**Table 2** Comparison of the suppressor of cytokine signaling 3 (SOCS3) immunostaining groups

| | SOCS3 high 31 cases | SOCS3 low 36cases | *P*-value |
|---|---|---|---|
| Age | 59.5 ± 8.1 | 54.5 ± 9.8 | 0.028 |
| Gender (male) | 16 (53%) | 21 (58%) | 0.581 |
| BMI (kg/m$^2$) | 23.3 ± 2.2 | 23.6 ± 3.5 | 0.719 |
| Viral load (KIU/mL) | 2139 ± 1367 | 2475 ± 1950 | 0.427 |
| White blood cell (/uL) | 4935 ± 1386 | 5039 ± 1384 | 0.765 |
| Hemoglobin (mg/dL) | 14.1 ± 1.1 | 14.0 ± 1.3 | 0.570 |
| Platelet (×10$^3$/uL) | 141.6 ± 41.3 | 189.5 ± 90.0 | 0.009 |
| AST (IU/L) | 94.5. ± 56.0 | 62.1 ± 33.5 | 0.003 |
| ALT (IU/L) | 119.0 ± 56.3 | 85.8 ± 52.4 | 0.015 |
| γGTP (IU/L) | 94.7 ± 70.6 | 48.8 ± 53.5 | 0.004 |
| Core 70 wild | 17 (55%) | 23 (63%) | 0.451 |
| Core 91 wild | 23 (74%) | 27 (75%) | 0.939 |
| Steatosis | 25 (81%) | 12 (33%) | 0.001 |
| Activity (severe)† | 21 (67%) | 10 (27%) | 0.001 |
| Fibrosis (severe)‡ | 26 (84%) | 19 (52%) | 0.006 |
| IL28 TT rs8099917 | 22 (71%) | 29 (80%) | 0.358 |

†Severe activity was defined as A2 or A3.
‡Severe fibrosis was defined as F2, F3, or F4.
ALT, alanine aminotransferase; AST, aspartate aminotransferase; BMI, body mass index; γGTP, gamma-glutamyl transpeptidase; HCV, hepatitis C virus.

immunostaining group than in the SOCS3 low immunostaining group. No significant difference was observed between the SOCS3 low and high groups in any of the other clinical factors (age, body mass index [BMI], viral load, white blood cell count, hemoglobin, substitution of the core 70, 91) (Table 2).

## Comparison of SOCS3 expression and the genetic variation of IL28B gene

No significant difference in the genetic variation of the IL28 TT genotype was observed between the SOCS3 low and high immunostaining groups (low : high = 80%: 71%, $P = 0.250$).

## Assessment of SOCS3 expression and genetic variation in IL28 as predictors of a sustained virological response

The age of patients in the non responder (NR) group was significantly higher than that in sustained virological response (SVR) group (SVR : NR = 52.3 ± 11.5: 59.6 ± 6.1, $P = 0.003$).

The incidence of the IL28 TT genotype was significantly lower, and that of SOCS3 high immunostaining group was significantly higher in the NR group than in the SVR group (Table 3).

As determined by a logistic regression analysis, the significant predictor of an SVR was high age (≥65 years old) (odds ratio 0.221 [0.120–0.966], $P = 0.045$), the IL28 TT genotype (odds ratio 5.422 [1.254–23.617], $P = 0.024$) and SOCS3 (high) (odds ratio 0.308 [0.104–0.948], $P = 0.040$) (Table 4).We found that two of nine (22%) patients with the IL28 TG genotype and SOCS3 high immunostaining showed a SVR, while one of seven (14%) patients with the IL28 TG genotype and SOCS3 low immunostaining, six of 22 (27%) patients with the IL28 TT genotype and SOCS3 high immunostaining, and 20 of 29 (69%) patients with the IL28 TG genotype and SOCS3 low immunostaining showed a SVR (Fig. 3).

## DISCUSSION

RECENT IMPROVEMENTS IN the efficiency of antiviral therapy have led to approximately 50% of patients with HCV genotype 1 achieving sustained viral clearance.[1–5] However, some patients are refractory to interferon therapy. A recent study reported that the presence of genetic variation near the IL28B gene (rs8099917, rs1297860) can be used as a pretreatment predictor of virlogical response to a 48-week PEG-IFN plus combination therapy in patients with HCV geno-

**Table 3** Factors associated with the response to peginterferon-α (PEG-IFN) and ribavirin

| | SVR 29 cases | NR 38 cases | *P*-value |
|---|---|---|---|
| Age | 52.8 ± 11.0 | 59.8 ± 6.4 | 0.002 |
| Gender (male) | 17 (58%) | 20 (52%) | 0.625 |
| BMI (kg/m$^2$) | 23.9 ± 3.1 | 22.9 ± 3.1 | 0.190 |
| Viral load (KIU/mL) | 2188 ± 1764 | 2420 ± 1689 | 0.587 |
| White blood cell (/uL) | 4816 ± 1427 | 5225 ± 1287 | 0.242 |
| Hemoglobin (mg/dL) | 14.1 ± 1.1 | 14.0 ± 1.3 | 0.626 |
| Platelet (×10$^3$/uL) | 176.5 ± 52.8 | 160.3 ± 89.2 | 0.350 |
| AST (IU/L) | 75.5 ± 36.1 | 78.3 ± 51.5 | 0.795 |
| ALT (IU/L) | 108.9 ± 56.8 | 95.3 ± 56.0 | 0.333 |
| γGTP (IU/L) | 63.9 ± 61.9 | 75.7 ± 68.6 | 0.464 |
| Core 70 wild | 20 (69%) | 20 (53%) | 0.176 |
| Core 91 wild | 21 (72%) | 29 (71%) | 0.173 |
| IL28 TT rs8099917 | 26 (90%) | 25 (65%) | 0.022 |
| steatosis | 14 (47%) | 23 (61%) | 0.452 |
| Activity (severe)† | 10 (34%) | 21 (64%) | 0.091 |
| Fibrosis (severe)‡ | 18 (62%) | 27 (71%) | 0.437 |
| SOCS3 (Positive) | 8 (27%) | 23 (61%) | 0.015 |

†Severe activity was defined as A2 or A3.
‡Severe fibrosis was defined as F2, F3, or F4.
ALT, alanine aminotransferase; AST, aspartate aminotransferase; BMI, body mass index; γGTP, gamma-glutamyl transpeptidase; HCV, hepatitis C virus; NR, non responder; SOCS3, suppressor of cytokine signal 3; SVR, sustained virological response.

type 1.[13–15] We previously reported that SOCS3 was a factor associated with the response to PEG-IFN treatment.[16] We compared these factors and clarified their usefulness as predictors of PEG-IFN plus combination therapy.

In the laboratory data from our patients, a significant difference between the groups with weak and strong SOCS3 staining was found in the level of AST, ALT, and platelets. These laboratory data suggested that the SOCS3 immunostained area was significantly associated with the presence of inflammation and the fibrosis stage. Indeed, in a pathological study, the inflammation and fibrosis stage were significantly different between the low and high SOCS3 immunostaining groups. This finding was consistent with our previous study that showed that the SOCS3 immunostained area was influenced by inflammation and the fibrosis stage.[16]

**Table 4** Results of a multilogistic regression analysis

| | Odds ratio | *P*-value |
|---|---|---|
| Age (>65 years) | 0.221 (0.120~0.966) | 0.045 |
| IL28 TT | 5.422 (1.254~23.617) | 0.024 |
| SOCS3 (low) | 0.308 (0.104~0.948) | 0.040 |

SOCS3, suppressor of cytokine signal 3.

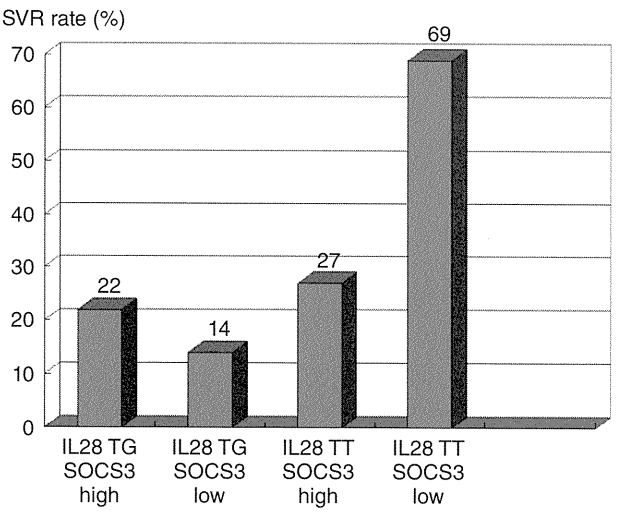


**Figure 3** A total of 12.5% of patients with IL28 TG and suppressor of cytokine signaling 3 (SOCS3) high immunostaining showed a sustained virological response (SVR), 20% of patients with IL28 TG and SOCS3 low immunostaining, 31% of patients with IL28 TT and SOCS3 high immunostaining, and 68% of patients with IL28 TG and SOCS3 low immunostaining showed a SVR.

Moreover, a significant difference between the low and high SOCS3 groups was also found in the level of γGTP. Several previous reports showed that the level of γGTP was correlated with steatosis in the liver.[7,17] In this study, the presence of steatosis also was significantly different in the low and high SOCS3 immunostaining groups. Together with our results, these results demonstrated that the SOCS3 immunostained area in the liver was associated with obesity, insulin resistance, and hepatic steatosis.[18,19]

Although recent reports showed that genetic variation of IL28B was also associated with liver inflammation and fibrosis,[20] this was not associated with the SOCS3 immunostained area in the present study. The SOCS3 proteins are known for their role as negative regulators and inhibitors of Janus kinase-signal transducer and activator of transcription (JAK-STAT) signaling, where they mediate a classical negative feedback loop in the IFN-α/β receptor signaling pathway.[21,22] The mechanism that leads to the association between genetic variation of IL28B and the effect of interferon therapy is clear, because it has been demonstrated that IL28B inhibits hepatitis C virus replication through the JAK-STAT pathway.[23] Taken together, both the SOCS3 immunostained area and IL28B polymorphisms were associated with the JAK-STAT pathway, but the different factors might interfere with JAK-STAT signaling in different ways.

The NR rate to combination PEG-IFN plus ribavirin therapy in patients with the non-TT genotype was 10–20%. The value of NR for the prediction of the genetic variation of IL28B was therefore very high. On the other hand, the SVR rate in patients with the TG genotype was about 50%. The value of SVR prediction based only on the genetic variation of IL28B was therefore not as strong for this genotype.

The substitution of core amino acids was also reported to be a predictive factor for the response to interferon therapy and was significantly associated with the genetic variation of IL28B.[24] On the other hand, the SOCS3 immunostained area was independent of both of these factors. Thus, we suggested that using a combination of the SOCS3 immunostained area with the IL28B genotype can provide the best prediction of the response to PEG-IFN plus ribavirin therapy.

Indeed, in TT genotype patients, the SVR rate in the SOCS3 weak group was about 70%, and NVR rate in the SOCS3 low immunostained group was 27%. If a liver biopsy was performed, immunostaining for SOCS3 was easy, and provided a useful predictor of the response to interferon therapy.

Our study has some limitations. Our sample size was too small. Further large-scale studies are necessary to confirm the present results and to provide a better understanding of the interactions between the SOCS3 immunostained area and the genetic variation of IL28B.

In conclusion, a combination of the SOCS3 immunostained area in the liver and the assessment of the genetic variation of IL28B seem to be good predictors of the response to PEG-IFN plus ribavirin therapy.

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***Original Article***

# Data mining reveals complex interactions of risk factors and clinical feature profiling associated with the staging of non-hepatitis B virus/non-hepatitis C virus-related hepatocellular carcinoma

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*Aim:* Non-hepatitis B virus/non-hepatitis C virus-related hepatocellular carcinoma (NBNC-HCC) is often detected at an advanced stage, and the pathology associated with the staging of NBNC-HCC remains unclear. Data mining is a set of statistical techniques which uncovers interactions and meaningful patterns of factors from a large data collection. The aims of this study were to reveal complex interactions of the risk factors and clinical feature profiling associated with the staging of NBNC-HCC using data mining techniques.

*Methods:* A database was created from 663 patients with NBNC-HCC at 20 institutions. The Milan criteria were used as staging of HCC. Complex associations of variables and clinical feature profiling with the Milan criteria were analyzed by graphical modeling and decision tree algorithm methods, respectively.

*Results:* Graphical modeling identified six factors independently associated with the Milan criteria: diagnostic year of HCC; diagnosis of liver cirrhosis; serum aspartate aminotransferase (AST); alanine aminotransferase (ALT); α-fetoprotein (AFP); and des-γ-carboxy prothrombin (DCP) levels. The decision trees were created with five variables to classify six groups of patients. Sixty-nine percent of the patients were

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within the Milan criteria, when patients showed an AFP level of 200 ng/mL or less, diagnosis of liver cirrhosis and an AST level of less than 93 IU/mL. On the other hand, 18% of the patients were within the Milan criteria, when patients showed an AFP level of more than 200 ng/mL and ALT level of 20 IU/mL or more.

*Conclusion:* Data mining disclosed complex interactions of the risk factors and clinical feature profiling associated with the staging of NBNC-HCC.

**Key words:** data mining, disease progression, hepatoma, non-viral hepatitis, tumor marker

## INTRODUCTION

HEPATOCELLULAR CARCINOMA (HCC) is the fifth most common cancer and the third most common cause of cancer-related deaths worldwide.[1–3] Chronic infection with hepatitis B virus (HBV) or hepatitis C virus (HCV) is a risk factor for HCC. Recent developments in the management of patients with viral hepatitis have resulted in early detection of HCC and improvement of prognosis.[4–8]

The number of patients with non-HBV/non-HCV-related HCC (NBNC-HCC) has been increasing, and NBNC-HCC now accounts for 12–16% of all the HCC cases in Japan.[8,9] A variety of factors are involved in the development and progression of this cancer including age, sex, alcoholic liver disease and diabetes mellitus.[10–12] Therefore, neither early detection nor improved prognosis has been achieved in NBNC-HCC.[6] Radical treatment is applicable to patients with NBNC-HCC who meet the Milan criteria;[13] however, this cancer is often detected at an advanced stage. For earlier detection, it is important to understand the complex interactions of the risk factors and clinical feature profiling associated with the Milan criteria, a staging system for NBNC-HCC.

Data mining, a set of statistical techniques, uncovers meaningful patterns and interactions of variables from a large data collection even when there is no a priori hypothesis imposed.[13] Graphical modeling is an exploratory multivariate analysis of data mining that reveals complex associations between variables.[14] This analysis assumes that the response variable is influenced by multiple factors.[15] Therefore, different from results of univariate analysis, an association between a risk factor and an outcome variable may disappear or appear because of the effects of another set of variables known as "confounding factors".[16,17] Furthermore, its findings are visualized as a graph, which provides an idea of how variables interact and denotes the conditional independence structure between random variables.[15] Therefore, graphical modeling is now identified as a new approach to model clinical data.[18]

Decision tree making is another exploratory technique of data mining that represents a series of rules for classification by identifying priorities.[19–21] It is an explicit, quantitative and systematic approach to decision-making under conditions of uncertainty and allows clinicians to choose an option that maximizes the net benefit to the patient.[22] Recently, decision trees were used to reveal the clinical feature profiling for staging of pancreatic cancer[23] and ovarian cancer.[24] However, decision trees have never been applied to identify the clinical feature profiling associated with the staging of NBNC-HCC.

The aims of this study were to reveal complex interactions of the risk factors and clinical feature profiling associated with the staging of NBNC-HCC using data mining techniques.

## METHODS

### Patient database

BETWEEN 1995 AND 2006, a total of 10 133 patients were diagnosed with HCC at 23 institutions located in Kyushu, a high morbidity area of HCC in Japan. Among them, 1363 patients were diagnosed with NBNC-HCC according to the negative results of both serum hepatitis B surface antigen and serum anti-HCV antibody or HCV RNA.

In order to examine the clinical variables associated with the staging of NBNC-HCC, a database of 663 patients with NBNC-HCC at 20 institutions was created on the basis of the following variables: diagnostic year of HCC; age; sex; family history of liver disease; past history of blood transfusion; alcohol intake; diagnosis of liver cirrhosis; diagnosis of liver disease; diagnosis of diabetes mellitus; serum aspartate aminotransferase (AST) level; serum alanine aminotransferase (ALT) level; serum α-fetoprotein (AFP) level; serum des-γ-carboxy prothrombin (DCP) level; size of HCC; and number of HCC.

For practical use, alcohol intake, serum AFP level and serum DCP level were categorized as follows. Alcohol intake: none; 60 g/day or less; 60–100 g/day; or more than 100 g/day. AFP level: 20 ng/mL or less; 20–200 ng/mL; or more than 200 ng/mL. DCP level: 40 mAU/mL or less; 40–100 mAU/mL; or more than 100 mAU/mL.

The study protocol conformed to the ethical guidelines of the 1975 Declaration of Helsinki, as reflected by the approval of the Ethics Committee of the Kurume University School of Medicine.

### Diagnosis and staging of HCC

The diagnosis of HCC was based on the clinical practice manual proposed by the Japan Society of Hepatology,[25] by using serum AFP and DCP levels and imaging techniques including ultrasonography, computerized tomography, magnetic resonance imaging, hepatic angiography and/or tumor biopsy. The Milan criteria (single nodule ≤5 cm or three nodules <3 cm) were used for the staging of HCC.[26]

### Data mining

An association between the Milan criteria and each risk factor was examined by Student's *t*-test and $\chi^2$-test. Because of the insufficient scientific evidence for testing specific clinical hypotheses, graphical modeling and decision trees were employed to explore complex associations between the Milan criteria and a set of risk factors.

MIM software (http://www.hypergraph.dk/) was used for graphical modeling. R package rpart (recursive partitioning and regression trees by Terry Therneau and Beth Atkinson; http://www.mayo.edu/biostatistics) was used to construct a decision tree algorithm. In order to evaluate the prediction error, the original data ($n = 663$) were randomly divided into a training dataset ($n = 442$) and a test dataset ($n = 221$). Ten-hold cross-validation was conducted to construct the initial tree on the basis of the training dataset; then, the optimal-size tree was constructed by examining a set of cost–complexity parameters. The overall prediction error rate as well as the sensitivity and specificity were calculated by applying the results of the decision tree algorithm to the test dataset.

## RESULTS

### Characteristics of patients with NBNC-HCC

THE PATIENTS' CHARACTERISTICS are summarized in Table 1. Family history of liver disease and history of blood transfusion were not noted in more than 80% of the patients. Approximately 40% of the patients did not have any etiology of chronic liver disease.

### Univariate analysis of variables associated with the Milan criteria

Univariate analysis showed that diagnosis of liver cirrhosis, serum AST level, serum ALT level, serum AFP level and serum DCP level were significantly associated with the Milan criteria (Table 2).

Table 1 Characteristics of all patients

| Variable | |
|---|---|
| *n* | 663 |
| Diagnostic year of HCC (years) | 2002 ± 3 |
| Age (years) | 68.1 ± 9.9 |
| Male/female | 480/183 |
| Family history of liver disease (yes/no/unclear) | 79/547/37 |
| History of blood transfusion (no/before 1989/after 1989/unclear) | 584/29/22/28 |
| Daily alcohol intake (none/<60 g/60–100 g/>100 g) | 254/183/141/85 |
| Etiology of chronic liver disease (none/alcohol/others) | 296/188/179 |
| Diagnosis of liver cirrhosis (yes/no) | 260/403 |
| Diagnosis of diabetes mellitus (no/yes without medication/yes with medication) | 396/109/158 |
| Serum AST level (U/L) | 53.3 ± 51.3 |
| Serum ALT level (U/L) | 51.8 ± 49.9 |
| Serum AFP level (ng/mL) | 9397 ± 71066 |
| Serum DCP level (mAU/mL) | 8003 ± 37377 |
| Size of HCC (cm) | 5.0 ± 3.4 |
| Number of HCC | 2.8 ± 2.9 |

Data are expressed as the mean ± standard deviation or the number of patients.
AFP, α-fetoprotein; ALT, alanine aminotransferase; AST, aspartate aminotransferase; DCP, des-γ-carboxy prothrombin; HCC, hepatocellular carcinoma.

### Graphical modeling

Complex interactions of the risk factors associated with the Milan criteria were visualized graphically (Fig. 1). Graphical modeling identified six independent factors directly associated with the Milan criteria: diagnostic year of HCC; diagnosis of liver cirrhosis; serum AST level; serum ALT level; serum AFP level; and serum DCP level (Fig. 1). Although alcohol intake, diagnosis of liver disease and diagnosis of diabetes mellitus were not directly associated with the Milan criteria, they were associated with the Milan criteria through diagnosis of liver cirrhosis (Fig. 1).

### Decision tree algorithm

With the training dataset ($n = 442$), a decision tree algorithm was created by using five variables to classify six groups of patients (Fig. 2). A serum AFP level of 200 ng/mL or less was the cut-off value for the initial

Table 2 Univariate analysis of the variables associated with the Milan criteria

| Variable | Statistical method | Test statistics | Degree of freedom (df) | *P* |
|---|---|---|---|---|
| Diagnostic year of HCC (years) | $\chi^2$ | 13.4013 | 11 | 0.2679 |
| Age (years) | Pooled | −1.07 | 661 | 0.2843 |
| Sex | $\chi^2$ | 0.2975 | 1 | 0.5854 |
| Family history of liver disease | $\chi^2$ | 1.7412 | 1 | 0.187 |
| History of blood transfusion | $\chi^2$ | 4.9527 | 2 | 0.084 |
| Daily alcohol intake | $\chi^2$ | 2.4158 | 3 | 0.4907 |
| Liver cirrhosis | $\chi^2$ | 28.9521 | 1 | <0.0001 |
| Diabetes mellitus | $\chi^2$ | 0.926 | 2 | 0.6294 |
| AST level (U/L) | Satterthwaite | 3.06 | 387.51 | 0.0023 |
| ALT level (U/L) | Satterthwaite | 4.79 | 546.95 | <0.0001 |
| AFP level (ng/mL) | $\chi^2$ | 63.1357 | 2 | <0.0001 |
| DCP level (mAU/mL) | $\chi^2$ | 47.7161 | 2 | <0.0001 |

Associations between the variables and the Milan criteria were analyzed by the indicated statistical methods. $P < 0.05$ was considered significant.
AFP, α-fetoprotein; ALT, alanine aminotransferase; AST, aspartate aminotransferase; DCP, des-γ-carboxy prothrombin; HCC, hepatocellular carcinoma.

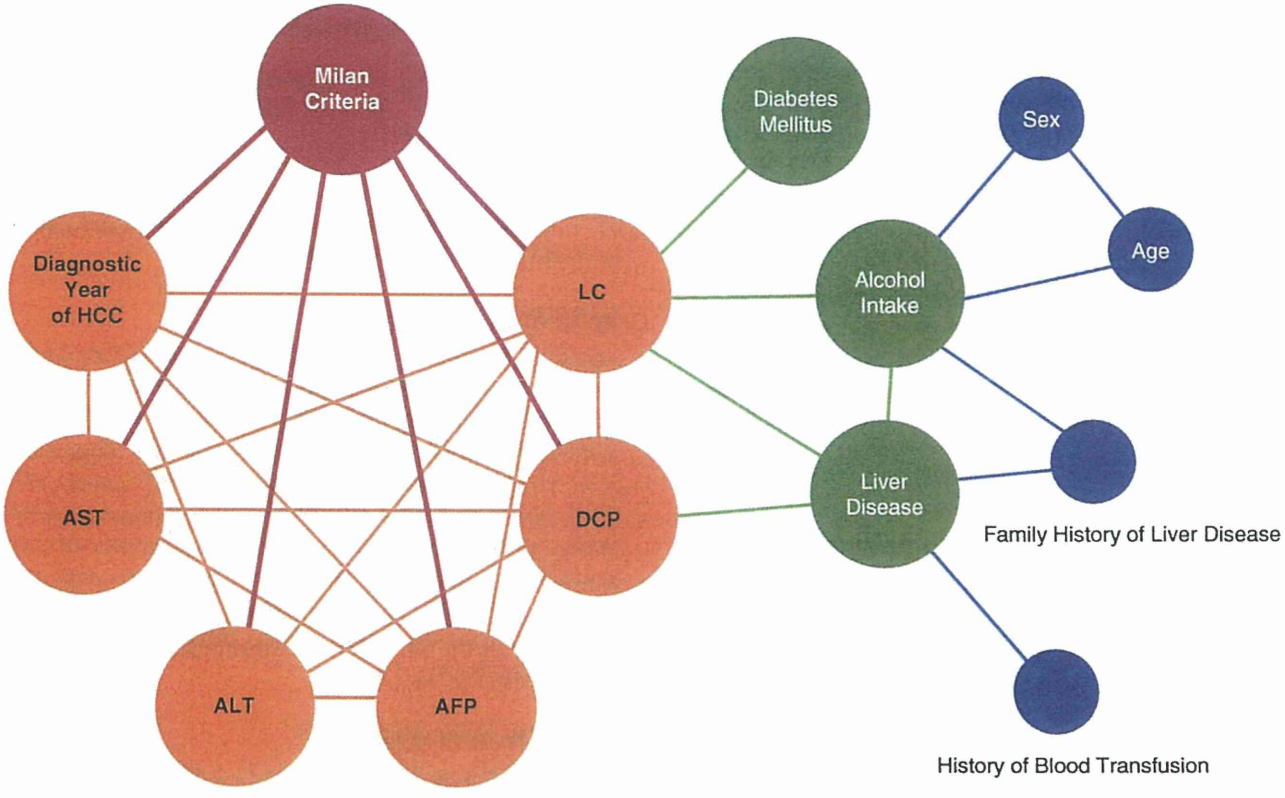


Figure 1 Graphical modeling of the interactions of the risk factors associated with the Milan criteria. AFP, α-fetoprotein; ALT, alanine aminotransferase; AST, aspartate aminotransferase; DCP, des-γ-carboxy prothrombin; HCC, hepatocellular carcinoma; LC, liver cirrhosis.

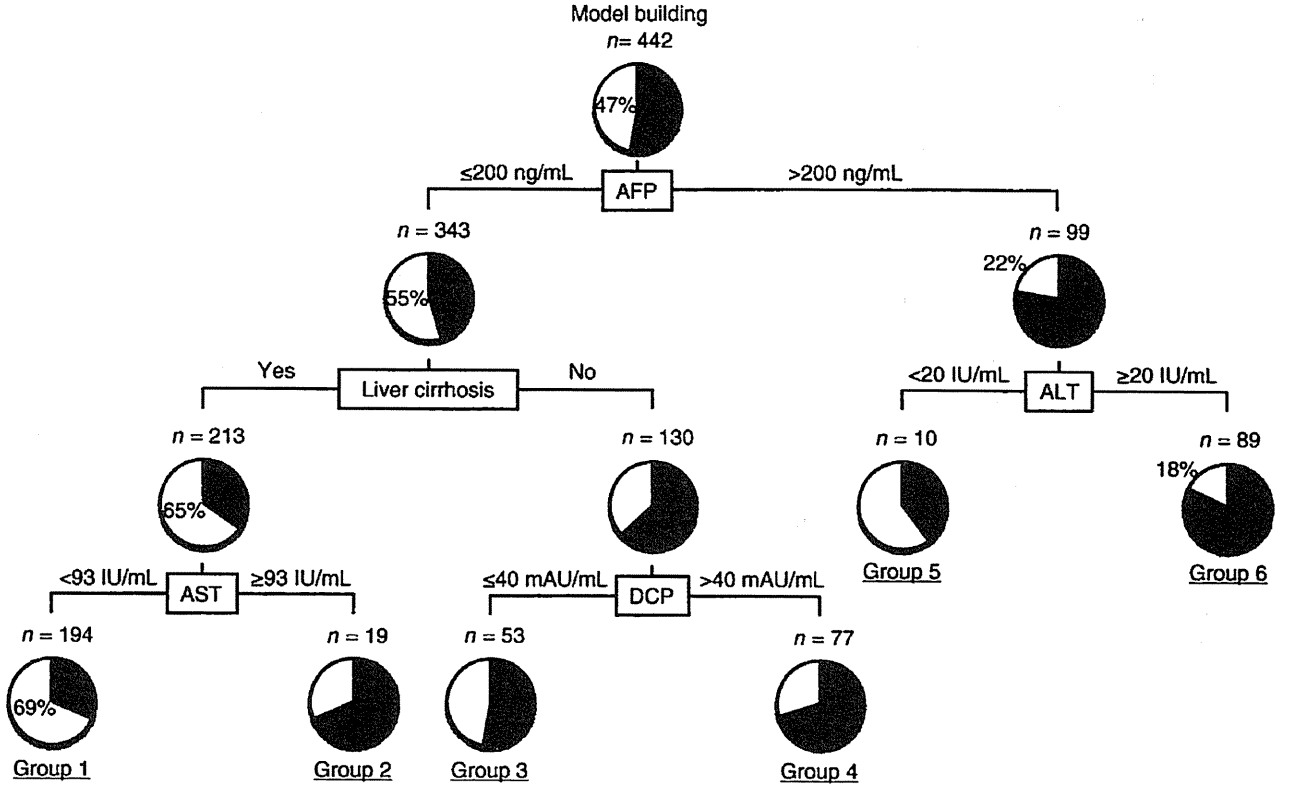


Figure 2 Decision tree algorithm of the variables associated with the Milan criteria. The patients were classified according to the indicated cut-off values of the variables. The pie graphs indicate the percentage of patients with HCC within (white)/beyond the Milan criteria in each group. AFP, α-fetoprotein; ALT, alanine aminotransferase; AST, aspartate aminotransferase; DCP, des-γ-carboxy prothrombin; HCC, hepatocellular carcinoma.

classification. Among the patients with an AFP level of 200 ng/mL or less, diagnosis of liver cirrhosis was used as the variable for the second division. Among the patients with liver cirrhosis, a serum AST level of less than 93 IU/mL was the cut-off value for the third division. Thus, 69% of the patients were within the Milan criteria, when the patients met all of the following conditions: AFP of 200 ng/mL or less; diagnosis of liver cirrhosis; and AST of less than 93 IU/mL (group 1; Fig. 2). On the other hand, only 18% of the patients were within the Milan criteria, when patients showed an AFP level of more than 200 ng/mL and an ALT level of 20 IU/mL or more (group 6; Fig. 2).

There were no significant differences in the patients' characteristics between the training dataset and the test dataset. Prediction error was obtained by applying the results of the decision tree algorithm to the test dataset. The sensitivity (proportion of patients with HCC correctly classified as beyond the Milan criteria) and specificity (proportion of patients with HCC correctly classified as within the Milan criteria) were 72.1% (75/104) and 68.4% (80/117), respectively; the overall prediction error rate was 29.8% (66/221).

## DISCUSSION

IN THIS STUDY, we revealed the complex interactions of the risk factors associated with staging of NBNC-HCC using graphical modeling. In addition, we presented a decision tree algorithm to identify clinical feature profiling associated with the staging of NBNC-HCC.

Various factors seem to be intricately related to the progression of NBNC-HCC. In this study, by graphical modeling, we identified six variables directly associated with the Milan criteria: serum AST level; serum ALT level; serum AFP level; serum DCP level; diagnosis of liver cirrhosis; and diagnostic year of HCC. Chronic hepatic inflammation modulates many of the signaling cascades involved in cell proliferation, survival and invasion of

HCC.[27,28] Further, AFP and DCP are directly associated with HCC progression through the induction of cancer cell proliferation and angiogenesis, respectively.[29,30] Thus, our results are in good accordance with previous basic investigations and suggest that hepatic inflammation as well as elevated AFP and DCP levels independently accelerate the progression of NBNC-HCC.

Diagnostic year of HCC was also directly associated with the Milan criteria in this study. Although the reason for this association is unclear, a progress in serum tumor markers is a possible explanation. Because sensitivities of AFP and DCP were improved during this study period (1995–2006),[31–33] one would think that serum AFP and DCP levels are confounding factors for an association between diagnostic year of HCC and the Milan criteria.

Recently, lifestyle-related factors including alcohol intake and diabetes mellitus have been noted as risk factors for the development of NBNC-HCC.[2,10–12,34–38] Previous *in vitro* studies showed that ethanol and glucose stimulate the proliferation and migration of HCC,[39,40] indicating the direct association of alcohol intake and diabetes mellitus with NBNC-HCC progression. However, in this study, these factors were not directly associated with the Milan criteria. Although the reason for this discrepancy remains unclear, alcohol intake and diabetes mellitus were associated with the Milan criteria through diagnosis of liver cirrhosis in this study. Both ethanol ~~co~~nsumption and diabetes mellitus can activate fibroblasts,[41,42] which are crucial components of the tumor microenvironment promoting the growth and invasion of cancer cells.[43,44] Thus, alcohol intake and diabetes mellitus may be associated with the clinical progression of NBNC-HCC through the tumor microenvironment.

Then, we created a decision tree algorithm to identify the clinical feature profiling associated with the staging of NBNC-HCC; the reproducibility of this model was confirmed by the independent validation datasets. Serum AFP level was selected for the initial classification, and serum DCP level was selected for the third division, creating groups 3 and 4. Although it is still unclear why the serum AFP level was associated with the Milan criteria to a greater extent than the serum DCP level, an association of the serum AFP level with the pathological features of HCC is a possible explanation. The AFP level is related to the number of HCC, whereas the DCP level is more specific to vascular invasion.[45–47] In this study, the staging of HCC was evaluated by using the Milan criteria, which include number and size of HCC but not vascular invasion,[26] explaining why serum AFP level was selected for the initial classification.

Diagnosis of liver cirrhosis was selected for the second division in the decision tree algorithm. Although liver cirrhosis is a well-known major risk factor for the development of HCC,[5,10,12,25,34,42] our result indicates that liver cirrhosis may suppress the progression of NBNC-HCC. We do not have any data accounting for the association between diagnosis of liver cirrhosis and suppression of the NBNC-HCC progression, the following is, however, a possible explanation for this contradiction. HCC surveillance may be performed more often in patients with liver cirrhosis than in those without liver cirrhosis,[12,25] so HCC could be identified at an early stage in patients with liver cirrhosis.

A limitation of this study is that a relationship between progression of NBNC-HCC and non-alcoholic steatohepatitis (NASH) was not evaluated. The reason is that NASH-related HCC is often diagnosed as cryptogenic cirrhosis-related HCC because of reduction of hepatic triglycerides according to the progression of NASH, so-called "burned-out NASH".[48] However, NASH is deeply involved in the development of HCC and a major reason for the increase in number of NBNC-HCC patients.[8,49,50] Recently, visceral fat accumulation is also reported to be an independent risk factor for HCC recurrence after curative treatment.[51] Thus, further study will be focused on a relationship between the progression of NBNC-HCC and NASH.

In conclusion, data mining disclosed complex associations of risk factors and clinical feature profiling associated with the staging of NBNC-HCC.

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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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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: $1605.2 \pm 478.78$ 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 $\chi^2$ 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).

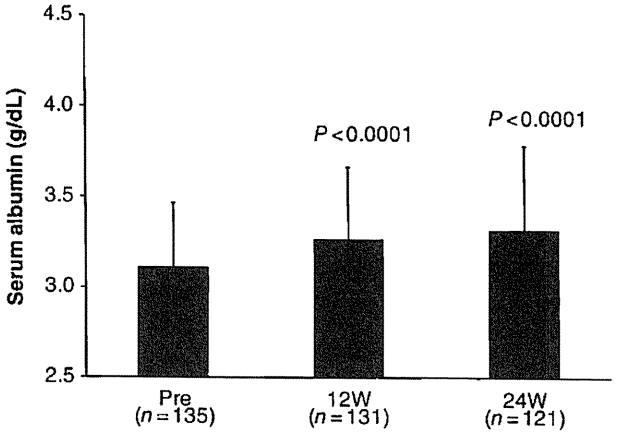


**Figure 1** Changes in serum albumin levels (mean, SD). Data were assessed using paired *t*-test (in comparison to the baseline level) and *P*-values are presented.

## 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

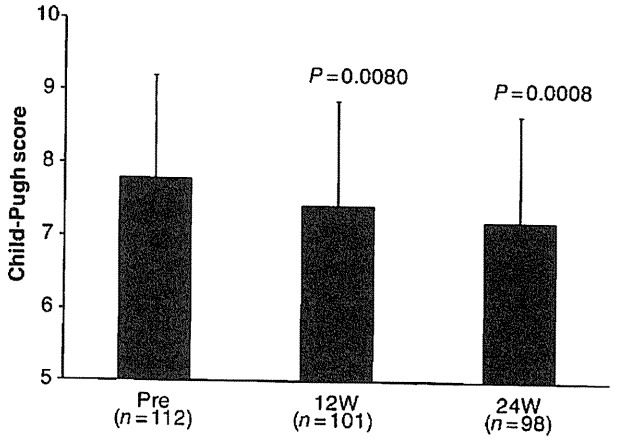


**Figure 2** Changes in Child–Pugh score (mean, SD). Data were assessed using Wilcoxon signed ranks test (in comparison to the baseline score) and *P*-values are presented.

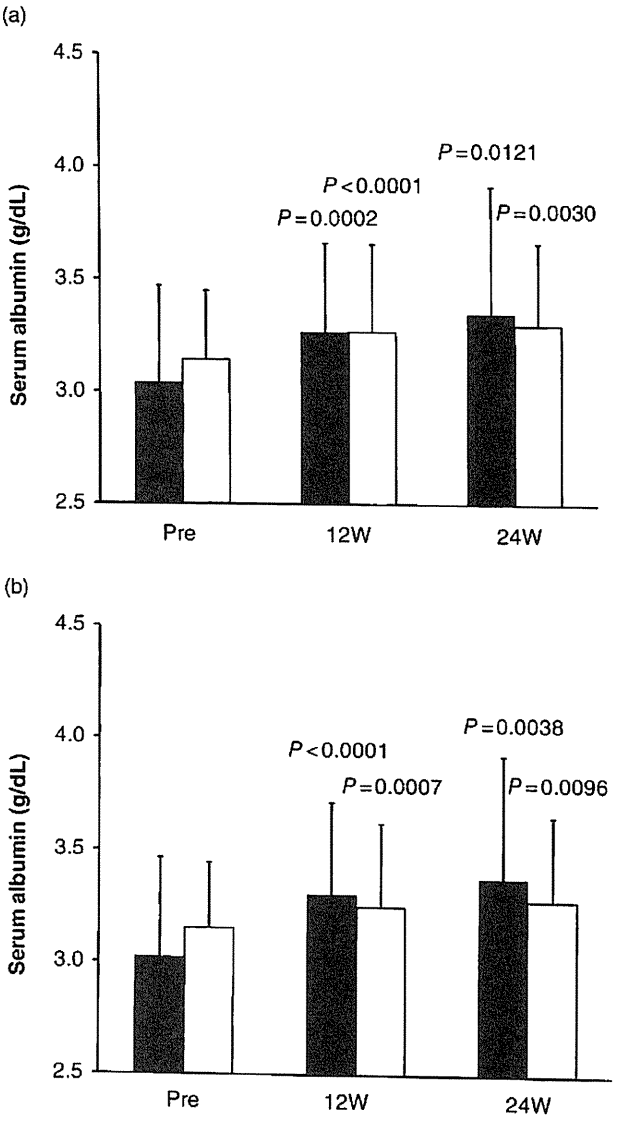


**Figure 3** (1) Changes in serum albumin levels by energy intake (mean, SD). Data were assessed using paired *t*-test (in comparison to the baseline level) by energy intake, and *P*-values are presented. The numbers of patients with less than 25 kcal/kg and 25 kcal/kg or more of energy intake were 41 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.

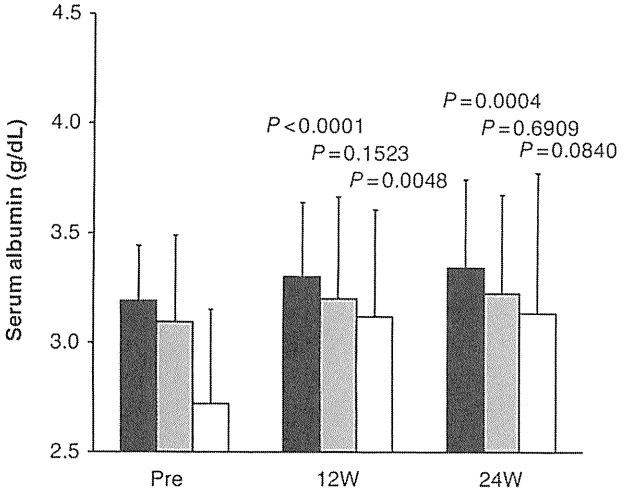


**Figure 4** Changes in serum albumin levels by degree of ascites (mean, SD). Data were assessed using paired *t*-test (in comparison to the baseline status) by degree of ascites, and *P*-values are presented. The number of patients with no ascites, mild ascites, and moderate ascites were 89, 23, and 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 $0.47 \pm 0.37$ at week 12 ($P = 0.0028$) and $0.48 \pm 0.74$ 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

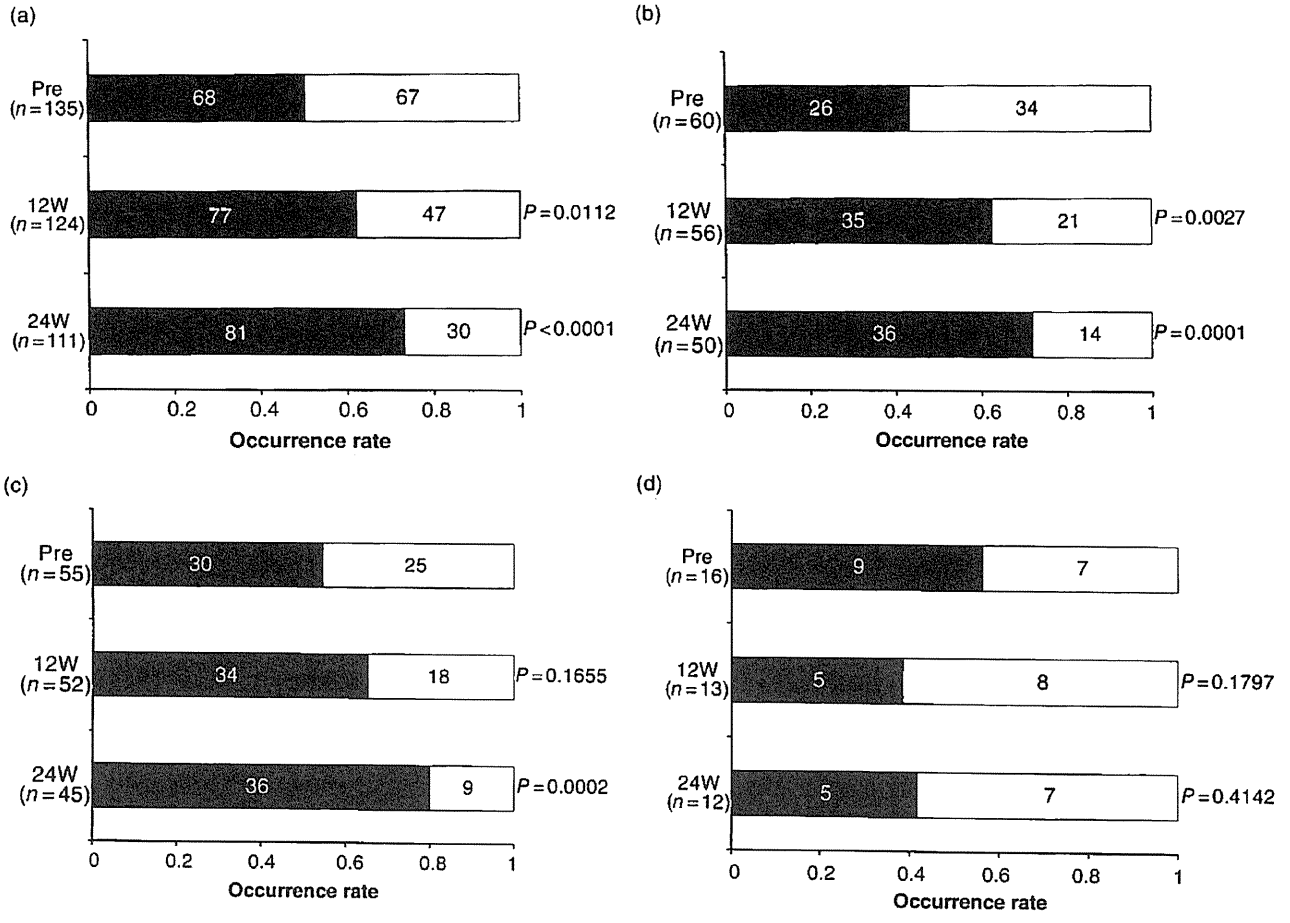


**Figure 5** (1) Incidence of ascites/edema. Figures below each horizontal bar represent the number of patients falling into pertinent categories. Data were assessed using McNemar's test (in comparison to the baseline incidence) and *P*-values are presented. ■ none; □ ascites/edema. (2) Incidence of 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;