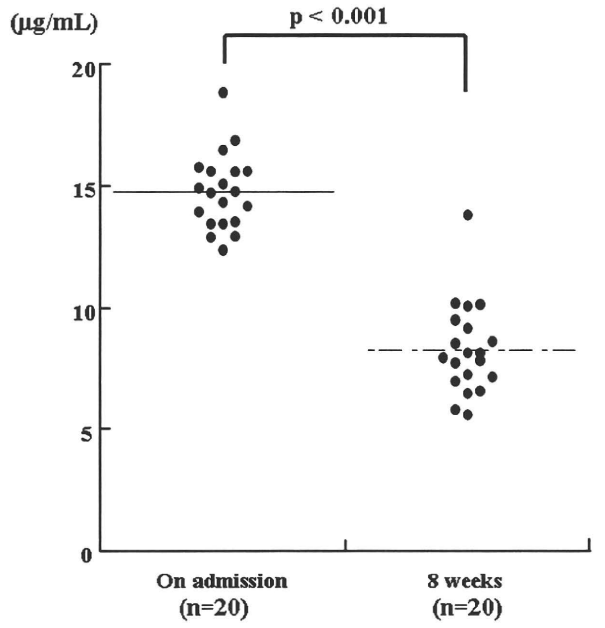

Fig. 3. Serum PEDF levels, as determined by ELISA, in 20 alcoholic patients on admission and after 8 weeks of abstinence. The serum PEDF levels are significantly decreased after 8 weeks of abstinence ($p < 0.001$). ELISA, enzyme-linked immunosorbent assay; PEDF, pigment epithelial-derived factor.

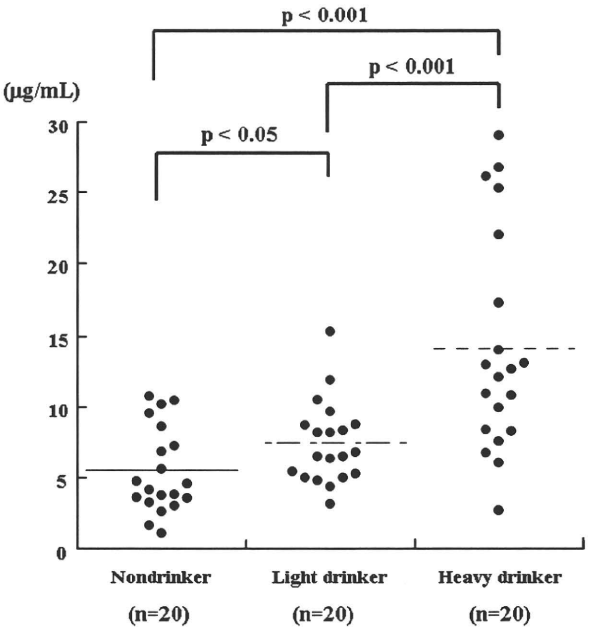

Fig. 4. Serum PEDF levels, as determined by ELISA, in 60 apparently healthy subjects with various drinking habits. The levels are significantly greater in the light habitual drinkers (7.5 ± 2.9 μg/ml) and in the heavy habitual drinkers (14.2 ± 7.7 μg/ml), compared with the nondrinkers (5.5 ± 3.0 μg/ml). ELISA, enzyme-linked immunosorbent assay; PEDF, pigment epithelial-derived factor.

ELISA

ELISA further confirmed an increase in serum PEDF levels in 20 male patients with alcoholic dependency. The PEDF level was 14.6 ± 1.9 μg/ml on admission and decreased significantly to 8.7 ± 2.3 μg/ml after 8 weeks of abstinence from drink ($p < 0.001$) (Fig. 3).

The serum PEDF levels in nondrinkers and habitual drinkers are shown in Fig. 4. The serum PEDF levels in light habitual drinkers (7.5 ± 2.9 μg/ml) and in heavy habitual drinkers (14.2 ± 7.7 μg/ml) were significantly greater than in nondrinkers (5.5 ± 3.0 μg/ml).

Serum PEDF levels were also measured in subjects with nonalcoholic chronic liver diseases of viral and nonviral origin. The PEDF levels in subjects with chronic nonalcoholic liver diseases were comparable to the PEDF levels in normal subjects without a drinking habit, as indicated in Fig. 5.

Serum PEDF levels of the control subjects younger than 50 yrs were 9.4 ± 7.3 μg/ml and the levels in those older than 50 years were 8.7 ± 5.1 μg/ml, indicating that there are no age-related differences in serum PEDF levels. In a total of 120 patients with nonalcoholic liver diseases, there were no gender-related differences (4.2 ± 2.1 μg/ml in males and 4.5 ± 2.4 μg/ml in females).

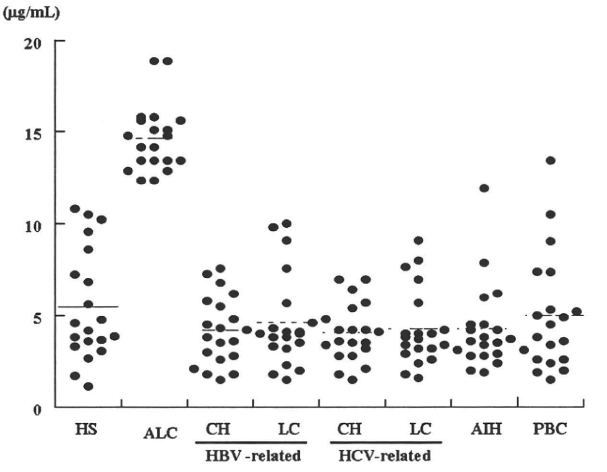

Fig. 5. Serum PEDF levels, as determined by ELISA, in alcoholic patients and in patients with chronic liver diseases of nonalcoholic etiology. The PEDF levels are significantly greater in alcoholic patients than in the healthy subjects. However, the PEDF levels in other patient groups are comparable to those in the healthy controls. AIH, autoimmune hepatitis; ALC, alcohol dependency; CH, chronic hepatitis; ELISA, enzyme-linked immunosorbent assay; HS, healthy subjects; LC, liver cirrhosis; PBC, primary biliary cirrhosis; PEDF, pigment epithelial-derived factor.

DISCUSSION

Recent advances in proteomic technology have provided promising ways to discover and identify novel biomarkers in various fields of clinical medicine. The application of various gel-based and gel-free methods has facilitated the discovery of potential clinical biomarkers, although there has been a long

and uncertain path from marker discovery to clinical utility. We previously discovered a 5.9-kDa peptide as a novel biomarker of alcohol abuse using mass spectrometry–based methods (Nomura et al., 2004; Sogawa et al., 2007). We also used fluorescent two-dimensional difference gel electrophoresis (2D-DIGE) for serum proteome analysis and found a change before and after abstinence in the serum levels of relatively abundant proteins (including alpha 1-antichymotripsin and haptoglobin) in subjects with alcohol abuse (Wu et al., 2007).

A technical challenge in serum proteome analysis has been that serum contains thousands of proteins and peptides that are present in a large dynamic range. Indeed, 22 abundant proteins (e.g., albumin, immunoglobulins, and transferrin) constitute up to 99% of the protein content of plasma (Anderson and Anderson, 2002; Tirumalai et al., 2003). Depletion of these abundant proteins and further fractionation of samples will be necessary in future proteomic studies searching for low-abundant serum proteins or peptides.

We previously used the three-step method and detected 3 proteins, including YKL-50, as promising novel biomarkers of sepsis (Hattori et al., 2009). We assessed reproducibility of the three-step method. To assess between-days precision, 4 aliquots of 40 μl from the same serum samples were subjected to the three-step proteome analyses on 4 different days, and silver-stained SDS–PAGE results of the 4 fractions were compared. As shown in Fig. S1, the between-day differences were minimal. In the present study, we applied the three-step method to search for biomarkers for excessive alcohol drinking. We performed three-step proteome analysis on 2 serum samples collected from each of 8 patients (16 samples in total) with alcohol dependence—one sample was collected on admission and one sample was collected after 8 weeks of abstinence from drink. Three-step serum proteome analysis revealed that the serum levels of 5 proteins—alpha2-HS glycoprotein, apolipoprotein A-I, glutathione peroxidase 3, heparin cofactor II, and PEDF were significantly greater on admission than after 8 weeks of abstinence. On the other hand, serum levels of apolipoprotein III were downregulated on admission. Although the data were shown only for PEDF, the results of Western blotting confirmed the changes in the expression levels of all the 6 proteins by heavy drinking. As alterations of serum levels of apolipoprotein A-I, alpha-2-HS glycoprotein, apolipoprotein C-III, glutathione peroxidase 3, and heparin cofactor II associated excessive alcohol consumption have been reported in the literature (Andersson and Bell, 1988; Kaku et al., 1982; Nanchahal et al., 2000; Peng et al., 2005; Robinson and Quarfordt, 1981), we focused on PEDF, alterations of which by heavy drinking are not well characterized. In the present study, these changes were initially detected by SDS–PAGE, the final step of the three-step proteome analysis. Western blotting and ELISA further confirmed these changes. After patients with alcohol abuse abstained from alcohol for 2 months, their elevated serum PEDF levels (noted on admission) returned to the levels found in the control subjects. This suggests that active and excessive drinking, rather than liver injury per se, could play a role in the upregulation of PEDF.

This notion is partially supported by data showing that the serum PEDF levels in people with nonalcoholic chronic liver diseases, including liver cirrhosis, are comparable to the serum PEDF levels in normal controls without a drinking history. Furthermore, serum PEDF levels in habitual drinkers are significantly greater than serum PEDF levels in nondrinkers.

PEDF is a glycoprotein belonging to the serine protease inhibitor superfamily. It was originally purified from the culture supernatant of retinal pigment epithelial cells as a factor that inhibited vascularization (Leung et al., 1989) and exhibited potent neurosecretory activity for human retinoblastoma cells (Tombran-Tink et al., 1991). The PEDF gene contains 8 exons and 7 introns and is located on chromosome 17p13.1. Its transcript is widely expressed in various tissues such as the eye, brain, spinal cord, skeletal muscle, adipose tissue, liver, and bone (Rychli et al., 2009). PEDF is reportedly also present in human plasma at a concentration of around 5 μg/ml (Petersen et al., 2003), which is very similar to the levels obtained in normal controls without drinking history in the present study.

Serum or plasma PEDF levels have been determined in several pathological conditions. Plasma PEDF levels are significantly elevated in diabetic patients, especially in patients with proliferative diabetic retinopathy (Ogata et al., 2007). Elevated serum levels of PEDF in metabolic syndrome have also been reported (Yamagishi et al., 2006). Matsumoto and colleagues (2004) measured serum PEDF levels in various chronic liver diseases. They reported that serum PEDF levels in patients with liver cirrhosis because of hepatitis C virus are significantly lower than the serum PEDF in controls subjects. These results do not agree with the findings of this study. The reasons for this discrepancy are not clear at the moment. It should be noted, however, that Matsumoto and colleagues used a particular ELISA kit from Chemicon International, which is good for measuring the PEDF level of vitreous fluid, but is reportedly not appropriate for measuring the serum PEDF level (Yamagishi et al., 2006). Indeed, the PEDF levels in the controls in Matsumoto's study were around 6 ng/ml, which is lower by almost three-orders magnitude than the levels obtained in this study (5 μg/ml).

The biological significance of the increase in serum PEDF after excessive alcohol drinking is of great interest. In this context, the antiangiogenic, antitumorigenic, antioxidant, antithrombotic, and anti-inflammatory properties of PEDF have to be considered (Rychli et al., 2009; Tombran-Tink and Barnstable, 2003; Uehara et al., 2004; Yamagishi et al., 2009). It has recently been suggested that PEDF plays a protective role in atherosclerosis and that the antiatherothrombogenic property of PEDF may be a therapeutic target in cardiovascular disease (Rychli et al., 2009; Yamagishi and Matsui, 2010). The reduced risk of fatal coronary diseases in habitual drinkers is well documented, but the underlying

mechanisms are complex (Renaud et al., 2004). It is tempting to speculate that the upregulation of PEDF may (at least partly) play a role in this protective action. Thus, three-step serum proteome analysis reveals that serum PEDF levels are significantly increased after excessive drinking. The exact diagnostic and pathophysiological roles of this phenomenon remain to be investigated.

GRANT SUPPORT

Ministry of Education, Culture, Science, Sports, and Technology of Japan.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Fig. S1. Silver-stained SDS–PAGE shows the reproducibility of the method. Four aliquots of 40 μl from the same serum sample underwent the three-step method. One-sixteenth of each sample (corresponding to the proteins from 2.5 μl serum) is loaded in each lane. SDS–PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis

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Journal of Medical Virology 83:201–207 (2011)

Hepatitis A Viral Load in Relation to Severity of the Infection

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A correlation between hepatitis A virus (HAV) genomes and the clinical severity of hepatitis A has not been established. The viral load in sera of hepatitis A patients was examined to determine the possible association between hepatitis A severity and HAV replication. One hundred sixty-four serum samples from 91 Japanese patients with sporadic hepatitis A, comprising 11 patients with fulminant hepatitis, 10 with severe acute hepatitis, and 70 with self-limited acute hepatitis, were tested for HAV RNA. The sera included 83 serial samples from 20 patients. Viral load was measured by real-time RT-PCR. The detection rates of HAV RNA from fulminant, severe acute, and acute hepatitis were 10/11 (91%), 10/10 (100%), and 55/70 (79%), respectively. Mean values of HAV RNA at admission were 3.48 ± 1.30 logcopies/ml in fulminant, 4.19 ± 1.03 in severe acute, and 2.65 ± 1.64 in acute hepatitis. Patients with severe infection such as fulminant hepatitis and severe acute hepatitis had higher initial viral load than patients with less severe infection ($P < 0.001$). Viremia persisted for 14.2 ± 5.8 days in patients with severe infection and 21.4 ± 10.6 days in those with acute hepatitis after clinical onset ($P = 0.19$). HAV RNA was detectable quantitatively in the majority of the sera of hepatitis A cases during the early convalescent phase by real-time PCR. Higher initial viral replication was found in severely infected patients. An excessive host immune response might follow, reducing the viral load rapidly as a result of the destruction of large numbers of HAV-infected hepatocytes, and in turn severe disease might be induced. ***J. Med. Virol. 83:201–207, 2011.*** © 2010 Wiley-Liss, Inc.

KEY WORDS: hepatitis A; fulminant hepatitis; severe acute hepatitis; hepatitis A virus; real-time PCR

INTRODUCTION

Hepatitis A virus (HAV) is a major etiologic agent of acute hepatitis, and it still poses a considerable problem worldwide. HAV causes self-limited acute hepatitis in most cases, but fulminant hepatitis develops in 0.1–0.2% of the cases.

Although the virological aspects of HAV have been studied extensively, a correlation between viral characteristics and clinical severity has still not been demonstrated. A sensitive nested RT-PCR method was therefore developed to detect serum HAV RNA in the early convalescent phase of hepatitis A with high frequency [Fujiwara et al., 1997], and the viral genomes in the sera from hepatitis A patients with a variety of clinicopathological features were analyzed and the associations between certain viral regions and clinical severities were documented. It has been reported that both viral and host factors should be considered when discussing the mechanisms responsible for the severity of hepatitis A [Fujiwara et al., 2000, 2001, 2002], and that several portions of the HAV genome including the 5′ non-translated region (5′NTR) and non-structural protein 2B and 2C [Fujiwara et al., 2003, 2007a,b, 2009], whose mutations have been shown to be important for enhanced replication and virulence in cell culture systems [Zhang et al., 1995] and simians [Raychaudhuri et al., 1998], should be examined.

The association between the severity of hepatitis A and serum HAV load has not yet been established because of the nature of acute illness and the difficulty of HAV quantitation [Fujiwara et al., 2005, 2009]. In the

Disclosures: All authors have nothing to disclose.

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Accepted 23 August 2010

DOI 10.1002/jmv.21958

Published online in Wiley Online Library (wileyonlinelibrary.com).

present study, HAV load and viremia of serial serum samples were examined during various clinical courses of hepatitis A both qualitatively and quantitatively by real-time RT-PCR method in an attempt to determine the association between severity of hepatitis A and HAV replication.

PATIENTS AND METHODS

Patients

One hundred sixty-four serum samples were obtained from 91 Japanese patients with sporadic hepatitis A recruited at six university hospitals located in various regions of Japan, comprising 11 patients with fulminant hepatitis, 10 with severe acute hepatitis, and 70 with self-limited acute hepatitis. The sera were collected between 1990 and 2002. They were kept at 100 μl per tube and stored at −20°C until analysis. Among them were 83 serial serum samples from 20 patients, 3 with fulminant hepatitis, 4 with severe acute hepatitis, and 13 with acute hepatitis. These patients were diagnosed on the basis of IgM anti-HAV antibody (IgM-HA) positivity in conjunction with compatible symptoms and laboratory findings. Informed consent was obtained from patients or appropriate family members. The study protocol conformed to the ethical guidelines of the 1975 Declaration of Helsinki as reflected in a priori approval by the appropriate institutional review committee.

Patients with prothrombin time (PT) <40% but without hepatic encephalopathy were defined as severe acute hepatitis, and those with hepatic encephalopathy as fulminant hepatitis. IgM anti-HBc antibody (IgM-HBc), HBsAg, and second-generation anti-HCV antibody were examined in all cases. 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-smooth muscle antibody, liver kidney microsomal antibody-1 and anti-mitochondrial antibody were also examined in patients with fulminant hepatitis and severe acute hepatitis as well as in some patients with acute hepatitis showing an atypical course. In addition, histories of recent exposure to drugs and chemical agents as well as of heavy alcohol consumption (>50 g/day for >5 years) were examined. None of the patients had clinical or laboratory evidence of acquired immune deficiency syndrome.

In hepatitis A, early symptoms including fever, general malaise, fatigue, nausea, vomiting and right upper quadrant discomfort are frequently observed, so we defined the beginning of these symptoms as clinical onset.

Serological markers

IgM-HA, IgM-HBc antibody, and HBsAg were measured by commercial radioimmunoassay kits (Abbott Laboratories, Chicago, IL), and second-generation HCV antibody was measured by enzyme immunoassay kit (Ortho Diagnostics, Tokyo, Japan). In fulminant hepatitis and severe acute hepatitis, HCV RNA was measured by nested RT-PCR. IgM-EBV, IgM-CMV, and IgM-HSV were examined by enzyme-linked immunosorbent assays, and anti-nuclear antibody, anti-smooth muscle antibody, anti-mitochondrial antibody, and anti-liver kidney microsomal antibody-1 were examined by fluorescent antibody method.

Quantitation of HAV RNA by Real-Time RT-PCR

Serum viral RNA was extracted using a High Pure Viral RNA Kit (Roche Diagnostics GmbH, Mannheim, Germany). One-step RT-PCR was carried out with a Hepatitis A Virus Quantification Kit (Roche Diagnostics GmbH) according to the manufacturer's instructions, which enable quantitation of RNA encoding HAV and simultaneous detection of HAV-specific internal control. Briefly, 20 μl of PCR mixture contained 15 μl of master mix from the Hepatitis A Virus Quantification Kit (Roche Diagnostics GmbH) and 5 μl of template RNA solution extracted from 20 μl of serum. LightCycler capillaries were sealed, centrifuged, and transferred to a LightCycler instrument (Roche Diagnostics GmbH). Reverse transcription was done for 10 min at 55°C, followed by 30-sec denaturing at 95°C. The corresponding cDNA was amplified by PCR of 45 cycles at 95°C for 5 sec, 55°C for 15 sec, and 72°C for 12 sec. The HAV RNA standards come supplied with this kit. All reactions were performed in the LightCycler (Roche Diagnostics GmbH). The CT values from clinical samples were plotted on a standard curve, and the number of copies was calculated automatically. This method provides a dynamic range of HAV RNA quantitation between 1.40 and 8.40 logcopies/ml.

Statistical Analysis

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

RESULTS

Clinical Characteristics

The characteristics of the 91 patients at admission are summarized in Table I. None of them was epidemic related.

Differences in male-to-female ratio and mean age among fulminant hepatitis, severe acute hepatitis, and acute hepatitis cases were not statistically significant. Mean alanine aminotransferase (ALT) level, mean total bilirubin (T-Bil) level, days from onset to serum sampling and presence of chronic liver disease were not significantly different among fulminant hepatitis, severe acute hepatitis and acute hepatitis cases. Mean PT activity was lower in fulminant hepatitis than in acute hepatitis and severe acute hepatitis and lower in severe acute hepatitis than in acute hepatitis.

Five (45%) of 11 patients with fulminant hepatitis died of hepatic failure, 1 (10%) of 10 with severe acute hepatitis died of sepsis, and all acute hepatitis patients

TABLE I. Characteristics of All Patients at Admission

	Fulminant hepatitis	Severe acute hepatitis	Acute hepatitis
n	11	10	70
Sex (M/F)	5/6[1)]	6/4[1)]	36/34[1)]
Age[a]	43.5 ± 15.2[2)]	40.0 ± 13.2[2)]	40.1 + 11.3[2)]
PT (%)[a]	17 ± 9[3)]	33 ± 8[3)]	75 ± 11[3)]
ALT (IU/L)[a]	5,538 ± 3,528[4)]	5,618 ± 3,186[4)]	4,353 ± 1,592[4)]
T-Bil (mg/dl)[a]	10.1 ± 6.5[5)]	6.0 ± 7.0[5)]	6.0 ± 3.8[5)]
Serum sampling (days from onset)[a]	11.3 ± 9.7[6)]	10.2 ± 10.5[6)]	15.5 ± 17.1[6)]
CLD	2[7)]	1[7)]	4[7)]
Death	5[8)]	1[8)]	0[8)]

PT, prothrombin time; ALT, alanine aminotransferase; T-Bil, total bilirubin; CLD, presence of chronic liver disease.
[1), 2), 4), 5), 6), 7)] Statistically not significant. [3)] Statistically significant between FH and AHs ($P < 0.001$), FH and AH ($P < 0.001$) and AHs and AH ($P < 0.001$) by Student's t-test. [8)] Statistically significant between FH and AH ($P < 0.001$) by Fisher's exact probability test.
[a]Mean ± SD.

recovered. The difference in mortality between fulminant hepatitis and acute hepatitis was statistically significant ($P < 0.001$ by Fisher's exact probability test).

Two patients with acute hepatitis were positive for HBsAg and anti-HBe antibody, and one patient with acute hepatitis was positive for antinuclear antibody but showed a typical hepatitis A clinical course. One patient with acute hepatitis was diagnosed as autoimmune hepatitis triggered by HAV infection. HCV RNA was negative in all patients with fulminant hepatitis and severe acute hepatitis. One patient with fulminant hepatitis was positive for HCV antibody on admission, and this patient had a previously documented HCV infection, although data for previous liver function tests were not available. One with fulminant hepatitis and one with severe acute hepatitis had alcoholic liver disease. IgM-EBV, IgM-HSV, IgM-CMV, anti-nuclear antibody, anti-smooth muscle antibody, liver kidney microsomal antibody-1, and anti-mitochondrial antibody were negative in all examined fulminant hepatitis and severe acute hepatitis patients.

Detection and Quantitation of HAV RNA at Admission

At admission, the detection rates of HAV RNA from fulminant hepatitis, severe acute hepatitis, and acute hepatitis were 10/11 (91%), 10/10 (100%), and 55/70 (79%), respectively. The differences between the respective groups were not significant.

Serum sampling at admission was 10.0 ± 8.6 days after onset in HAV RNA positive cases and 35.2 ± 23.7 days in HAV RNA negative ones, with the difference in timing being significant ($P < 0.001$) (Fig. 1).

The mean values of HAV RNA at admission were 2.92 ± 1.62 logcopies/ml, with 3.48 ± 1.30 logcopies/ml in fulminant hepatitis, 4.19 ± 1.03 in severe acute hepatitis, 3.82 ± 1.20 in fulminant hepatitis and severe

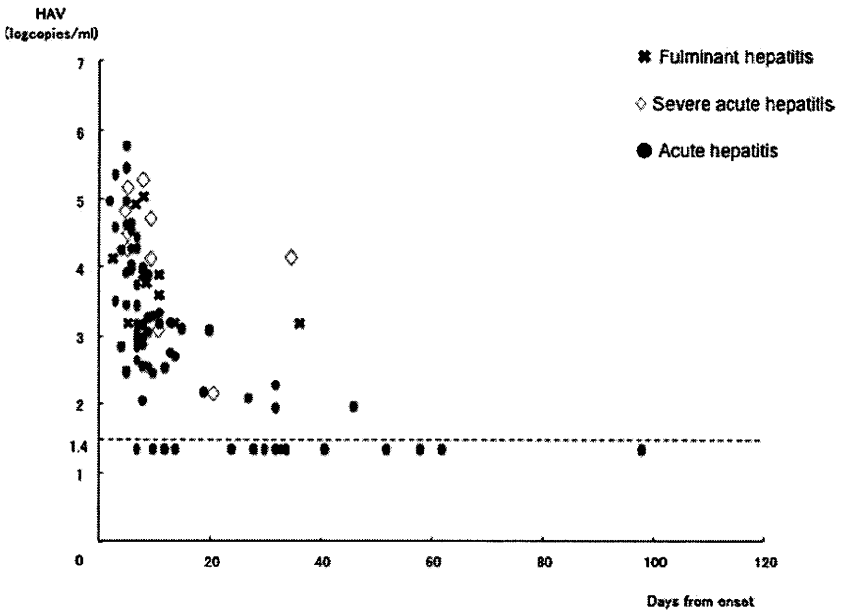

Fig. 1. Association between the duration from onset and initial HAV load in various severities of patients with hepatitis A.

TABLE II. Patients Analyzed for Serial Serum Hepatitis A virus

Patient	Age/sex	Diagnosis	Onset	The last day of HAV (+)	The first day of HAV (−)	Initial viral load (logcopies/ml)
1	38/M	Acute hepatitis	1990	27	36	2.1
2	37/M	Acute hepatitis	1992	21	—	4.0
3	33/M	Severe acute hepatitis	1993	11	25	4.8
4	35/M	Fulminant hepatitis	1994	13	33	3.3
5	46/M	Severe acute hepatitis	1994	21	23	2.1
6	29/F	Acute hepatitis	1995	20	34	2.5
7	41/M	Acute hepatitis	1995	15	—	4.0
8	42/M	Acute hepatitis	1995	—	62	—
9	23/M	Acute hepatitis	1997	13	19	3.2
10	52/F	Fulminant hepatitis	1999	19	20	4.7
11	43/F	Acute hepatitis	2000	15	20	5.8
12	60/M	Acute hepatitis	2000	16	23	3.0
13	54/F	Fulminant hepatitis	2001	7	27	3.7
14	23/F	Severe acute hepatitis	2001	12	—	4.6
15	42/M	Acute hepatitis	2001	14	19	4.3
16	51/M	Acute hepatitis	2001	15	21	2.9
17	38/M	Acute hepatitis	2001	27	34	3.1
18	36/M	Acute hepatitis/autoimmune hepatitis	2001	46	51	2.0
19	60/M	Acute hepatitis	2001	32	—	5.5
20	61/M	Severe acute hepatitis	2002	15	—	3.3

HAV, hepatitis A virus.

acute hepatitis, and 2.65 ± 1.64 in acute hepatitis. The differences were significant between severe acute hepatitis and acute hepatitis ($P < 0.001$), and fulminant hepatitis + severe acute hepatitis and acute hepatitis ($P < 0.001$) (Fig. 1). In fulminant hepatitis and severe acute hepatitis, the HAV RNA levels were 3.21 ± 1.65 in 6 who died and 4.06 ± 0.93 in 15 who recovered ($P = 0.28$).

Time Course of Viral Loads

The characteristics and time courses of HAV load and ALT levels of patients examined by serial serum samples are shown in Table II and Figures 2 and 3. Viral loads ranged between 2.0 and 5.8 logcopies/ml. The dynamics of viremia were correlated with those of ALT.

In a 52-year-old female patient with fulminant hepatitis (patient 10), viral loads fluctuated and then declined gradually in concordance with the ALT level (Fig. 2A). This might be related to the fact that she was treated with steroid pulse therapy, although the viremia duration was 19 days after onset and it was not prolonged.

A 36-year-old Japanese male (patient 18) presenting with jaundice was admitted to a local hospital in China (Fig. 2B). IgM anti-HAV antibody was positive and he was diagnosed as hepatitis A. After a month of hospitalization, liver function tests improved and he came back to Japan. Two weeks after his return, he was hospitalized with general malaise and itching. Laboratory tests revealed re-elevation of ALT and T-Bil levels (peak T-Bil 8.2 mg/dl), IgG 2,190 mg/dl, ANA < 40×, and ASMA < 40×. Liver histology showed centrilobular necrosis and marked plasma cell accumulation, leading to a diagnosis of his repeated and prolonged liver injury of acute onset of autoimmune hepatitis triggered by HAV infection [Fujiwara et al., 2008]. Corticosteroid was introduced and liver function tests normalized within 2 weeks. HAV was detected only on admission, but not at severe exacerbation upon histological examination, suggesting that the severe liver injury was not induced by HAV.

Duration of Viremia

HAV did not disappear during the observation period in 5 (25%; 2 severe acute and 3 acute hepatitis) of 20 patients examined by serial serum samples, with the last time point of HAV detection being 12, 15, 15, 21, and 32 days after onset, respectively. In one of the other 15 patients (patient 8), HAV was not detected at all during follow-up, and HAV disappeared in 14 (70%). Viremia persisted for 18.9 ± 9.6 (7–46) days after clinical onset, similar to the duration of the previous qualitative detection by RT-nested PCR [Fujiwara et al., 1997].

The time courses of the HAV loads of all patients are shown in Figure 3. In the 14 patients in whom HAV disappeared, viremia persisted for 14.2 ± 5.8 days in fulminant hepatitis and severe acute hepatitis, and 21.4 ± 10.6 days in acute hepatitis ($P = 0.19$ by Student's t-test), with the first day of negative HAV being 25.6 ± 4.9 days from onset in fulminant hepatitis and severe acute hepatitis, and 28.6 ± 11.0 in acute hepatitis ($P = 0.50$ by Welch's t-test) (Table II). Therefore, the duration of viremia was not associated with disease severity statistically, although it was relatively longer in cases with non-severe infection than in those with fulminant and severe infections.

DISCUSSION

The reason why the severity of hepatitis A varies among patients is not clear. It has been suggested that

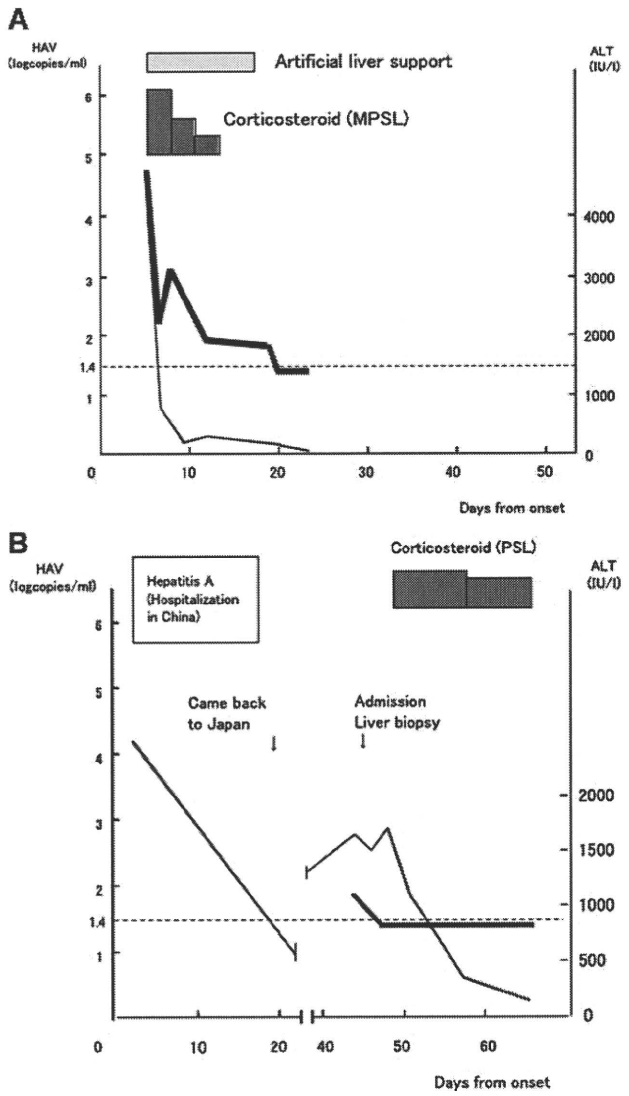

Fig. 2. **A**: Time course of HAV load and ALT of a patient 10 with fulminant hepatitis. Thick solid and thin solid lines denote HAV load and ALT, respectively. **B**: Time course of HAV load and ALT of a patient 18 with autoimmune hepatitis triggered by hepatitis A. Thick solid and thin solid lines denote HAV load and ALT, respectively.

disease severity depends on host factors including age and underlying chronic liver disease [Muraoka, 1990; Vento et al., 1998]. On the other hand, it was reported that young healthy persons were also at risk for severe complications, based on the outbreak of an urban epidemic of hepatitis A in the United States [Willner et al., 1998], in which a cluster of fulminant hepatitis A was reported [Durst et al., 2001]. Analysis of factors contributing to disease severity revealed no significant differences in patients' factors including age [Fujiwara et al., 1995, 2000], suggesting that viral factors and the individual immune responses might determine the severity.

Some articles have reported that the pathogenicity of HAV could be related to cooperative mutations within 5′NTR and P2 protein including non-structural protein

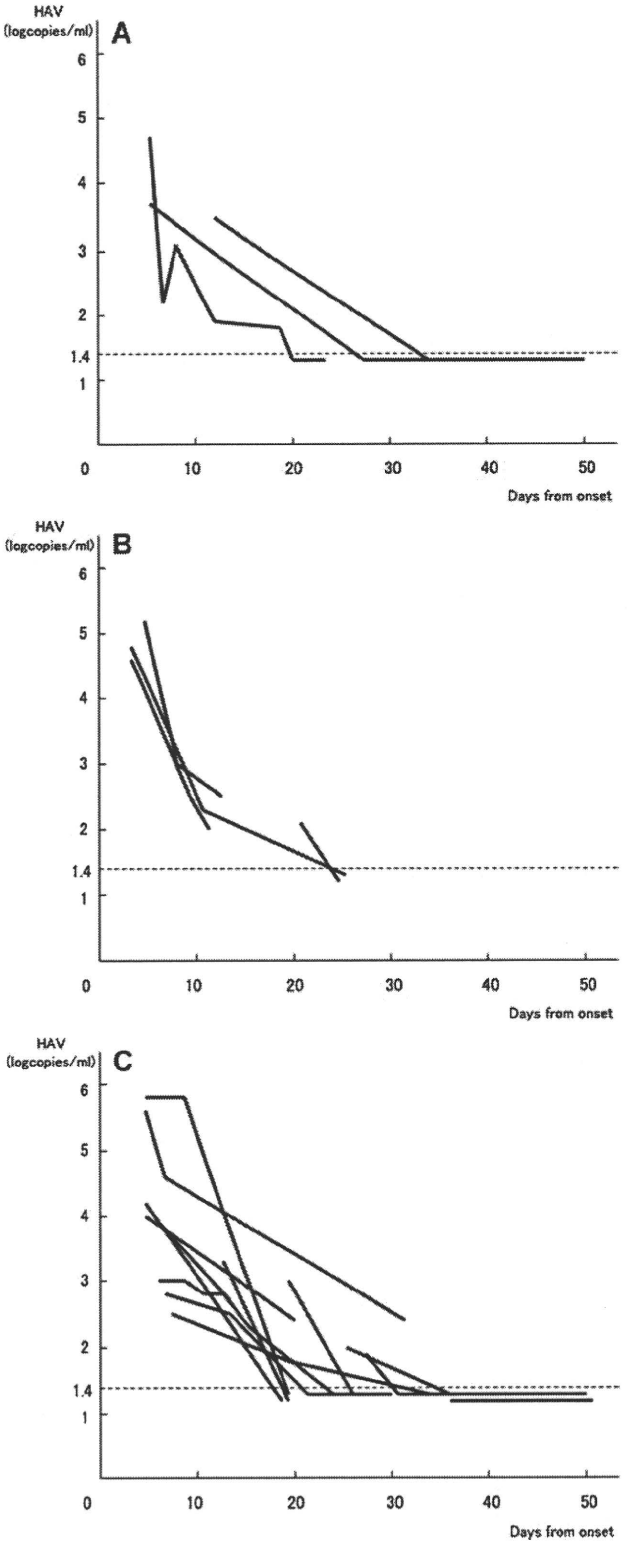

Fig. 3. **A**: Time courses of HAV loads of three patients with fulminant hepatitis. **B**: Time courses of HAV loads of four patients with severe acute hepatitis. **C**: Time courses of HAV loads of 13 patients with acute hepatitis.

2B and 2C, based on the study of cultured cells [Zhang et al., 1995] and simians [Raychaudhuri et al., 1998]. However, association between the clinical severity of hepatitis A and HAV genome had not yet been examined. Therefore, to identify differences in HAV for different severities of hepatitis, the HAV genome in the sera from hepatitis A patients with various clinicopathological features was analyzed. Past studies also suggested an association between infection severity and genomic variations in certain parts of the HAV genome including 5′NTR [Fujiwara et al., 2002, 2009], and non-structural protein 2B and 2C [Fujiwara et al., 2007a,b, 2009].

The detection of HAV in clinical samples started after 1990 using the RT-PCR method. Yotsuyanagi et al. [1993] reported that serum HAV RNA was detected only before ALT reached peak levels, as determined by RT-PCR. As ALT levels have already passed their peak in most patients prior to admission, a more sensitive detection method was needed. A technique of nested RT-PCR was previously developed with primers located in 5′NTR for detecting HAV RNA in the majority of patients' sera in the early convalescent phase of hepatitis A [Fujiwara et al., 1997]. In the present study, HAV RNA in serum was quantitatively detectable in almost all patients with hepatitis A in their early convalescent phase by real-time RT-PCR.

It is usually impossible to determine the exact duration of viremia in human HAV infection, as samples can obviously not be readily obtained before clinical onset. Under the special condition of using serum specimens obtained from participants in a clinical trial of hepatitis B vaccine in the United States, Bower et al. [2000] reported that HAV viremia was detected 17 days before ALT peak and persisted for 79 days beyond, as determined by nested RT-PCR. They used three pairs of primers for the VP3–VP1 junction region, the VP1–P2A junction region and 5′NTR, a method more sensitive than ours or those of others. They also demonstrated the presence of high concentrations of HAV during the period before the start of ALT elevation, but the concentrations were low during the convalescent phase: HAV concentrations were 10^4–10^5 plaque-forming units (pfu)/ml before ALT peak, 10^2–10^4 pfu/ml at the peak, 10–100 pfu/ml after the peak, and 1–10 pfu/ml 30 days later.

In a Korean study, using nested RT-PCR with primer sets located at the VP1 region, Kwon et al. [2000] reported a mean duration of HAV viremia of 30 days. In an Italian study, using nested RT-PCR with primers for 5′NTR, Sagnelli et al. [2003] reported that clearance of HAV viremia was observed within 20 days of clinical onset in all patients with a typical self-limiting course. A Dutch study reported that HAV viremia was detected for a median period of 42 days after onset, based on nested RT-PCR with primers at the VP3–VP1 and VP1–P2A regions [Tjon et al., 2006].

Regarding the quantitation of HAV by real-time RT-PCR, several reports have been published. In a French study using real-time RT-PCR quantifying 5′NTR of HAV, viremia was observed to persist for 60 days after onset [Costa-Mattioli et al., 2002]. Hepatitis A viral loads on admission ranged from 1.8×10^3 to 7.71×10^6 copies/ml, with a mean value of 6.38×10^5 copies/ml. Normann et al. [2004] described the time course of HAV viremia and serum viral load in 11 German patients by real-time RT-PCR, reporting long durations of viremia, from 45 days to 490 days after the onset of jaundice, although the viral dynamics of the described patients seemed to be quite different from those in other reports, including ours. Their measured viral loads ranged from 2.0×10^3 to 3.1×10^5 genome equivalents/ml. In an Indian study, six of seven patients with non-severe infection had peak viral loads at admission, whereas three patients with severe infection had peak loads at 15 or 30 days after admission, with the authors suggesting that severe HAV infection may be triggered by diminished cellular immunity [Hussain et al., 2006].

In the current study, initial HAV load was higher in severely infected cases (fulminant hepatitis and severe acute hepatitis) than in cases with non-severe infection, although there was no relation to outcome in the severely infected ones. Interestingly, Rezende et al. [2003] reported that HAV-related liver failure results from an excessive host response associated with a marked reduction in viral load, which represents a distinct discrepancy between their data and ours. As they did not report their schedule of serum sampling, which would provide critical data about viremia in acute hepatitis, a reasonable explanation for this discrepancy cannot be offered. In their study, a higher bilirubin level at admission was associated with fulminant hepatitis A, suggesting an advanced stage of illness of their patients. It is supposed that higher viral replication at onset may induce excessive host responses and severe diseases, and that viral load would be reduced rapidly in the advanced stage of illness as a result of the destruction of large numbers of HAV-infected hepatocytes. In contrast, a longer duration of viremia may be associated with a mild host immune response of non-severe infection, even in the absence of a statistically significant difference.

In summary, HAV RNA was detected quantitatively in the majority of the sera of hepatitis A cases in the early convalescent phase by real-time PCR, and examination of HAV viremia by real-time PCR was useful for analyzing the clinical severity of liver injury during the course of hepatitis A. It might be suggested in part that genetic variations in some regions of HAV including 5′NTR, 2B and 2C might influence cooperatively the replication of the virus and the cellular immune response of the human host, and in large part that vigorous clearance of HAV-infected hepatocytes by the immune response might be the cause of severe disease. Of course, this concept is still at a speculative level and awaits more concrete evidence.

ACKNOWLEDGMENTS

We are indebted to all our colleagues at the liver unit of our hospital who cared for the patients described

herein. We thank Dr. Makoto Miki (Yokohama-Higashi National Hospital), Professor Kazuyuki Suzuki (Iwate Medical University), Professor Isao Sakaida, Professor Kiwamu Okita (Yamaguchi University), and Professor Eiji Tanaka (Shinshu University) for clinical samples.

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Journal of Gastroenterology and Hepatology

JGHF

doi:10.1111/j.1440-1746.2010.06322.x

HEPATOLOGY

Long-term follow-up of patients with hepatitis B e antigen negative chronic hepatitis B

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Key words
HBe antibody, hepatitis B virus, long-term follow-up.

Accepted for publication 5 March 2010.

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Abstract

Background and Aim: After hepatitis B virus (HBV) e antigen (HBeAg) seroconversion, HBV-DNA continues to replicate, and HBeAg-negative patients still face the risk of liver disease progression. We investigated the predictive factors for alanine aminotransferase (ALT) elevation, antiviral drug use, and hepatocellular carcinoma (HCC) occurrence in HBeAg-negative patients.

Methods: Age, sex, ALT, platelet counts, HBV-DNA levels, genotype, antidiabetic drug use, body mass index, smoking, and alcohol consumption were analyzed for a total of 244 HBV carriers who were HBeAg-negative.

Results: Of 244 HBeAg-negative patients, 158 (64.8%) showed normal ALT levels at baseline. Multivariate Cox hazard regression analysis identified high HBV-DNA levels and high ALT at baseline as independent risk factors for ALT elevation in the patients with normal ALT at baseline. The threshold ALT and HBV-DNA levels were determined to be 31 IU/L and 5.3 logcopies/mL, respectively. Seventeen (7.0%) patients used antiviral drugs. Multivariate Cox hazard regression analysis identified high HBV-DNA levels (threshold, 5.7 log copies/mL), the use of antidiabetic drugs, and daily alcohol consumption at baseline as an independent risk factor for the use of antiviral drugs in HBeAg-negative patients. In 10 patients (4.1%), HCC was detected, and a low platelet count (threshold, $10.0 \times 10^4/mm^3$) was associated with the occurrence of HCC.

Conclusion: This study identified predictors of future active liver disease in HBeAg-negative patients, i.e. ALT elevation, unavoidable use of antiviral drugs, and occurrence of HCC.

Introduction

Chronic hepatitis caused by hepatitis B virus (HBV) often follows a fluctuating course characterized by periods of active hepatitis interspersed with quiescence. Therefore, close follow-up is necessary to understand the natural history of HBV patients. On the other hand, patients in which HBV is truly inactive have persistently quiescent disease with an excellent prognosis. Determining an accurate prognosis for HBV carriers based on clinical presentation is important for clinical management of the disease. Various studies have been performed to distinguish the positive and negative prognostic factors among HBV carriers.[1–3]

Hepatitis e antigen (HBeAg) seroconversion is an important event in the natural history of HBV infection. HBV-infected patients usually have a very good prognosis after HBeAg seroconversion.[4] Therefore, HBeAg seroconversion has become an important treatment goal during follow-up of HBV carriers.[5] However, it has also been shown that HBV-DNA replication and hepatic inflammation in seroconverted patients continue despite the persistent loss of HBeAg; thus, HBeAg-negative patients are likely to develop liver cirrhosis or hepatocelluar carcinoma.[6] In this study, we focused on the natural history of patients with HBeAg-negative chronic hepatitis B, particularly with respect to alanine aminotransferase (ALT) elevation, antiviral drugs, and hepatocellular carcinoma (HCC).

Recently, prognostic factors for HBeAg-negative patients have been investigated in Taiwan and Canada.[7,8] We expect to identify a unique constellation of prognostic factors for HBeAg-negative chronic hepatitis B in the Japanese population, due to differences in race and HBV genotype.

Methods

Patients

Between January 1985 and April 2007, all patients visiting the Chiba University Hospital with HBV infection were approached for participation in the study. This study was carried out only at

one institute, Chiba University Hospital and was approved by ethical the committee of Chiba University. Written informed consent was obtained from all of the patients in accordance with the Declaration of Helsinki. New patients since 1985 and those who were already being followed-up in 1985 were eligible for inclusion in the study. A total of 881 patients were enrolled; of which, 862 were HBsAg positive at enrollment, and 319 were hepatitis B e antibody (HBeAb) positive. Patients who were positive for hepatitis C virus antibody or hepatitis D virus antibody or who had other potential cause of chronic liver diseases (autoimmune hepatitis, primary biliary cirrhosis) were excluded. Patients followed for less than 12 months were also excluded from the analysis. In total, 244 patients were included in the analysis. Serum samples from patients were stored at −20°C and the oldest sample for each patient was used for defining the level of HBV-DNA. The date of evaluation of HBV DNA level by PCR was defined as the baseline. Patient consent was obtained for storage and analysis of serum samples.

Laboratory methods

Serum ALT level was measured using a routine automated method. HBeAg and HBeAb were measured by standard enzyme-linked immunosorbent assays. Patients were screened for hepatitis C virus, hepatitis delta virus, and human immunodeficiency virus antibodies by a third-generation enzyme-linked immunosorbent assay.

HBV-DNA quantitative assay and genotyping

To investigate the level of HBV-DNA in serum, we chose polymerase chain reaction (PCR) assay with an accurate range of 500–200 000 copies/mL (Amplicor HBV monitor test, Roche Diagnostic Systems, Basel, Switzerland). The six major genotypes of HBV (A–F) were determined by enzyme-linked immunosorbent assay (ELISA) (HBV Genotype EIA, Institute of Immunology, Co., Ltd, Tokyo, Japan).

Statistical analysis

ALT elevation was defined as a change from normal ALT (< 42 IU/L) to elevated ALT (≥ 42 IU/L), and normalization was defined as a change from elevated ALT to normal from one visit to the next. Baseline data are presented as mean ± standard deviation (SD). Differences in clinical parameters between groups were analyzed by unpaired *t*-test, Welch *t*-test, and χ^2 tests. The Cox proportional hazards model was used to identify predictive factors for future ALT elevation/normalization, use of antviral drugs, and HCC occurrence using SPSS version 16.1 software (SPSS Inc., Chicago, IL, USA).

Results

Patient characteristics

To investigate the natural course of HBV carriers with HBeAb, 244 carriers (HBeAg-negative and HBeAb-positive) were enrolled in the study. Follow-up was terminated when the use of antiviral drugs was started or the occurrence of HCC. The baseline clinical and virological characteristics of the 244 HBeAg-negative carriers are shown in Table 1. Because liver biopsy was performed only in 44 (18.0%) out of 244 patients, liver biopsy results could not be analyzed further. Age, sex, ALT, platelet count (PLT), HBV-DNA level, genotype, antidiabetic drug use, body mass index, smoking, and alcohol consumption were analyzed. The average (± SD) period of follow-up was 103.6 ± 74.8 months. Seventeen (7.0%) patients used antiviral drugs (lamivudine in eight and entecavir in nine) and HCC was detected in 10 (4.1%) patients. Two (0.82%) patients died of HCC. In addition, one died of intrahepatic cholangiocarcinoma, one of liver failure due to gastrointestinal bleeding, and one of tongue cancer during the follow-up period. In Japan, the majority of HBV cases are genotype C and B and these genotypes do not cause HBV carrier by way of horizontal infection in adults; therefore, the HBV infection in our HBV carriers mainly occurred by vertical infection or infection during childhood.[9] Thus, the period of HBV infection roughly coincided with the age of HBV carriers in Japan.

ALT and HBV-DNA levels

One hundred and fifty-eight of 244 (64.8%) HBeAg-negative patients had normal ALT levels at baseline. Of these 158 subjects, 85 (53.8%) continued to have normal ALT levels during follow-up, whereas 73 (46.2%) showed fluctuation of ALT levels with intermittently elevated ALT (Fig. 1). A total of 34 (21.5%) patients had ALT ≥ 84 IU/L (more than double the normal limit). Of the 86 patients who had elevated ALT levels at baseline, ALT elevation persisted in 10 (11.6%) and 76 (88.4%) showed ALT fluctuations with intermittently elevated ALT. Although HBV-DNA levels were associated with higher ALT levels in general, correlation was weak ($r^2 = 0.13$).

Platelet count

Patients were sub classified based on PLT as follows: (I) < 100 000 (II) 100 000–149 000 (III) 150 000–199 000 (IV) 200 000–249 000 (V) > 250 000 and more (/mm^3). The numbers of patients in groups I, II, III, IV, and V were 17, 28, 73, 68, and 58, respectively. A total of 84 (34.4%) patients reached a lower platelet count at the end of follow-up.

Risk factors for future ALT elevation in patients with normal ALT levels

Although 158 (64.8%) out of 244 HBeAb-positive patients had normal ALT levels at baseline, 73 patients showed fluctuation of ALT levels with intermittently elevated ALT. We investigated the risk factors for future ALT elevation in these patients. The predictive factors of ALT elevation (ALT > 42 IU/L) in patients with normal ALT levels were HBV-DNA and ALT levels at baseline (Table 2). We carried out an additional univariate analysis changing the threshold of HBV DNA from 3.5 to 7.0 log copies/mL in 0.1 log increments and that of ALT from 15 to 41 IU/L in 1.0 increments. We determined the threshold when the value of probability was smallest; the thresholds for ALT and HBV-DNA levels were 31 IU/L and 5.3 logcopies/mL, respectively. The time

Table 1 Baseline characteristics of hepatitis B virus (HBV) e antigen (HBeAg)-negative patients

	Total	Normal ALT	Elevated ALT	*P*
Number	244	158	86	
Age(years) : (mean ± SD)	44.1 ± 12.5	44.1 ± 13.1	44.0 ± 11.4	NS*
<30	35 (14.3%)	24 (15.2%)	11 (12.8%)	
30–39	52 (21.3%)	32 (20.3%)	20 (23.2%)	
40–49	66 (27.0%)	44 (27.8%)	22 (25.6%)	
50–	91 (37.3%)	58 (36.7%)	33 (38.4%)	
Sex				<0.001**
Male	141 (57.8%)	76 (48.1%)	66 (75.9%)	
Female	103 (42.2%)	82 (51.9%)	21 (24.1%)	
Alanine aminotransferase (ALT) (IU/L) (mean ± SD)	58.9 ± 108.1	20.9 ± 8.7	127.9 ± 160	<0.001*
<20	84			
21–30	47			
31–40	27			
42–84	47			
85–	39			
Platelet count ($\times 10^4/mm^3$) (mean ± SD)	205.5 ± 69.6	211.4 ± 60	193.3 ± 81.8	NS*
HBV-DNA (log copies/mL) (mean ± SD)	4.3 ± 1.5	3.8 ± 1.1	5.1 ± 1.7	<0.001*
<4.0	116 (47.5%)	91 (57.6%)	25 (29.1%)	
4.0–4.9	54 (22.1%)	38 (24.1%)	16 (18.6%)	
5.0–5.9	27 (11.1%)	18 (11.4%)	9 (10.5%)	
6.0–6.9	26 (10.7%)	5 (3.2%)	21 (24.4%)	
7.0–	16 (6.6%)	3 (1.9%)	13 (15.1%)	
Genotype				NS**
A	3 (1.2%)	2 (1.3%)	1 (1.2%)	
B	30 (12.3%)	16 (10.1%)	14 (16.3%)	
C	87 (35.7%)	49 (31%)	38 (44.2%)	
Not detected	124 (50.8%)	91 (57.6%)	33 (38.4%)	
Liver Histology ($n = 44$)				
Fibrosis 4/3/2/1	7/8/9/20	0/1/4/13	7/7/5/7	NS**
Activity 3/2/1	7/16/21	1/4/13	6/12/8	NS**
Use of anti-Diabetes drug	20 (8.2%)	3 (1.9%)	6 (7.0%)	NS**
Body mass index (kg/m^2) (mean ± SD)	23.3 ± 3.3	23.1 ± 3.2	24.0 ± 3.5	NS**
Smoker/ ever smoker/ non-smoker	32/15/89	16/5/56	16/10/33	NS**
Daily alcohol consumption	46 (27.1%)	24 (23.1%)	22 (33.3%)	NS**
Follow-up (months) (mean ± SD)	103.6 ± 74.8	109.5 ± 76.1	101.8 ± 74.6	NS*

*Unpaired *t*-test and **χ^2 test. NS, not significant difference.

interval from a visit with a normal ALT to a visit with an elevated ALT was used for Kaplan–Meier and Cox regression analysis. Kaplan–Meier curves were constructed for ALT and HBV-DNA levels (Fig. 2).

Risk factors for future use of antiviral drugs for HBV in HBeAg-negative patients

Seventeen (7.0%) patients used an antiviral drug (lamivudine in 8 and entecavir in 9). We investigated the risk factors for future use of antiviral drugs for HBV. The time interval from baseline to the use of an antiviral drug for HBV was used for Cox regression analysis. HBV-DNA levels, use of antidiabetic drugs, and daily alcohol consumption were predictive of future antiviral drug use for HBV, according to the results of multivariate Cox hazard regression analysis. Hazard ratios for HBV-DNA levels, antidiabetic drug use, and daily alcohol consumption were 1.519 (1.130–2.042, 95% confidence interval [CI]), 3.769 (1.203–11.81), and 3.011 (1.086–8.348), respectively. We repeated the univariate analysis, changing the threshold for HBV DNA from 3.5 to 7.0 log copies/mL in 0.1 log increments. We determined the threshold when the probability value was lowest; the HBV-DNA threshold level was 5.7 log copies/mL. Kaplan–Meier curves were constructed for HBV-DNA levels, antidiabetic drug use, and daily alcohol consumption (Fig. 3).

Risk factors for hepatocellular carcinoma in HBeAg-negative patients

In 10 patients (4.1%), HCC was detected. We investigated the risk factors for HCC in HBeAg-negative patients. The time interval from baseline to occurrence of HCC was used for Cox regression analysis. According to the results of multivariate Cox regression analysis, PLT was predictive of the development of HCC. The hazard ratio for PLT was 0.807 (0.724–0.899, 95% CI). We performed univariate analyses, changing the PLT threshold from 8.0 to $30.0 \times 10^4/mm^3$ in $1.0 \times 10^4/mm^3$ increments. We determined the threshold when the value of probability was smallest; the

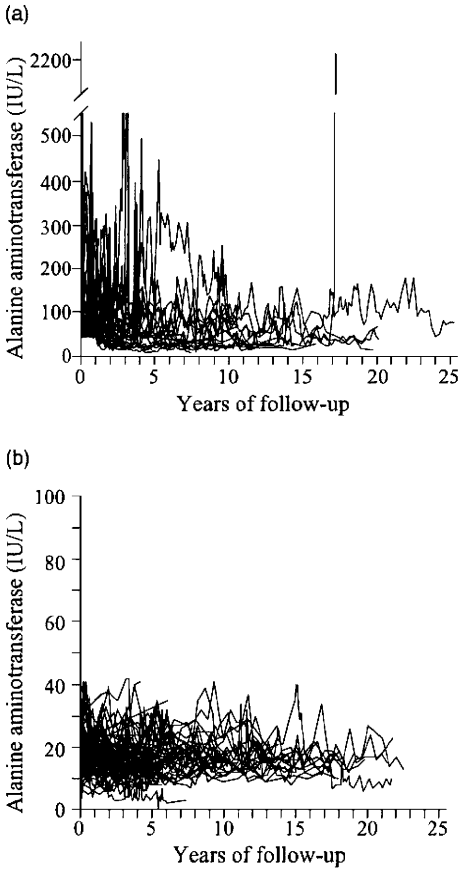

Figure 1 Level of alanine aminotransferase (ALT) in (a) patients with normal ALT at baseline and intermittently elevated ALT during follow-up ($n = 73$) and (b) patients with normal ALT at baseline and during follow-up ($n = 85$).

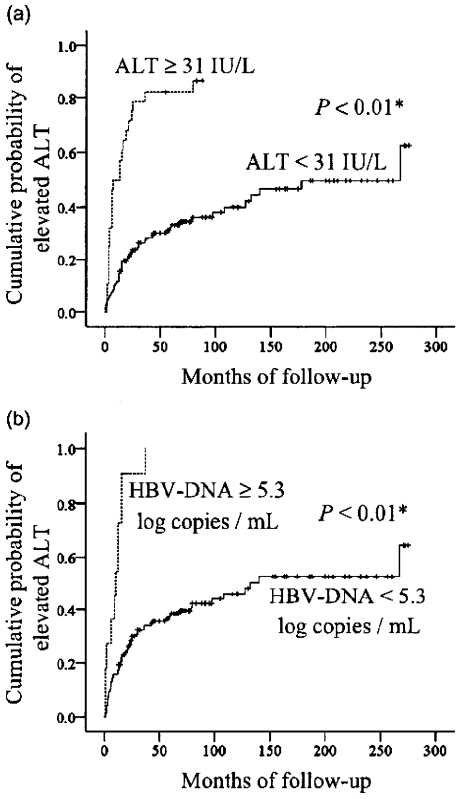

Figure 2 Cumulative occurrence of abnormal alanine aminotransferase (ALT) in HBeAg-negative patients with normal ALT based on (a) ALT and (b) HBV-DNA levels. We determined the threshold for ALT and HBV-DNA levels when the probability value was lowest in the univariate analysis. Kaplan–Meier curves show the time to ALT elevation. Solid lines indicated the control group. *A significant difference was determined by log-rank test.

Table 2 Univariate and multivariate analysis of factors associated with alanine aminotransferase (ALT) elevation in hepatitis B virus (HBV) e antigen (HBeAg)-negative patients with normal ALT levels

	Univariate analysis				Multivariate analysis			
	Standard error	Wald statistic	*P*-value	Hazard ratio (95% confidence interval)	Standard error	Wald statistic	*P*-value	Hazard ratio (95% confidence interval)
Sex (Male)	0.263	0.203	0.652	1.126 (0.673–1.885)				
Age (years)	0.011	5.704	0.017	1.027 (1.005–1.049)	0.252	0.068	0.794	1.015 (0.572–1.534)
HBV-DNA	0.109	17.773	<0.001	1.587 (1.280–1.966)	0.111	10.602	0.001	1.437 (1.155–1.788)
Genotype								
B	0.459	0.22	0.639	0.806 (0.328–1.982)				
C	0.435	0.055	0.815	1.107 (0.472–2.600)				
Alanine aminotransferase	0.014	42.440	<0.001	1.097 (1.067–1.128)	0.015	29.496	<0.001	1.086 (1.054–1.119)
Platelet count	0.019	5.928	0.015	0.955 (0.920–0.991)	0.021	0.754	0.385	0.982 (0.942–1.023)
Use of anti-diabetes drug	0.427	0.470	0.493	1.340 (0.581–3.091)				
Body mass index (kg/m^2)	0.042	0.033	0.855	0.992 (0.913–1.078)				
Smoker and ever smoker	0.374	0.111	0.739	1.133 (0.544–2.359)				
Daily alcohol consumption	0.333	0.512	0.474	1.269 (0.661–2.435)				

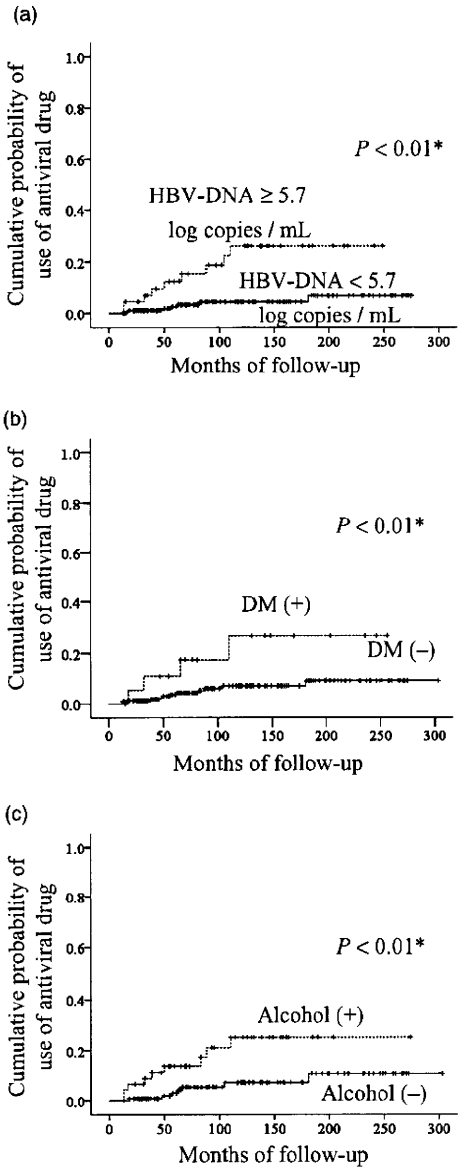

Figure 3 Cumulative occurrence of antiviral drug use for hepatitis B virus (HBV) in HBeAg-negative patients based on (a) HBV-DNA levels (b) use of antidiabetic drug, and (c) daily alcohol consumption. We determined the threshold for HBV-DNA levels when the probability value was lowest in the univariate analysis. Kaplan–Meier curves show the time to use of antiviral drugs for HBV. Solid lines indicated the control group. *A significant difference was determined by log-rank test.

PLT threshold was $10.0 \times 10^4/mm^3$. Kaplan–Meier curves were constructed for PLT (Fig. 4).

Stratification analyses of risk factors for clinical outcomes in HBeAg-negative patients by age, sex, and HBV genotype

The stratification analyses by age, sex, and HBV-genotype were performed to evaluate the risk factors for future ALT elevation in patients with normal ALT levels, future use of antiviral drugs for HBV, and HCC in HBeAg-negative patients (Table 3). The age threshold was 45 years, which was the average age of all the patients. We did not perform stratification analysis for patients infected with HBV genotype B because the number of such cases was very small.

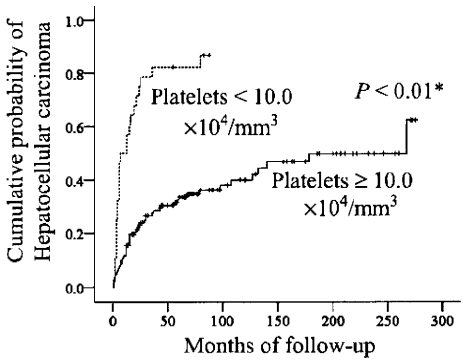

Figure 4 Cumulative occurrence of hepatocellular carcinoma (HCC) based on the platelet counts. We determined the threshold for HBV-DNA levels when the probability value was lowest in the univariate analysis. Kaplan–Meier curves show the time to HCC. Solid lines indicated the control group. *A significant difference was observed by log-rank test.

Discussion

Most patients who have undergone HBeAg seroconversion have normal serum ALT levels, which is indicative of a good clinical outcome.[10] Therefore, various therapies for early seroconversion have been used.[5] Recently, HBeAg-negative viral mutants have been shown to be responsible for continuous HBV-DNA replication.[7] That is, there exists the possibility that liver disease will get worse after HBeAg seroconversion. In fact, previous reports revealed that HBeAg status is not a predictive factor for HCC,[11,12] and fulminant hepatitis can occur by the infection of HBV with HBeAg-negative.[13] HBeAg-negative patients should be monitored closely, even though most of these patients show normal ALT levels and no progressive liver disease.[14] Therefore, predictive factors for active liver disease in HBeAg-negative patients need to be identified in order to facilitate optimal disease management. This study provides data regarding the prediction of future active liver disease, i.e. ALT elevation, unavoidable use of antiviral drugs, and occurrence of HCC.

Many previous reports have attempted to define a threshold HBV-DNA level that corresponds to the presence of active liver disease.[15] A National Institute of Health workshop demonstrated that an HBV-DNA level of 10^5 copies/mL could be used to distinguish active HBV infection from inactive HBV infection.[16] Other studies also suggested that the threshold HBV-DNA level lies somewhere between 10^4 and 10^6 copies/mL.[8] In this study, in order to clarify the natural course of HBeAg-negative patients with normal ALT levels, we used a HBV-DNA threshold of $10^{5.3}$ copies/mL. By log rank analysis, the ALT levels in patients with $>10^{5.3}$ copies/mL HBV-DNA level were significantly higher than in patients with HBV-DNA below this level. In HCV patients, ALT is

Table 3 Stratification analysis multivariate analysis of factors associated with alanine aminotransferase (ALT) elevation in hepatitis B virus (HBV) e antigen (HBeAg)-negative patients with normal ALT levels, future use of antiviral drugs for HBV, and occurrence of hepatocellular carcinoma

		Age (years)						Sex				Genotype			
		≥45 years		<45 years				Male		Female		C			
		$n = 126$		$n = 118$				$n = 141$		$n = 103$		$n = 87$			
	Factors	Hazard ratio (95% CI)	*P*-value	Factors	Hazard ratio (95% CI)	*P*-value	Factors	Hazard ratio (95% CI)	*P*-value	Factors	Hazard ratio (95% CI)	*P*-value	Factors	Hazard ratio (95% CI)	*P*-value
Future ALT elevation in the patients with normal ALT level	HBV-DNA	1.535 (1.146–2.057)	0.004	ALT	1.106 (1.059–1.156)	<0.001	ALT	1.194 (1.075–1.325)	<0.001	ALT	1.060 (1.015–1.108)	0.008	ALT	1.149 (1.075–1.228)	<0.001
	ALT	1.077 (1.035–1.122)	<0.001							HBV-DNA	1.739 (1.213–2.492)	0.003	HBV-DNA	1.902 (1.223–2.956)	0.004
Future use of anti-viral drugs for HBV	Alcohol	4.744 (1.362–16.52)	0.014	HBV-DNA	2.238 (1.107–4.526)	0.025	HBV-DNA	1.486 (1.053–2.098)	0.024	DM†	86.14 (1.842–4027.3)	0.023	Alcohol	5.617 (1.431–22.05)	0.013
										BMI†	0.408 (0.187–0.890)	0.024			
Occurrence of hepatocellular carcinoma	PLT	0.772 (0.659–0.905)	0.001	(–)†			PLT	0.832 (0.731–0.948)	0.006	PLT†	0.775 (0.635–0.947)	0.013	PLT	0.833 (0.732–0.948)	0.006

†Three patients were used in subgroup for HBV antiviral drugs and HCC occurrence.
Alcohol, Daily alcohol consumption; BMI, Body mass index; CI, confidence interval; DM, use of antidiabetic medication; PLT, platelet count.

a poor surrogate marker for inflammation and fibrosis.[17] Therefore, even if the patient's ALT level was within normal limits, they should still be monitored closely, and HCV eradication therapy is recommended under certain circumstances. Similarly, even if the ALT levels are within normal limits in HBV-infected patients who are HBeAg-negative, the higher their ALT levels were, the more frequently their ALT levels would be high in the future, which might cause progressive liver disease.[18]

Some of the patients with progressive liver disease caused by HBV infection were treated with the antiviral drugs lamivudine and entecavir. The use of lamivudine or entecavir might result in mutant HBV resistance to antiviral drugs[19,20] and the associated costs are not trivial. The baseline levels of HBeAg, ALT, and HBV-DNA, and the presence of either chronic hepatitis or cirrhosis have been established as determinants for eligibility for antiviral treatment.[21] According to treatment guidelines in the Unites States (National Guideline Clearinghouse, http://www.guideline.gov), patients with HBeAg-negative chronic hepatitis B should be considered for antiviral treatment based on their HBV-DNA and ALT levels (serum HBV-DNA >20 000 IU/mL and elevated ALT >2 times normal). In this study, only four out of 17 patients treated with an antiviral drug showed normal ALT levels at baseline, and all four patients showed elevated ALT levels in 8–57 months later. Therefore, this study revealed that patients with high HBV-DNA levels tended to have high ALT levels at baseline or in the future; as a result, such patients have a tendency for future treatment with and antiviral drug.

Hepatocellular carcinoma occurrence was noted in only 10 cases (4.1%). The only predictive factor for HCC occurrence was PLT, which meant that patients with advanced liver disease tended to develop HCC later, because the decrease in PLT corresponded to the extent of liver fibrosis. Four patients (1.6%) died of liver-related diseases and one (0.4%) died of cancer in another organ. The number of deaths was too small to determine the predictive factors for death of HBeAg-negative HBV carriers. Further analysis is needed to properly address this factor.

Stratification analyses of risk factors for clinical outcomes by age, sex, and HBV-genotype were performed. Because the numbers of female patients with future use of antiviral drugs for HBV ($n = 3$), HCC occurrence ($n = 3$), or who were under 45 years old with HCC occurrence ($n = 3$) were very small, it was not possible to properly evaluate these subgroups. The risk factors among subgroups for future ALT elevation in patients with normal ALT levels, and for HCC were almost equal to those of the entire patient population. However, daily alcohol consumption, not HBV-DNA level, was predictive of future use of antiviral drugs for HBV in patients ≧45 years old or in patients infected with HBV genotype C. In these subgroups, alcohol consumption was an important factor for predicting the clinical course of HBV carriers; i.e. advising patients to abstain from drinking might reduce the need for antiviral drugs in the future.

Coffee or caffeine consumption is reported to be strongly related to ALT levels and HCC occurrence.[22–24] In our study, we did not survey caffeine consumption; therefore, further analysis is needed to determine the importance of coffee or caffeine consumption as a predictive factor of the clinical course in HBeAg-negative HBV carriers.

In conclusion, we established that low HBV-DNA levels and ALT levels at baseline were good predictors for future ALT eleva-

tion in HBeAg-negative HBV carriers with normal ALT levels. In addition, this study provides data on the prediction of unavoidable antiviral drug use and HCC occurrence.

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© Med Sci Monit, 2010; 16(4): PI7-12
PMID: 20357731

Received: 2009.06.03
Accepted: 2009.12.11
Published: 2010.04.01

Aminofeel® improves the sensitivity to taste in patients with HCV-infected liver disease

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Source of support: This study was supported in part by Health and Labour Sciences Research Grants for Research on Hepatitis from the Ministry of Health, Labour and Welfare of Japan

Authors' Contribution:
A Study Design
B Data Collection
C Statistical Analysis
D Data Interpretation
E Manuscript Preparation
F Literature Search
G Funds Collection

Summary

Background: Patients with chronic liver diseases have a taste disorder and altered zinc metabolism. We investigated the effects of a supplement enriched with branched-chain amino acids (BCAA) (Aminofeel®) on sensitivity to different tastes in patients with hepatitis C virus (HCV) infected liver disease.

Material/Methods: Nine patients (mean age 63.3±9.1 years) with HCV-related liver diseases were identified and examined for sensitivity to different tastes. Eight patients had no awareness of taste disorders, and 3 patients had oral lichen planus. We examined 4 tastes (sweet, salty, sour, and bitter) using a Taste Disk® and sensitivity to different tastes was rated on a 6-point scale (I, II, III, IV, V, and VI). Each patient was given one sachet of Aminofeel® after breakfast and another at bedtime for 90 days.

Results: Only one patient was aware of a taste disorder before administration of Aminofeel®, but 4 patients had decreased gustatory sensitivity in the sour taste test, and 2 had it in the bitter taste test. Sensitivity to sour tastes significantly increased after the administration of Aminofeel® (P=0.03). Sensitivity to sweet tastes increased after the administration of Aminofeel® (P=0.06). Zinc value significantly increased after the administration of Aminofeel® (P=0.02).

Conclusions: Patients with HCV-infected liver disease have decreased sensitivity to different tastes and decreased zinc levels. Some patients were unaware that they had a taste disorder. Aminofeel® improved sensitivity to different tastes and increased zinc values. Thus, Aminofeel® is a useful therapeutic agent for taste disorders.

key words: **taste disorder • zinc • hepatitis C virus (HCV) • lichen planus • branched-chain amino acids (BCAA) • Aminofeel®**

Abbreviations: **BCAA** – branched-chain amino acids; **Anti-HCV** – anti-bodies to HCV; **CLEIA** – chemiluminescent enzyme immunoassay; **HBsAg** – hepatitis B surface antigen; **HCC** – hepatocellular carcinoma; **HCV** – hepatitis C virus; **IFN** – interferon

Full-text PDF: http://www.medscimonit.com/fulltxt.php?ICID=878481
Word count: 1517
Tables: 4
Figures: 5
References: 21

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BACKGROUND

In Japan, the number of patients seeking treatment from otolaryngologists for taste disorders is approximately 240,000/year; the number has almost doubled in the last 13 years [1]. The main treatment for taste disorders is zinc administration. And many studies have found zinc deficiency in patients with liver diseases [2–4]. Therefore, patients with chronic liver disease may have taste disorders and altered zinc metabolism [5,6]. Several factors, such as poor dietary intake, impaired intestinal absorption, or excessive urinary losses may be responsible for the reduced whole-body zinc content [7].

Decreases in levels of serum branched-chain amino acids (BCAA) are often seen in patients with chronic liver diseases and these decreases lead to a decline in production of detoxified ammonia and albumin. Therefore, BCAAs are used for the treatment of hepatic encephalopathy and hypoalbuminemia [8,9]. The Department of Digestive Disease Information & Research, Kurume University School of Medicine and Seikatsu Bunkasya Co. Inc. (Tokyo, Japan) developed the BCAA-enriched supplement (Aminofeel®) and facilitated the commercialization of the product. On March 1, 2007, Seikatsu Bunkasya Co. Inc. released Aminofeel®. A dose of Aminofeel® contains 5.0 mg zinc as well as 3200.0 mg BCAA [10]. We previously reported that Aminofeel® is a useful therapeutic agent for decreasing insulin resistance in male patients with chronic viral diseases of the liver [10,11]. The administration of Aminofeel® in men for 90 days increases serum albumin levels significantly and also increases serum zinc levels [10].

There are few reports that have used objective outcomes to show that the sense of taste is disordered in patients with liver disease. Accordingly, in this study, we conducted objective, gustatory tests and objectively studied sensitivity to different tastes before and after administration of Aminofeel® in patients with hepatitis C virus (HCV)-infected liver disease.

MATERIAL AND METHODS

Subjects

A prospective, consecutive-patient entry study was conducted. Eligibility criteria were chronic viral liver disease, sufficient food intake, and serum albumin concentrations >3.5 g/dl and <4.0 g/dl. Patients with hepatic encephalopathy, ascites, hepatocellular carcinoma (HCC) or renal failure were excluded.

This study included 9 Japanese patients (3 males and 6 females) with HCV-infected liver disease. Eight patients, who had visited our clinic at the Kurume University Hospital in Japan between September 2006 and December 2006, had no awareness of taste disorders associated with their HCV-infected liver disease. The other patient visited our clinic with the main complaint of a taste disorder on December 2008. Patients ranged in age from 51 to 78 years, with an average age of 63.3±9.1 years.

Informed consent for participation in the study was obtained from each patient. The study protocol conformed to the ethical guidelines of the 1975 Declaration of Helsinki as reflected in prior approval by the Ethics Committee of the Kurume University School of Medicine. None of the subjects were institutionalized.

Methods

Taste test

Taste functions were analyzed using a Taste Disk® (Sanwa Kagaku Kenkyusho Co. Inc, Nagoya, Japan).

The region of taste buds are located is on the front two-thirds of the tongue, which is innervated by chorda tympani; the area located on the rear one-third of the tongue is innervated by the glossopharyngeal nerve, and the region located on the soft palate is innervated by the greater petrosal nerve. We used a taste kit to check the different dominant nerves involved in taste in each area. The type and concentration of test fluid and the are as shown in Table 1. To stimulate tastes we put a paper 5 millimeters in diameter and moistened with a reagent in the measurement site (Figure 1). Gustatory criteria for 4 tastes (sweet, salty, sour, and bitter) were examined using a 6-point scale (I, II, III, IV, V, and VI). Numbers I, II, and III were standard values (I – minimum standard value, II – the median of the standard value, III – the upper limit of the standard value). Numbers IV, V, and VI were abnormal values (IV – a slight taste disorder, V – a medium taste disorder, VI – a severe taste disorder). Taste disorders were evaluated in the area of the right chorda tympani.

Liver function tests

Sera from all 9 patients were used for the following liver function tests: serum aspartate aminotransferase (AST), alanine aminotransferase (ALT), albumin (Alb), and zinc. Sera were also examined for the presence or absence of HCV or HBV infection. Anti-bodies to HCV (anti-HCV) antibodies and hepatitis B virus surface antigen (HBsAg) were measured by a chemiluminescent enzyme immunoassay (CLEIA) kit and a chemiluminescent immunoassay (CLIA), respectively. Ultrasonography for all subjects was done to examine the shape of the liver and lesions in the liver.

Design of the administration of Aminofeel®

Each patient was given one sachet of Aminofeel®, a BCAA-enriched supplement including zinc, after breakfast and another at bedtime for 90 days.

Statistical analysis

All data are expressed as mean ± standard error. Differences between two groups were analyzed using the Mann-Whitney U test. Statistical comparisons before administration of the Aminofeel® and after 90 days were done using Wilcoxon's test. All statistical analyses were conducted using JMP Version 6 (SAS Institute, Cary, NC, USA). The level of statistical significance was defined as 0.05.

RESULTS

The characteristics of the 9 patients studied are shown in Table 2. The diagnosis of liver disease included: chronic hepatitis C (n=5), liver cirrhosis (n=3), and post interferon

Table 1. A gustatory criterion by the kind of test fluid and the concentration.

Taste		I	II	III	IV	V	VI
Sweet taste	(Sucrose)	0.30%	2.50%	10.00%	20.00%	80.0%	Unobservable V
Salty taste	(Sodium chloride)	0.30%	1.25%	5.00%	10.00%	20.00%	Unobservable V
Sour taste	(Acidum tartaricum)	0.02%	0.20%	2.00%	4.00%	8.00%	Unobservable V
Bitter taste	(Quinine)	0.001%	0.02%	0.10%	0.50%	4.00%	Unobservable V

Table 2. Characteristics of 9 patients with HCV-related liver diseases before administration of Aminofeel®.

No	Sex	Age	Liver disease	Subjective symptom of taste disorder	Systemic disease	Oral lichen planus		Score of taste				Laboratory data				
						Occurrence	Site	Sweet	Salty	Sour	Bitter	AST (IU/L) St. value; 13–33	ALT (IU/L) St. value; 8–42	Alb (g/dL) St. value; 4.0–5.0	PLT (/mm³) St. value; 13.0–36.0	Zinc (μg/dL) St, value; 80–130
1	F	66	LC-C	Negative	Hypertenstion	Positive	Buccal mucosa	III	III	V	III	92	40	3.54	13.7	71
2	F	78	CH-C	Negative	Negative	Negative		II	I	III	II	45	44	3.88	8.7	73
3	F	56	CH-C	Negative	Hypertenstion	Negative		II	II	III	II	24	18	3.94	25.0	84
4	M	58	CH-C	Negative	Negative	Negative		II	II	II	II	46	70	3.92	15.8	85
5	F	69	CH-C	Negative	Negative	Negative		I	II	IV	II	46	45	3.99	18.1	71
6	F	54	LC-C	Negative	Negative	Negative		II	II	II	II	33	24	3.88	18.0	126
7	F	72	LC-C	Negative	Hypertenstion, Hyperlipidemia	Negative		II	II	III	II	34	29	3.87	20.5	93
8	M	51	CH-C	Negative	Diabetes mellitus	Positive	Buccal mucosa and tongue	II	VI	V	V	65	104	3.68	13.8	87
9	M	66	CH-C post IFN (SVR)	Positive	Hypothyroidism	Positive	Buccal mucosa	VI	II	VI	V	21	15	4.3	8.6	67

CH-C – chronic hepatitis C; LC-C – HCV-related liver cirrhosis; SVR – Sustained virological response; AST – serum aspartate aminotransferase; ALT – alanine aminotransferase; Alb – albumin; PLT – platelets.

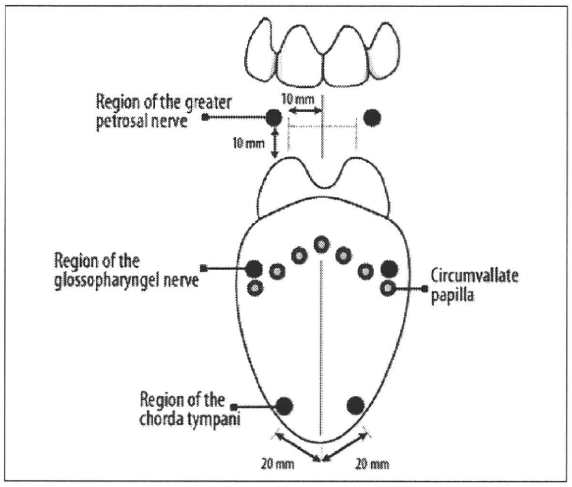

Figure 1. Measurement of tastes using a filter-paper disc method.

(IFN) treatment for chronic hepatitis C (n=1). After we succeeded in eliminating HCV by IFN treatment, one patient, a 66 year old man, developed a taste disorder. Of the 9 patients, 3 had oral lichen planus. There was one patient with oral lichen planus of the tongue. The serum zinc value of the 66 year old man with a complaint of a taste disorder was decreased (67 μg/dL).

The distributions of gustatory sensitivity before and after administration of Aminofeel® are as shown in Figures 2–5. There was only one patient who was aware of a taste disorder before administration of Aminofeel®, but 4 patients had decreased gustatory sensitivity in the sour taste test, and 2 had it in the bitter taste test. Sensitivity to the sour taste was significantly increased 90 days after the administration of Aminofeel® (P=0.03, Figure 4). Sensitivity to the sweet taste was increased 90 days after the administration of Aminofeel® (P=0.06, Figure 2).