compared between 37 asymptomatic carrier patients with HCV infection and 56 patients whose HCV had cleared after interferon treatment. Asymptomatic carriers with HCV infection showed a smaller proportion of male gender (27 vs 68%, $P < 0.0005$), a higher level of ALT (23 ± 9 vs 19 ± 9, $P < 0.05$) and a greater proportion of HCV genotype 1 (80 vs 47.8%, $P < 0.05$) compared with patients whose HCV infection had cleared, but the proportion of patients with a HOMA-IR above 2.0 was similar in the two groups (32 vs 36%, $P = 0.8$). These results suggest that HCV infection alone might not be associated with IR.

Discussion

This cross-sectional study showed a weak and not statistically significant higher association of diabetes with HCV infection compared with HBV infection, and advanced liver disease such as cirrhosis and other traditional risk factors for diabetes such as older age and male gender were more closely involved than HCV infection. This result also held true for the association between HCV infection and IR in patients without overt diabetes.

The prevalence of DM increased according to the progression of liver disease in patients with either HBV or HCV infection, and it was higher in patients with HCV than in those with HBV in every clinical stage except for asymptomatic carrier (Table 2). The prevalence of DM was similar in asymptomatic carrier patients with HCV and those with HBV (6.4 and 6.0%, $P = 0.67$) and, furthermore, it was also similar in

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asymptomatic carrier patients with HCV and those whose HCV had cleared (6.4 and 9.0%, $P=0.60$), with both groups showing almost normal ALT levels. These observations suggest that not HCV infection itself but the resultant ongoing inflammation and fibrosis of the liver might determine a higher risk for DM. This was also applicable to IR in patients without overt DM, as the present study showed that liver enzyme abnormalities such as elevation of ALT, γ-glutamyl transpeptidase and BMI over 25.0 were independent risk factors for IR but HCV infection itself was not. These results are discordant with the recent report of a transgenic mouse model, in which the HCV core protein was shown to contribute directly to the development of IR by disturbing tyrosine phosphorylation of insulin receptor substrate 1 without inflammation and fibrosis during the observation period (29).

Treatment with interferon or interferon plus ribavirin is now a standard regimen for hepatitis C, and IR was reported to be improved in patients whose HCV had cleared and was not affected in those with relapse or no response after interferon treatment (30). In the present study, we found the prevalence of IR to be higher in patients infected with HCV than in those whose HCV had been cleared, but multivariate logistic regression analysis did not extract HCV infection as an independent risk factor for IR after adjusting for age, BMI and ALT (Table 6). Furthermore, we found no difference in the prevalence of IR between HCV-infected asymptomatic carrier patients and patients whose HCV had cleared (32 vs 36%, $P=0.83$), both with normal ALT levels.

Table 6. Clinical factors associated with insulin resistance in HCV+chronic or HCV+cleared patients using multivariate logistic regression analysis in insulin resistance substudy

Variables	Odds ratio	95% confidence interval	*P*-value
Male (vs female)	0.870	0.473–1.603	0.6550
Age (vs ≤ 49)			
50–59	1.715	0.700–4.202	0.2376
≥60	2.392	1.093–5.236	0.0290
Body mass index (vs < 25)			
≥25	4.749	2.170–10.393	< 0.0001
HCV-RNA (+) [vs (−)]	1.518	0.646–3.570	0.3383
AST ≥40 (vs < 40 IU/L)	1.101	0.502–2.417	0.8090
ALT ≥40 (vs < 40 IU/L))	4.634	2.153–9.973	< 0.0001
Platelet (vs ≥200 × 10^9/L)			
< 200	1.155	0.602–2.213	0.6647
Total cholesterol (vs ≥180 mg/dl)			
< 180	1.042	0.564–1.925	0.8946

ALT, alanine aminotransferase; AST, aspartate aminotransferase; HCV, hepatitis C virus; HCV-RNA, hepatitis C virus ribonucleic acid.

There are six major genotypes of HCV classified in the world (31). Among them, genotype 3 has been reported to be closely associated with steatosis in the liver (32), but genotype 3 is not common in Japan. There have been several reports on the association between genotype 1 or 2 and DM (13, 16, 21). In the present study, we could not confirm these findings because there were no differences in the prevalence of DM or IR between patients with genotypes 1 and 2.

The diabetes study is a retrospective one, and hence some important confounding risk factors for diabetes such as BMI were defective. However, similar results were obtained in the prospective IR substudy on the prevalence of IR, antecedent to the development of diabetes, after adjustment for additional confounding risk factors. In the diabetes study, BMI data were available in 396 (48%) of 830 patients and the prevalence of diabetes in these patients was higher in those with HCV infection than in those with HBV infection (33/266 (12.4%) vs 7/130 (5.4%), $P=0.033$). Multivariate logistic regression analysis showed that the OR of HCV infection was 2.07 after adjustment for age, gender, BMI and clinical stage without statistical significance ($P=0.1347$).

Previous studies by Caronia *et al.* (15) and Mason *et al.* (21) demonstrated a higher prevalence of DM in patients with HCV-related disease than in those with HBV-related disease in agreement with our findings. Although the present study failed to ascertain HCV infection as an independent factor for diabetes by multivariate logistic regression analysis in discordance with the previous studies (15, 21), the relative OR for diabetes was 1.67 times higher in patients with HCV than in those with HBV. Considering that there is no normal control group, we could not deny the association of HCV infection with DM. Furthermore, HBV infections occur vertically from their mothers at birth in most Japanese patients with chronic hepatitis B, indicating that the duration of infection is almost the same as the age of the patients, but in this study of community-acquired HCV the duration of infection could not be estimated and it is difficult to establish the temporality of the development of hepatitis and diabetes.

In conclusion, this study showed a higher prevalence of DM and IR in patients with HCV infection than in those with HBV infection. However, other factors such as age, male gender, BMI and cirrhosis seemed to be more important risk factors for the development of glucose abnormalities in Japan.

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J Gastroenterol 2008; 43:711–719
DOI 10.1007/s00535-008-2222-5

Journal of Gastroenterology

Efficacy of combination therapy of antiviral and immunosuppressive drugs for the treatment of severe acute exacerbation of chronic hepatitis B

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Background. Patients with severe exacerbation of chronic hepatitis B, sometimes developing into fulminant liver failure, are at high risk for mortality even with antiviral therapy. The efficacy of immunosuppressive therapy in clinically severe exacerbation of chronic hepatitis B has not been well demonstrated. In this study, we evaluated the efficacy of the early introduction of immunosuppressive therapy in combination with antiviral therapy in such patients. ***Methods.*** Forty-two patients, 29 men and 13 women, were defined as having severe exacerbation of chronic hepatitis B based on our uniform criteria, and were enrolled in this study. Sixteen patients between 1982 and 1996 were analyzed retrospectively. We defined the criteria of severe disease in 1997, and then began to introduce sufficient doses of corticosteroids prospectively. Nucleoside analogs were administered in combination with corticosteroids after 1999. Twenty-six patients between 1997 and 2007 were analyzed prospectively. ***Results.*** In the retrospective study between 1982 and 1996, four of 16 (25%) patients recovered. In the prospective study between 1997 and 2007, 17 of 26 (65%) patients recovered; 15 of 17 patients treated with corticosteroids with or without antiviral drugs within 10 days after the diagnosis of severe disease recovered, none of five treated similarly but later than 10 days after the diagnosis recovered, and two of three treated with antiviral drugs recovered. ***Conclusions.*** The early introduction of sufficient doses of corticosteroids and nucleoside analogs could be one option for reversing the potential deterioration of patients with clinically severe, life-threatening exacerbation of chronic hepatitis B.

Key words: chronic hepatitis B, severe exacerbation, immunosuppressive therapy, antiviral therapy

Received: March 1, 2008 / Accepted: May 17, 2008
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Introduction

It is well recognized that exacerbation of hepatitis B may occur in chronic hepatitis B virus (HBV) carriers, spontaneously or in relation to cytotoxic or immunosuppressive therapy. A clinical picture of acute hepatitis, and even severe exacerbation, and sometimes fulminant liver failure, may develop that is associated with high mortality.[1] In a retrospective survey in Japan, a 53% incidence of severe hepatitis with a 24% mortality rate (mortality rate of 45% in severe hepatitis) has been reported in relation to chemotherapy in HBV carriers with hematologic malignancies.[2] For the treatment of patients with severe exacerbation without malignancies who progress to serious deterioration, liver transplantation may be a viable option. However, the problems of a shortage of donor livers in Japan and the high cost of the procedure still remain. Thus, therapies other than transplantation must be further investigated for chronic hepatitis B patients with severe exacerbation.

In HBV infection, liver injury is considered to be induced mainly by cytotoxic T lymphocyte-mediated cytolytic pathways of infected hepatocytes.[3] Sjogren et al.[4] suggested that corticosteroids modulate the activity of chronic hepatitis B by suppression of the host immune response to hepatitis B virus antigens, based on a comparison of alanine aminotransferase (ALT) and IgM anti-hepatitis B core antibody (IgM-HBc) levels during the course of short-term high-dose prednisolone therapy. Accordingly, treating chronic hepatitis B patients with corticosteroids to inhibit excessive immune response and prevent cytolysis of infected hepatocytes is a reasonable treatment decision.

Corticosteroids have been administered to treat active chronic hepatitis B since the 1970s. However, in recent years, because the advantage of their use has not been confirmed by controlled studies, their use for the routine management of chronic hepatitis B has fallen

out of favor.[5–7] However, these previous studies dealt mainly with cases of clinically nonsevere hepatitis that was not urgently life threatening, and the effects of corticosteroid treatment for severe, potentially life-threatening exacerbation of chronic hepatitis B, as well as the timing and dose of such treatment, have not been well demonstrated. Lau et al.[8] reported that the reintroduction of long-term, high-dose corticosteroids in the early phase of reactivation after withdrawal of immunosuppressive therapy prevents both progressive clinical deterioration and potentially the need for orthotopic liver transplantation.

In our previous study, we investigated the clinicopathological features of patients with severe exacerbation selected by uniform criteria and treated by the early introduction or reintroduction of sufficient doses of corticosteroids, and reported that the introduction of high-dose corticosteroids can significantly reverse deterioration in patients with clinically severe, life-threatening exacerbation of chronic hepatitis B, compared with historical controls, when used in the early stage of illness.[9] Recently, nucleoside analogs have been administered safely even to patients with severe disease,[10–15] but their benefits in terms of the clinical outcome have still to be clarified.

In this study, we treated patients with severe disease by early initiation of sufficient doses of corticosteroids and nucleoside analogs prospectively to clarify the benefits and limitations of the therapy for amelioration of clinically severe exacerbation of chronic hepatitis B.

Patients and methods

Patients

Forty-two patients with severe exacerbation of chronic hepatitis B who were admitted to our liver unit (Chiba University Hospital and related hospitals) between 1982 and 2007 were studied. The diagnosis of chronic hepatitis B viral carrier was made based on either hepatitis B surface antigen (HBsAg) positivity for at least 6 months before entry, or, in patients with a follow-up period of less than 6 months before entry, on hepatitis B surface antigen positivity, a high anti-hepatitis B core antibody (HBcAb) titer, and IgM-HBc negativity or a low titer. The patients fulfilling all of the following three criteria during the disease course were defined as having severe exacerbation: prothrombin time (PT) activity less than 60% of normal control, total bilirubin (T-Bil) greater than 3.0 mg/dl, and ALT greater than 300 IU/l. All patients were in poor general condition, including general malaise, fatigue, jaundice, edema, ascites, and encephalopathy. A histological examination was performed in patients during the convalescent phase or after their death.

All patients were negative for IgM anti-hepatitis A virus (HAV) antibody, anti-hepatitis D antibody, anti-hepatitis C virus (HCV) antibody, HCV RNA, IgM anti-Epstein-Barr virus antibody (IgM-EBV), IgM anti-herpes simplex virus antibody (IgM-HSV), IgM anti-cytomegalovirus antibody (IgM-CMV), anti-nuclear antibody, anti-smooth muscle antibody, liver kidney microsomal antibody 1, and anti-mitochondrial antibody. Patients with histories of recent exposure to drugs and chemical agents as well as of recent heavy alcohol intake were excluded. One patient was human immunodeficiency virus (HIV) positive, but had no clinical evidence of acquired immune deficiency syndrome.

Protocols for treatment

Twenty-six patients treated after 1997 were examined prospectively and 16 treated before 1996 were examined retrospectively. Of the prospectively studied group, informed consent was obtained from the patients or appropriate family members. Patients were treated by early introduction of corticosteroids (CS) (early CS) as follows: 60 mg or more of prednisolone daily was administered within 10 days after the diagnosis of severe disease using the above-mentioned criteria. This dose was maintained for a minimum of 4 days. When the patient showed a trend toward remission in PT, the dose was reduced by 10 mg at least every 4 days to 30 mg. Then, the dose was further tapered by 2.5 or 5 mg every 2 weeks or longer, depending on the decreasing trend of the ALT level, in the period of immunosuppressive monotherapy before nucleoside analogs were administered. Afterward, lamivudine (LMV), adefovir (ADV), and entecavir (ETV), nucleoside analogs with significant inhibition of HBV DNA polymerase, could be used safely in patients with severe disease.[10–15] In Japan, LMV for HIV and chronic hepatitis B became available in 1997 and 2000, respectively. The CS dose was reduced more rapidly and tapered off while monitoring the viral load reduction after 1999, when we began to administer nucleoside analogs.

Patients who had already passed more than 10 days after the diagnosis before being admitted to our unit were treated by the delayed introduction of corticosteroids (delayed CS). Patients with marked prolongation of PT were treated with 1000 mg of methylprednisolone daily for 3 days followed by the same prednisolone therapy as described above. After 1998, LMV was administered at a daily dose of 100–300 mg. ADV was administered at a daily dose of 10 mg to LMV-breakthrough hepatitis. ETV was administered at a daily dose of 0.5–1.0 mg. Patients who showed a trend toward remission or irreversible hepatic failure at admission

were treated with intravenous glycyrrhizin (stronger neominophagen C, SNMC), an aqueous extract of licorice root, at 60–100 ml/day. SNMC is reported to have anti-inflammatory activity and has been used for the treatment of chronic viral hepatitis in Japan.[16]

In the retrospective study before 1996, two patients with deep hepatic coma on admission were treated with a combination therapy of CS and interferon (IFN). IFN β was administered at 3 million units/day. Cyclosporin A (CyA) was administered to one patient, and IFN monotherapy to one.

Serological markers

HBsAg, hepatitis B e antigen (HBeAg), anti-HBe antibody (HBeAb), HBcAb, IgM-HBc, IgM anti-HAV antibody, and anti-hepatitis D antibody were detected by commercial radioimmunoassay (Abbott Laboratories, Chicago, IL, USA), and HCV RNA was measured by nested reverse transcriptase-polymerase chain reaction.[17] Second generation anti-HCV antibody was measured by enzyme immunoassay (Ortho Diagnostics, Tokyo, Japan). IgM-EBV, IgM-CMV, and IgM-HSV were examined by enzyme-linked immunosorbent assay. Anti-nuclear antibody, anti-smooth muscle antibody, anti-mitochondrial antibody, and anti-liver kidney microsomal 1 antibody were examined by the fluorescent antibody method. HBV DNA polymerase was assayed according to the method of Kaplan et al.[18] The HBV DNA level was measured by hybridization assay (Abbott), branched DNA hybridization assay (Chiron, Emeryville, CA, USA), transcription-mediated amplification and hybridization protection assay (Chugai Diagnosis Science, Tokyo, Japan), or Amplicor HBV monitor (Roche Diagnostics, Tokyo, Japan).

Statistical analysis

Differences in proportions among the groups were compared by Fisher's exact probability test, Student's *t* test, or Welch's *t* test.

Results

Clinical features of severe chronic hepatitis B patients on admission

Of the 42 patients fulfilling the criteria of severe exacerbation, 29 were men and 13 were women. Mean age at the time of admission was 50.3 ± 13.5 years. Mean PT activity was 32 ± 14%, mean ALT was 820 ± 860 IU/l, and mean T-Bil was 12.1 ± 7.5 mg/dl. HBeAg/HBeAb status was +/– in 15, –/+ in 23, +/+ in two and –/– in two. Sixteen patients (38%) had primary diseases or conditions (five, non-Hodgkin's lymphoma; two, ulcerative colitis; and one each, acute lymphocytic leukemia, breast cancer, rheumatoid arthritis, pemphigoid, aplastic anemia, alcoholic hepatitis, HIV-positive, schizophrenia, Down's syndrome, and mental retardation), and 11 (26%) had been treated with immunosuppressive or cytotoxic drugs and suffered exacerbation after their withdrawal.

In the 1982–1996 period, the main treatment was delayed CS (period I), retrospectively. We defined the criteria of severe disease in 1997, and in the 1997–1998 period began to introduce CS as soon as possible after reaching a diagnosis; the main treatment was early CS (period II). After 1999, we administered LMV in combination with CS, and ETV after 2007; the main treatment was early CS and nucleoside analogs (NA) (period III). The three periods included 16, 10, and 16 patients, respectively (Table 1). Mean ages were 53.7 ± 14.0 years in 1982–1996, 43.0 ± 13.6 in 1997–1998, and 51.4 ± 12.0 in 1999–2007. Mean PT activities were 28 ± 14%, 33 ± 12%, and 36 ± 14%, mean ALT levels were 593 ± 853 IU/l, 1290 ± 1005, and 753 ± 693, and mean T-Bil levels were 15.8 ± 7.8 mg/dl, 9.9 ± 5.4, and 9.8 ± 7.2, respectively. Differences in age, sex, PT, ALT, HBe Ag/Ab status, and use of preimmunosuppressive or cytotoxic therapies for primary diseases were not significant in any of the periods. The T-Bil level was higher in period I than in period II ($P = 0.047$) or period III ($P = 0.03$). The time between the diagnosis of severe disease and the introduction of immunosuppressive drugs was longer in period I than in period II ($P = 0.02$) or period III ($P = 0.02$) (Table 1).

All surviving patients except one in period III were free of hepatic encephalopathy during the treatment course. Six patients (two in period I and four in period III) had hepatic encephalopathy at admission, and five did not respond to any therapy, including artificial liver supports such as plasma exchange and hemodiafiltration. One surviving patient in period III had grade II encephalopathy at admission. Twenty failed to respond to any therapy, including artificial liver supports, and gradually developed hepatic failure and died. One with mental retardation and recurrent pneumonia was treated with SNMC in period I and died of sepsis.

Therapies in each period

During 1982–1996, seven patients were treated with delayed CS, three with SNMC, two with delayed CS and IFN, two with early CS, one with CyA, and one with IFN. During 1997–1998, seven patients were treated with early CS, two with delayed CS, and one with delayed CS and LMV. During 1999–2007, seven patients were treated with early CS and LMV, two with early CS

Table 1. Clinical and biochemical features of patients according to study period

	1982–1996 (period I) Retrospective study	1997–1998 (period II) Prospective study	1999–2007 (period III) Prospective study
n	16	10	16
Age[a]	53.7 ± 14.0	43.0 ± 13.6	51.4 ± 12.0
Sex (M/F)	10/6	7/3	12/4
PT (%)[a]	28 ± 14	33 ± 12	36 ± 14
ALT (IU/l)[a]	593 ± 853	1290 ± 1005	753 ± 693
T-Bil (mg/dl)[a]	15.8 ± 7.8*	9.9 ± 5.4*	9.8 ± 7.2*
HBeAg/Ab	5/11[d]	5/5[d]	7/9[d]
Duration[a,b]	34.8 ± 43.0**	6.3 ± 5.4**	6.1 ± 4.1**
Pretherapy[c]	4	3	5
Recovery	4***	7***	10***

PT, prothrombin time; ALT, alanine aminotransferase; T-Bil, total bilirubin; HBeAg, hepatitis B e antigen; Ab, antibody
* $P = 0.047$, between periods I and II; $P = 0.03$ between periods I and III; Student's t test
** $P = 0.02$ between periods I and II, and between periods I and III; Welch's t test
*** $P = 0.03$ between periods I and II; $P = 0.04$ between periods I and III; Fisher's exact probability test
[a] Mean ± SD
[b] Time between the diagnosis of severe disease and introduction of corticosteroids
[c] Use of preimmunosuppressive or cytotoxic therapies for primary diseases
[d] Statistically not significant

Table 2. Therapies and clinical outcomes in each period

Period	Therapy Early CS	Non-early CS	Recovery rate
I. 1982–1996 (retrospective)			4/16
	Early CS		2/2
		Delayed CS	0/7
		Delayed CS + IFN	0/2
		Cyclosporine	0/1
		IFN	0/1
		SNMC	2/3
II. 1997–1998 (prospective)			7/10
	Early CS		7/7
		Delayed CS	0/2
		Delayed CS + LMV	0/1
III. 1999–2007 (prospective)			10/16
	Early CS + LMV		6/7
	Early CS + ETV		2/2
	Early CS + IFN, LMV		0/1
		Delayed CS + LMV	0/2
		LMV	1/2
		IFN, ADV	1/1
		SNMC	0/1

CS, corticosteroid; IFN, interferon; SNMC, stronger neominophagen C; LMV, lamivudine; ETV, entecavir; ADV, adefovir

and ETV, two with delayed CS and LMV, two with LMV, one with early CS, IFN, and LMV, one with IFN and ADV, and one with SNMC (Table 2).

Clinical outcome according to period

Overall, 21 (50%) of 42 patients survived. Four (25%) patients survived among those in 1982–1996 (period I), seven (70%) in 1997–1998 (period II), and ten (63%) in 1999–2007 (period III). The recovery rate was lower in period I than in period II ($P = 0.03$) or period III ($P = 0.04$) (Tables 1 and 2, Fig. 1).

Clinical outcomes according to therapy

In the retrospective study between 1982 and 1996, none of the ten patients survived who received delayed immunosuppressive therapy with or without IFN; two (100%) who received early CS, none who received IFN, and two (67%) who received SNMC survived. In the prospective

study between 1997 and 2007, 15 (88%) patients survived who were treated with early CS with or without antiviral drugs, but none of five who were treated with delayed CS with or without antiviral drugs, two (67%) who received antiviral drugs, and none who received SNMC (Table 2) survived.

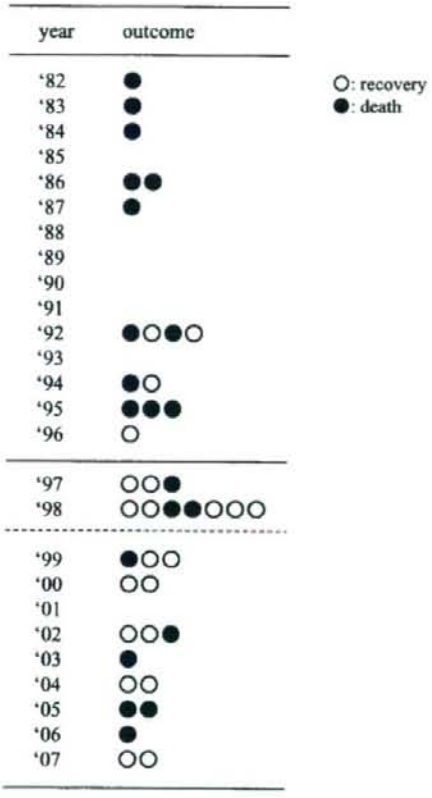

Fig. 1. Serial changes and clinical outcomes of patients

Comparison of CS monotherapy and CS and NA combination therapy in the prospective study

In the prospective study, the clinical features of patients treated with CS monotherapy and CS and NA combination therapy were compared. The differences in age, sex, PT, ALT, T-Bil, HBe Ag/Ab status, time between the diagnosis of severe disease and introduction of corticosteroids, and recovery rate were not significant between these groups (Table 3).

Clinical outcomes according to the time between the diagnosis of severe disease and the introduction of immunosuppressive drugs

Overall, immunosuppressive drugs were introduced to 19 patients within 10 days after the diagnosis of severe disease, and 17 (89%) recovered. In contrast, when they were introduced later than 10 days after the diagnosis of severe disease, none of 12 recovered (Fig. 2). The exact number of days before immunosuppressive drug introduction was not known in three patients, but they were obviously introduced later than 10 days after the diagnosis.

In the retrospective study between 1982 and 1996, both of two patients in whom immunosuppressive drugs were initiated within 10 days after the diagnosis of severe disease recovered, and all eight with initiation 11 days or longer after diagnosis died. In the prospective study between 1997 and 2007, 15 of 17 patients administered immunosuppressive drugs within 10 days after the diagnosis recovered, and all four treated 11 days or longer after diagnosis died.

Viral kinetics during therapies in CS and NA combination therapy

Among eight patients receiving early CS and NA therapy of the prospective study group, the hepatitis B

Table 3. Comparison of CS monotherapy and CS and NA combination therapy in the prospective study

	CS monotherapy	CS and NA combination therapy
n	9	13
Age[a]	44.6 ± 13.4	47.6 ± 13.3
Sex (M/F)	6/3	10/3
PT (%)[a]	34 ± 12	35 ± 14
ALT (IU/l)[a]	1392 ± 1010	863 ± 691
T-Bil (mg/dl)[a]	9.7 ± 5.7	8.2 ± 5.2
HBeAg/Ab	4/5	6/8
Duration[a,b]	6.3 ± 5.4	5.8 ± 4.0
Recovery	7	8

No statistically significant differences were observed
NA, nucleoside analog
[a]Mean ± SD
[b]Time between the diagnosis of severe disease and introduction of corticosteroids

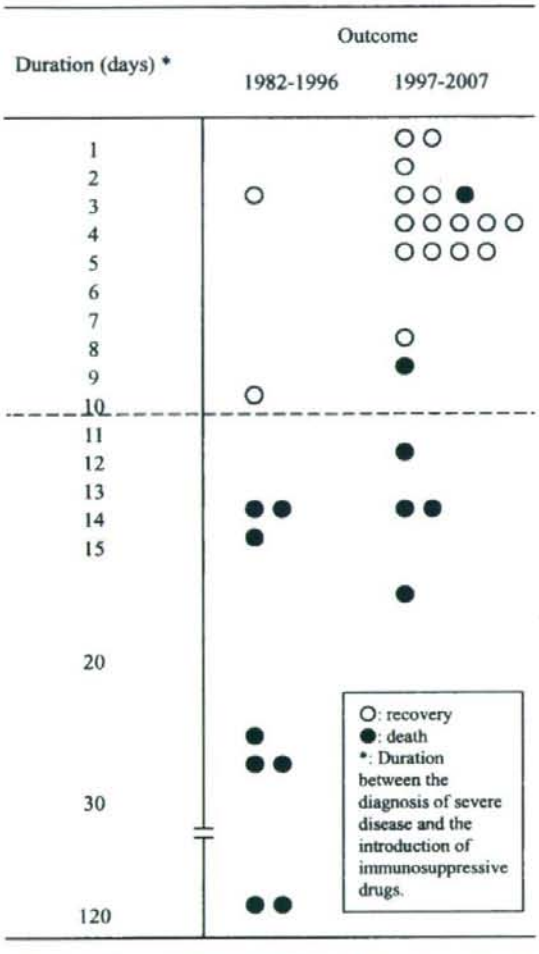

Fig. 2. Clinical outcomes according to the time between the diagnosis of severe disease and the introduction of immunosuppressive drugs

viral load was 6.5 ± 1.7 log copies/ml before treatment initiation (at week 0), 5.1 ± 1.2 at 2 weeks after treatment initiation, and 4.0 ± 1.3 at 4 weeks. The difference between the load at week 0 and at 4 weeks was significant ($P < 0.01$) (Fig. 3). Figure 3 shows the viral kinetics in the eight patients receiving early CS and NA therapy, one receiving delayed CS and NA therapy, and two receiving NA therapy.

Long-term outcomes of survivors

Of the nine survivors in the early CS group, two had LMV introduced afterward and three did not (four

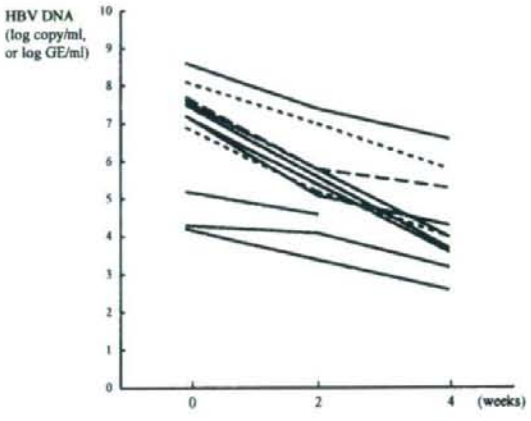

Fig. 3. Viral kinetics during therapies in the prospective study. *Solid*, *dashed*, and *dotted* lines denote early corticosteroid (CS) and nucleoside analog (NA) therapy, delayed CS and NA therapy, and NA therapy alone, respectively. *HBV*, hepatitis B virus; *GE*, genome equivalents

unknown), and corticosteroids were tapered to zero within a year in all five of these patients.

One patient with liver cirrhosis in the LMV group was introduced to ADV 2 years later, and the viral load decreased, but liver failure developed gradually. He received a living donor-liver transplantation from his son 3 years later.

Discussion

The prognosis of severe exacerbation of chronic hepatitis B is poor if signs of liver failure appear.[1,2,19] Recently, nucleoside analogs (NA), which exhibit a strong inhibitory effect on HBV replication, have been administered to patients with chronic hepatitis B, and dramatic improvements have been achieved. NA can be administered safely even to patients with severe disease,[10–15] but mortality is still high in patients demonstrating liver failure. A nationwide survey of fulminant hepatitis and late-onset hepatic failure between 1998 and 2003 in Japan revealed that the prognosis was especially poor in HBV carriers even after the introduction of LMV.[20] Tsubota et al.[21] reported that LMV monotherapy conferred no significant protection against rapid progression of the disease to liver failure in cases of severe acute exacerbation of chronic hepatitis B. Similar results were reported by Chan et al.[22] and Chien et al.[23] With the administration of NA, HBV DNA is reduced rapidly, but the improvement in liver function is delayed by a few weeks to a few months.[12] During the time lag, exces-

sive immunological reaction may continue and liver cell injury may progress. If in this phase effective therapeutic approaches were available, they would certainly be beneficial for these patients.

In our previous study, we described that the introduction of high-dose CS can reverse deterioration in patients with clinically severe, life-threatening exacerbation of chronic hepatitis B, when used in the early stage of illness.[9] We defined the criteria of severe disease in 1997, treated patients with severe disease with early initiation of CS of sufficient doses after 1997 prospectively, and after 1999 we used the combination of early and sufficient doses of CS and NA. In the present study, we examined the effect of the combination therapy of CS and NA.

One reason for not using CS for the treatment of chronic hepatitis B is that CS might enhance HBV replication through a steroid responsive element in the HBV genome.[24] In our previous study, none of the patients given high doses of CS showed increases in HBV replication during short-term observation periods.[9] In this study, HBV DNA decreased significantly during the 4-week period from the start of the CS and NA therapy. Gregory et al.[25] reported that in their study steroids would likely have proved beneficial if treatment had been started "much earlier" in the course of the illness. We are also convinced that timing is very important for optimum CS treatment.

In the 1982–1996 period, CS was introduced in the advanced stage of liver failure in most patients, but the recovery rate was low (25%). After we established the criteria of severe disease in 1997, CS was introduced at an earlier stage and the recovery rate improved (70% in 1997–1998). After we could use NA in combination with CS from 1999, we shortened the treatment period of CS while monitoring the viral load, but, contrary to our expectations, the recovery rate did not improve (63%).

Regarding the timing of the therapies, none of the patients recovered when delayed immunosuppressive therapy, with or without antiviral drugs such as NA, were implemented. In contrast, 89% of patients who received early immunosuppressive therapy with or without NA recovered ($P < 0.001$). The importance of the early introduction of immunosuppressive therapy was shown again in the presence of effective NA. Regarding the time between the diagnosis of severe disease and the start of immunosuppressive therapy, 89% of patients administered immunosuppressive therapy within 10 days recovered, but none administered the therapy 11 days or longer after diagnosis recovered. When the start of the treatment is delayed beyond 10 days, large numbers of hepatocytes are likely already destroyed and inhibition of the inflammatory reaction might not be effective. Our cutoff point of 10 days to define "early introduction" seems to be very close to the mark.

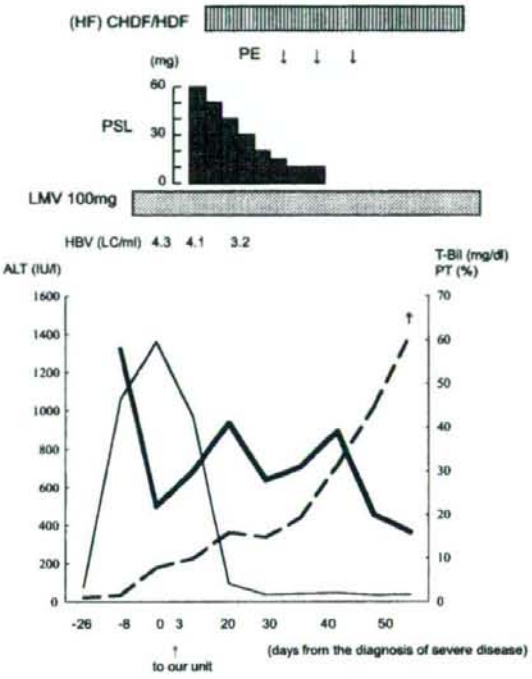

Fig. 4. Clinical course of a 63-year-old female patient. She suffered from chronic hepatitis B with hepatitis B e antigen (HBeAg). She had natural exacerbation, and lamivudine (*LMV*) was administered before the criteria of severe disease were fulfilled. Eight days after the start of LMV, she showed hepatic encephalopathy, marked prolonged prothrombin time (*PT*) activity, and elevated alanine transaminase (*ALT*) and total bilirubin (*T-Bil*). A corticosteroid was administered 9 days after the diagnosis of severe disease, but she did not respond to the therapies. *Thick solid*, *thin solid*, and *dashed lines* denote PT, ALT, and T-Bil, respectively. *PE*, plasma exchange; *(HF) CHDF*, (high flow) continuous hemodiafiltration; *HDF*, hemodiafiltration; *PSL*, prednisolone

Two patients died despite early CS and NA. One was an HIV-positive man who was hospitalized 10 days after the onset of jaundice. Three days after the diagnosis of severe disease, CS and NA were administered, but he did not respond to the therapy. The other was a woman suffering from acute exacerbation in a related hospital, and LMV was administered before the criteria of severe disease were fulfilled. Eight days after the start of LMV, she showed hepatic encephalopathy, marked prolonged PT activity, and elevated ALT and T-Bil. CS was administered 9 days after the diagnosis of severe disease, but she did not respond to the therapy. In both cases, the timing of the diagnosis of severe disease was delayed,

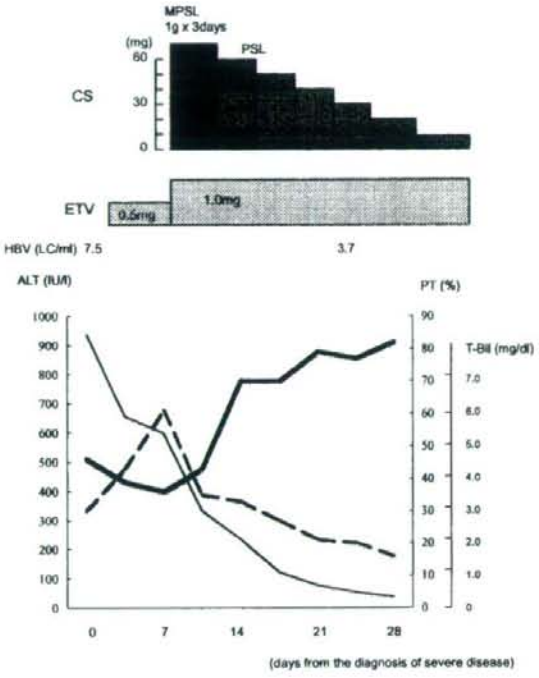

Fig. 5. Clinical course of a 51-year-old male patient. He suffered from chronic hepatitis B with HBeAg. He had natural exacerbation, and entecavir (*ETV*) was administered the day after admission, but he did not show a trend toward remission in PT or T-Bil. A double dose of ETV, together with CS, were introduced 5 days after the diagnosis of severe disease, and he responded to the therapy. *Thick solid*, *thin solid*, and *dashed lines* denote PT, ALT, and T-Bil, respectively. *MPSL*, methylprednisolone

although CS and NA therapy was started within 10 days after this diagnosis. These results emphasize that an even earlier diagnosis of severe disease is required.

Our results highlight the importance of immunosuppressive therapy for preventing the progression to liver failure. As Tsubota et al.[21] reported that an effective therapeutic strategy should be aggressively combined with LMV because LMV lacks the capability of suppressing a hyperimmune reaction, it seems that antiviral therapy is not sufficient to stop progressive deterioration and additional therapy to suppress liver cell degeneration may be necessary. Combination treatment with early high-dose CS and NA might be effective in suppressing the excessive host immune response in the early period. Additionally, NA can make it possible to shorten the term of CS therapy.

In summary, our study demonstrated that the early introduction of high-dose CS treatment in combination with NA may be beneficial for cases of clinically severe acute exacerbation of chronic hepatitis B. We were unable to include placebo-controlled patients, considering the current knowledge of the poor prognosis of such patients and our historical control patients between 1982 and 1996. Nevertheless, delay in treatment may result in fatal liver failure even when these treatment protocols are used, suggesting that an early diagnosis of such patients is urgently required.

Acknowledgments. We are indebted to all our colleagues at the liver units of our hospitals who cared for the patients described herein. In addition, we thank Dr. Toshiki Ehata (Ehata Clinic), Dr. Kazuo Suzuki (Takushinkai Tatsumi Hospital) and Professor Masao Omata (University of Tokyo) for clinical contributions.

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Liver International ISSN 1478-3223

CLINICAL STUDIES

Phylogenetic analysis of hepatitis A virus in sera from patients with hepatitis A of various severities

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Keywords
2B – 2C – 5′NTR – fulminant hepatitis – hepatitis A – hepatitis A virus

Abbreviations
AH, acute hepatitis; AHs, acute hepatitis severe type; FH, fulminant hepatitis; HAV, hepatitis A virus; PT, prothrombin time.

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Received 13 August 2008
Accepted 4 October 2008

DOI:10.1111/j.1478-3231.2008.01919.x

Abstract
Background: We analysed the association of the 5′ nontranslated region (5′NTR), nonstructural proteins 2B and 2C of the hepatitis A virus (HAV) genome, whose mutations have previously been shown to be important for enhanced replication in cell culture systems, in order to align all our data and examine whether genomic differences in HAV are responsible for the range of clinical severities. *Methods:* Our accumulated HAV strains of 5′NTR [nucleotide(nt) 200 and 500], entire 2B and 2C from 25 Japanese patients with sporadic hepatitis A, consisting of seven patients with fulminant hepatitis (FH), five with severe acute hepatitis (AHs) and 13 with self-limited acute hepatitis (AH), in whom the sequences of all three regions were available, were subjected to phylogenetic analysis. *Results:* Fulminant hepatitis patients had fewer nucleotide substitutions in 5′NTR, had a tendency to have more amino acid (aa) substitutions in 2B and had fewer aa substitutions in 2C than AH patients. Four FH and two AHs with a higher viral replication were located in the near parts of the phylogenetic trees, indicating the association between the severity of hepatitis A and genomic variations in 5′NTR, 2B and 2C of HAV. *Conclusions:* Our study suggests that genetic variations in HAV not in one specific region but in 5′NTR, 2B and 2C might cooperatively influence replication of the virus, and thereby affect virulence. Viral factors should be considered and examined when discussing the mechanisms responsible for the severity of hepatitis A.

Hepatitis A is still a major problem worldwide, not only in underdeveloped countries but also in industrialized nations. Because of improvements in sanitation, there have been no hepatitis A epidemics in Japan in recent years. However, sporadic cases of hepatitis A have not been rare of late. Although the majority of hepatitis A cases are self-limited acute hepatitis (AH), some develop into severe forms of hepatitis (1). In fact, in the past several years, there has been an increase in the numbers of patients with sporadic hepatitis A, especially the more severe kind, visiting our hospital. Our analysis of the possible factors responsible for the disease severity in our patients revealed no significant differences in terms of background including age, suggesting that viral factors might be involved in determining the severity of the disease (2, 3).

Hepatitis A virus (HAV) is the sole member of the hepatovirus genus and a member of the Picornavirus family. Virological studies have revealed that HAV is a positive-strand RNA virus comprising approximately 7500 nucleotides and containing a 5′ nontranslated region (NTR), a single long open reading frame encoding a large polyprotein and a 3′NTR. A large polyprotein is cleaved by the viral protease to produce the P1, P2 and P3 regions. The P1 region encodes four structural proteins – VP4, VP2, VP3 and VP1. The P2 and P3 regions encode nonstructural proteins 2A, 2B and 2C, and 3A, 3B, 3C and 3D respectively (4). As far as is known, nonstructural protein 2A participates in virion morphogenesis (5). 2B and 2C play important roles in the replication of viral RNA. 2C is a multifunctional protein and is considered to have helicase and NTPase activities. 2C or 2BC have membrane- and RNA-binding properties (6). 3B is considered to be a genome-linked viral protein (Vpg), 3A a pre-Vpg, 3C a viral protease and 3D an RNA-dependent RNA polymerase.

It was reported that the strains adapted to cell culture systems have mutations in 5′NTR and the P2 region of HAV (7, 8). Zhang *et al.* (9) reported that rapidly replicating, cytopathic variants of HAV isolated from cultured cells required mutations within 5′NTR, 2B and 2C, and these mutations acted cooperatively. Raychaudhuri *et al.* (10) reported that the simian virus 2C gene could confer the phenotype of virulence to an otherwise attenuated virus, and clusters of residues near both ends of the 2C protein were required for virulence using chimeras between human and simian strains of hepatitis A virus in tamarins.

Despite advances in the understanding of HAV, a correlation between the HAV genome and the clinical status of hepatitis A has not been established. Durst *et al.* (11) reported a cluster of fulminant hepatitis A, in which the severity of the infection in three siblings was related to the virulence of HAV. To examine the possibility of differences in hepatitis A viruses in terms of the different categories of hepatitis, we have analysed the viral genomes in sera from hepatitis A patients with a variety of clinicopathological features and reported the associations between some viral regions and clinical severities (3, 12–18).

When analysing the viral genome, rather than focusing on one specific region, perhaps several portions of the HAV genome should be investigated. In the present study, we examined the clinicopathological features of hepatitis A and possible correlations with variations in the three regions of 5′NTR, 2B and 2C of the HAV genome, whose mutations have previously been shown to be important for enhanced replication and virulence in cell culture systems and simians, in the same patients using phylogenetic analysis.

Materials and methods

Patients

Serum samples from 25 patients with hepatitis A in Japan were collected between 1986 and 1999 and stored at −20 °C until analysis. Informed consent was obtained from the patients or appropriate family members. These patients were diagnosed based on the positivity of the IgM antibody to HAV (IgM anti-HAV) in conjunction with compatible symptoms and laboratory findings.

Among the patients seven had fulminant hepatitis (FH), five had severe acute hepatitis (AHs) and 13 had self-limited AH. Patients with a prothrombin time < 40% of control were defined as AHs, and those with hepatic encephalopathy as FH. Patients with significant increases in serum blood urea nitrogen and creatinine (more than three times the upper level of the normal range) were judged to be undergoing acute renal failure. The patients were also investigated for histories of recent exposure to drugs and chemical agents as well as heavy alcohol consumption (> 50 g/day for > 5 years).

None of the patients had clinical or laboratory evidence of acquired immune deficiency syndrome.

Serological markers

IgM antibody to HBc(IgM anti-HBc), HBsAg and second-generation antibody to hepatitis C virus (HCV) were examined in all cases. IgM anti-HAV, IgM anti-HBc and HBsAg were measured by commercial radioimmunoassay kits (Abbott Laboratories, Chicago, IL, USA); second-generation HCV antibody was measured by the enzyme immunoassay kit (Ortho Diagnostics, Tokyo, Japan). In the FH and AHs patients, HCV RNA, IgM antibody to Epstein–Barr virus (IgM anti-EBV), IgM antibody to herpes simplex virus (IgM anti-HSV), IgM antibody to cytomegalovirus (IgM anti-CMV), anti-smooth muscle antibody, liver kidney microsomal antibody-1 and anti-mitochondrial antibody were also examined. HCV RNA was measured by nested reverse transcriptase-polymerase chain reaction (RT-PCR) as described by the authors (19). IgM anti-EBV, IgM anti-CMV and IgM anti-HSV were examined by enzyme-linked immunosorbent assays. Anti-nuclear antibody, anti-smooth muscle antibody, anti-mitochondrial antibody and anti-liver kidney microsomal-1 antibody were examined by the fluorescent antibody method.

Quantification of hepatitis A virus RNA by real-time reverse transcriptase-polymerase chain reaction

Serum viral RNA was extracted by the High Pure Viral RNA Kit (Roche Diagnostics GmbH, Mannheim, Germany). RT-PCR was carried out with a Hepatitis A Virus Quantification Kit (Roche Diagnostics) according to the manufacturer's instructions. Twenty microliters of the PCR mixture contained 15 μl of master mix from the kit and 5 μl of template RNA. The standards of HAV RNA are supplied with this kit. All reactions were performed in a LightCycler (Roche Diagnostics). The C_T values from clinical samples were plotted on the standard curve, and the number of copies wes calculated automatically. This method has a dynamic range of HAV RNA quantification between 0.5 and 5×10^6 copies/μl.

Amplification of serum hepatitis A virus RNA and direct sequencing

Hepatitis A virus RNA was examined by nested RT-PCR and direct sequencing as described previously (14, 17, 18).

Nucleotide sequence accession numbers

The nucleotide sequence data reported herein appear in DDBJ/EMBL/GenBank nucleotide sequence databases with the following accession numbers:

5′NTR

AB045327 for A1, AB045336 for A5, AB045330 for A204, AB045331 for A205, AB045332 for A206, AB045334 for A414, AB045338 for A601, AB045342 for A159, AB045344 for A160, AB045345 for A161, AB045350 for A302, AB045353 for A811, AB045672 for A7, AB045692 for A9, AB045568 for A20, AB045671 for A68, AB045680 for A75, AB045681 for A77, AB045366 for A162, AB045572 for A303, AB045646 for A304, AB045648 for A306, AB045649 for A307, AB045678 for A712 and AB045679 for A713.

2B

AB047652 for A1, AB047671 for A5, AB047660 for A204, AB047661 for A205, AB047662 for A206, AB047669 for A414, AB047673 for A601, AB047654 for A159, AB047655 for A160, AB047656 for A161, AB047663 for A302, AB047680 for A811, AB047675 for A7, AB047681 for A9, AB047658 for A20, AB047674 for A68, AB047678 for A75, AB047679 for A77, AB047657 for A162, AB047664 for A303, AB047665 for A304, AB047666 for A306, AB047667 for A307, AB047676 for A712 and AB047677 for A713.

2C

AB082174 for A1, AB082130 for A5, AB0821323 for A204, AB082133 for A205, AB082134 for A206, A082135 for A414, AB082137 for A601, AB082139 for A159, AB082140 for A160, AB082141 for A161, AB082145 for A302, AB082147 for A811, AB082148 for A7, AB082149 for A9, AB082150 for A20, AB082154 for A68, AB082155 for A75, AB082156 for A77, AB082160 for A162, AB082165 for A303, AB082166 for A304, AB082167 for A306, AB082168 for A307, AB082171 for A712 and AB082172 for A713.

Phylogenetic analysis

To determine the heterogeneity of the viral sequences obtained from the 25 patients, a phylogenetic tree was constructed by the neighbour-joining method. To confirm the reliability of the phylogenetic tree, bootstrap resampling tests were performed

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1000 times. These analyses were conducted using a computer program, GENETYX-MAC version 10.1 (Software Development, Tokyo, Japan).

Statistical analysis

Differences in proportions among the groups were compared by Fisher's exact probability test, Student's *t*-test and Welch's *t*-test (DA STATS version 1.0, Nagata O, Tokyo, Japan).

Results

Clinicopathological characteristics of the patients

The characteristics of the 25 patients with hepatitis A analysed for HAV 5′NTR, 2B and 2C at admission are summarized in Table 1. None of the cases was associated with an epidemic.

Differences in the mean age, sex and presence of chronic liver disease among FH, AHs and AH, and between FH+AHs and AH, were not statistically significant. Serum was sampled 2–17 days after clinical onset. The mean ALT level was higher in AHs than that in AH ($P = 0.002$), and in FH+AHs than that in AH ($P = 0.003$). The mean prothrombin time was prolonged in FH compared with AHs ($P = 0.002$), FH compared with AH ($P < 0.001$), AHs compared with AH ($P < 0.001$) and FH+AHs compared with AH ($P < 0.001$). The mean total bilirubin level was higher in FH than that in AHs ($P = 0.049$).

Four of seven patients with FH died of hepatic failure, and all patients with AHs and AH recovered ($P = 0.007$). All seven FH cases needed artificial liver support (plasma exchange and haemodiafiltration). Four (16%) patients – two (28%) with FH and two (15%) with AH – had acute renal failure and were treated by haemodiafiltration.

Two patients with AH were positive for HBsAg and antibody to HBe, and one patient with AH was positive for anti-nuclear antibody, but they showed a typical hepatitis A course. IgM anti-EBV, IgM anti-HSV, IgM anti-CMV, anti-nuclear antibody, anti-smooth muscle antibody, liver kidney microsomal antibody-1 and anti-mitochondrial antibody were negative in all examined cases of FH and AHs. One FH patient and one AHs patient had histories of heavy alcohol consumption. One male patient with AH was homosexual.

Histological examination was performed in all seven FH cases, two of five AHs cases and seven of 13 AH cases in the convalescent phase or postmortem. In the FH cases, liver histology revealed massive necrosis in three patients and submassive necrosis in one. Liver histology in the two patients with histories of heavy alcohol consumption showed pericellular fibrosis, consistent with alcoholic liver disease. The histological findings of the other cases showed AH to be in a residual phase or subsiding.

Phylogenetic analysis

The results of phylogenetic analysis are shown in Figures 1 and 2. Four FH (A204, A601, A414 and A1) and two AHs (A160 and A159) were located in the near parts of the phylogenetic trees (Fig. 2).

The clinical backgrounds, and the biochemical and viral characteristics are shown in Table 2. As described above, none of them were associated with an epidemic. Two of the FH patients died and the others recovered. HAV RNA was quantified by real-time RT-PCR in five of these six patients. Our other recent study of HAV RNA quantification revealed that the mean viral load in > 60 AH at admission was 2.75 ± 1.55 log copies/ml (20), and so these five patients had comparatively higher viral loads (4.35 ± 0.81 log copies/ml) ($P = 0.03$). The HAV genotype was IA in all patients, similar to the majority of Japanese patients in general.

Table 1. Characteristics of patients

	FH	AHs	AH
n	7	5	13
CLD	1†	1†	3†
Recovery/death	3/4‡	5/0‡	13/0‡
Sex (M/F)	3/4†	5/0†	7/6†
Age*	44.1 ± 13.5†	36.8 ± 12.9†	39.5 ± 9.1†
PT (%)*	16 ± 7§	34 ± 8§	63 ± 20§
ALT (IU/L)*	6337 ± 3838¶	6165 ± 1718¶	2873 ± 1733¶
T-Bil (mg/dl)*	9.4 ± 7.6‖	2.3 ± 0.8‖	5.0 ± 2.3‖

*Mean ± SD.

†Statistically not significant.

‡Statistically significant between FH and AH ($P = 0.007$) by Fisher's exact probability test.

§Statistically significant between FH and AHs ($P = 0.002$) by Student's *t*-test, FH and AH ($P < 0.001$) by Welch's *t*-test, and AHs and AH ($P < 0.001$) by Welch's *t*-test.

¶Statistically significant between AHs and AH ($P = 0.002$) by Student's *t*-test.

‖Statistically significant between AHs and FH ($P = 0.049$) and AHs and AH ($P = 0.002$) by Welch's *t*-test.

AH, acute hepatitis; AHs, severe acute hepatitis; ALT, alanine aminotransferase; CLD, chronic liver disease; FH, fulminant hepatitis; PT, prothrombin time; T-Bil, total bilirubin.

Discussion

Although the severity of hepatitis A varies, it is not clear why it is more severe in some patients than that in others. It is thought that disease severity may be dependent on certain characteristics of the individual patients. It has been reported that ageing and underlying chronic liver disease could be factors that increase hepatitis A severity (21). Vento *et al.* (22) reported that patients with chronic hepatitis C had a substantial risk of FH and death associated with HAV superinfection.

During an urban epidemic in the US, it was described that hepatitis A caused serious illness and death and that complications were more frequent in patients 40 years of age and older, but that young healthy persons were also at risk for severe complications (23). A cluster of fulminant hepatitis A was reported, relating the severity of the infection in three siblings to the virulence of HAV, as the patients were all healthy before the infection and their illness followed a similar course (11).

In the past several years, increasing numbers of patients with sporadic hepatitis A, especially the more severe forms, have visited our hospital, but our analysis of factors possibly contributing to the severity of the disease failed to reveal any significant differences in patient characteristics including age (2, 3), suggesting that viral factors might determine the severity of the disease. To identify possible differences in hepatitis A viruses for different types of hepatitis, we analysed the HAV genome in sera from hepatitis A patients with various clinicopathological features. Our analysis of whole HAV genomes from three cases of FH and three cases of AH indicated possible associations between the severity of hepatitis A and the nucleotide substitutions in 5′NTR and the amino acid (aa)

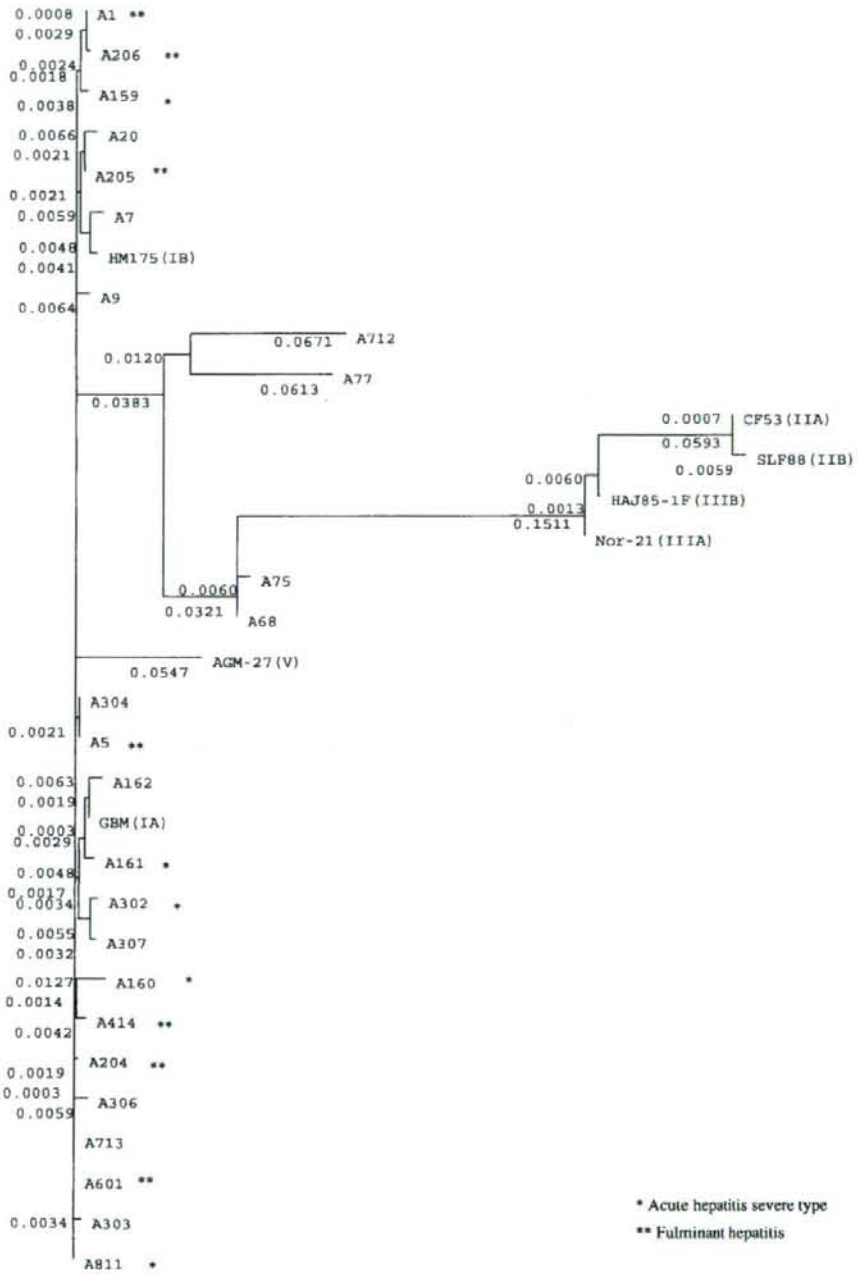

Fig. 1. Genetic relatedness between individual hepatitis A virus (HAV) strains between nucleotides 200 and 500 of the 5′ nontranslated region recovered from 25 patients and HAV reference strains GBM (subgenotype IA), HM175 (subgenotype IB), CF53 (subgenotype IIA), SLF88 (subgenotype IIB), Nor-21 (subgenotype IIIA), HAJ85-1F (subgenotype IIIB) and AGM27 (genotype V). Numbers beside the phylogenetic roots are the results of bootstrap analyses.