

Table 1 Characteristics of the 28 cirrhotic patients

Characteristic	n (%) ^a [Range]
Age (years)	58.4±10.2[36–66]
Male	18 (64)
Etiology	
Alcohol	9 (32)
Hepatitis C (HCV)	12 (43)
Hepatitis B (HBV)	1 (4)
Alcohol and HCV	4 (14)
Primary biliary cirrhosis	1 (4)
Unknown	1 (4)
Laboratory parameters	
Serum albumin (g/dl)	3.03±0.52
Total bilirubin (mg/dl)	0.86±0.55
Prothrombin time (%)	59.4±20.5
Platelet count (×10 ⁴ μl)	9.1±5.2
Blood ammonia (μg/dl)	76.8±36.7
Tyrosine (nmol/ml)	136.4±45.6
Phenylalanine (nmol/ml)	91.5±24.5
Branched chain amino acids (nmol/ml)	352.1±166.5
Severity of liver disease (Child–Pugh score)	
A	9 (32)
B	14 (50)
C	5 (18)
History of overt hepatic encephalopathy	6 (21)
History of ascites	11 (39)

^a Values are means±SD

The study protocol was approved by the Human Ethics Review Committee of Iwate Medical University, Morioka, Japan.

Methods

Blood samples were collected from all patients in the morning after an overnight fast. Biochemical parameters including serum total bilirubin (T.Bil), serum albumin (Alb), platelet count (Plt), blood ammonia (NH₃), prothrombin time (PT%) as well as branched chain amino acids, BCAA (valine, leucine and isoleucine) and aromatic amino acids, AAA (phenylalanine and tyrosine) were measured. The severity of the hepatic function impairment was evaluated according to the Child–Pugh classification (Hanje and Patel 2007).

Dopamine D₂ receptor binding was measured in each region of brain between cirrhotic patients and healthy subjects. Patients were subdivided into groups according to the presence/absence of a history of encephalopathy, alcohol dependence or ascites.

Measurement of biochemical parameters

Peripheral blood counts were measured by routine auto-analyzer (Siemens, ADVIA 120 Hematology Analyzer). Biochemical parameters (T.Bil, Alb, NH₃) were measured by routine auto-analyzer (Biomajesty JCA-BM 2250, JEOL, Ltd.) using commercial kits (Total Bilirubin E-HA; Wako Pure Chemical Industries, Ltd., Aqua-

auto KAINOS: KAINOS Laboratories, Inc., Ammonia-L; Serotec Co., Ltd., respectively). PT was measured by automated coagulation analyzer (ACL-TOP, Mitsubishi Kagaku Iatron, Inc.) using a commercial kit (RecombiPlasTin, Instrumentation Laboratory, Ltd.). Plasma amino acids were measured by high performance liquid chromatography on Amino acid analyzer (L-8500, Hitachi, Ltd.).

Measurement of D₂ binding sites and designation of ROIs

A SET-1400W10 PET scanner (Shimadzu, Japan, at the Cyclotron Research Center, Iwate Medical University) was used for the study. Static scans were obtained for 80 min after intravenous injection of 75 Bq of ¹¹C-*N*-MSP. Regional brain site levels were divided by those in the D₂ receptor-free cerebellum. The pixel values in twelve ROIs corrected for the pixel value of the cerebellum were measured to determine dopamine D₂ receptor binding.

ROIs were analyzed using three dimensional stereotaxic ROI template (3DSRT). 3DSRT is a fully automated ROI-based analysis program whereby ROIs grouped into 12 segments are designated on each hemisphere of the brain by computer processing, and the use of this template program has been shown to allow objective and reproducible assessment of SPECT data in the designated ROIs. The template is described in detail elsewhere (Takeuchi et al. 2002). A typical image using ¹¹C-*N*-MSP PET with 3DSRT in a healthy control subject is presented in Fig. 1.

Statistical analysis

Mann–Whitney's *U* test was employed for testing the significant of the differences between subgroups of cirrhotic patients. Correlation analyses were carried out for the biochemical data, prothrombin data, amino acid analyses, dopamine D₂ receptor binding as well as other laboratory parameters (Alb, T.Bil, NH₃, PT%, Plt, Tyr, Phe, BCAA), Child–Pugh scores and age. For analysis of continuous variables, Pearson's correlation coefficient was used, and $p < 0.05$ was considered to indicate statistical significance. For the two continuous variables which cannot be considered to follow a normal distribution (T.Bil and NH₃) values were log-transformed prior to the analysis. For discrete variables (Child–Pugh score and age), Spearman's rank and sum correlation coefficient was determined, and $p < 0.05$ was considered to indicate statistical significance.

For multivariate analysis, linear multiple regression analysis was carried out, with the binding of dopamine D₂ receptors in each ROI of the brain serving as the dependent variable. The following were adopted as independent variables based on the results of the simple correlation analysis: Alb, T.Bil, NH₃, PT%, Plt, Tyr, Phe and BCAA, age, sex, history of alcohol dependence, encephalopathy and ascites. For multivariate analysis, dummy variables were incorporated into the history of encephalopathy, history of ascites and gender of the patients.

Statistical analyses were carried out with Dr SPSS II for Windows 11.0.1.J standard version (SPSS Inc.).

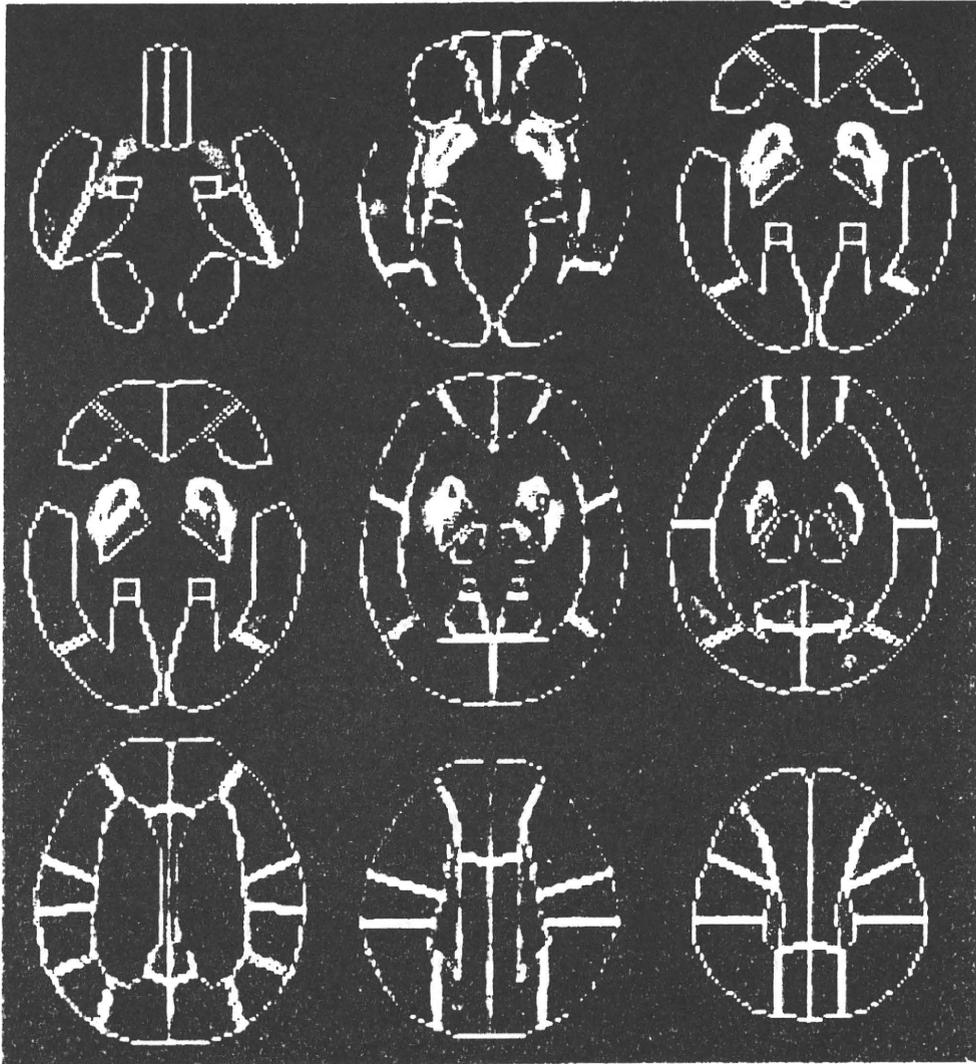


Fig. 1 Positron emission computed tomography, (PET) images using ^{11}C -N-methylspiperone in a healthy volunteer subject with ROI delineation (*white*) by 3DSRT. Number (*red*) shows each region of the brain. 1: Callosomarginal, 2: Precentral, 3: Central, 4: Parietal, 5: Angular, 6: Temporal, 7: Occipital, 8: Pericallosal, 9: Lenticular Nucleus, 10: Thalamus, 11: Hippocampus.

Results

Dopamine D_2 receptor binding in each ROI of the brain in healthy controls and liver cirrhosis patients

The dopamine D_2 receptor binding was lower in cirrhotic patients compared to that in the healthy controls in all ROI's investigated and in the thalamus and hippocampus these reductions were statistically significant (Table 2).

D_2 receptor binding was also lower in cirrhotic patients with a history of alcohol dependence where, again, the difference was statistically significant in thalamus

Table 2 Comparison of regional brain dopamine D₂ receptor binding in cirrhotic patients and healthy controls

Region of interest	D ₂ receptor binding		<i>p</i>
	Cirrhosis (<i>n</i> =28)	Control (<i>n</i> =3)	
Callosomarginal	0.71±0.15	0.81±0.17	0.24
Precentral	0.90±0.15	1.00±0.18	0.26
Central	0.36±0.23	0.50±0.27	0.23
Parietal	0.36±0.26	0.45±0.20	0.26
Angular	0.63±0.48	0.80±0.59	0.23
Temporal	1.07±0.09	1.16±0.09	0.09
Occipital	0.93±0.23	1.03±0.18	0.42
Pericallosal	0.66±0.29	0.72±0.27	0.18
Lenticular nuclei	1.74±0.21	1.96±0.18	0.12
Thalamus	0.86±0.13*	1.11±0.11	0.01
Hippocampus	0.88±0.10*	1.04±0.05	0.01

Values are means±SD

**p*<0.01 by Mann–Whitney *U* test, significantly different from control

(*p*<0.01) and hippocampus (*p*<0.02) (Table 3). Dopamine D₂ receptor binding was higher in all ROI in cirrhotic patients with history of overt HE and the difference was statistically significant for the pericallosal region and the hippocampus (Table 4). On the other hand, receptor binding between cirrhotic patients with a history of ascites was not significantly different from patients with no such history (data not shown).

Correlations between D₂ binding sites and clinical biochemical data in cirrhotic patients

Simple correlation analysis was performed in the 28 patients except for plasma amino acid analysis where data was unavailable in nine of these patients.

Table 3 Brain D₂ receptor binding in cirrhotic patients with or without alcohol dependence

Region of interest	History of alcohol dependence		<i>p</i>
	Yes (<i>n</i> =13)	No (<i>n</i> =15)	
Callosomarginal	0.68±0.11	0.73±0.17	0.39
Precentral	0.86±0.16	0.93±0.15	0.45
Central	0.30±0.20	0.41±0.26	0.32
Parietal	0.29±0.22	0.41±0.28	0.28
Angular	0.51±0.52	0.73±0.45	0.28
Temporal	1.04±0.08	1.10±0.09	0.15
Occipital	0.85±0.24	1.01±0.20	0.11
Pericallosal	0.56±0.30	0.74±0.26	0.20
Lenticular nuclei	1.69±0.16	1.78±0.24	0.27
Thalamus	0.80±0.10*	0.92±0.14	0.01
Hippocampus	0.82±0.07*	0.92±0.10	0.02

Values are means±SD

*Significantly different from patients with no history of alcohol dependence by Mann–Whitney *U* test (*p* values shown in right hand column)

Table 4 Brain D₂ receptor binding in cirrhotic patients with or without history of overt hepatic encephalopathy

Region of interest	History of overt hepatic encephalopathy		<i>p</i>
	Yes (<i>n</i> =6)	No (<i>n</i> =22)	
Callosomarginal	0.81±0.24	0.68±0.10	0.16
Precentral	1.00±0.18	0.87±0.14	0.07
Central	0.55±0.35	0.31±0.17	0.07
Parietal	0.57±0.37	0.30±0.19	0.06
Angular	0.98±0.31	0.53±0.49	0.12
Temporal	1.13±0.11	1.06±0.08	0.15
Occipital	1.12±0.20	0.88±0.22	0.12
Pericallosal	0.92±0.29*	0.59±0.25	0.02
Lenticular nuclei	1.89±0.24*	1.70±0.18	0.05
Thalamus	0.96±0.19	0.83±0.11	0.12
Hippocampus	0.97±0.12*	0.85±0.08	0.02

Values are means±SD

*Significantly different from patients with no history of hepatic encephalopathy by Mann–Whitney U test (*p* values shown in right hand column)

Dopamine D₂ receptor binding in thalamus and hippocampus showed significant positive correlations with serum total bilirubin and negative correlations with prothrombin times. D₂ receptor binding in thalamus was positively correlated with serum phenylalanine levels and Child–Pugh scores whereas D₂ receptor binding in callosomarginal, precentral, central, parietal, occipital, pericallosal cortices and hippocampus showed significant positive correlations with the age of the patient (Table 5). Using multiple linear regression analysis, D₂ receptor binding in hippocampus was significantly associated with two variables (plasma tyrosine concentrations and a history of HE). D₂ receptor binding in thalamus revealed a

Table 5 Correlations between clinical and laboratory parameters and D₂ receptor binding in cirrhotic patients

Variables	Region of Interest	Correlation coefficient	<i>P</i>
Child–Pugh scores ^a	Thalamus	0.452	0.016
Serum bilirubin	Thalamus	0.431	0.022
	Hippocampus	0.419	0.027
Prothrombin time activity	Thalamus	–0.480	0.010
Phenylalanine (plasma)	Thalamus	0.457	0.049
Age ^a	Callosomarginal	0.408	0.031
	Precentral	0.439	0.019
	Central	0.419	0.027
	Parietal	0.392	0.039
	Occipital	0.402	0.034
	Pericallosal	0.429	0.023
	Hippocampus	0.451	0.016

No significant correlations were observed between D₂ receptor binding and serum albumin, ammonia, tyrosine, branched chain or aromatic amino acids or platelet count.

Order Correlation coefficient. Association of other continuous variables to cerebral D₂ receptor binding activities was examined by Pearson's correlation coefficient

^a Association of Age and Child–Pugh scores to D₂ receptor binding was examined by Spearman Rank

significant positive correlation with plasma phenylalanine and a negative correlation with the plasma levels of BCAA. D₂ receptor binding in the lenticular nuclei was correlated with prothrombin time.

Discussion

The present study is the first to describe using PET, significant alterations of binding sites for ¹¹C-*N*-MSP in the brains of cirrhotic patients indicative of alterations of dopamine D₂ receptors in these patients. Binding site densities were found to be heterogeneous in distribution with up to five-fold differences observed between lenticular nuclei (known to be rich in D₂ receptors) and other cortical and subcortical structures. ¹¹C-*N*-MSP binding sites in thalamus and hippocampus of cirrhotic patients were significantly reduced compared to healthy controls. A previous study using SPECT and ¹²³I-iodobenzamide as ligand revealed decreased D₂ receptor sites in striatum of a cirrhotic patient (Weissenborn et al. 2000). However, in contrast to the patient population in the present study in which no patients had overt neurological symptoms, the patient studied by SPECT showed clear extrapyramidal symptoms. Earlier neurochemical studies in autopsied brain tissue from cirrhotic patients who died in hepatic coma also showed a significant loss of D₂ sites in globus pallidus/putamen (Mousseau et al. 1993) and it was suggested that loss of these sites could be the consequence of manganese deposition in the brains of these patients (Spahr et al. 1996). Manganese accumulation is the most likely cause of T₁-weighted signal hyper-intensities observed in pallidum by magnetic resonance imaging (Pomier-Layrargues et al. 1995) and such signal hyperintensities were observed in all patients enrolled in the present study. However, findings from the present study of a lack of decrease in D₂ sites in lenticular nuclei do not support the notion of a toxic effect of manganese on D₂ sites suggesting that the toxic effects of manganese occur at later stages of liver decompensation associated with overt encephalopathy. Findings from the present study of a selective loss of thalamic D₂ sites in cirrhotic patients could be expected to lead to altered levels of brain excitability and relate to previous PET findings of increased cerebral blood flow and glucose utilization in thalamus of similar patients (Lockwood et al. 1991).

Decreased D₂ binding site decreases were found to be more severe in alcoholic cirrhotic patients compared to non-alcoholic cirrhotics suggesting that alcohol (or one of its metabolites) could play a contributory role. A substantial body of evidence suggests that the brain dopamine system is implicated in the central nervous system effects of alcohol (Heinz et al. 2004) and results of a previous SPECT study suggest a role for decreased D₂ binding sites in alcohol dependence (Ebert et al. 2002). In the autopsy study of Mousseau et al. (1993), showing loss of D₂ sites, the etiology of cirrhosis was alcoholic in all cases. Taken together, these findings strongly suggest that exposure to alcohol (or its metabolites) in addition to liver-derived toxins contributes to the loss of D₂ sites in the brains of alcoholic cirrhotics.

In contrast to the apparent effects of alcohol, a previous history of overt HE did not result in greater decreases of D₂ sites. On the contrary, D₂ binding sites were significantly increased in lenticular nuclei, pericallosal area and hippocampus of patients who manifested previous episodes of HE compared to those patients who

did not. This apparently counter-intuitive finding might suggest the presence of an alternative or additional mechanism to explain the pathogenesis of HE in these patients. On the other hand, these finding could relate to decreased synaptic concentrations of dopamine resulting in upregulation of postsynaptic D₂ receptors in the brains of these patients. Studies in autopsied brain tissue from cirrhotic patients who died in hepatic coma provide evidence for a dopamine deficit; such evidence includes increased activities of monoamine oxidase (the enzyme responsible for dopamine degradation) (Mousseau et al. 1997) and increased brain concentrations of dopamine metabolites (Bergeron et al. 1989). The notion of dopaminergic deficit has clinical correlates in HE. Both the dopamine precursor amino acid and L-DOPA (Lunzer et al. 1974) and the dopamine receptor agonist bromocriptine (Morgan et al. 1980) improve the motor coordination and performance in speed-based psychometric tests in patients with chronic HE.

The magnitude of D₂ sites in thalamus of cirrhotic patients in the present study were significantly correlated with indices of severity of liver failure such as Child–Pugh scores, serum bilirubin and prothrombin times. No significant correlations were observed between D₂ site densities and serum ammonia, albumin or amino acids with the exception of phenylalanine (correlation $p < 0.05$ with D₂ sites). Phenylalanine is a precursor amino acid for the synthesis of catecholamines. However, in the case of dopamine, the rate-limiting enzyme is tyrosine hydroxylase and results of the present study showed no correlation between D₂ sites and circulating levels of tyrosine. It is unlikely therefore that alterations of D₂ sites in the brain of cirrhotic patients is a consequence of altered availability of dopamine precursor amino acids.

D₂ binding sites in cirrhotic patients showed a clear correlation with patient age in both cortical and hippocampal structures, a finding that could relate to loss of cholinergic neurons in these brain structures that are known to express D₂ receptors (Finch and Roth 1999). A greater loss of D₂ sites from these neurons could explain the more severe cognitive dysfunction that is observed in older cirrhotic patients following portal decompression by TIPS or in aged portacaval-shunted animals (Audet and Butterworth 1998). If confirmed, these findings might indicate a potentially beneficial effect of D₂ receptor agonists in this population of patients.

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Diagnosis of sub-clinical hepatic encephalopathy by Neuropsychological Tests (NP-tests)

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Aim: At present, there are no generally accepted diagnostic criteria or methods for sub-clinical hepatic encephalopathy (SHE) associated with liver cirrhosis. We therefore developed an easily conducted computer-aided quantitative neuropsychological function test system for use in routine medical practice.

Methods: The system was used to prepare basic values according to age in 542 healthy subjects, and the results were compared with 292 liver cirrhosis patients. The software is composed of eight tests: NCT-A, NCT-B, Figure Design Test, Digit Symbol Test, Block Design Test, and the Reaction Time-A, Reaction Time-B, and Reaction Time-C.

Results: Performance time is approximately 15 to 20 min. There is no need to select a specific test location and it is

convenient to use even without a professional examiner. When the top and bottom 10%, which correspond to the outlier values statistically in the healthy subjects, were used as the cutoff values, abnormal results were observed in approximately 25% of the liver cirrhosis patients. Moreover, 58% of the patients had abnormal values according to the results of at least one of the tests.

Conclusion: It is expected that this test will be used to further assess the diagnosis and pathology of SHE and that it will be utilized as a routine method of diagnosis.

Key words: liver cirrhosis, quantitative neuropsychological function test, sub-clinical hepatic encephalopathy

INTRODUCTION

HEPATIC ENCEPHALOPATHY IS a neuropsychiatric disease that develops as a result of serious liver disease, such as in fulminant hepatitis or cirrhosis, or a portosystemic shunt. Hepatic coma is used almost as a synonym, and there is a broad spectrum ranging from mild to deep coma. There is also a sub-clinical form of hepatic encephalopathy with unclear neuropsychiatric manifestations that it is only detected by quantitative neuropsychological function tests. This article reviews sub-clinical hepatic encephalopathy (SHE), including its diagnosis, the pathophysiology of the consciousness disorder and the classification of hepatic encephalopathy.

CONCEPT AND CLASSIFICATION OF HEPATIC ENCEPHALOPATHY

Overt encephalopathy

BASED ON ITS clinical course and the pattern of onset of the encephalopathy, hepatic encephalopathy is classified into an acute type, a chronic type, and a special type. The acute type is represented by fulminant hepatitis and the chronic type by liver cirrhosis in which portosystemic shunt has developed. The chronic type is subdivided into a type in which the portosystemic shunt factor is more prominent, and a type in which the hepatocellular damage factor is more prominent. Citrullinemia is a frequent cause of the special type in congenital urea-cycle enzyme abnormalities.

Problems such as cases in which hepatic encephalopathy develops as a result of a portosystemic shunt alone in the absence of liver cirrhosis, and cases of the acute type in which it is difficult to distinguish between whether the patient has hepatic encephalopathy secondary to acute liver failure or an acute onset of encephalopathy in liver cirrhosis, have been pointed out, and

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new classifications of hepatic encephalopathy have been devised, mainly in Western countries.¹

Sub-clinical hepatic encephalopathy

Abnormalities of neuropsychological function have sometimes been detected by conducting sensitive quantitative neuropsychological tests in liver cirrhosis patients who have no clear neuropsychiatric manifestations or evidence of hepatic encephalopathy clinically, and such cases are referred to as SHE. This concept lies in the part of the classification of the severity of encephalopathy that would correspond to grade 0. Decreases in motor ability and attentiveness have been pointed out clinically in SHE, and assuming that approximately half of the liver cirrhosis patients in Japan exhibit SHE, the estimated number of patients would be about 150 000.

It has not yet been determined whether SHE should be perceived as a precursor of overt hepatic encephalopathy, but 23% (5/22) of the patients in the author's study² manifested grade II or more encephalopathy within 6 months of being diagnosed with SHE for the first time, and in some of the cases diagnosed as SHE it can be viewed as a precursor of overt hepatic encephalopathy.

Although there have been criticisms that the significance of a diagnosis of SHE is not very important clinically,³ based on studies showing a clear decrease in quality of life (QOL) of patients with SHE,⁴ their difficulty in performing complex actions, such as driving maneuvers, etc.,⁵ and a negative impact on the outcome of liver cirrhosis,^{6–8} Sub-clinical hepatic encephalopathy is of diagnostic significance clinically, and in Western countries the term minimal hepatic encephalopathy has been proposed.¹

DIAGNOSIS AND DIFFERENTIAL DIAGNOSIS OF HEPATIC ENCEPHALOPATHY

Diagnosis and differential diagnosis of overt hepatic encephalopathy

THE DIAGNOSIS OF coma is evaluated on the basis of the Inuyama classification, however, judging grade I coma is often problematic. Amodio *et al.*⁹ have proposed a diagnostic evaluation method from grade I to grade IV based on tests described below that include quantitative neuropsychological function tests (Table 1).

When there is no clear preceding liver disease and when the patient has liver cirrhosis but the diagnosis of hepatic encephalopathy based on the results of clinical

Table 1 Modification of West Haven Criteria for the grading of mental state in patients with cirrhosis

Grade	Proposed operative definition
0	
I	<ul style="list-style-type: none"> • Not able to complete Trail-Making test A in 120 sec, or naming <7 animals in 120 sec • Oriented in time and space
II	<ul style="list-style-type: none"> • Disoriented in time: (>3 items incorrect) Day of the week, day of the month, the month, the year • Orientated in place
III	<ul style="list-style-type: none"> • Disoriented in place: (>3 items incorrect) state/country, region/country, city, place, floor/ward • Disorientated in time and reduction of Glasgow score (8–14)
IV	<ul style="list-style-type: none"> • Unresponsive to pain stimuli Glasgow score (<8)

tests is not definitive, additional tests must be performed to differentiate it from other diseases that give rise to consciousness disorders. Brain computed tomography (CT) scans, cerebrospinal fluid findings, etc., are useful in making the differential diagnosis from central nervous system diseases, and blood glucose, urinary ketone body, blood gas, and serum electrolyte values are useful in making the differential diagnosis from diabetic ketoacidosis. Since alcohol-dependent patients who have chronic liver disease sometimes have subdural hematomas as a result of head injuries or have alcohol withdrawal syndrome, it is especially important to make the differential diagnosis from hepatic encephalopathy.

Diagnosis of sub-clinical hepatic encephalopathy

In order to make the diagnosis of SHE strictly, (i) it seems necessary to ask questions related to QOL, including changes in behavior patterns of daily living, such as appetite, sleep, activity level, etc., and (ii) questions related to changes in mental status, such as in memory, concentration, ability to concentrate, cognitive ability, etc. (iii) It also appears important to modify the conventional classification of coma severity in overt hepatic encephalopathy and make the diagnosis by adding relatively convenient quantitative neuropsychological function tests. In addition, (iv) it is important to conduct comprehensive neuropsychological function tests, and tests for both linguistic cognitive function disorders, such as dysarthria, and for motor cognitive function disorders, such as of reaction time, spatial perception, etc.⁹

Table 2 Diagnosis of sub-clinical hepatic encephalopathy

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- Quantitative neuropsychological function test
 - Trail making test A and B
 - Reaction time to light and sound
 - Wechsler Adult Intelligence Scale (WAIS-R)
 - Block design test
 - Digit Symbol test
 - Electrophysiological examination
 - Electroencephalogram
 - Auditory and visual evoked potentials
 - P300 evoked potentials
 - Radiological examination
 - Magnetic resonance spectroscopy
 - Positron emission tomography
-

Nevertheless, it is difficult to perform all of the above in ordinary clinical practice, and usually an attempt is made to make the diagnosis on the basis of a combination of quantitative neuropsychological function tests (trail making test, reaction time to light and sound), the Wechsler Adult Intelligence Scale (WAIS), etc., and the EEG and cerebral evoked potentials (auditory, visual), which are electrophysiological tests¹⁰ (Table 2).

Quantitative neuropsychological function tests

The WAIS and other quantitative neuropsychological function tests are used as convenient so-called “paper and pencil” diagnostic tests to make the diagnosis of SHE. Intelligence tests assume that intelligence is a set of several abilities, and they determine the degree to which the cognitive functions of the brain have been impaired based on the level of successful completion of the tests. When intelligence test items encompass a diversity of diverse tests, such as tests of spatial perception, psychomotor ability (psychomotor function, eye-hand coordination), memory, attentiveness, etc, they are difficult to conduct in ordinary clinical practice.

Since SHE is characterized by a marked decrease in motor cognitive ability, whereas linguistic cognitive ability in the form of knowledge, counting, and words is relatively preserved, three tests are conducted, the Block Design Test of the WAIS value, the Digit Symbol Test, and the Number Connection Test Part A or Part B (NCT-A, NCT-B). Sub-clinical hepatic encephalopathy is often diagnosed when the results of any one of the tests are abnormal.

The frequency of SHE when the WAIS and Symbol Connection Test were used in combination was 25–80%, and thus there was a wide range in the fre-

quency of abnormal results, and the fact that the frequency of pathology termed “sub-clinical” varied with the test that was conducted was a problem. With the aim of resolving these problems, Weissenborn *et al.*¹¹ recommended five tests, including the NCT-A, NCT-B, and Digit Symbol Test, but since two of the tests in the combination, the Line Drawing Test and the Serial Dotting Test, are not very well known in Japan, it would not necessarily be easy to include them.

With paper-and-pencil type neuropsychological function tests, it is considered necessary to have a trained examiner conduct them under standardized environmental conditions in order to achieve reproducibility, and while they can be conveniently conducted in ordinary clinical practice, caution is required when evaluating them.

Moreover, when making comparisons between countries, differences in education level and cultural background must basically be taken into consideration and assessed when evaluating neuropsychological functions.^{12,13}

Electroencephalography mapping (topography; isopotential maps)

Changes in responses to electrophysiological tests occur in liver cirrhosis as a result of the effect of toxic substances, including ammonia, and of electrolyte imbalances and energy metabolism abnormalities, and electroencephalography (EEG) and evoked potential alterations are often observed even in SHE in which clinical, overt encephalopathy is not observed.¹⁴

Numerous EEG analyses of overt hepatic encephalopathy have been carried out in the past, but most of them have been based on visual evaluations (Parson-Smith classification),¹⁵ and a subjective element entered into them. When the EEG in SHE is analyzed in the usual manner, a decrease in α waves (12–8 c/s) and increase in θ waves (7–4 c/s) is observed in both cerebral hemispheres, and so-called slow-wave activity is occasionally seen, but the frequency of the abnormality is 9% to 33% and not very high.¹⁶

By contrast, EEG mapping by frequency analysis has made it possible to analyze the EEG objectively, and there is a report of a high rate of brain wave slowing of 83%.¹⁷ Our own results¹⁸) showed a decrease in the frequency of α waves ($\% \alpha$) in the occipital area in 86% and an increase in θ -wave frequency ($\% \theta$) and δ -wave frequency ($\% \delta$) in 40% to 70%.

Amodio *et al.*¹⁶ claimed that it is possible to diagnose SHE based on EEG abnormalities, and they have even reported that there is a rough correlation between

Table 3 Composition of neuropsychological test (NP-test)

1	Number connection test A	(NCT-A)
2	Number connection test B	(NCT-B)
3	Figure position test	(FPT)
4	Digit symbol test	(DST)
5	Block design test	(BDT)
6	Reaction time test A	(RTT-A)
7	Reaction time test B	(RTT-B)
8	Reaction time test C	(RTT-C)

ammonia concentrations and SHE becoming overt, but their theory has not always been accepted.¹⁹

Cerebral evoked potentials

Diagnosis of SHE on the basis of evoked potentials has also been reported, and prolongations of the latency of auditory evoked potentials, etc., has been reported. More specifically, visual, auditory, and somatosensory evoked potentials and p300 event-related potentials have been used, but investigators' views differ as to which potential is the most useful for diagnosing SHE.^{20–22}

Moreover, because of the fact that the abnormalities are not specific for SHE, that similar changes are also seen in alcohol-dependency²³ and diabetics,^{24,25} and that there is no clear association with the degree of liver damage or outcome, they do not necessarily appear to be useful.

Quantitative neuropsychological function testing by computer

Based on multicenter cooperative research the authors have developed new software for the conventional WAIS

revised version (WAIS-R) with a personal computer.²⁶ The software is composed of 8 tests, the NCT-A, NCT-B, Figure Design Test, Digit Symbol Test, Block Design Test, and the Reaction Time-A, Reaction Time-B, and Reaction Time-C tests (Table 3). Performance time is approximately 15 to 20 min. There is no need to select a specific test location, and it is convenient to use even without a professional examiner. The system was used to prepare basic values according to age in 542 healthy subjects, and the results were compared with 292 liver cirrhosis patients (Table 4). When the top and bottom 10%, which correspond to the "outlier values" statistically in the healthy subjects, were used as the cutoff values abnormal results were observed in approximately 25% of the liver cirrhosis patients. Moreover, 58% of the patients had abnormal values according to the results of at least one of the tests. Whether to make abnormal results on just one of the tests or on more than one of the tests the criterion for the diagnosis of SHE will require further study, but it is expected that this test will be used to further assess the diagnosis and pathology of SHE and that it will be utilized as a routine method of diagnosis.

CONCLUSIONS

SCHOMERUS *ET AL.*²⁷ conducted the neuropsychological function testing described above in 40 liver cirrhosis patients without overt hepatic encephalopathy and then assessed them in regard to fitness to drive. The results showed that 60% of the patients were unfit to drive a motor vehicle. The results also showed that 25%

Table 4 Clinical and laboratory characteristics of patients and healthy subjects

	Cirrhotic patients (n = 292)	Healthy subjects (n = 542)	P-value
Male/female ratio	65 : 32	51 : 49	P < 0.0001*
Age (year), mean ± SD	59.0 ± 7.3	52.6 ± 7.9	P < 0.0001**
Age (year) ratio, (n)			
40–44	5 (14)	18 (98)	
45–49	9 (27)	21 (112)	
50–54	11 (33)	20 (112)	
55–59	19 (54)	18 (99)	
60–64	28 (82)	15 (79)	
65–69	28 (82)	8 (45)	
Total bilirubin (mg/dL)	1.6 ± 1.9		
ALT (IU)	70.1 ± 50.0		
Child (A/B/C) ratio	45/46/9		
Virus/alcohol/others ratio	81/13/6		

*Chi-square test, **students-t test.

of the patients were capable of limited driving only. The investigators also found significantly more persons unfit to drive in an alcoholic liver cirrhosis group than in a non-alcoholic liver cirrhosis group. By contrast, Srivastava *et al.*²⁸ compared 15 patients with non-alcoholic liver cirrhosis with abnormal results on neuropsychological function tests and healthy subjects with the same age composition in regard to actual driving ability on the road and claimed to have found no significant differences between the results in the two groups. It is impossible to make simple comparisons between the two groups, but such results have a great impact on society, and further study appears necessary.

Groeneweg *et al.*⁴ and Marchesini *et al.*²⁹ evaluated the QOL of SHE patients by the Sickness Impact Profile questionnaire and the SF-36, respectively, but they both found a reduction in QOL. Schomerus *et al.*³⁰ reported that ability to perform manual labor and learning ability were reduced in SHE, but they were not prospective studies, and investigating changes in response to treatment also appears to be a future task.

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Supplementation with Branched-chain Amino Acids Inhibits Azoxymethane-induced Colonic Preneoplastic Lesions in Male C57BL/KsJ-*db/db* Mice

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Abstract Purpose: Obesity and related metabolic abnormalities, including insulin resistance and activation of the insulin-like growth factor (IGF)/IGF-I receptor (IGF-IR) axis, are risk factors for colon cancer. Supplementation with branched-chain amino acids (BCAA) reduces the risk of liver cancer in cirrhotic patients who are obese, and this has been associated with an improvement of insulin resistance. The present study examined the effects of BCAA on the development of azoxymethane (AOM)-initiated colonic premalignant lesions in C57BL/KsJ-*db/db* (*db/db*) mice that were obese and had hyperinsulinemia.

Experimental Design: Male *db/db* mice were given 4 weekly s.c. injections of AOM (15 mg/kg of body weight) and then they were fed a diet containing 3.0% BCAA or casein, a nitrogen content – matched control diet, for 7 weeks.

Results: Feeding with BCAA caused a significant reduction in the number of total aberrant crypt foci and β -catenin accumulated crypts, both of which are premalignant lesions of the colon, compared with the control diet – fed groups. BCAA supplementation caused a marked decrease in the expression of IGF-IR, the phosphorylated form of IGF-IR, phosphorylated glycogen synthase kinase 3 β , phosphorylated Akt, and cyclooxygenase-2 proteins on the colonic mucosa of AOM-treated mice. The serum levels of insulin, IGF-I, IGF-II, triglyceride, total cholesterol, and leptin were also decreased by supplementation with BCAA.

Conclusion: BCAA supplementation in diet improves insulin resistance and inhibits the activation of the IGF/IGF-IR axis, thereby preventing the development of colonic premalignancies in an obesity-related colon cancer model that was also associated with hyperlipidemia and hyperinsulinemia. BCAA, therefore, may be a useful chemoprevention modality for colon cancer in obese people.

Colorectal cancer (CRC) is a major health problem worldwide. Recent evidence indicates that the risk of CRC is elevated in patients with metabolic syndrome, also called insulin resistance syndrome, which is commonly associated with obesity and related metabolic abnormalities (1, 2). Obesity is the main determinant of insulin resistance and hyperinsulinemia, which is also a possible risk factor for CRC (3). CRC occurs more frequently in patients with diabetes mellitus, a condition associated with hyperinsulinemia (4, 5). Insulin has growth-

promoting properties in CRC cells, and exogenous insulin injection stimulates the growth of CRC precursors in rodent models (6–8). In addition, elevated circulating levels of insulin causes alterations in the insulin-like growth factor (IGF)/IGF-I receptor (IGF-IR) axis, which is involved in the development and progression of CRC (9, 10). Therefore, increased insulin resistance and abnormalities in the IGF/IGF-IR axis might be a critical target to prevent the development of obesity-related malignancies, including CRC. For instance, (-)-epigallocatechin gallate, the major biologically active component of green tea, inhibited the development of colonic premalignant lesions in an obesity-related colon cancer that was associated with improvement in insulin resistance and inhibition of the IGF/IGF-IR axis (11).

Diet supplementation with branched-chain amino acids (BCAA; leucine, isoleucine, and valine) has been suggested to improve protein malnutrition in patients with liver cirrhosis (12). Recent studies have revealed that BCAA is useful for both preventing progressive hepatic failure and improving event-free survival in patients with chronic liver diseases, such as liver cirrhosis, and these beneficial effects are associated with the improvement of insulin resistance by BCAA (13–15). In addition, oral supplemental treatment with BCAA can reduce the risk of hepatocellular carcinoma in cirrhotic patients who

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Translational Relevance

Obesity and related metabolic abnormalities, including insulin resistance and the activation of the insulin-like growth factor (IGF)/IGF-I receptor axis, are associated with colorectal cancer (CRC) development. Therefore, the prevention of CRC by targeting the dysregulation of energy homeostasis might be a promising strategy for obese people who are at increased risks of CRC. We believe that this study is novel and clinically relevant because this article is the first report indicating that supplementation with branched-chain amino acids (BCAA) effectively suppressed the development of azoxymethane-induced putative precursor lesions of colonic adenocarcinoma in C57BL/KsJ-*db/db* mice that are obese and developed diabetes mellitus. Our studies indicate that this suppressing effect of BCAA was associated with improvement of hyperlipidemia and hyperleptinemia. BCAA supplementation could also improve insulin resistance and exert a depressant effect on the IGF/IGF-IR axis. The current findings suggest the possibility of using BCAA as a chemopreventive agent for obesity-related malignancies.

are obese (with a body mass index ≥ 25 ; ref. 16). Obesity, hyperinsulinemia, and diabetes mellitus are possible risk factors for hepatocellular carcinoma, which commonly develops in cirrhotic livers (16–18). Based on these findings, BCAA supplementation in diet may also reduce the risk of other obesity-related human malignancies, including CRC, by improving insulin resistance. However, no detailed studies on whether BCAA can prevent the development of obesity-related CRC have yet been conducted.

In previous studies, we have established a useful preclinical animal model to determine the possible underlying mechanisms of how specific agents prevent the development of obesity-related CRC with the use of C57BL/KsJ-*db/db* (*db/db*) mice with obesity, hyperinsulinemia, and hyperleptinemia (19–21). The mice are susceptible to the colonic carcinogen azoxymethane (AOM) because the development of AOM-induced aberrant crypt foci (ACF) and β -catenin-accumulated crypts (BCAC), both of which are putative precursor lesions for colonic adenocarcinoma (22, 23), is enhanced in *db/db* mice compared with *db/+* or *+/+* mice (19, 20). In the present study, we investigated in detail the effects of BCAA on the development of colonic premalignant lesions, ACF and BCAC, in *db/db* mice initiated with AOM, focusing on the improvement of hyperinsulinemia, hyperlipidemia, and hyperleptinemia. In addition, we also determined whether BCAA supplementation in the diet inhibits the activation of the IGF/IGF-IR axis in this animal model.

Materials and Methods

Animals, chemicals, and diets. Four-week-old male homozygous *db/db* mice were obtained from Japan SLC, Inc. All mice were maintained at the Gifu University Life Science Research Center according to the Institutional Animal Care Guidelines. AOM was purchased from Sigma Chemical Co. BCAA and casein were obtained from Ajinomoto Co., Ltd. The BCAA composition (2:1:1.2, leucine/isoleucine/valine) was set at the

clinical dosage that is used for the treatment of hypoalbuminemia in patients with decompensated liver cirrhosis in Japan.

Experimental procedure. The animal experiment was approved by the Institutional Committee of Animal Experiments of Gifu University. A total of 54 male *db/db* mice were divided into 6 groups. At 5 wk of age, the mice in groups 1 to 3 were s.c. injected with AOM (15 mg/kg of body weight) weekly for 4 wk. As controls, the mice in groups 4 to 6 were given s.c. injections of saline. Groups 1 (12 mice) and 4 (6 mice) were fed a basal diet, corticotropin-releasing factor (CRF)-1 (Oriental Yeast Co., Ltd.), throughout the experiment. Groups 3 (12 mice) and 6 (6 mice) were given a basal diet containing 3.0% BCAA (weight for weight) for 7 wk, starting 1 wk after the last injection of AOM. The BCAA concentration (3.0%) was determined by the previous study, which indicated the same intake to improve insulin resistance in C57BL/6J mice (24). The mice in groups 2 (12 mice) and 5 (6 mice) were given a basal diet containing 3.0% casein (weight for weight). The casein-fed groups were served as nitrogen content-matched controls for the BCAA-treated groups to eliminate the possibility that the nitrogen content itself affects the promotion or the prevention of colonic premalignant lesions. At the termination of the study (16 wk of age), the mice were sacrificed by CO₂ asphyxiation to analyze the number of colonic ACF and BCAC.

Counting the number of ACF and BCAC. The ACF and BCAC were determined according to the standard procedures described previously (20, 21, 25). ACF are defined as single or multiple crypts that have altered luminal openings, exhibit thickened epithelia, and are larger than adjacent normal crypts (22). BCAC, which have high frequency mutations in the β -catenin gene, show histologic dysplasia with a disruption of the cellular morphology and an accumulation of this protein (Fig. 1A; ref. 23). BCAC do not have a typical ACF-like appearance because the lesion is not recognized on the mucosal surface like ACF and is only identified in the histologic sections of en face preparations. Both of these lesions are utilized as biomarkers to evaluate a number of agents for their potential chemopreventive properties (26). After the colons were fixed flat in 10% buffered formalin for 24 h, the mucosal surface of the colons were stained with methylene blue (0.5% in distilled water), and then the number of ACF were counted under a light microscope. Thereafter, the distal parts (5 cm from the anus) of the colon were cut to count the number of BCAC. To identify BCAC intramucosal lesions, the distal part of the colon (mean area, 0.7 cm² per colon) was embedded in paraffin, and then a total of 20 serial sections (4- μ m thick each) per colon were made by an en face preparation (20, 21, 25). For each case, 2 serial sections were used to analyze BCAC.

Histopathology and immunohistochemical analyses for β -catenin and PCNA. Three serial sections were made from paraffin-embedded tissue blocks. Two sections were subjected to H&E staining for histopathology and β -catenin immunohistochemistry to count the number of BCAC. The other section was used for the proliferating cell nuclear antigen (PCNA), a G₁-to-S phase marker, immunohistochemistry to estimate the cell proliferative activity in the colonic mucosa. Immunohistochemical analyses for β -catenin and PCNA were done with the labeled streptavidin-biotin method (LSAB kit; DAKO) as previously described (20, 21). Anti- β -catenin antibody (1:1,000 final dilution) was obtained from Transduction Laboratories (catalogue no. 610154). Anti-PCNA antibody (1:100 final dilution) was from Santa Cruz Biotechnology, Inc. (sc-7907). Negative control sections were immunostained without the primary antibody. PCNA-positive cells in the colonic mucosa, which seemed normal by H&E staining, were counted and expressed as a percentage of the total number of normal crypt cells. The PCNA labeling index (%) was determined by counting at least 200 crypt cells in each mouse (a total of 1,000 crypt cells per group). Two experienced pathologists (Y. Hirose and T. Tanaka) immunohistologically determined the BCAC and PCNA-positive cells.

Protein extraction and western blot analysis. Total proteins were extracted from the scraped mucosa from the remaining colon of the AOM-treated mice (groups 1 to 3), and equivalent amounts of proteins

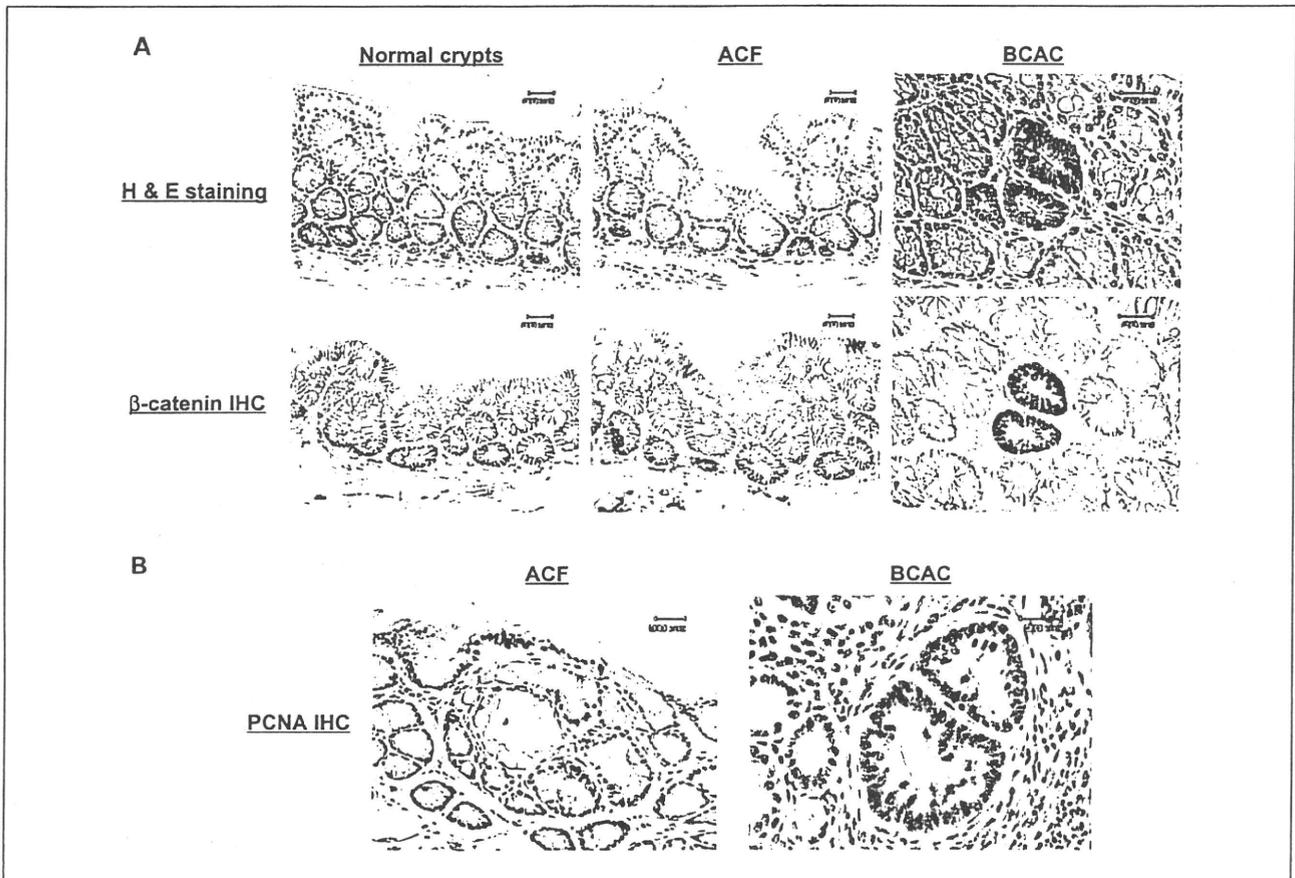


Fig. 1. Histopathology and immunohistochemical expression of β -catenin and PCNA proteins in ACF and BCAC. *A*, representative photographs of ACF and BCAC induced by AOM in *db/db* mice. Top, H&E staining; bottom, β -catenin immunohistochemistry. Left, normal crypts; middle, ACF; right, BCAC. The localization of the accumulated β -catenin protein is apparent in the cytoplasm and nucleus of atypical cryptal cells in BCAC. *B*, immunohistochemical pattern of PCNA protein in ACF and BCAC. The nuclear expression of the PCNA protein significantly increased in BCAC compared with ACF and surrounding normal crypts. Bar, 20 or 30 μ m, respectively.

(40 μ g per lane) were examined by a western blot analysis with the use of the primary antibodies for IGF-IR, phosphorylated IGF-IR (p-IGF-IR), phosphorylated glycogen synthase kinase 3 β (p-GSK-3 β), Akt, phosphorylated Akt (p-Akt), cyclooxygenase-2 (COX-2), and glyceraldehyde-3-phosphate dehydrogenase as described previously

(11, 27, 28). An antibody to glyceraldehyde-3-phosphate dehydrogenase served as a loading control. The intensities of the blots were quantified with the NIH Image software version 1.62. The intensities of the blots found at the CRF-fed mice in each antibody was set at 1, and the changes in expression were shown as the fold difference.

Table 1. Body, liver, kidney, and white adipose tissue weights of the experimental mice

Group no.	Treatment	Diet	No. of mice	Final body weight (g)	Body length (cm)	BMI	Absolute organ weight		
							Liver	Kidney	White adipose tissue
1	AOM 15 mg/kg	CRF-1	12	49.7 \pm 8.3 ^{*†}	9.25 \pm 0.77	0.58 \pm 0.05	2.64 \pm 0.76	0.38 \pm 0.04 [†]	2.67 \pm 0.64
2	AOM 15 mg/kg	Casein	12	51.7 \pm 4.8	9.43 \pm 0.37	0.58 \pm 0.02	2.75 \pm 0.48 [†]	0.41 \pm 0.04	2.74 \pm 0.37
3	AOM 15 mg/kg	BCAA	12	50.3 \pm 5.0 [§]	9.47 \pm 0.25	0.56 \pm 0.04	2.58 \pm 0.64 [§]	0.40 \pm 0.05	2.51 \pm 0.42
4	Saline	CRF-1	6	58.1 \pm 2.5	9.63 \pm 0.22	0.63 \pm 0.02	3.35 \pm 0.72	0.45 \pm 0.06	3.02 \pm 0.32
5	Saline	Casein	6	58.0 \pm 2.1	9.70 \pm 0.18	0.62 \pm 0.01	3.87 \pm 1.04	0.44 \pm 0.04	2.70 \pm 0.38
6	Saline	BCAA	6	58.5 \pm 2.5	9.63 \pm 0.17	0.63 \pm 0.01	3.83 \pm 0.86	0.44 \pm 0.01	2.60 \pm 0.35

* Mean \pm SD.

[†]Significantly different from group 4 ($P < 0.05$).

[‡]Significantly different from group 5 ($P < 0.05$).

[§]Significantly different from group 6 ($P < 0.05$).

Table 2. Effects of BCAA on AOM-induced ACF and BCAC formation in the experimental mice

Group no.	Treatment	Diet	No. of mice	Length of colon (cm)	Total no. of ACFs per colon	Total no. of BCACs/cm ²
1	AOM 15 mg/kg	CRF-1	12	12.4 ± 1.4*	85.9 ± 8.1	11.7 ± 8.4
2	AOM 15 mg/kg	Casein	12	12.5 ± 0.5	83.4 ± 11.2	8.3 ± 3.9
3	AOM 15 mg/kg	BCAA	12	12.0 ± 0.7	54.5 ± 8.6 ^{†, ‡}	4.2 ± 6.7 [§]
4	Saline	CRF-1	6	12.5 ± 1.0	0	0
5	Saline	Casein	6	11.5 ± 0.7	0	0
6	Saline	BCAA	6	11.3 ± 0.5	0	0

*Mean ± SD.

[†]Significantly different from group 1 ($P < 0.001$).[‡]Significantly different from group 2 ($P < 0.001$).[§]Significantly different from group 1 ($P < 0.05$).

Clinical chemistry. At sacrifice, blood samples were collected from the AOM-treated mice (groups 1-3) to measure the serum concentrations of insulin, leptin, triglyceride, total cholesterol, IGF-I, IGF-II, and BCAA. The serum triglyceride, total cholesterol, and BCAA levels were assayed as described previously (20, 29). The serum insulin, leptin, IGF-I, and IGF-II were determined by an enzyme immunoassay according to the manufacturer's protocol (R&D Systems).

Statistical analysis. The results were presented as the mean ± SD and were analyzed with the use of the GraphPad InStat software program version 3.05 (GraphPad Software) for Macintosh. Differences between groups were analyzed by one-way ANOVA or, as required, by two-way ANOVA. When ANOVA showed a statistically significant effect ($P < 0.05$), comparisons of each experimental group with the control group were then made with the use of the Tukey-Kramer multiple comparisons test. The differences were considered significant when the two-tailed P was < 0.05 .

Results

General observations. As shown in Table 1, the average body weights of groups 1 (CRF-1) and 3 (BCAA) in the AOM-injected mice at the termination of this experiment were smaller than those of the saline-injected groups 4 (CRF-1; $P < 0.05$) and 6 (BCAA; $P < 0.05$). The mean liver weights in the AOM-treated groups 2 (casein) and 3 (BCAA) were significantly lower than those in the saline-treated groups 5 (casein; $P < 0.05$) and 6 (BCAA; $P < 0.05$). Among CRF-1-fed mice, the mean kidney weight in the AOM-treated group 1 was also significantly lower than that of the saline-treated group 4 ($P < 0.05$). No significant difference was observed in the body length, body mass index, and mean white adipose tissue weight among the experimental mice. A histopathologic examination also

revealed no alteration, thus suggesting the absence of toxicity of BCAA in the liver and kidney of the mice in groups 3 and 6 (data not shown).

Effects of BCAA supplementation on AOM-induced ACF and BCAC formations in db/db mice. Table 2 summarizes the total number of ACF and BCAC (Fig. 1) in the mice of all groups. ACF and BCAC developed in the colons of all the mice that received AOM (groups 1 to 3) but not in the colons of the mice that did not receive AOM (groups 4 to 6). Dietary supplementation with BCAA significantly decreased the number of total ACF compared with those of the CRF-1-fed (37% reduction; $P < 0.001$) and casein-supplemented groups (35% reduction; $P < 0.001$). Compared with the CRF-1-fed group, the administration of BCAA also significantly reduced the number of total BCAC (64% reduction; $P < 0.05$).

Effects of BCAA supplementation on the serum levels of BCAA in AOM-treated db/db mice. Because the colonic premalignant lesions developed only in the AOM-injected mice (Table 2), the following experiments were done among the mice that received AOM (groups 1 to 3). BCAA supplementation caused a significant increase in the serum concentrations of total BCAA (valine, isoleucine, and leucine; 1736 ± 179 nmol/mL) compared with the CRF-1-fed (882 ± 160 nmol/mL; $P < 0.001$) and casein-supplemented groups (853 ± 51 nmol/mL; $P < 0.001$). These findings suggest that supplementation with 3.0% BCAA is sufficient to raise the serum concentration of BCAA.

Effects of BCAA supplementation on the serum levels of total cholesterol, triglyceride, and leptin in AOM-treated db/db mice. As shown in Table 3, the serum levels of total cholesterol in the BCAA-supplemented mice were significantly lower than

Table 3. Serum levels of total cholesterol, triglyceride, and leptin in AOM-treated db/db mice

Group no.	Treatment	Diet	No. of mice	Total cholesterol (mg/dL)	Triglyceride (mg/dL)	Leptin (ng/dL)
1	AOM 15 mg/kg	CRF-1	12	185 ± 34*	244 ± 49	117 ± 18
2	AOM 15 mg/kg	Casein	12	186 ± 40	229 ± 40	133 ± 32
3	AOM 15 mg/kg	BCAA	12	141 ± 48 ^{†, ‡}	187 ± 48 [†]	99 ± 23 [§]

*Mean ± SD.

[†]Significantly different from group 1 ($P < 0.05$).[‡]Significantly different from group 2 ($P < 0.05$).[§]Significantly different from group 2 ($P < 0.01$).

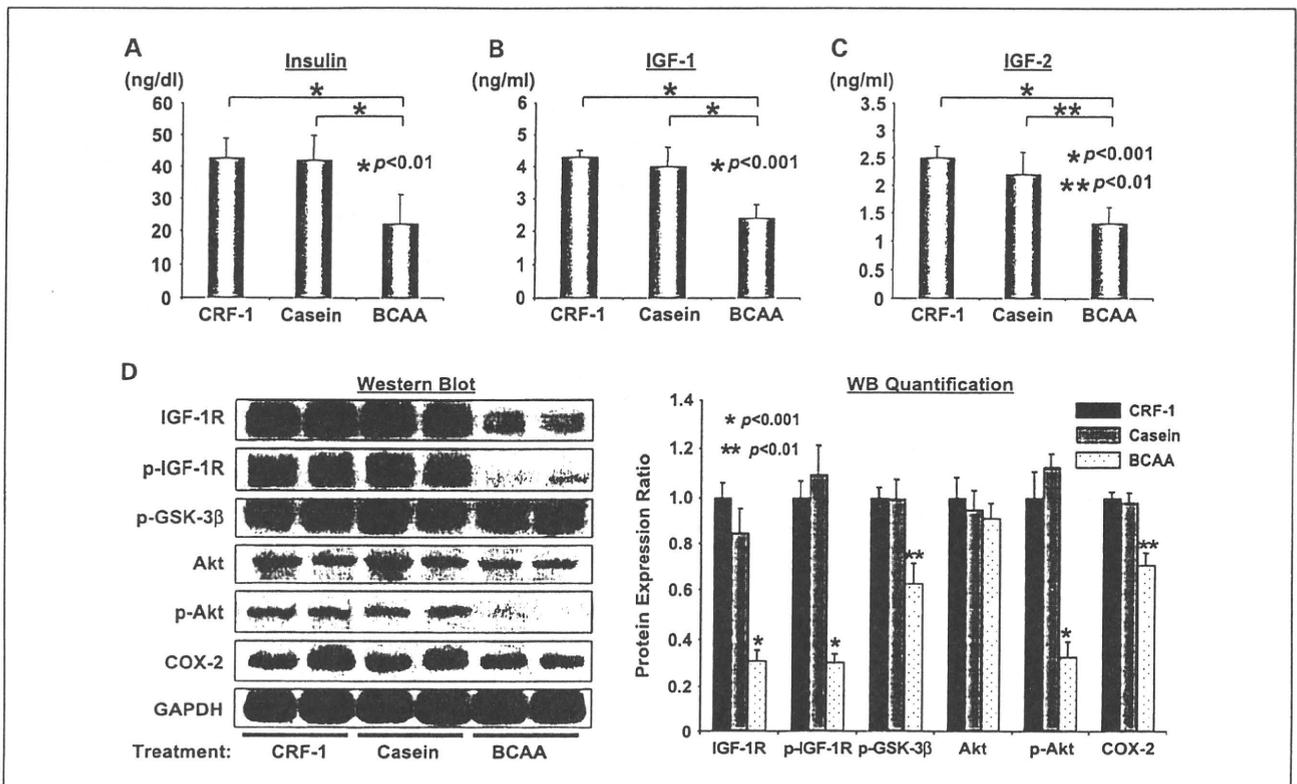


Fig. 2. The effect of BCAA supplementation on the serum levels of insulin, IGF-I, and IGF-II, and on the expression levels of the IGF-IR, p-IGF-1R, p-GSK-3β, Akt, p-Akt, and COX-2 proteins in AOM-treated *db/db* mice. A to C, the serum concentration of insulin (A), IGF-I (B), and IGF-II (C) were measured by an enzyme immunoassay. Bars, SD of triplicate assays. D, total proteins were extracted from the scraped colonic mucosa, and equivalent amounts of proteins were examined by a western blot analysis as described in Materials and Methods. Lanes, protein samples from two different mice in each group (left). The intensities of blots were quantitated by densitometry (right). Repeat western blots gave similar results. Values, mean ± SD. *, $P < 0.001$ and **, $P < 0.01$; significant differences obtained by comparison with CRF-1-treated or casein-treated mice, respectively.

those in the CRF-1-fed ($P < 0.05$) and casein-supplemented mice ($P < 0.05$). The mice supplemented with BCAA showed a significant decrease in the serum levels of triglyceride compared with the CRF-1 fed ($P < 0.05$). The serum leptin level of group 3 (BCAA) was also significantly lower than that of group 2 (casein; $P < 0.01$).

Effects of BCAA supplementation on the serum levels of insulin, IGF-I, and IGF-II in AOM-treated *db/db* mice. Supplementation with BCAA caused a significant decrease in the serum levels of insulin (Fig. 2A) compared with the CRF-1-fed ($P < 0.01$) and casein-supplemented mice ($P < 0.01$). Similarly, there was a significant decrease in the serum levels of both IGF-I (Fig. 2B) and IGF-II (Fig. 2C) in BCAA-supplemented mice compared with the CRF-1-fed ($P < 0.001$ for each comparison) and casein-supplemented mice ($P < 0.001$ and $P < 0.01$, respectively).

Effects of BCAA supplementation on the expression levels of IGF-IR, p-IGF-1R, p-GSK-3β, p-Akt, and COX-2 proteins, and on cell proliferative activity in the colonic mucosa of AOM-treated *db/db* mice. Hyperinsulinemia and abnormal activation of the IGF/IGF-IR axis play a critical role in obesity-related CRC development (3, 6–10). Therefore, the effects of BCAA on the levels of IGF-IR and the phosphorylated (i.e., activated) form of IGF-IR proteins, and cell proliferation were examined in the colonic mucosa of AOM-treated mice. As shown in Fig. 2D, western blot analyses showed that BCAA supplementation

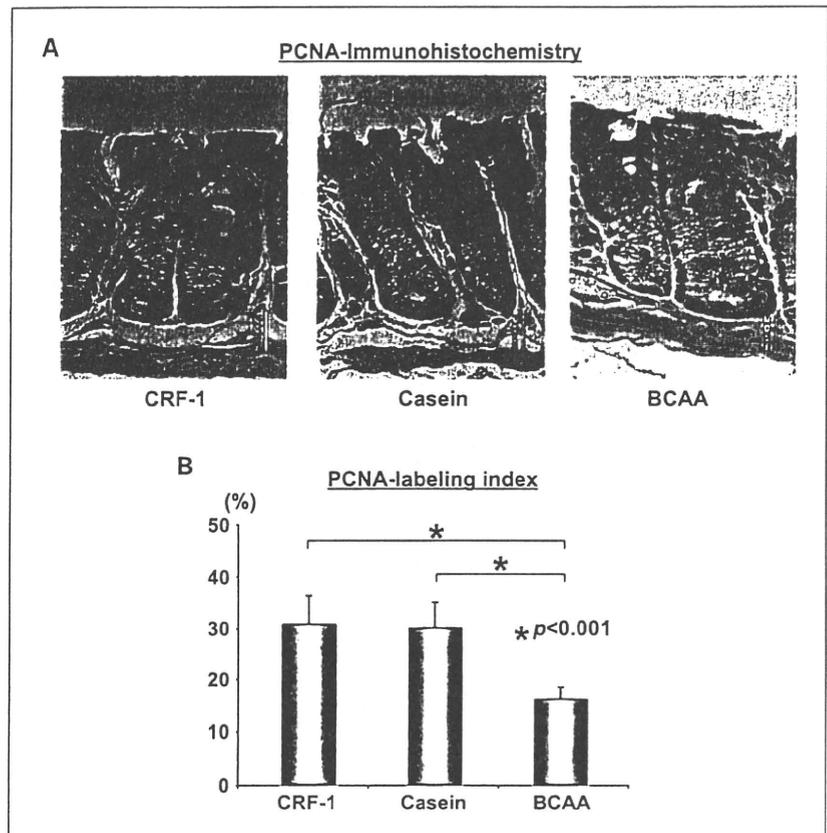
caused a decrease in the levels of IGF-IR ($P < 0.001$ for each comparison) and p-IGF-1R ($P < 0.001$ for each comparison) proteins compared with the CRF-1-fed and casein-supplemented mice. Supplementation with BCAA also decreased the expression levels of the phosphorylated (i.e., inactivated) form of GSK-3β ($P < 0.01$ for each comparison), the phosphorylated (i.e., activated) form of Akt ($P < 0.001$ for each comparison), and COX-2 ($P < 0.01$ for each comparison) proteins compared with the control groups. The finding that BCAA supplementation inhibited the phosphorylation of Akt is considered to be significant because the activation of this protein is one of the critical targets in the constitutive activation of the IGF/IGF-IR axis in colorectal carcinogenesis (30).

In addition, as shown in Fig. 3, the PCNA labeling index of nonlesional crypts in the BCAA-supplemented mice was significantly smaller than that of the CRF-1-fed and casein-supplemented mice ($P < 0.001$ for each comparison), thus indicating that BCAA supplementation significantly inhibits cell proliferation in the colonic mucosa of the AOM-treated *db/db* mice.

Discussion

The present study clearly indicated that dietary supplementation with BCAA effectively suppressed the development of putative precursor lesions, ACF and BCAC (Fig. 1), for CRC

Fig. 3. The effect of BCAA supplementation on colonic epithelial cell proliferation in AOM-treated *db/db* mice. **A**, immunohistochemical staining of the normal crypts in the colon of AOM-treated *db/db* mice with anti-PCNA antibody. Sections of the colon were analyzed from CRF-1 – fed, casein-supplemented, and BCAA-supplemented mice, respectively. They were stained with anti-PCNA monoclonal antibody as described in Materials and Methods. Representative photographs from each group are shown. Bar, 20 μ m. **B**, PCNA labeling index in the normal crypts in the colon of AOM-treated *db/db* mice. Bars, SD of triplicate assays.



(Table 2) by improving hyperlipidemia and hyperleptinemia in *db/db* mice (Table 3). The suppressive effect of BCAA in the early phase of obesity-related colorectal carcinogenesis was also associated, most likely, with the improvement of hyperinsulinemia (Fig. 2A) and the inhibition of cell proliferation on the colonic mucosa of experimental mice (Fig. 3). BCAA supplementation has also been reported to significantly decrease the incidence of hepatocellular carcinoma in patients with chronic liver disease if they had a body mass index score ≥ 25 , and this effect might be associated with improvement of insulin resistance (15, 16, 31). Thus, BCAA might effectively prevent cancer development, at least in several organs, in obese subjects who are considered to have insulin resistance syndrome (3).

How can BCAA exert chemopreventive effects on obesity-related colorectal carcinogenesis? As described above, insulin resistance might be a critical target of BCAA in this beneficial effect because insulin has oncogenic properties on CRC cells. For instance, insulin stimulates the proliferation of CRC cells and promotes colorectal tumor growth in animal models (6–8). These reports, therefore, suggest that BCAA inhibits the development of colonic premalignant lesions (Table 2) and excessive cell proliferation in the colonic mucosa of AOM-injected *db/db* mice (Fig. 3) by improving insulin resistance (Fig. 2A). Recent studies by others have indicated that BCAA improves glucose tolerance by modulating insulin-independent glucose uptake into skeletal muscle in rodent models (32, 33). An improvement of insulin resistance and glucose tolerance by BCAA has also been shown by certain clinical trials (15, 31).

In addition, it is widely accepted that insulin resistance causes alterations in the IGF/IGF-IR axis, which may be closely associated with the development of CRC (9, 10, 30). For instance, the IGF-IR protein is overexpressed in BCAC compared with the surrounding normal cryptal cells (11). Therefore, the IGF/IGF-IR system is regarded as one of the effective targets with respect to the prevention of CRC (11). Our observations described herein comprise the first report showing that BCAA decreases the serum levels of IGF-I and IGF-II (Fig. 2B and C), thereby inhibiting the expression and activation of IGF-IR on the colonic mucosa of AOM-treated *db/db* mice (Fig. 2D). Our findings suggest that not only the improvement of insulin resistance but the inhibition of IGF/IGF-IR activation by BCAA plays a critical role in suppressing obesity-related and diabetes mellitus-related colorectal carcinogenesis.

The present study revealed that BCAA supplementation in the diet prevents the development of BCAC (Table 2), which is characterized by abundant β -catenin protein expression (23) and also accumulates the IGF-IR protein (11) while decreasing the expression levels of p-Akt and p-GSK-3 β proteins on the colonic mucosa of AOM-treated *db/db* mice (Fig. 2D). Recent *in vitro* studies have indicated that insulin and the IGF/IGF-IR axis stabilize and activate the Wnt/ β -catenin pathway, which is involved in the development of CRC (34, 35). GSK-3 β , which can be phosphorylated by phosphatidylinositol 3-kinase/Akt via insulin or IGF treatment, is considered to be a key kinase for CRC development because the inactivation of GSK-3 β leads to the dissociation of the adenomatous polyposis coli/axin/ β -catenin complex and cytosolic β -catenin accumulation (36).