cerous liver tissue.[2,3] Thus, the virus-derived angiogenic drive is thought to be less pronounced in overt HCC than in noncancerous livers. Indeed, clinical data supporting the predominant upregulation of VEGF in patients infected with HCV as compared with those infected with HBV are unavailable.

Taken together, it can be speculated that preventing the inflammation and subsequent fibrosis caused by persistent HCV infection is crucial to suppressing the hypoxia-mediated oncogenic pressure on HCV core-harboring hepatocytes.

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ORIGINAL ARTICLE

Hepatitis B virus strains of subgenotype A2 with an identical sequence spreading rapidly from the capital region to all over Japan in patients with acute hepatitis B

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ABSTRACT

Objective To examine recent trends of acute infection with hepatitis B virus (HBV) in Japan by nationwide surveillance and phylogenetic analyses.

Methods During 1991 through 2009, a sentinel surveillance was conducted in 28 national hospitals in a prospective cohort study. Genotypes of HBV were determined in 547 patients with acute hepatitis B. Nucleotide sequences in the preS1/S2/S gene of genotype A and B isolates were determined for phylogenetic analyses.

Results HBV genotype A was detected in 137 (25% (accompanied by genotype G in one)) patients, B in 48 (9%), C in 359 (66%), and other genotypes in the remaining three (0.5%). HBV persisted in five with genotype A including the one accompanied by genotype G; another was co-infected with HIV type 1. The genotype was A in 4.8% of patients during 1991–1996, 29.3% during 1997–2002, and 50.0% during 2003–2008 in the capital region, as against 6.5%, 8.5% and 33.1%, respectively, in other regions. Of the 114 genotype A isolates, 13 (11.4%) were subgenotype A1, and 101 (88.6%) were A2, whereas of the 43 genotype B isolates, 10 (23.3%) were subgenotype B1, 28 (65.1%) were B2, two (4.7%) were B3, and three (7.0%) were B4. Sequences of 65 (64%) isolates of A2 were identical, as were three (23%) of A1, and five (18%) of B2, but none of the B1, B3 and B4 isolates shared a sequence.

Conclusions Acute infection with HBV of genotype A, subgenotype A2 in particular, appear to be increasing, mainly through sexual contact, and spreading from the capital region to other regions in Japan nationwide. Infection persisted in 4% of the patients with genotype A, and HBV strains with an identical sequence prevailed in subgenotype A2 infections. This study indicates the need for universal vaccination of young people to prevent increases in HBV infection in Japan.

Significance of this study

What is already known about this subject?

- In Japan, a national prevention programme was started in 1986 with selective vaccination of babies born to mothers who carry hepatitis B virus (HBV). Since then, the prevalence of hepatitis B surface antigen among younger generations has decreased sharply.
- However, retrospective studies indicate that the frequency of HBV genotype A is increasing among patients with acute hepatitis B (AHB) within the capital region of Japan.
- Infection with genotype A more often persists than infection with other genotypes.
- Because there is no reliable and comprehensive surveillance system for AHB in Japan, the incidence of AHB and factors responsible for changes over many years are not known.

What are the new findings?

- This is a prospective cohort study for surveillance of AHB throughout Japan in a national research programme.
- The incidence of AHB in Japan has not decreased, because genotype A infections have increased over time.
- Genotype A infections started to increase in the capital region of Japan, and then spread to other regions 5–6 years later.
- About 90% of genotype A found in AHB patients in Japan is subgenotype A2.
- Subgenotype A2 isolates from patients with AHB tend to preserve sequence identity over time, indicating that particular subgenotype A2 strains have been transmitted without undergoing mutations.

Hepatitis B virus (HBV) has been classified into 10 genotypes, designated A–J, based on a >8% divergence in the full-genome sequence.[1–7] Different genotypes are associated with distinct clinical manifestations, such as severity and progression of liver disease, as well as response to antiviral treatments.[8–10] Some genotypes are subclassified: genotype A into at least two subgenotypes, A1 (Asian/African type) and A2 (European type)[11–13];

Significance of this study

How might it impact on clinical practice in the foreseeable future?

- It needs to be noted that subgenotype A2 infections are spreading among sexually active generations in Japan.
- Although selective vaccination has prevented mother-to-baby transmission of HBV since 1986, it does not contain sporadic infections in Japan.
- Herd vaccination of younger generations needs to be considered in Japan.

B into B1 (Japanese type) and B2 (Asian type)[14 15]; and C into C1 (Southeast-Asian type) and C2 (East-Asian type).[16] Subgenotypes also influence the replication of HBV and clinical manifestation.[15 17 18]

According to a report from Japan in 2001,[19] genotype C was the most prevalent (84.7%), followed by genotype B (12.2%) and A (1.7%), among patients with chronic hepatitis B. In 2002, genotype A became the most prevalent in patients with acute hepatitis B (AHB) around Tokyo, the capital region of Japan.[20 21] Several reports have shown that infection with HBV genotype A is associated with particular sexual behaviours, such as homosexual activity and promiscuous sexual contacts, and tends to persist longer than that with HBV genotype C.[22 23] These reports have raised concerns about the horizontal HBV infection in adults, which, in general, is considered to resolve spontaneously. However, adult-acquired HBV infection may result in chronic HBV infection in some instances.

Information on changes in genotype distribution over time, as well as genotype-specific clinical manifestations, may help in planning preventive measures and antiviral therapy strategies. Therefore it is important to examine how genotype A infection has spread in Japan, and what clinical and virological characteristics it possesses.

We have been conducting a nationwide, sentinel surveillance on acute viral hepatitis for more than 30 years. As part of this surveillance, a prospective cohort study has been conducted on 547 patients with AHB in 28 medical centres over the 19 years from 1991 to 2009. Geographical and longitudinal distributions of HBV genotypes/subgenotypes were surveyed, and their influence on clinical outcome was evaluated.

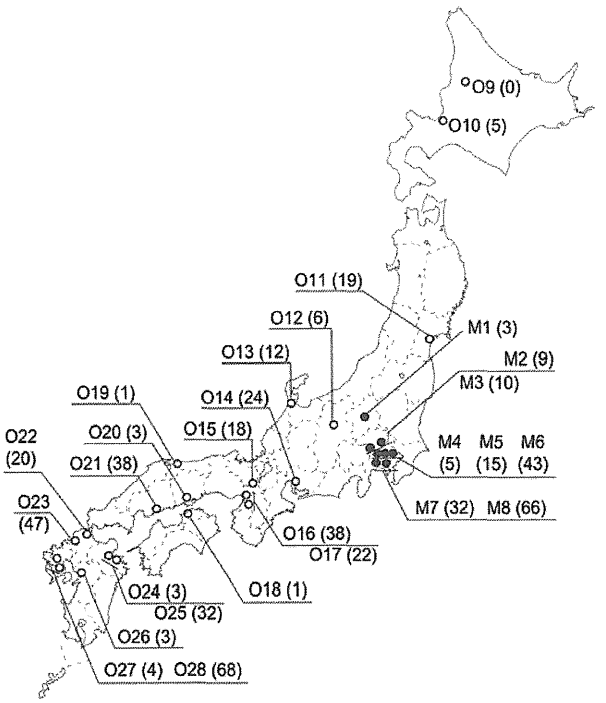

Figure 1 Locations of participating hospitals in Japan. Hospitals in the capital region (M1—M8) are indicated by eight closed circles, and those in other regions (O9—O28) by 20 open circles. Numbers in parentheses indicate the total number of enrolled subjects for each site. The hospitals are: M1, Nishigunma Hospital, Gunma; M2, Nishisaitama-Chuo Hospital, Saitama; M3, National Disaster Medical Center, Tokyo; M4, Tokyo Hospital, Tokyo; M5, Tokyo Medical Center, Tokyo; M6, National Center for Global Health and Medicine, Tokyo; M7, Sagamihara Hospital, Kanagawa; M8, Yokohama Medical Center, Kanagawa; O9, Asahikawa Medical Center, Hokkaido; O10, Hokkaido Medical Center, Hokkaido; O11, Sendai Medical Center, Miyagi; O12, Matsumoto Medical Center, Nagano; O13, Kanazawa Medical Center, Ishikawa; O14, Nagoya Medical Center, Aichi; O15, Kyoto Medical Center, Kyoto; O16, Osaka National Hospital, Osaka; O17, Osaka-Minami Medical Center, Osaka; O18, Zentsuji Hospital, Kagawa; O19, Yonago Medical Center, Tottori; O20, Okayama Medical Center, Okayama; O21, Kure Medical Center and Chugoku Cancer Center, Hiroshima; O22, Kokura Medical Center, Fukuoka; O23, Kyushu Medical Center, Fukuoka; O24, Beppu Medical Center, Oita; O25, Oita Medical Center, Oita; O26, Kumamoto Medical Center, Kumamoto; O27, Ureshino Medical Center, Saga; and O28, Nagasaki Medical Center, Nagasaki.

PATIENTS AND METHODS

Patients

A total of 681 patients with sporadic AHB were enrolled consecutively in a survey carried out by the Japan National Hospital Acute Hepatitis Study Group (JNHAHSG). They were admitted to 28 national hospitals from January 1991 to the end of December 2009. They were grouped geographically into two areas: the capital region (Gunma, Saitama, Tokyo and Kanagawa) and other regions (figure 1). Patients were also longitudinally categorised into three periods: 1st (1991—1996), 2nd (1997—2002) and 3rd (2003—2008). In addition, the year 2009 provided the most recent data. Of the 681 patients, 547 (80.3%) entered the study, for whom serum samples were available on admission and had been stored at −20°C.

The diagnosis of AHB was based on the following criteria: (1) acute onset of liver injury without a history of liver dysfunction; (2) detection of hepatitis B surface antigen (HBsAg) in the serum; (3) positivity for IgM antibody to HBV-core antigen (IgM anti-HBc) in high titres (detectable in sera diluted 10-fold); and (4) absence of past or family history of chronic HBV infection. Severe acute hepatitis (SAH) was defined as prothrombin time (PT) ≤40% and hepatic encephalopathy of grade ≤I. Fulminant hepatitis (FH) was diagnosed from PT ≤40% and hepatic encephalopathy of grade ≥II. Patients in whom HBsAg remained in the serum for >6 months after onset were considered to have acquired chronic HBV infection. The following information was collected from each patient: year and age at onset, gender, residential area, HBsAg, IgM anti-HBc, alanine aminotransferase, total bilirubin, PT, severity of liver disease, mortality, routes of transmission, sexual behaviours, travelling abroad in recent past, HBV genotype, mutations in precore (PreC) and core promoter (CP) regions, and RNA of hepatitis D virus. Antibody to HIV type 1 (anti-HIV) was

determined in patients who were at high risk and gave consent to testing.

Informed consent was obtained from each patient. The study protocol conforms to the ethical guidelines of the 1975 Declaration of Helsinki and the Ministry of Education, Culture, Sports Science and Technology of Japan, and was approved by the ethics committee of each institution.

Extraction of HBV DNA

HBV DNA was extracted from serum (100 μl) by the SMITEST EX-R&D Nucleic Acid Extraction Kit (MBL Co, Nagoya, Japan) and used for genotyping/subgenotyping and detecting mutations in PreC and CP regions.

HBV genotypes

Genotypes were determined in Nagasaki Medical Center with the SMITEST HBV Genotyping Kit (MBL) by hybridisation with type-specific probes immobilised on a solid-phase support.[24]

Determination of HBV subgenotypes

For subgenotyping, HBV DNA was amplified by PCR with TaKaRa Ex Taq (Takara Bio, Shiga, Japan). PCR was performed with appropriate nested primers to amplify a ~1.2 kb sequence in the preS1/S2/S gene (nucleotides 2854–835 in the reference isolate (AB116077)). PCR products were purified, subjected to cycle sequencing reaction with the BigDye Terminator v1.1 (Applied Biosystems, Tokyo, Japan), and applied to the DNA sequencer (3100-Avant; Applied Biosystems).

Mutations in the PreC and CP regions

The A1896 mutation in the PreC region was detected by the enzyme-linked minisequence assay (SMITEST HBV PreC ELMA; Roche Diagnostics, Tokyo, Japan), and mutations in the CP region for T1762/A1764 by the enzyme-linked specific probe assay (SMITEST HBV Core Promoter Mutation Detection Kit; Roche Diagnostics). The results were recorded as 'wild-type' and 'mutant types' dominantly expressed by HBV isolates.[25]

Phylogenetic analyses

Nucleotide sequences were aligned, and phylogenetic trees were constructed by the CLUSTAL W program v1.83 (DDBJ homepage: http://clustalw.ddbj.nig.ac.jp/top-j.html). The statistical validity was assessed by bootstrap resampling with 1000 replicates. Reference HBV strains were retrieved from the GenBank database.

Statistical analysis

Results were expressed as percentage or mean±SD. Statistical differences were evaluated by χ^2 and Fisher exact tests for categorical variables, and analysis of variance and Scheffe's test for quantitative variables, using the SPSS software. The 95% CI, for the difference in means, was calculated in analyses for quantitative variables. $p<0.05$ was considered significant.

RESULTS

Distribution of HV genotypes

HBV genotypes were determined in the 547 patients with AHB. The genotype was A in 137 (25.0%) patients (accompanied by G in one (0.2%)), B in 48 (8.8%), C in 359 (65.6%), D in one (0.2%), E in one (0.2%), and H in one (0.2%). Because HBV genotype G is a defective virus and cannot replicate by itself,[26 27] the single patient with mixed genotypes A and G was included in the 137 patients with genotype A in further analyses. RNA of hepatitis D virus was detected in three of the 453 (0.7%) patients. Anti-HIV was examined in patients at high risk of infection and detected in 14 of the 53 (26.4%) who gave consent to testing.

Demographic and clinical differences among patients infected with HBV of distinct genotypes

Demographic and clinical characteristics of patients with different genotypes are compared in table 1. There was no difference in mean age among patients with genotypes A, B and C. The proportion of men was higher in patients with genotype A than B or C (94.2% vs 79.2%, $p<0.05$; or 56.0%, $p<0.0001$), and in those with genotype B than C (79.2% vs 56.0%, $p<0.05$).

Maximum levels of total bilirubin were higher in patients with genotype A than C (9.6±7.6 vs 7.1±6.2 mg/dl, $p<0.05$), with a difference of 2.5 mg/dl (95% CI 0.93 to 4.08), whereas the highest alanine aminotransferase activity and lowest PT values did not differ among patients with distinct genotypes.

SAH developed in four (2.9%) patients with genotype A, four (8.3%) with genotype B, and 26 (7.2%) with genotype C. FH developed in one (2.1%) patient with genotype B and eight (2.2%) with genotype C; no patients with genotype A developed FH. Eight (1.5%) patients died, including one with genotype B and seven with genotype C. There were no significant differences among patients with different genotypes in the frequency of SAH or FH or mortality.

The outcome of AHB was traceable in 514 of the 547 (94.0%) patients. Chronic infection with persistence of HBsAg for >6 months developed in five of the 123 (4.1%) patients with genotype A (including the one accompanied by genotype G), none of the 46 (0%) with genotype B, and none of the 342 (0%) with genotype C; it was more common in patients with genotype A than C ($p<0.05$). HBV infection persisted exclusively in the patients with genotype A, either alone (four patients) or together with genotype G (one).

Among the five patients who acquired chronic HBV infection, four (three with genotype A and one with mixed genotypes A and G) were examined for anti-HIV, and one with genotype A was found to be positive. HBV infection persisted in three (including the one with anti-HIV) of the five patients for >1 year after the onset, and the remaining two (both without anti-HIV) cleared HBsAg from the serum after retaining it for >6 months.

Mutations in the PreC and/or CP region were detected in 3.7% (4/109) of patients with genotype A, 15.4% (6/39) of those with genotype B, and 25.5% (79/310) of those with genotype C. They were significantly less common in patients with genotype A than B or C (A vs B, $p<0.05$; A vs C, $p<0.0001$). The only patient with genotype A who had the PreC mutation was simultaneously infected with genotype G.

Routes of transmission were identifiable in 275 of the 547 (50%) patients, and the main route was heterosexual contacts; those in the remaining patients could not be disclosed. The frequency of heterosexual activity did not differ among patients with distinct genotypes. However, homosexual activity was more common in patients with genotype A than B or C (21.2%, 0% and 0.8%, respectively (A vs B, $p<0.001$; A vs C, $p<0.0001$)). Among the 32 homosexual men, HBV genotype A was detected in 29 (91%). Consent to anti-HIV testing was given by 10 of the 29 patients, and four of these (40%) were positive.

Longitudinal changes in the distribution of genotypes

Figure 2 illustrates changes in the distribution of HBV genotypes through three 6-year periods over 18 years (1991–2008). In addition, data from 2009 are shown. HBV genotype A accounted

Table 1 Demographic and clinical characteristics of patients with acute hepatitis who were infected with HBV of different genotypes (1991–2009)

Feature	Total (n=547)	HBV genotypes A (n=137)† (25.0%)	B (n=48) (8.8%)	C (n=359) (65.6%)	Others (n=3)‡ (0.5%)
Age (years)	35.6±14.8	35.2±12.2	39.6±15.6	35.1±15.5	49.7±13.6
Male	367 (67.1%)	129 (94.2%)¶ * †† ***	38 (79.2%)†† *	201 (56.0%)	3 (100%)
ALT (IU/l)§	2553±1563	2289±1069	2557±1412	2342±1728	3333±2406
T-Bil (mg/dl)§	7.8±6.7	9.6±7.6†† *	7.7±7.4	7.1±6.2	9.0±2.5
PT (%)§	74.6±22.6	75.2±15.9	73.8±24.5	74.7±24.5	15.8‡‡
Severe hepatitis	34 (6.2%)	4 (2.9%)	4 (8.3%)	26 (7.2%)	0 (0.0%)
Fulminant hepatitis	10 (1.8%)	0 (0.0%)	1 (2.1%)	8 (2.2%)	1 (33.3%)
Mortality	8 (1.5%)	0 (0.0%)	1 (2.1%)	7 (1.9%)	0 (0.0%)
HBsAg persisting >6 months	5/514 (1.0%)	5/123 (4.1%)†† *	0/46 (0.0%)	0/342 (0%)	0/3 (0.0%)
PreC/CP mutations					
PreC	43/461 (9.3%)	1/109 (0.9%)¶ * †† *	6/39 (15.4%)	34/310 (11.0%)	2/3 (66.7%)
CP	69/461 (15.0%)	3/109 (2.8%)†† ***	0/39 (0.0%)†† *	63/310 (20.3%)	3/3 (100%)
PreC and/or CP	92/461 (20.0%)	4/109 (3.7%)¶ * †† ***	6/39 (15.4%)	79/310 (25.5%)	3/3 (100%)
Transmission route					
Homosexual	32 (5.9%)	29 (21.2%)¶ ** †† ***	0 (0.0%)	3 (0.8%)	0 (0.0%)
Heterosexual	217 (39.5%)	52 (38.0%)	25 (52.1%)	139 (39.6%)	1 (33.3%)
Medical procedure	16 (2.9%)	2 (1.5%)	2 (4.2%)	12 (3.3%)	0 (0.0%)
Other	10 (1.8%)	1 (0.7%)	1 (2.1%)	7 (1.9%)	1 (33.3%)
Undetermined	272 (49.7%)	53 (38.7%)†† *	20 (41.7%)	198 (55.2%)	1 (33.3%)
Anti-HIV	14/53 (26.4%)	11/35 (31.4%)	0/3 (0.0%)	3/15 (20.0%)	0/0

Values are mean±SD or number (%).
†One patient with genotype A was simultaneously infected with genotype G.
‡Each patient was infected with genotype D, E or H.
§Highest values during the clinical course are shown for ALT and T-Bil, and lowest values for PT.
Statistical analysis was performed to compare genotypes A, B and C.
¶Significantly different compared with genotype B.
††Significantly different compared with genotype C.
*p<0.05, **p<0.001, ***p<0.0001.
‡‡Data from the patient with genotype E only.
ALT, alanine aminotransferase; CP, core promoter; HBsAg, hepatitis B surface antigen; HBV, hepatitis B virus; PreC, precore; PT, prothrombin time; T-Bil, total bilirubin.

for 6% (9/150) in the 1st period, 15.4% (19/123) in the 2nd, and 39.4% (89/226) in the 3rd, with significant differences between 1st and 2nd (p<0.05), 2nd and 3rd (p<0.0001), and 1st and 3rd (p<0.0001). Conversely, AHB associated with genotype C decreased through three periods with significant differences, while AHB associated with genotype B did not change appreciably.

On the basis of these results, the yearly incidence in each of the three 6-year periods is calculated to be: 25.0 cases including 1.5 with genotype A in the 1st period; 20.5 cases including 3.2 with genotype A in the 2nd; and 37.7 cases including 14.8 with genotype A in the 3rd. Hence, the incidence of AHB had not changed markedly over the 12 years from 1991 to 2002, but increased thereafter until 2008. Of the increment in the 3rd period of 17.2 (37.7 minus 20.5) cases, there were 11.6 (14.8 minus 3.2) with genotype A; they accounted for 67% (11.6/17.2) of the recent increase in AHB.

Regional distributions and longitudinal changes in genotype A

Among the 183 patients from the capital region, the genotype was A in 65 (35.5%), B in 22 (12.0%), C in 94 (51.4%), E in one (0.5%), and H in one (0.5%) (table 2). Of the remaining 364 (66.5%) patients from other regions, by contrast, the genotype was A in 72 (19.8%), B in 26 (7.1%), C in 265 (72.8%), and D in one (0.3%). Genotype A was significantly more common in the capital than in other regions (35.5% vs 19.8%, p<0.0001). In the capital region, genotype A accounted for 4.8% (2/42) in the 1st period, 29.3% (12/41) in the 2nd, and 50.0% (42/84) in the 3rd. There were significant differences between the 1st and 2nd periods (p<0.05), 2nd and 3rd (p<0.05), and 1st and 3rd (p<0.0001). In other regions, by contrast, genotype A accounted for 6.5% (7/108) in the 1st period, 8.5% (7/182) in the 2nd, and 33.1% (47/142) in the 3rd. For the first time in other regions, genotype A increased in the 3rd period, in comparison with the 1st and 2nd (1st vs 3rd, p<0.0001; 2nd vs 3rd, p<0.0001).

Subgenotypes of genotype A

Of the 137 genotype A isolates, amplification and sequencing of HBV DNA were feasible in 114 (83.2%); the isolate from the single patient with genotypes A and G was excluded. A phylogenetic tree was constructed, on the entire preS1/S2/S genes of ~1.2 kb, for these 114 isolates along with 34 genotype A isolates retrieved from the database (figure 3).

Of the 114 isolates in this study, 101 (88.6%) were subgenotype A2, and the remaining 13 (11.4%) were subgenotype A1. In a pair-wise comparison, the sequence divergence among the 101 subgenotype A2 isolates was 0–1.3%, and that among the 13 subgenotype A1 isolates spanned 0% to 2.3%. The sequence divergence between subgenotype A2 and A1 isolates ranged from 2.6% to 4.7%.

A sequence of 1203 nucleotides was possessed in common by three of the 101 (3%) isolates of subgenotype A2. For convenience, the group comprising these three isolates was labelled 'identical group I'. Likewise, an additional six 'identical groups' were found, and numbered from 'II' to 'VII'. They comprised 35 (35%), seven (7%), two (2%), three (3%), 12 (12%) and three (3%) of the 101 isolates of subgenotype A2. In contrast, only one identical group, designated 'VIII', was constructed by three of the 13 (23%) isolates of subgenotype A1.

Some isolates of subgenotype A1 and A2 were obtained from patients who had travelled to foreign countries in the recent past (5/13 (38.5%) patients with A1 to Africa, Philippines, Myanmar and China; and 5/101 (5.0%) patients with A2 to Europe, Thailand, Brazil and the USA).

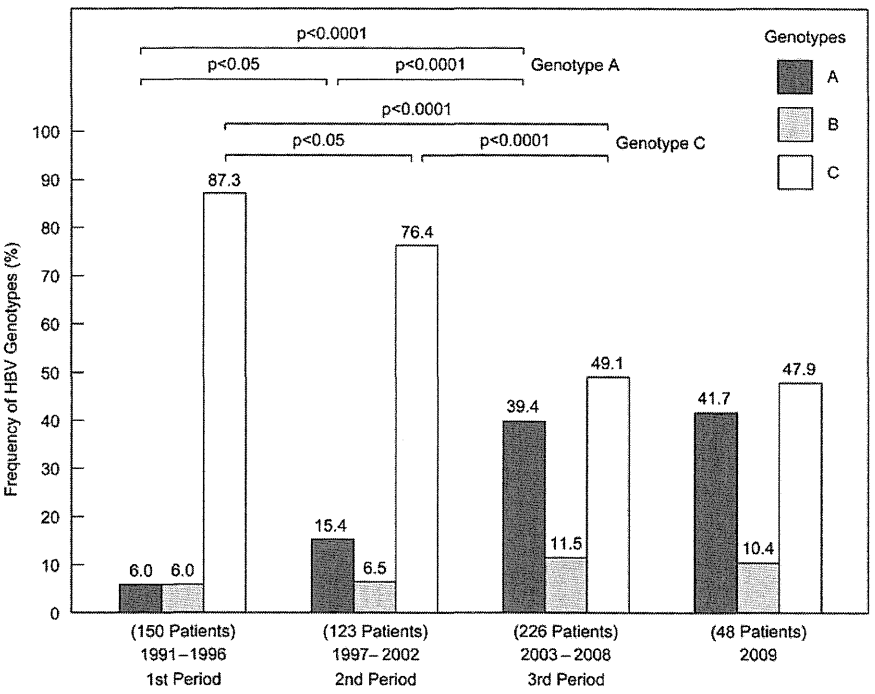

Figure 2 Distribution of hepatitis B virus (HBV) genotypes in three periods.

Subgenotypes of genotype B

Of the 48 isolates of genotype B, subgenotyping was feasible in 43 (90.0%). A phylogenetic tree was constructed on preS1/S2/S-gene sequences from these 43 isolates, along with those from 25 isolates of genotype B retrieved from the database (figure 4). Of the 43 isolates in this study, 10 (23.3%) were subgenotype B1, 28 (65.1%) were B2, two (4.7%) were B3, and three (7.0%) were B4. In a pair-wise comparison, the sequence divergence among 10 subgenotype B1 isolates ranged from 0.4% to 1.4%, and that among 28, two and three isolates of subgenotypes B2, B3 and B4 spanned 0–1.7%, 0.5% and 0.6–0.8%, respectively. The inter-subgenotype divergence among B1–B4 ranged from 0.6% to 4.4%.

One 'identical group' made up of five isolates was detected among the 28 of subgenotype B2; it was named 'IX'. In contrast, no 'identical group' was found in 10, two or three isolates of subgenotype B1, B3 or B4.

Some isolates of subgenotypes B2, B3 and B4 were obtained from patients who had travelled to foreign countries in the recent past (7/28 (25.0%) patients with B2 to China and other countries; 1/2 (50.0%) patients with B3 to a country unknown; and 1/3 (33.3%) patients with B4 to Vietnam). However, none of the 10 subgenotype B1 isolates was associated with travel to foreign countries.

Identical groups

The proportion of isolates that shared a sequence in identical groups was higher for subgenotype A2 (64.4%) than for A1, B1, B2, B3 or B4 (23.1%, 0%, 17.9%, 0% or 0%, respectively (A2 vs A1, $p<0.001$; A2 vs B1, $p<0.0001$; A2 vs B2, $p<0.0001$)).

Homosexual activity was more common in patients belonging to the seven identical groups than the non-identical group of subgenotype A2 (17/65 (26.2%) vs 3/36 (8.3%), $p<0.05$). Among the isolates in the seven identical groups of subgenotype A2, those in groups I, III and VII clustered locally during short periods of 2–7 years. In contrast, subgenotype A2 isolates in groups II and VI were scattered widely over longer periods of 11–16 years.

DISCUSSION

In Japan, as in most Asian countries, the persistent HBV carrier state had been established mainly through perinatal transmission from mother to baby and horizontal infection during infancy. In 1986, a national prevention programme was launched in Japan with selective vaccination of babies born to carrier mothers with hepatitis B e antigen (HBeAg). In 1995, this was extended to babies born to HBeAg-negative carrier mothers. As a result, the prevalence of HBsAg among younger people born since 1986 has decreased dramatically.[28 29] However, there are an

Table 2 Changes in the distribution of genotype A compared between the capital region and other regions over three periods

Area	n	1st Period (1991–1996)	2nd Period (1997–2002)	3rd Period (2003–2008)	2009
Capital region	65/183 (35.5%)†***	2/42 (4.8%)‡* §***	12/41 (29.3%)†* §*	42/84 (50.0%)†*	9/16 (56.3%)
Other regions	72/364 (19.8%)	7/108 (6.5%)§***	7/82 (8.5%)§***	47/142 (33.1%)	11/32 (34.4%)
Total	137/547 (25.0%)	9/150 (6.0%)‡* §***	19/123 (15.4%)§***	89/226 (39.4%)	20/48 (41.7%)

Statistical analysis of the differences between the capital and other regions was performed, as well as through the 1st, 2nd and 3rd periods.
†Significantly different compared with other regions.
‡Significantly different compared with the 2nd period.
§Significantly different compared with the 3rd period.
*$p<0.05$, ***$p<0.0001$.

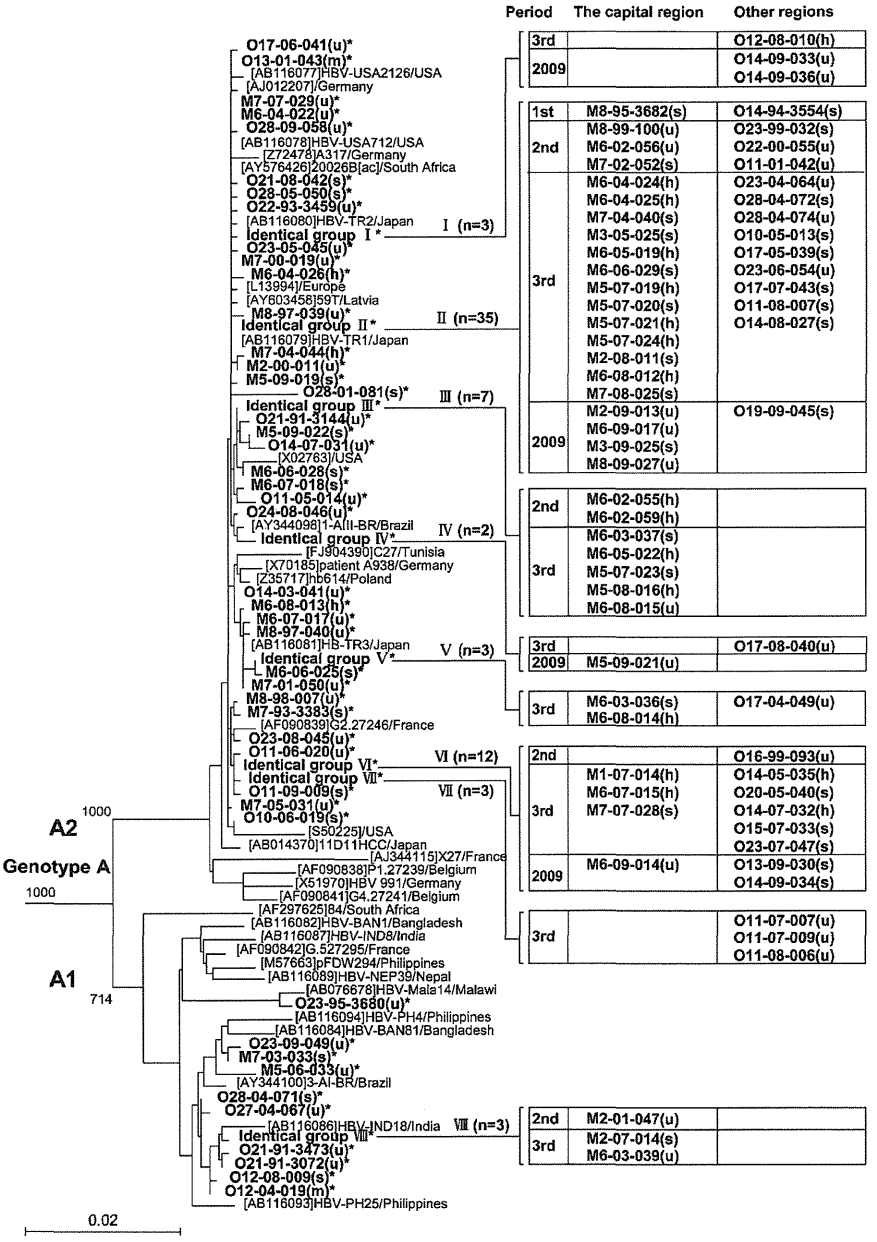

Figure 3 Phylogenetic analysis of genotype A strains by the neighbour-joining method. Isolates obtained in this study are shown in bold with asterisks. Hospitals in the capital region are labelled M1–M8 and those in other regions O9–O28 (corresponding to those in figure 1). Year of onset is indicated by the last two digits after the first hyphen. Numbers after the second hyphen represent the identification numbers of patients in each year (not always consecutive). Transmission routes are shown in lower-case letters in parentheses: h, homosexual; s, heterosexual; m, medical procedure; o, others; and u, undetermined. Isolates with identical sequences are bracketed in 'Identical groups I through VIII' on the tree. Each bracket is divided by areas and periods. Reference hepatitis B virus (HBV) isolates, including 12 of subgenotype A1 and 22 of subgenotype A2, were obtained from the database and specified by their accession numbers, isolate names and countries of origin. Bootstrap values are indicated on major phylogenetic branches.

estimated one million HBV carriers in Japan at present.[30] Furthermore, many Japanese remain at increased risk of horizontal infection with HBV, because they have not received selective vaccination and therefore do not have the antibody to HBsAg. Because AHB is extremely under-reported and no national surveillance data are available in Japan, the incidence has not been determined accurately. In the USA, the incidence of AHB has decreased markedly since the adoption of a comprehensive immunisation strategy in 1991.[31 32]

In the present study over 1991–2009, we conducted a nationwide, sentinel surveillance on AHB in Japan. In the 547 patients recruited over 19 years, genotype C was the most prevalent (65.6%), followed by genotype A (25.0%) and genotype B (8.8%). Demographic and clinical differences were observed among patients with genotypes A, B and C (table 1). The proportion of men reached 94.2% for genotype A infection, higher than that for genotype B (79.2%) or C (56.0%) infection. In the analysis of the route of transmission, homosexual activity was reported by 21.2% of patients with genotype A; all were male. In general, sexual activity tends to be higher in men than women. The predominance of genotype A in men may be attributable to a high frequency of homosexual activity among men.

Although adult-acquired HBV infection persists at a high frequency of ~10% in European countries and the USA,[33] it rarely, if ever, becomes chronic in Japan. Recent studies suggest that the chance of a chronic outcome of AHB may differ by HBV genotype[21 34]; it is more common for genotype A than other genotypes.[22 35 36] In the present study, HBV infection persisted in 4.1% of patients with genotype A, in comparison with 0% of

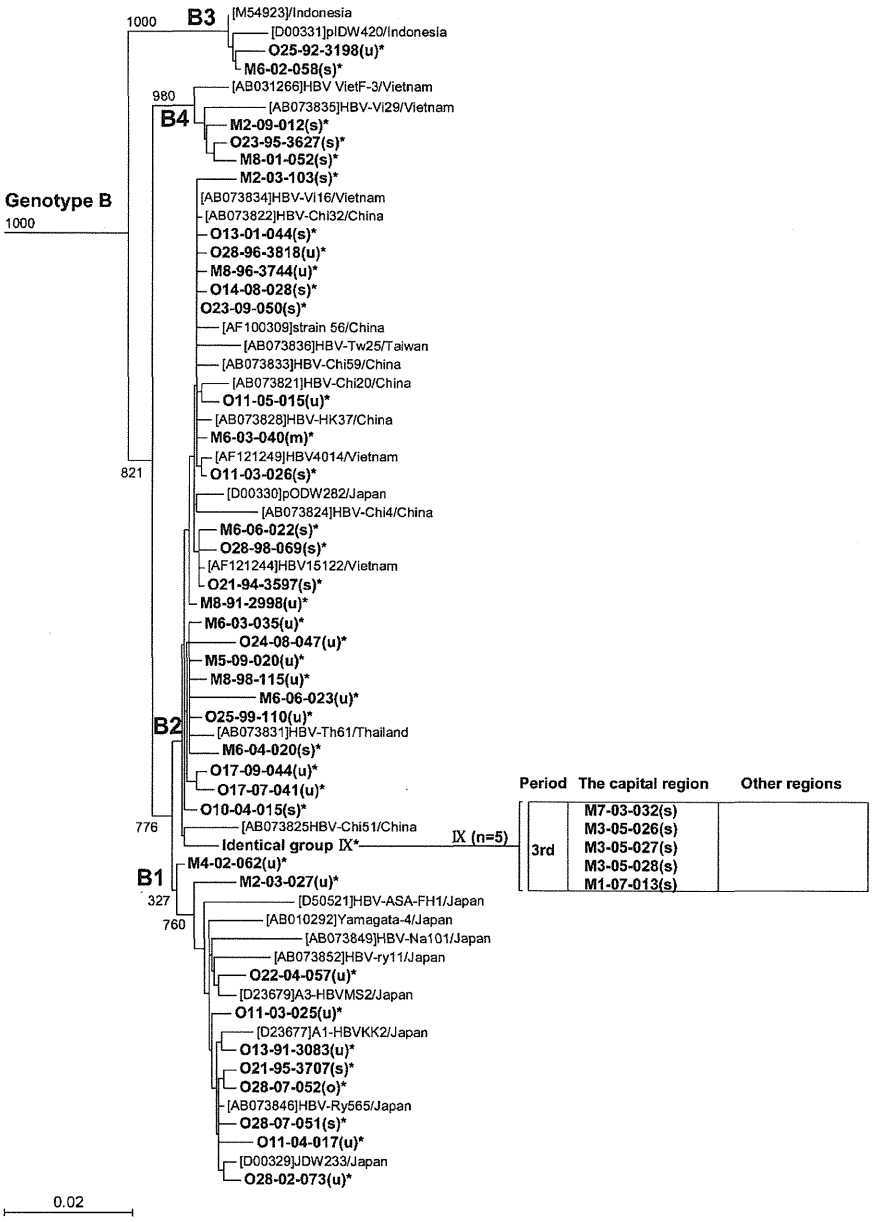

Figure 4 Phylogenetic analysis of genotype B strains by the neighbour-joining method. Hepatitis B virus (HBV) isolates obtained in the present study are specified in the same manner as in figure 3, and isolates with an identical sequence are bracketed in 'Identical group IX' on the tree. Of them, 10 reference isolates of subgenotype B1 and 13, two and two of those of B2, B3 and B4, respectively, were retrieved from the database; they are specified as in figure 3.

those with genotype C. Remarkably, all five patients with AHB who acquired chronic infection possessed HBV genotype A, either alone (four patients) or together with HBV genotype G (one). Increasing genotype A infections may have changed the genotype distribution in patients with AHB and those with chronic HBV infection. In Japanese patients with chronic hepatitis B, the proportion of genotype A has doubled, from 1.7% in 1999–2000 to 3.5% in 2005–2006.[37]

The genotype was A in 29 of the 32 (91%) homosexual men. Of the 29 homosexuals with genotype A, 10 gave consent to anti-HIV testing, and four of these (40%) were found to be positive. Of the five patients who acquired chronic HBV infection, anti-HIV was tested in four (three with genotype A and one with genotypes A and G), and one with genotype A was found to be positive. There is a possibility that co-infecting HIV in this patient with genotype A may have promoted chronic HBV infection; HIV is known to prolong and aggravate HBV infection by compromising immune responses.[38]

Patients with FH in this study were infected with either HBV genotype B (1/48 (2.1%)) or C (8/359 (2.2%)); no patients with genotype A developed FH. PreC and/or CP mutations were significantly less common in genotype A (1/109 (3.7%)) than B (6/39 (15.4%)) or C (279/310 (5.5%)) infection. The single patient with genotype A who had PreC mutation was simultaneously infected with HBV genotype G. There is a possibility that the PreC mutation in this patient was from HBV genotype G.[26] FH did not develop in any patients with genotype A, which may be attributable, at least in part, to the lack of PreC mutation in genotype A infections.[39]

Previous reports have shown that genotype A is common in patients with AHB in Metropolitan Tokyo,[20 21 40] as well as around Aichi located in the middle of Mainland Japan.[22]

Yotsuyanagi *et al*[23] reported that genotype A is more common in patients with AHB in the metropolitan region than in other regions. Sugauchi *et al*[41] found that, in patients with AHB, the proportion with genotype A has increased over time. The present study indicates that the number of patients with AHB in Japan would not have decreased. We found that the proportion of patients with genotype A infection is increasing in the 28 national hospitals in Japan (6.0% in the 1st period, 15.4% in the 2nd, and 39.4% in the 3rd (figure 2)), with the prevalence much higher in the capital than other regions (35.5% vs 19.8% (table 2)).

In this study, there was a time lag in the increase in genotype A infection between the capital region and other regions of Japan (table 2). In the capital region, the prevalence of genotype A started to increase in the late 1990s, and kept increasing through the early 2000s (4.8% in the 1st period, 29.3% in the 2nd, 50.0% in the 3rd, and 56.3% in 2009). In other regions, by contrast, the frequency of genotype A did not change during the late 1990s, and increased significantly in the 2000s (6.5% in the 1st period, 8.5% in the 2nd, 33.1% in the 3rd, and 34.4% in 2009). Thus infiltration of genotype A infection into other regions occurred 5–6 years behind the epidemic in the capital region. This indicates that genotype A infection originated in the capital region and then spread to other areas of Japan.

Some genotypes are classified into several subgenotypes, and they have distinct geographical distributions.[42] Hence, subgenotypes are useful in tracing the route of HBV infection. By phylogenetic analysis (figures 3 and 4), 88.6% of genotype A isolates had the European–American type (A2), and the remaining 11.4% possessed the Asian–African type (A1). Likewise, 76.7% of genotype B isolates had Asian types (B2–B4), and the remaining 23.3% possessed the type endemic to Japan (B1). Of the 157 HBV isolates of genotype A or B, 147 (93.6%) had subgenotypes foreign to Japan. They are thought to have been transmitted from foreign sex workers, and spread among certain populations who share particular sexual behaviours in Japan.[41]

Of note, some HBV isolates of distinct subgenotypes possessed an identical sequence in the preS1/S2/S gene. The isolates of subgenotype A2 were prominent in this regard, and more often had the same sequence than those of other subgenotypes, such as A1, B1 and B2. The high prevalence of subgenotype A2 isolates with an identical sequence would not have been caused by cross-contamination. If cross-contamination had occurred, it would have affected isolates of all subgenotypes, and not influenced subgenotype A2 isolates preferentially. As many as 35% of subgenotype A2 isolates had an identical sequence, and those with the same sequence increased to 56.3% in the recent 2009 survey in Metropolitan Tokyo. Furthermore, some subgenotype A2 isolates in groups I, III and VII clustered locally within short periods, whereas others in groups II and VI were scattered widely over a long period of time. On the basis of these results, it is tempting to speculate that some subgenotype A2 strains would have been transmitted from person to person without undergoing mutations for many years.

In summary, the present study indicates the following. (1) AHB in the 28 national hospitals in Japan has not decreased, because genotype A infections are increasing. (2) Genotype A infections started to increase in the capital region, and then spread to local areas 5–6 years later. (3) Approximately 90% of genotype A in patients with AHB is subgenotype A2. (4) Subgenotype A2 strains with an identical sequence are spreading among younger generations with high sexual activity. (5) On the basis of the results obtained, AHB in Japan is not decreasing, because HBV of subgenotype A2 is prevailing in particular subpopulations at high risk. Finally, in order to prevent further increases in AHB in Japan, universal vaccination of young people deserves consideration.

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Ethics approval Approved by the ethics committee of each institution.

Contributors YT, HY and HI designed data collection tools, monitored data collection for the whole study, wrote the statistical analysis plan, cleaned and analysed the data. YT, HY and YM drafted and revised the paper. HY, NM, MN, EM, TK, YW, TM, MS, TH, TS, YM, TK, MT, HK, HO, SH and SA collaborated in data and sample collection.

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Original Article

Anti-hypoalbuminemic effect of branched-chain amino acid granules in patients with liver cirrhosis is independent of dietary energy and protein intake

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Aim: A multicenter prospective intervention study was conducted in 204 patients with uncompensated liver cirrhosis to explore the influence of dietary intake and patient clinical characteristics on improvement of hypoalbuminemia at weeks 12 and 24 of treatment with branched-chain amino acid (BCAA) granules.

Methods: The primary endpoint set in this study was improvement of hypoalbuminemia in patients with liver cirrhosis. The dietary energy and protein intake per day were estimated based on the results of a survey on diet during a 3-day period preceding the start of the study.

Results: As for the primary endpoint, the mean serum albumin level increased significantly at weeks 12 and 24 of BCAA treatment, compared with the baseline level. The mean Child–Pugh score decreased significantly at weeks 12 and 24 of treatment as compared to the mean baseline score. There was a significant increase in the serum albumin level following treatment with BCAA granules regardless of energy intake and of protein intake. The incidence of ascites and edema significantly decreased in the overall patient population both at weeks 12 and 24 of treatment, compared with the baseline incidence. A subgroup analysis conducted in patients stratified according to changes in the serum albumin level at week 12 of treatment as against baseline showed that the incidence of ascites/edema was significantly reduced not only in the increased albumin group but in the unchanged albumin group.

Conclusion: The present data suggest that the anti-hypoalbuminemic effect of BCAA treatment in patients with liver cirrhosis is independent of dietary intake.

Key words: albumin, branched-chain amino acids, food intake, hepatic failure, liver cirrhosis

INTRODUCTION

ALBUMIN IS THE most abundant circulating protein in serum at concentrations as high as 4.2 to 5.1 g/dL. The known physiological functions of albumin include maintenance of colloid osmotic pressure, transport of numerous substances, supply of amino acids, pH buffering, and radical scavenging actions.[1,2]

Serum albumin is the protein synthesized in and secreted by parenchymal cells of the liver into blood and has long been used as an indicator of protein nutrition. In patients with chronic hepatic disorders, especially in liver cirrhosis with concurrent hypoalbuminemia, the serum albumin level is regarded as important not merely as an indicator of protein nutrition but also as factors to estimate hepatic functional reserve and prognosis.[3]

In recent years branched-chain amino acid (BCAA) granules have become available and prescribed for improvement of hypoalbuminemia in patients with

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Participating investigators are listed in Appendix.
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uncompensated liver cirrhosis who have adequate dietary intake in Japan. It is recommended as grade-A treatment for improvement of hypoalbuminemia in patients with liver cirrhosis in the Japanese guidelines for the treatment of liver cirrhosis published by the Japanese Society of Gastroenterology in 2010.[4] Furthermore, it has been reported in Italy[5] and in Japan[6] that multicenter randomized controlled clinical trials demonstrated not only improvement of hypoalbuminemia but also suppression of the development of serious complications such as aggravation of hepatic failure, that is, prolongation of time to onset of events related to prognosis for survival, in patients with liver cirrhosis receiving BCAA treatment.

Improvement of hypoalbuminemia brought about by treatment with BCAA granules owes primarily to supplementation of deficient BCAA as substrates required for protein synthesis. A recent clarification of the intracellular signal transduction mechanism showed that BCAA activates the mTOR signaling pathways to stimulate initiation of albumin protein translation, thereby enhancing albumin synthesis.[7,8]

Despite the established efficacy of BCAA granules in the treatment of hypoalbuminemia in patients with liver cirrhosis as above, treatment with BCAA granules is not necessarily associated with elevation of the serum albumin level in all patients with liver cirrhosis, and conducting further investigation was considered to determine factors involved in the improvement of hypoalbuminemia brought on by BCAA treatment. We therefore conducted a multicenter prospective intervention study to explore the influence of energy intake and protein intake on the improvement of hypoalbuminemia brought on by BCAA therapy.

METHODS

Study design and protocol

THIS STUDY WAS designed as a multicenter prospective intervention study to clarify the actual state of treatment and dietary intake status in patients with uncompensated liver cirrhosis. Thirty-three medical institutions affiliated with the National Hospital Organization of Japan participated in this study, in which 204 patients with uncompensated liver cirrhosis were enrolled. The study subjects received oral treatment with BCAA granules (Livact Granules, Ajinomoto Pharmaceuticals Co., Ltd, Tokyo) at 4 g (containing 952 mg of L-isoleucine, 1904 mg of L-leucine and 1144 mg of L-valine) three times daily after meals.

Patients with uncompensated liver cirrhosis who had hypoalbuminemia were included in the study, except for those who violated any of the following exclusion criteria: (i) a history of treatment with BCAA granules within 24 weeks before enrollment in this study; (ii) suspected concurrent hepatocellular carcinoma; (iii) a history of hepatocellular carcinoma; and (iv) congenital amino acid metabolic abnormality.

The primary endpoint in this study was improvement of hypoalbuminemia at weeks 12 and 24 after the start of BCAA treatment. The effects of dietary intake and patient clinical characteristics were assessed as the secondary endpoints. Baseline patient clinical characteristics included gender, age, height, body weight, etiology of liver cirrhosis, previous medical history, complications, clinical manifestations, and routine laboratory test data. Child–Pugh scoring and grading of encephalopathy and ascites were carried out according to Pugh's modified classification.[9]

The energy intake per day and protein intake per day were estimated based on data obtained in a 3-day diet survey using a disposable camera conducted by Asahi Kasei Life Support Corporation at the start of BCAA treatment.[10]

Written informed consent to participate in this study was obtained from every patient. The protocol for this study was approved by the ethics committee of the National Hospital Organization, and the study was conducted in accordance with the Ethical Guidelines for Clinical Studies.[11]

Rationale for the number of study enrollees

The sample size (n) required for determining the mean of the study population is calculated using the following formula:

$$n = (\text{Standard deviation of the estimated study population})^2 \times (1.96/\text{precision})^2$$

To estimate the mean energy intake with the 95% confidence interval set within 10% of the mean according to a previous study:[12]

$$(478.78)^2 \times (1.96/(1605.2/10))^2 = 34.2 \text{ (35 patients)}$$

(Mean energy intake for 10 patients with liver cirrhosis and standard deviation of the mean: 1605.2 ± 478.78 kcal)

To estimate the mean protein intake with the 95% confidence interval set within 10% of the mean according to the above study:

$$(15.64)^2 \times (1.96/(64.7/10))^2 = 22.4 \text{ (23 patients)}$$

(Mean protein intake for 10 patients with liver cirrhosis and standard deviation of the mean: 64.7 ± 15.64 kcal)

Thus, approximately 35 patients were estimated to be required for estimating the mean energy intake and mean protein intake with the 95% confidence interval set within 10% of the mean. Inasmuch as patients whose pertinent values were below the standard energy intake (25 kcal/kg body weight) and/or below the standard protein intake (1 g/kg body weight) accounted for 25% of the study population in the above study, it was estimated that a total of 140 patients are needed in order to secure at least 35 study subjects for the population. Taking account of possible dropouts during the study period, we finally decided that 200 patients should be enrolled in the study.

Statistical analysis

The analyses were conducted using JMP9.01 and SAS9.2 (both from SAS Institute Inc.). Continuous data such as serum albumin levels over time were expressed as mean ± standard deviation of the mean. Statistical tests used included paired *t*-test, Wilcoxon signed-rank test and McNemar's test, and results were displayed in terms of the *P*-value. The level of significance was assessed as two-sided 5%; in the testing at individual time points, nevertheless, the level of significance was set as two-sided 2.5% by Bonferroni correction, taking multiplicity into account.

RESULTS

Disposition of patients and clinical characteristics

A TOTAL OF 204 patients were enrolled in this study. For the efficacy evaluation, 135 patients were eventually included in the analysis with the exception of the following 69 patients: three patients who were definitely diagnosed as having hepatocellular carcinoma after study enrollment, nine patients who took other amino acid or albumin products during the study period, 19 patients who withdrew from the study prior to week 12 after the start of the study, hence lacking in data other than baseline data, and 38 patients whose serum albumin level had exceeded 3.5 g/dL prior to the study. Table 1 shows clinical characteristics of patients included in the analysis.

Evaluation of efficacy

For the primary endpoint, the mean serum albumin level increased significantly both at week 12 (3.26 ± 0.40) and week 24 (3.31 ± 0.46), compared with the baseline level (3.11 ± 0.35) ($P < 0.0001$ and $P < 0.0001$, respectively, Fig. 1).

The mean Child–Pugh score decreased significantly both at week 12 (7.4 ± 1.4) and week 24 (7.2 ± 1.5) as compared to the baseline mean score (7.8 ± 1.4) ($P = 0.0080$ and $P = 0.0008$, respectively); indicating improvement of hepatic functional reserve (Fig. 2).

Energy intake and protein intake data were assessed according to the following cut-off values (energy intake: 25 kcal/kg or more (33.7 ± 5.7) and less than 25 kcal/kg (19.6 ± 4.0), and protein intake: 1.0 g/kg or more (1.35 ± 0.24) and less than 1.0 g/kg (0.78 ± 0.16)), respectively. For these categories of each parameter, the mean serum albumin level elevated significantly at weeks 12 and 24 as compared to the baseline level (Fig. 3-1,3-2).

A total of 131 patients whose serum albumin measured at both weeks 0 and 12 were classified into the following three groups according to changes in the serum albumin level at week 12 from the baseline: increasing group with changing the serum albumin level by 0.2 g/dL or more (60 patients), no-change group with a change between −0.1 g/dL and 0.1 g/dL (55 patients) and decreasing group with changing the serum albumin level by at least −0.2 g/dL (16 patients). The clinical characteristics among these groups were assessed for any bias, and there were significant differences with respect to ascites, energy intake and protein intake (Table 1). Among them, for energy intake and protein intake, the mean serum albumin level increased as compared to the baseline level for the categories as described above (Fig. 3-1,3-2). As for ascites, the response was assessed in three categories (ascites: none, mild, or moderate), and the mean serum albumin level increased as compared to the baseline level for all these categories (Fig. 4).

To explore the influence of the above factors on the serum albumin level, multiple regression and simple regression analyses were carried out using the absolute change in the serum albumin level at week 24 versus the baseline level as a response variable and the three factors as explanatory variables. None of the three factors proved to have any significant influence.

Of clinical manifestations, the proportion of patients who reported ascites/edema to the whole analysis population was 49.6% at baseline and then significantly

Table 1 Baseline characteristics of the study patients

Changes in the serum albumin level from 0 to 12 weeks	All	Increase ≥0.2	No change ≤0.1 and ≥−0.1	Decrease ≤−0.2	*P*-value
n	135	60	55	16	
Sex					
Male	64 (47.4%)	29 (48.3%)	22 (40.0%)	10 (62.5%)	$P = 0.2644$
Female	71 (52.6%)	31 (51.7%)	33 (60.0%)	6 (37.5%)	
Age (years)	69 (35–89)	67.5 (35–85)	69 (38–89)	70.5 (55–84)	$P = 0.7159$
Height (cm)	155.9 (136–183)	155.8 (136–183)	153.6 (140–177)	158 (142–176)	$P = 0.7610$
Weight (kg)	57.3 (32.0–113.0)	55.3 (32–113)	58 (38–99)	55 (36–89)	$P = 0.6447$
BMI	22.8 (15.7–42.9)	22.7 (15.7–33.7)	23.0 (17.6–42.9)	23.0 (16.0–28.7)	$P = 0.6902$
Cause of hepatic cirrhosis					
HCV	75 (55.6%)	26 (43.3%)	34 (61.8%)	11 (68.8%)	$P = 0.3833$
HBV	6 (4.4%)	3 (5.0%)	3 (5.5%)	0 (0.0%)	
AL	19 (14.1%)	11 (18.3%)	6 (10.9%)	2 (12.5%)	
Other	35 (25.9%)	20 (33.3%)	12 (21.8%)	3 (18.8%)	
Child–Pugh Score					
A	16 (14.3%)	5 (10.9%)	9 (18.8%)	1 (6.7%)	$P = 0.3695$
B	82 (73.2%)	33 (71.7%)	36 (75.0%)	12 (80.0%)	
C	14 (12.5%)	8 (17.4%)	3 (6.3%)	2 (13.3%)	
Diabetes					
None	103 (76.3%)	48 (80.0%)	40 (72.7%)	11 (68.8%)	$P = 0.5268$
Diabetes	32 (23.7%)	12 (20.0%)	15 (27.3%)	5 (31.3%)	
Hepatic encephalopathy					
None	121 (89.6%)	54 (90.0%)	49 (89.1%)	14 (87.5%)	$P = 0.5319$
Grade I	11 (8.1%)	4 (6.7%)	6 (10.9%)	1 (6.3%)	
Grade II	1 (0.7%)	1 (1.7%)	0 (0.0%)	0 (0.0%)	
Unknown	2 (1.5%)	1 (1.7%)	0 (0.0%)	1 (6.3%)	
Ascites					
None	89 (65.9%)	32 (53.3%)	44 (80.0%)	9 (56.3%)	$P = 0.0131$
Mild	23 (17.0%)	11 (18.3%)	7 (12.7%)	5 (31.3%)	
Moderate	16 (11.9%)	13 (21.7%)	1 (1.8%)	2 (12.5%)	
Unknown	7 (5.2%)	4 (6.7%)	3 (5.5%)	0 (0.0%)	
Edema					
None	99 (73.3%)	42 (70.0%)	40 (72.7%)	14 (87.5%)	$P = 0.3696$
Edema	36 (26.7%)	18 (30.0%)	15 (27.3%)	2 (12.5%)	
Serum albumin (g/dL)	3.2 (2.0–3.5)	3.15 (2.0–3.5)	3.2 (2.4–3.5)	3.3 (2.7–3.5)	$P = 0.5449$
Platelet (×10 000/μL)	8.1 (2.2–39.4)	8.3 (2.9–39.4)	7.9 (3.1–21.5)	8.05 (2.2–23.3)	$P = 0.3289$
AST (IU/L)	48 (17–336)	43 (19–336)	50 (17–123)	54 (20–107)	$P = 0.3262$
ALT (IU/L)	32 (7–320)	26.5 (9–320)	36 (10–113)	40 (7–136)	$P = 0.0949$
Total bilirubin (mg/dL)	1.2 (0.3–7.8)	1.22 (0.4–4.5)	1.2 (0.3–7.8)	1.09 (0.5–4.0)	$P = 0.7592$
PT (INR)	1.26 (0.90–2.77)	1.28 (0.90–2.77)	1.25 (0.96–1.71)	1.23 (1.01–1.46)	$P = 0.1984$
BTR	2.62 (1.40–20.70)	3.02 (1.44–6.32)	2.50 (1.40–20.70)	2.65 (2.06–9.12)	$P = 0.4635$
Calorie intake (kcal/kg)	29.3 (8.2–50.1)	27.1 (8.2–43.0)	30.9 (12.7–50.1)	33.3 (16.6–42.7)	$P = 0.0132$
Protein intake (g/kg)	1.16 (0.31–2.05)	1.07 (0.31–1.69)	1.17 (0.53–2.05)	1.39 (0.64–1.92)	$P = 0.0117$

Data were assessed using χ^2 test or Kruskal–Wallis test.
ALT, alannine aminotransferase; AST, aspartate aminotransferase; BMI, body mass index; BTR, molar ratio of total branched-chain amino acid to tyrosine; PT, prothrombin time.

decreased at week 12 (37.9%) and week 24 (27.0%) ($P = 0.0112$ and $P < 0.0001$, respectively). A subgroup analysis with stratification according to the absolute change in the serum albumin level at week 12 from baseline revealed that the percentage of patients with ascites/edema decreased in the increasing group and the no-change group, whereas in the decreasing group, no such improvement was noted (Fig. 5-1 to 5-4).

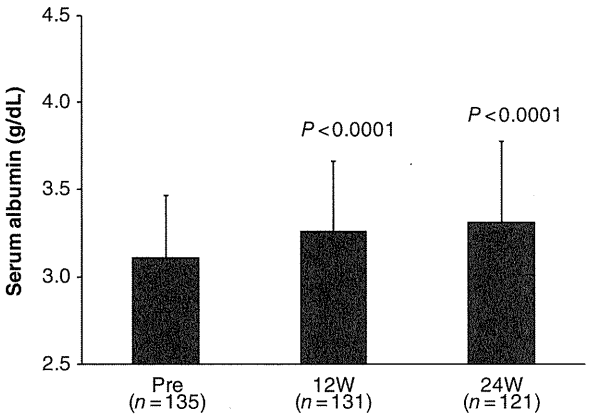

Figure 1 Changes in serum albumin levels (mean, SD). Data were assessed using paired *t*-test (in comparison to the baseline level) and *P*-values are presented.

DISCUSSION

AS REPORTED IN a previous study,[6] a significant elevation in the serum albumin level and a significant improvement in hepatic functional reserve in terms of the Child–Pugh score were observed following treatment with BCAA granules in the present study. At week 12 of study treatment, however, the serum albumin level elevated in 60 patients (45.8%), while it was unchanged in 55 patients (42.0%) and decreased in 16 patients (12.2%). We thus investigated whether these differences in therapeutic response to BCAA treatment are due to patient clinical characteristics. The investigation revealed significant differences among the above

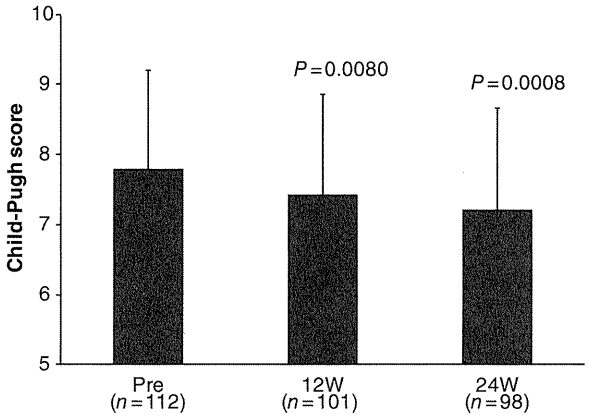

Figure 2 Changes in Child–Pugh score (mean, SD). Data were assessed using Wilcoxon signed ranks test (in comparison to the baseline score) and *P*-values are presented.

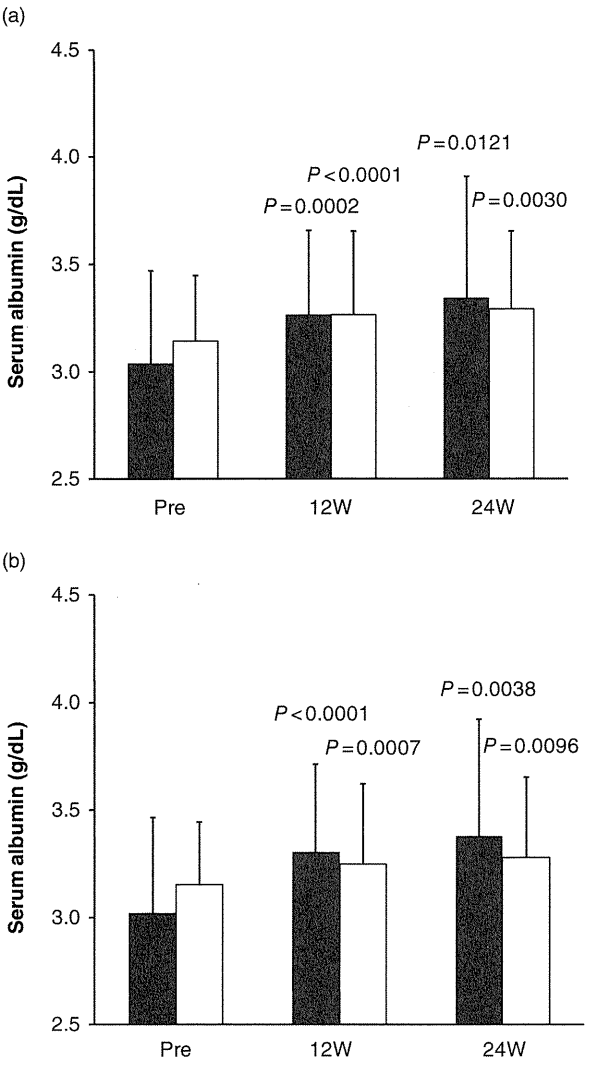

Figure 3 (1) Changes in serum albumin levels by energy intake (mean, SD). Data were assessed using paired *t*-test (in comparison to the baseline level) by energy intake, and *P*-values are presented. The numbers of patients with less than 25 kcal/kg and 25 kcal/kg or more of energy intake were 41 and 90 at baseline, 40 and 87 at week 12, and 37 and 80 at week 24. ■ <25 kcal/kg; □ ≥25 kcal/kg. (2) Changes in serum albumin levels by protein intake (mean, SD). Data were assessed using paired *t*-test (in comparison to the baseline level) by protein intake, and *P*-values are presented. The numbers of patients with less than 1.0 g/kg and 1.0 g/kg or more of protein intake were 41 and 90 at baseline, 39 and 88 at week 12, and 36 and 81 at week 24. ■ <1.0 g/kg; □ ≥1.0 g/kg.

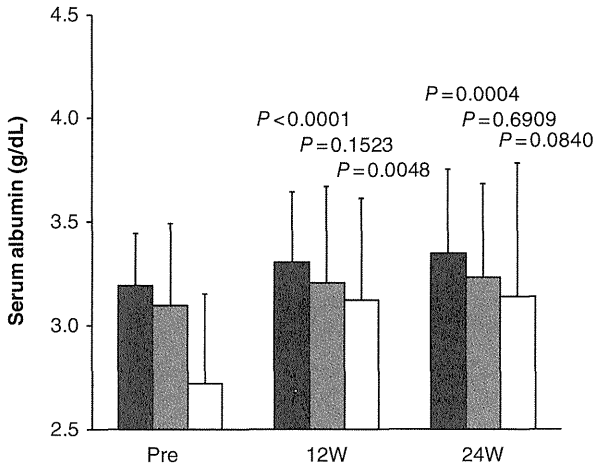

Figure 4 Changes in serum albumin levels by degree of ascites (mean, SD). Data were assessed using paired *t*-test (in comparison to the baseline status) by degree of ascites, and *P*-values are presented. The number of patients with no ascites, mild ascites, and moderate ascites were 89, 23, and 16 at baseline, 85, 23, and 16 at week 12, and 81, 21, and 13 at week 24. ■ none; ▩ mild; □ moderate.

three subgroups with respect to baseline ascites, energy intake and protein intake. Hypoalbuminemia improved irrespective of the degree of ascites. Regarding energy intake and protein intake, their influence was assessed in accordance with the European Society for Clinical Nutrition and Metabolism (ESPEN) guidelines[13] using the definition of an adequate energy intake and protein intake of 25 kcal/kg and 1.0 g/kg or more, respectively. In the present study, hypoalbuminemia improved even in patients with liver cirrhosis whose energy intake and/or protein intake was inadequate. We explored the influence in 10 patients (7.4%) whose baseline energy intake was below 1000 kcal, which is considered to be a minimal amount of energy intake. Hypoalbuminemia improved even in these patients with an absolute change in the serum albumin level of 0.47 ± 0.37 at week 12 ($P = 0.0028$) and 0.48 ± 0.74 at week 24 ($P = 0.0904$). The results suggest the possibility that the effect of BCAA treatment may be independent of dietary intake.

The dietary energy intake and protein intake that had been obtained from the 3-day diet survey at the start of BCAA treatment did not necessarily remain practically consistent throughout the study period. In view of this, we checked relevant data on 93 patients who underwent the diet surveys both at the beginning baseline and at week 24 of study treatment. The results showed that the dietary energy intake was either less than ≤25 kcal at both assessment time points or ≥25 kcal or more at both assessment time points in 72% of patients, hence consistent, and the protein intake was either less than ≤1.0 g/kg at both assessment time points or ≥1.0 g/kg or more at both assessment time points in 74% of patients, hence again consistent. It is thus considered reasonable to conclude that the anti-hypoalbuminemic effect of BCAA granules treatment is independent of dietary energy and protein intake. Nevertheless, it is beyond dispute that dietary counseling is important in satisfactorily maintaining the nutritional condition of liver cirrhosis patients, as has been widely advocated.

Taking a BMI of ≤18.5 as a protein-energy malnutrition state in order to discuss the effects of the nutritional condition closely related to dietary intake, there were as few as seven patients (5.3%) with BMI ≤18.5 among 132 patients whose BMI data were available; this percentage was approximate to 5.5% for patients with BMI ≤18.5 among patients enrolled in the Long-Term Survival Study (LOTUS) study. The conclusion that the effect of BCAA granules treatment is independent of dietary energy and protein intake may be ascribed to the fact that the present study population comprised liver cirrhosis patients in Japan where recent patients with protein-energy malnutrition constitute only a small proportion. As for the subgroups in Table 1, the percentage of patients with BMI ≤18.5 was 3.5% for the increasing group, 5.5% for the no-change group and 13.3% for the decreasing group; there was no significant difference among the three subgroups ($P = 0.3242$). The percentage of patients with BMI ≤18.5 tended to be higher in the decreasing group though there were fewer patients (15) in this subgroup; therefore, a relationship between the lack of serum albumin level response to BCAA treatment and the protein-energy malnutrition state cannot be completely ruled out.

The LOTUS study[6] and other studies reported that improvement of symptoms such as ascites/edema, encephalopathy, and jaundice was observed in addition to amelioration of hypoalbuminemia in patients treated with BCAA granules. As the percentage of ascites/edema, encephalopathy, and jaundice (total bilirubin: 2.0 mg/dL or more) was 49.6%, 8.9%, and 23.7% at baseline in the present study, we focused on ascites/edema with the highest incidence. The percentage of patients who reported ascites/edema significantly decreased in association with improvement of the serum albumin level following treatment with BCAA in the increasing group at week 12. This percentage also significantly decreased in the no-change group at week

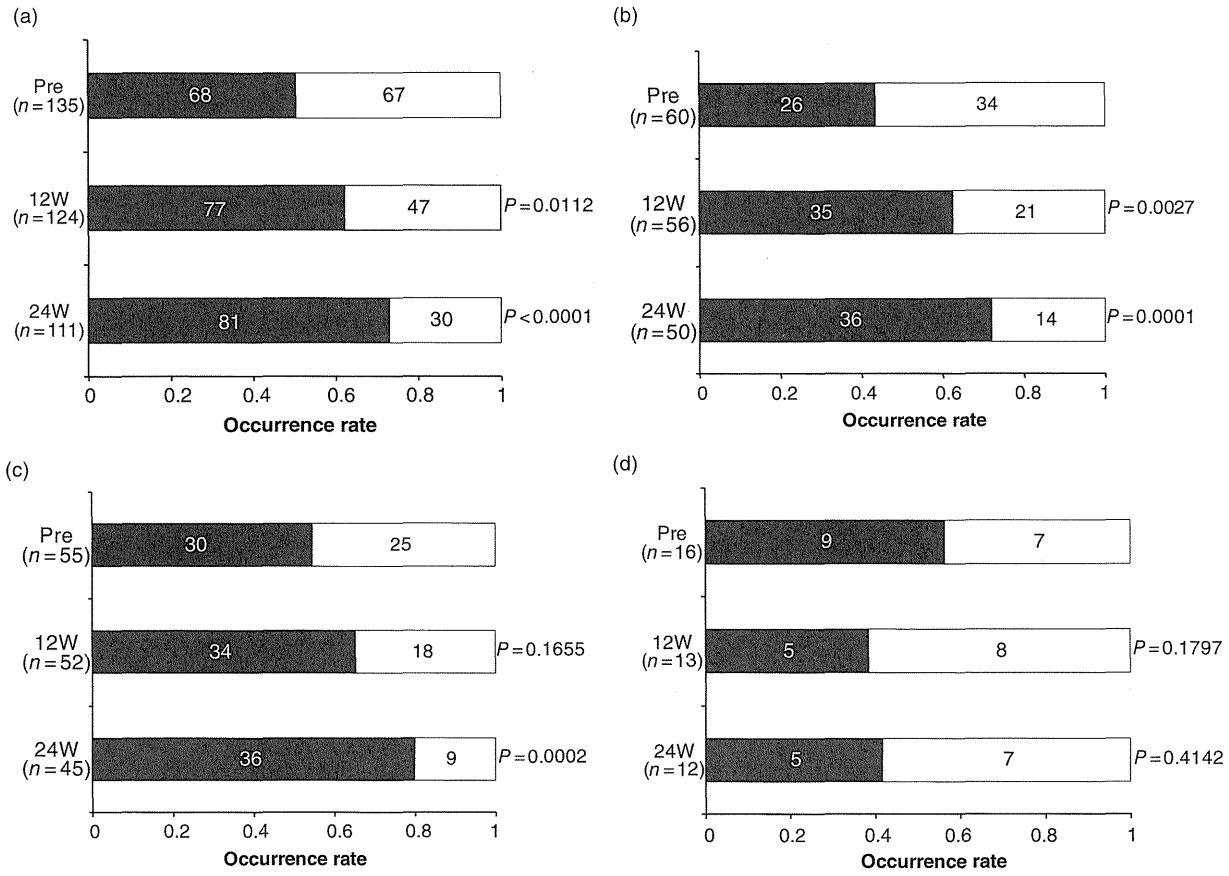

Figure 5 (1) Incidence of ascites/edema. Figures below each horizontal bar represent the number of patients falling into pertinent categories. Data were assessed using McNemar's test (in comparison to the baseline incidence) and *P*-values are presented. ■ none; □ ascites/edema. (2) Incidence of ascites/edema (Patients with increased serum albumin level at week 12). Figures below each horizontal bar represent the number of patients falling into pertinent categories. Data were assessed using McNemar's test (in comparison to the baseline incidence) and *P*-values are presented. ■ none; □ ascites/edema. (3) Incidence of ascites/edema (Patients with unchanged serum albumin level at week 12). Figures below each horizontal bar represent the number of patients falling into pertinent categories. Data were assessed using McNemar's test (in comparison to the baseline incidence) and *P*-values are presented. ■ none; □ ascites/edema. (4) Incidence of ascites/edema (Patients with decreased serum albumin level at week 12). Figures below each horizontal bar represent the number of patients falling into pertinent categories. Data were assessed using McNemar's test (in comparison to the baseline incidence) and *P*-values are presented. ■ none; □ ascites/edema.

12. Although the importance of the serum albumin level in assessing the development of ascites/edema has been reported,[14] the improvement of ascites/edema observed in the above no-change group cannot be explained by the serum albumin level alone since it remained unchanged even at week 24 in this group. The percentage of patients receiving concomitant diuretics for treatment of ascites/edema was 65.0% for the increasing group, 43.6% for the no-change group and 62.5% for the decreasing group, indicating a smaller percentage in the no-change group. This suggests that the use of concomitant diuretics might be unrelated to the improvement of ascites/edema in the no-change group. Watanabe *et al.*[15] reported that the oxidized albumin level increased with the progress of the disease state of hepatic disorders. Sakata *et al.*[16] showed that the oxidized albumin ratio correlated more positively with the extracellular fluid level than the serum albumin level. Therefore, the oxidized albumin ratio in patients with chronic hepatic disorders may be associated with body water retention such as ascites/edema. BCAA treatment reduced the oxidized/reduced albumin level once

elevated in patients with uncompensated liver cirrhosis.[17] It is thus suggested that the improvement of ascites/edema observed in the serum albumin no-change group in this study was brought about by a decreased oxidized albumin ratio following BCAA treatment, and exploration of this hypothesis is currently underway.

In conclusion, hypoalbuminemia significantly improved as well as the Child–Pugh score, an indicator for hepatic functional reserve, in patients receiving BCAA treatment. Improvement of hypoalbuminemia was also noted in patients with liver cirrhosis showing inadequate energy and protein intake. Furthermore, it is important to continue BCAA treatment beyond week 12 because the incidence of ascites/edema decreased at week 24 of treatment even in patients showing no change in the serum albumin level at week 12.

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APPENDIX

IN ADDITION TO the study authors, the investigators in the Livact study group included; Yukio Ohara, National Hospital Organization Nishi Sapporo National Hospital; Akira Saito, National Hospital Organization Nishi Saitama Chuo National Hospital; Takashi Yamaguchi, National Hospital Organization Mito Medical Center; Koutaro Kaneko, National Hospital Organization Kasumigaura Medical Center; Masakazu Kobayashi, National Hospital Organization Matsumoto Medical Center; Kiyoshi Furuta, National Hospital Organization Matsumoto Medical Center; Naohiko Masaki, National Center For Global Health and Medicine; Masahiko Takahashi, National Hospital Organization Tokyo Hospital; Toru Segawa, National Hospital Organization Murayama Medical Center;

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ORIGINAL ARTICLE—LIVER, PANCREAS, AND BILIARY TRACT

Long-term effect of lamivudine treatment on the incidence of hepatocellular carcinoma in patients with hepatitis B virus infection

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Abstract

Background Nucleotide analogues have recently been approved for the treatment of patients with hepatitis B virus (HBV) infection. However, it is still controversial whether the decrease of HBV-DNA amount induced by treatment with nucleotide analogues can reduce the risk of hepatocellular carcinoma (HCC) development in HBV patients.

Methods A total of 293 HBV patients without HCC who were treated with lamivudine (LAM) were enrolled in a multicenter trial. The incidence of HCC was examined after the start of LAM therapy, and the risk factors for liver carcinogenesis were analyzed. The mean follow-up period was 67.6 ± 27.4 months.

Results On multivariate analysis for HCC development in all patients, age ≥50 years, platelet count $<14.0 \times 10^4/mm^3$, cirrhosis, and median HBV-DNA levels of ≥4.0 log copies/ml during LAM treatment were significant risk factors. The cumulative carcinogenesis rate at 5 years was

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