雑誌

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Ⅲ．研究成果の刊行物・別冊

Original Article

Free fatty acid as a marker of energy malnutrition in liver cirrhosis

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Aim: Protein–energy malnutrition is frequently observed in patients with liver cirrhosis (LC). Non-protein respiratory quotient (npRQ) measured by indirect calorimetry is a good marker to estimate energy malnutrition, and predicts the prognosis of patients with LC. However, measurement of npRQ is limited because of the high cost of indirect calorimetry. Our aim was to find out an alternative marker to npRQ that can be used in the routine clinical setting.

Methods: One hundred and fifty-six patients with LC were enrolled in this study. Indirect calorimetry and blood examinations were conducted after overnight fasting, and anthropometry was performed by an expert dietician. The correlation between npRQ and other parameters were calculated by simple and multiple regression analysis. Receiver-operator curve (ROC) analysis was used to identify the cut-off value that would best predict the threshold npRQ of 0.85.

Results: Plasma levels of free fatty acid (FFA) was significantly correlated with npRQ value by simple ($r = -0.39$, $P < 0.0001$) and multiple regression analysis ($t = -2.96$, $P = 0.0052$). Free fatty acid rose in parallel with the increasing disease severity as defined by Child–Pugh classification ($P < 0.05$). FFA was also correlated with increasing oxidation rate of fat ($r = 0.38$, $P < 0.0001$) and decreasing oxidation rate of carbohydrate ($r = -0.39$, $P < 0.0001$). The cut-off value of FFA to predict npRQ = 0.85 was 660 μEq/L by ROC analysis.

Conclusion: FFA is a useful alternative marker to represent npRQ in patients with LC.

Key words: anthropometry, free fatty acid, indirect calorimetry, liver cirrhosis, non-protein respiratory quotient, protein–energy malnutrition

INTRODUCTION

THE LIVER PLAYS a central role in the nutrient metabolism of carbohydrate, fat, protein, vitamins and trace minerals, among others. Protein–energy malnutrition (PEM) is, therefore, a common manifestation in patients with liver cirrhosis (LC).[1–5] PEM is related to reduced dietary intake, impaired digestion and absorption in cirrhotic patients. PEM is also associated with impaired protein synthesis, disorder of glycolysis and glycogenesis, a negative nitrogen balance and an increasing lipolysis.[3] Several reports demonstrated that PEM is associated with a high morbidity and mortality due to an increased risk of complications, resulting in poor survival rate and quality of life (QOL) in patients with cirrhosis.[4–6] Thus, diagnosis of and intervention for PEM are important in the clinical management of LC, and nutritional support for cirrhotic patients is recommended in current guidelines.[7–11]

As an intervention for protein malnutrition, supplementation of oral branched-chain amino acid (BCAA) increases protein synthesis in cirrhotic patients.[12–14] Moreover, long-term oral supplementation with BCAA contributes to improve QOL and to raise the event-free survival in patients with LC.[15–17] As an intervention for energy malnutrition, frequent meals including a late-evening snack (LES) is recommended to prevent nocturnal starvation and to improve catabolic state during fasting in patients with LC.[17–21]

After an overnight fast, the energy generation shifts from glycolysis to an increased fat oxidation because patients with LC have insufficient glycogen stores due to

liver atrophy.[22–25] Such catabolic state in cirrhotic patients is similar to that observed in normal individuals after 2–3 days of starvation.[25–27] In such condition, indirect calorimetry shows reduced carbohydrate oxidation and elevated fat oxidation, leading to a decreased non-protein respiratory quotient (npRQ).[5,28,29] It has been reported that decrease in npRQ (<0.85) predicted a significantly lower survival rate in patients with LC.[5] Hence, npRQ measured by indirect calorimetry is a good marker to estimate energy malnutrition and to predict the prognosis of patients with LC. Where indirect calorimeters are available, measurement of npRQ after overnight fasting is recommended for nutritional management of LC.[8,10,11] However, measurement of npRQ is limited in daily clinical practice because of the high cost of indirect calorimetry. Thus, it is important to find out an alternative marker to npRQ that can be used in the routine clinical setting.

The aim of this study is to establish such markers, and to characterize their role in energy metabolism in patients with LC. For candidate markers, we screened both anthropometric indices and blood biochemical parameters. For the former, the significance of arm circumference has been suggested by, for example, Terakura *et al.*[29] For the latter, we particularly focused on free fatty acid (FFA), because several studies have described the association of FFA with malnutrition, nocturnal hypoglycemia and hepatic failure in patients with LC.[24,30]

Free fatty acid is supposed to rise extensively in the early morning in cirrhotic patients due to severe starvation state and hypoglycemia. Such a situation is brought about by the shortage of glycogen store in the cirrhotic/atrophic liver, and promotes breakdown of adipose tissue to release FFA for direct energy generation and glycerol as the source of gluconeogenesis.[30]

METHODS

Patients

WE ENROLLED 156 consecutive patients with LC, who were admitted in Gifu University Hospital between October 2011 and August 2012. Their demographic, clinical and blood biochemical features are described in Table 1. LC was diagnosed by clinical and laboratory data and by liver biopsy specimens. They consisted of 107 men and 49 women, with median age of 69 years. According to the Child–Pugh classification,[31] 105 patients were in class A, 40 were in class B and 11 were in class C. The etiology of LC was hepatitis B virus in 16 cases, hepatitis C virus in 100 cases, alcohol

in 27 cases, and others in 13 cases such as non-alcoholic steatohepatitis, primary biliary cirrhosis and autoimmune hepatitis. Ninety-three patients had hepatocellular carcinoma detected by imaging modalities including abdominal ultrasonography, dynamic computed tomography, dynamic magnetic resonance imaging or abdominal arteriography. Patients fasting for over a day within 2 weeks before calorimetry were excluded in this study.

We set up another cirrhotics group consisting of 10 patients, in order to validate the identified possible marker(s) by LES intervention. The baseline characteristics of the 10 patients are given in Table 2. All these patients had npRQ of less than 0.85. LES was provided for 7 days with 210 kcal BCAA-enriched enteral formula (Aminoleban; Otsuka Pharmaceutical, Tokyo, Japan), while total energy intake was kept at 30 kcal/kg standard bodyweight/day as recommended in the guideline for nutrition support of LC.[10]

The purpose of the study was fully explained, and informed consent was obtained from all participants. The study protocol was approved by the ethics committee of the Gifu University School of Medicine, and carried out in accordance with the 1975 Helsinki Declaration as revised in 1983.

Blood samples

Blood samples were obtained after overnight fasting on the day of metabolic studies. Serum total bilirubin, albumin, alanine aminotransferase, prothrombin time, triacylglycerol, total cholesterol, ketone body, FFA, fasting plasma glucose (FPG), immunoreactive insulin (IRI) and urinary nitrogen were measured by standard clinical methods (Department of Clinical Laboratory, Gifu University Hospital). Serum BCAA to tyrosine ratio (BTR) was measured by the enzymatic method (Diacolor BTR; Toyobo, Osaka, Japan).[32] 3-Methylhistidine content of urine was analyzed by high-performance liquid chromatography as previously reported.[33] Serum interleukin (IL)-6 level was measured by chemiluminescent enzyme immunoassay (Human IL-6 CLEIA; Fujirebio, Tokyo, Japan).

Indirect calorimetry

Metabolic parameters were measured by indirect calorimetry (Aeromonitor; Minato Medical Science, Osaka, Japan) to estimate oxygen consumption per minute (Vo_2) and carbon dioxide production per minute (Vco_2) in a similar manner to that explained in our previous reports.[34] Total urinary excretion of nitrogen was also measured. Resting energy expenditure, npRQ and sub-

Table 1 Baseline demographic characteristics, body composition, blood biochemistry and calorimetric data in patients with liver cirrhosis†

	Cirrhosis ($n = 156$)	Child A ($n = 105$)	Child B ($n = 40$)	Child C ($n = 11$)	P‡
Age (years)	69.2 ± 11.5	69.3 ± 11.0	70.4 ± 12.7	64.3 ± 12.2	0.294
Male/female	107/49	72/33	28/12	7/4	0.923
BMI (kg/m^2)	22.7 ± 3.2	22.8 ± 3.0	22.0 ± 2.7	25.0 ± 5.7	0.024*
Etiology (HBV/HCV/alcohol/others)	16/100/27/13	8/74/15/8	6/22/8/4	2/4/4/1	0.254
Hepatocellular carcinoma (+/−)	93/63	62/43	25/15	6/5	0.874
Arm circumference (%)	99.7 ± 13.1	100.9 ± 12.0	95.4 ± 11.7	102.0 ± 24.0	0.112
Triceps skinfold thickness (%)	92.8 ± 43.8	92.1 ± 41.9	96.7 ± 51.9	87.0 ± 35.7	0.809
Arm muscle circumference (%)	101.7 ± 11.1	102.3 ± 8.9	99.1 ± 13.2	104.2 ± 21.6	0.311
Total bilirubin (mg/dL)	1.32 ± 1.44	0.91 ± 0.41	1.75 ± 1.34	3.65 ± 3.85	<0.001***
Albumin (g/dL)	3.54 ± 0.67	3.86 ± 0.46	2.95 ± 0.32	2.67 ± 1.03	<0.001***
Alanine aminotransferase (IU/L)	41.5 ± 29.6	45.1 ± 32.4	34.5 ± 16.7	33.0 ± 34.3	0.097
Prothrombin time (%)	87.0 ± 16.6	94.1 ± 13.1	75.1 ± 10.5	63.1 ± 17.7	<0.001***
Triacylglycerol (mg/dL)	89.9 ± 39.1	98.8 ± 39.8	73.6 ± 26.3	65.3 ± 44.1	<0.001***
Total cholesterol (mg/dL)	146.6 ± 37.4	155.7 ± 30.9	127.5 ± 34.0	130.0 ± 68.1	<0.001***
Ketone body (μmol/L)	137.1 ± 136.4	123.0 ± 129.4	157.5 ± 160.5	198.5 ± 80.5	0.122
Free fatty acid (μEq/L)	628.2 ± 226.9	583.0 ± 199.5	680.9 ± 246.2	869.2 ± 226.8	<0.001***
3-Methylhistidine (μmoL/day)	147.8 ± 68.1	161.3 ± 70.5	124.9 ± 52.1	99.8 ± 55.3	0.002**
BTR	4.45 ± 1.53	4.95 ± 1.35	3.56 ± 1.37	2.82 ± 1.16	<0.001***
FPG (mg/dL)	106.4 ± 27.6	108.7 ± 29.9	102.6 ± 22.3	97.3 ± 20.1	0.261
IRI (μU/mL)	9.58 ± 6.61	9.21 ± 6.15	9.48 ± 5.87	13.47 ± 11.45	0.125
Interleukin-6 (pg/dL)	20.0 ± 28.8	9.8 ± 18.7	18.9 ± 17.5	54.6 ± 43.0	<0.001***
REE (kcal/day)	1238.9 ± 204.1	1258.0 ± 202.1	1177.5 ± 219.5	1282.0 ± 109.5	0.081
BMR (kcal/day)	1207.9 ± 207.0	1216.6 ± 182.2	1164.6 ± 210.2	1282.8 ± 362.1	0.186
npRQ	0.86 ± 0.07	0.87 ± 0.07	0.84 ± 0.05	0.79 ± 0.06	<0.001***
CHO (%)	43.1 ± 19.3	47.9 ± 18.7	37.2 ± 16.4	20.7 ± 14.0	<0.001***
FAT (%)	40.1 ± 19.2	35.8 ± 18.9	45.9 ± 17.0	59.4 ± 12.9	<0.001***
PRO (%)	16.8 ± 7.4	16.3 ± 6.2	16.9 ± 8.4	19.9 ± 13.0	0.337

†Values are presented as number of patients or mean ± standard deviation.
‡Compared among Child–Pugh grades by χ^2-test or one-way ANOVA. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.
BCAA, branched-chain amino acids; BMI, body mass index; BMR, basal metabolic rate; BTR, branched-chain amino acid to tyrosine ratio; CHO, substrate oxidation rate of carbohydrate; FAT, substrate oxidation rate of fat; FPG, fasting plasma glucose; HBV, hepatitis B virus; HCV, hepatitis C virus; IRI, immunoreactive insulin; npRQ, non-protein respiratory quotient; PRO, substrate oxidation rate of protein; REE, resting energy expenditure.

strate oxidation rates of carbohydrate, fat and protein were calculated as in Kato *et al.*[34] Calorimetry was performed between 07.00 and 09.00 hours while the patients were still in bed. The last meal was served at 18.00 hours on the previous day. The basal metabolic rate (BMR) was predicted by the Harris–Benedict formula.[35]

Anthropometric parameters

We measured bodyweight and height, and calculated body mass index. Anthropometry such as arm circumference (AC) and triceps skinfold thickness (TSF) were carried out by using a standard American Society for Parenteral and Enteral Nutrition procedure by an expert dietician, and arm muscle circumference (AMC) was calculated. AC, TSF and AMC were standardized according to age- and sex-stratified Japanese anthropometric reference data 2001 in a similar manner to our previous reports.[29]

Statistics

The continuous data were expressed as mean ±standard deviation, and comparisons among Child–Pugh class A, class B and class C were conducted by one-way ANOVA, Mann–Whitney U-test or Kruskal–Wallis test. The χ^2-test or Fisher's exact test was used to compare differences between groups for categorical variables. Pearson's correlation test and Spearman's rank correlation coefficient

Table 2 Baseline characteristics of patients†

	Cirrhosis ($n = 10$)
Age (y)	72.1 ± 9.4
Male/female	6/4
BMI (kg/m²)	22.9 ± 2.8
Etiology (HBV/HCV/alcohol/others)	0/7/3/0
Child (A/B/C)	6/4/0
Hepatocellular carcinoma (+/−)	5/5
Total bilirubin (mg/dL)	1.16 ± 0.54
Albumin (g/dL)	3.26 ± 0.36
Alanine aminotransferase (IU/L)	35.0 ± 18.1
Prothrombin time (%)	84.1 ± 5.8
Ketone body (μmol/L)	204.7 ± 159.6
Free fatty acid (μEq/L)	914.9 ± 312.3
3-Methylhistidine (μmoL/day)	123.9 ± 39.2
BTR	3.15 ± 0.83
FPG (mg/dL)	89.5 ± 6.2
IRI (μU/mL)	11.5 ± 5.3
REE (kcal/day)	1242.5 ± 215.1
BMR (kcal/day)	1170.0 ± 176.6
npRQ	0.770 ± 0.031
CHO (%)	19.2 ± 8.1
FAT (%)	65.0 ± 12.2
PRO (%)	15.8 ± 5.6

†Values are presented as number of patients or mean ± standard deviation.
BCAA, branched-chain amino acid; BMR, basal metabolic rate; BTR, BCAA to tyrosine ratio; CHO, substrate oxidation rate of carbohydrate; FAT, substrate oxidation rate of fat; FPG, fasting plasma glucose; HBV, hepatitis B virus; HCV, hepatitis C virus; IRI, immunoreactive insulin; npRQ, non-protein respiratory quotient; PRO, substrate oxidation rate of protein; REE, resting energy expenditure.

test were used to analyze the relation among blood test parameters and substrate oxidation rates or Child–Pugh grade. Multiple regression analysis was also performed to find the independent predictors of npRQ. The receiver–operator curve (ROC) analysis was used to identify the cut-off values that would best predict npRQ of 0.85. All analyses were carried out using JMP ver. 9.0.2 software (SAS Institute, Cary, NC, USA) and $P < 0.05$ was considered statistically significant.

RESULTS

Baseline data and energy metabolisms

THE CLINICAL AND laboratory features of the patients are given in Table 1. Anthropometric parameters such as AC, TSF and AMC were not correlated with the Child–Pugh classification (Table 1).

Cirrhotic patients showed significantly lower triacylglycerol, total cholesterol, 3-methylhistidine and BTR in parallel with the increasing grade of disease severity as defined by the Child–Pugh classification, indicating the presence of PEM in these subjects (Table 1). Serum FFA level correlated with the increasing Child–Pugh grade in patients with LC ($P < 0.001$), but ketone body did not. Serum IL-6 concentration also rose in advanced cirrhotic patients (Table 1).

The npRQ value in patients of Child B and C were significantly lower than that of Child A ($P < 0.01$). Decrease in npRQ indicated a higher oxidation rate of fat and lower oxidation rate of carbohydrate in patients with cirrhosis (Table 1, Fig. 1). An increase in oxidation rate of fat and a decrease in oxidation rate of carbohydrate significantly correlated with the progression of disease severity in patients with LC as defined by the Child–Pugh classification (Table 1, Fig. 1). There was no statistically significant difference in oxidation rates of fat, carbohydrate, protein or in npRQ between presence and absence of hepatocellular carcinoma in this study (data not shown).

Correlation between npRQ and other parameters

Correlation coefficients among clinical, laboratory, anthropometric and calorimetric parameters were analyzed, and those of significance by npRQ are described in Table 3. There was no significant correlation between npRQ and anthropometric parameters such as AC, TSF and AMC.
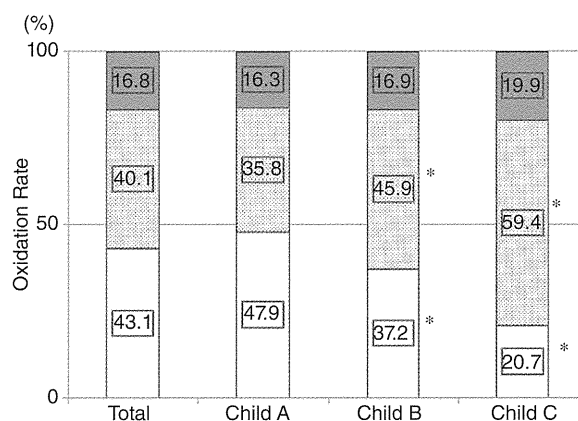

Figure 1 Substrate oxidation rates of protein, fat and carbohydrate in patients with liver cirrhosis ($n = 156$) graded by Child–Pugh classification (A = 105, B = 40 and C = 11). Values are presented by mean. *$P < 0.01$ compared with Child A. Child, Child–Pugh classification.

Table 3 Correlation coefficients between npRQ and other parameters

	r-value	P value
BMI	−0.03	0.7092
Arm circumference	0.05	0.5274
Triceps skinfold thickness	0.04	0.6735
Arm muscle circumference	0.07	0.4265
Total bilirubin	−0.27	0.0008***
Albumin	0.31	<0.0001***
Alanine aminotransferase	0.07	0.4175
Prothrombin time	0.25	0.0018**
Triacylglycerol	0.13	0.0990
Total cholesterol	0.09	0.2334
Ketone body	−0.22	0.0057**
Free fatty acid	−0.39	<0.0001***
3-Methylhistidine	0.21	0.0167*
BTR	0.18	0.0289*
FPG	0.13	0.0943
IRI	−0.03	0.7171
Interleukin-6	−0.38	0.0093**

$*P < 0.05$, $**P < 0.01$, $***P < 0.001$.
BCAA, branched-chain amino acids; BMI, body mass index; BTR, BCAA to tyrosine ratio; FPG, fasting plasma glucose; IRI, immunoreactive insulin; npRQ, non-protein respiratory quotient.

Table 4 Multiple regression analysis between npRQ and other parameters

	t-value	P-value
Prothrombin time	1.25	0.2181
Ketone body	1.21	0.2346
Free fatty acid	−2.96	0.0052*
Interleukin-6	−1.53	0.1343

$*P < 0.01$.
npRQ, non-protein respiratory quotient.

Non-protein respiratory quotient correlated with albumin, prothrombin time, 3-methylhistidine and BTR in patients with LC. Inverse correlation with npRQ was observed for total bilirubin, ketone body, FFA and IL-6 (Table 3). Multiple regression analysis showed that only FFA is a significant independent predictor of npRQ among them ($P < 0.01$) (Table 4).

Correlation between FFA and substrate oxidation rates

Serum FFA level rose in parallel with the increasing grade of disease severity as defined by Child–Pugh classification in patients with LC (Fig. 2).

Significant correlation was found between FFA and oxidation rate of fat ($r = 0.38$, $P < 0.0001$). Inverse correlation was observed between FFA and oxidation rate of carbohydrate ($r = −0.39$, $P < 0.0001$). However, there was no correlation between FFA and oxidation rate of protein ($r = 0.05$, $P = 0.5578$) (Fig. 3).

FFA as an alternative marker of npRQ

The cut-off value of FFA for npRQ of 0.85 was 660 µEq/L by ROC analysis, where the area under the ROC curve was 0.67 (Fig. 4). Table 5 shows the patients' baseline clinical, laboratory and calorimetric characteristics divided by the FFA level of 660 µEq/L.

The patients with FFA of 660 µEq/L or higher showed significantly higher total bilirubin, ketone body and IRI, and similarly lower BTR, than those with FFA of less than 660 µEq/L. However, serum albumin level, a protein nutritional parameter, did not differ significantly between the subgroups (Table 5). There was a significant correlation between FFA and blood ammonia level (Fig. 5). In contrast, FFA did not correlate with fasting plasma glucose, Homeostasis Model of Assessment – Insulin Resistance or the presence ($n = 45$) or absence ($n = 111$) of diabetes mellitus (DM) (data not shown).

In energy metabolism, higher oxidation rate of fat and lower oxidation rate of carbohydrate were observed in patients with FFA of 660 µEq/L or higher ($P < 0.001$) (Table 5).

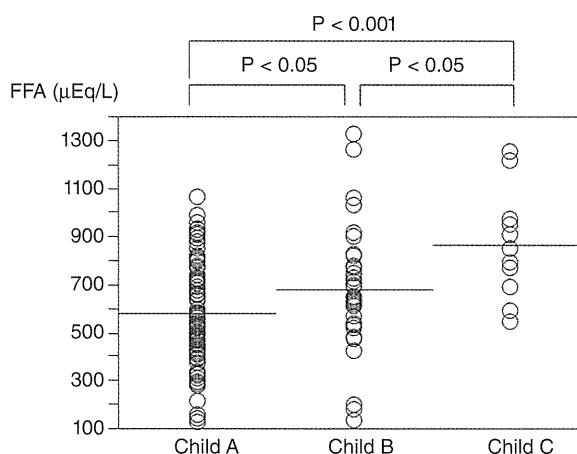There was no significant difference between FFA of less than 660 and 660 µEq/L or higher groups in

Figure 2 Free fatty acid (FFA) in patients with liver cirrhosis ($n = 154$) graded by Child–Pugh classification. Horizontal lines indicate the median.
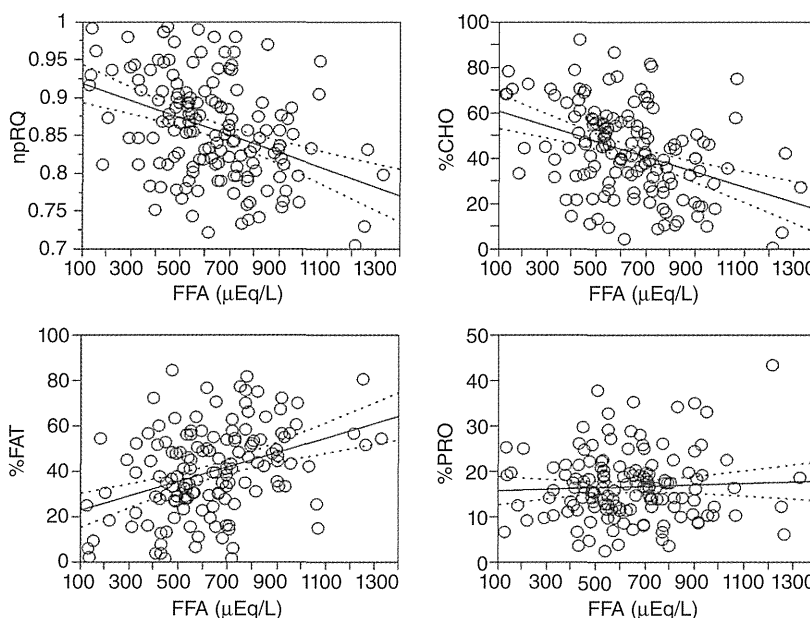
Figure 3 Correlation between free fatty acid (FFA) and non-protein respiratory quotient (npRQ; $r = -0.39$, $P < 0.0001$), carbohydrate (%CHO; $r = -0.39$, $P < 0.0001$), fat (%FAT; $r = 0.38$, $P < 0.0001$) and protein (%PRO; $r = 0.05$, $P = 0.5578$) in patients with liver cirrhosis ($n = 154$). Dotted lines indicate the 90% confidence range of the regression line.

anthropometric parameters such as AC, TSF and AMC (Table 5).

Effects of LES on npRQ and FFA

All 10 patients of the validation group showed npRQ of less than 0.85 at entry. Hence, only predictive

Figure 4 Receiver–operator curve for free fatty acid (FFA). The area under the curve was 0.67, which gave the cut-off value of FFA at 660 μEq/L.

sensitivity and accuracy of FFA for npRQ were available as 80% and 80%, respectively. LES recovered npRQ ($P < 0.01$) and reduced FFA ($P < 0.01$) in all cases (Table 6, Fig. 6).

DISCUSSION

IN THIS STUDY, we showed that FFA was a sole significant independent predictor of npRQ in patients with LC, and revealed that FFA of 660 μEq/L or higher represents npRQ of less than 0.85 by ROC analysis. In a fasting state, the liver contributes to approximately 50% of the body's total caloric requirements by releasing glucose derived from glycogen stores into the blood.[26,27] Cirrhotic patients have insufficient glycogen stores due to liver atrophy, and their energy generation pattern after an overnight fast (Fig. 1) is equivalent to that observed in healthy individuals after 2–3 days of starvation.[25–27] Thus, cirrhotic patients develop a catabolic state more rapidly than do the normal individuals, and should avoid long-term fasting state. In this study, energy metabolism showed a decrease in carbohydrate oxidation rate and an increase in fat oxidation rate, and such trend significantly correlated with the progression of disease severity as defined by the Child–Pugh classification. Decrease in carbohydrate oxidation in cirrhosis is explained by the lower production rate of glucose from glycogen due to shortage in hepatic glycogen stores, and decrease in peripheral glucose use due to
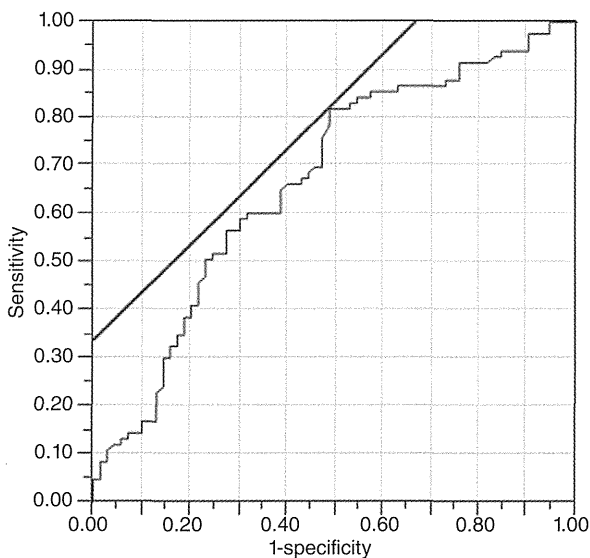
Table 5 Baseline clinical, laboratory and calorimetric characteristics classified according to free fatty acid level†

	FFA <660 μEq/L (*n* = 88)	FFA ≥660 μEq/L (*n* = 66)	*P*‡
Age (y)	68.9 ± 12.4	69.7 ± 10.5	0.705
Male/female	60/28	45/21	1.000
BMI (kg/m^2)	22.5 ± 3.1	23.1 ± 3.4	0.195
Etiology (HBV/HCV/alcohol/others)	9/62/10/7	6/37/17/6	0.246
Hepatocellular carcinoma (+/−)	52/36	41/25	0.704
Arm circumference (%)	99.1 ± 11.8	101.1 ± 14.7	0.383
Triceps skinfold thickness (%)	90.5 ± 44.7	96.7 ± 43.0	0.418
Arm muscle circumference (%)	102.1 ± 11.3	101.6 ± 10.8	0.784
Total bilirubin (mg/dL)	1.04 ± 0.90	1.69 ± 1.90	0.006**
Albumin (g/dL)	3.56 ± 0.66	3.52 ± 0.70	0.710
Alanine aminotransferase (IU/L)	39.8 ± 28.2	43.9 ± 31.8	0.396
Prothrombin time (%)	88.8 ± 16.2	84.8 ± 17.0	0.142
Triacylglycerol (mg/dL)	95.1 ± 42.0	83.7 ± 34.4	0.074
Total cholesterol (mg/dL)	144.1 ± 36.4	149.9 ± 39.3	0.349
Ketone body (μmol/L)	80.0 ± 69.0	213.3 ± 164.7	<0.001***
Free fatty acid (μEq/L)	474.6 ± 131.3	833.0 ± 153.4	<0.001***
3-Methylhistidine (μmoL/day)	147.5 ± 63.8	148.3 ± 74.9	0.946
BTR	4.75 ± 1.48	4.06 ± 1.54	0.007**
FPG (mg/dL)	104.3 ± 30.0	109.5 ± 24.3	0.254
IRI (μU/mL)	8.66 ± 5.85	10.93 ± 7.39	0.035*
Interleukin-6 (pg/dL)	16.2 ± 26.4	25.2 ± 32.7	0.322
REE (kcal/day)	1224.0 ± 213.0	1254.1 ± 193.6	0.369
BMR (kcal/day)	1210.0 ± 208.2	1205.7 ± 210.0	0.900
npRQ	0.875 ± 0.065	0.837 ± 0.063	<0.001***
CHO (%)	47.8 ± 18.8	37.0 ± 18.6	<0.001***
FAT (%)	35.6 ± 18.5	46.0 ± 18.6	0.001***
PRO (%)	16.6 ± 7.2	17.0 ± 7.5	0.739

†Values are presented as number of patients or mean ± standard deviation.
‡Compared between FFA of less than 660 and FFA of 660 μEq/L or higher by one-way ANOVA or contingency table analysis. *P < 0.05, **P < 0.01, ***P < 0.001.
BCAA, branched-chain amino acid; BMI, body mass index; BMR, basal metabolic rate; BTR, BCAA to tyrosine ratio; CHO, substrate oxidation rate of carbohydrate; FAT, substrate oxidation rate of fat; FPG, fasting plasma glucose; HBV, hepatitis B virus; HCV, hepatitis C virus; IRI, immunoreactive insulin; npRQ, non-protein respiratory quotient; PRO, substrate oxidation rate of protein; REE, resting energy expenditure.

insulin resistance.[36] Increase in fat oxidation is caused by an increased rate of lipolysis in fat tissue.[24]

Several studies showed that serum FFA concentration is higher in patients with LC than normal individuals after overnight fasting.[24,27,37] FFA is essential fuel for the liver, skeletal muscle, kidney and myocardium in the starvation state, and the liver is the central organ in the metabolism of FFA. It has been proposed that high level of FFA was caused by an increased rate of lipolysis in the fat tissue in the fasting state,[24] and there is a significant correlation between FFA and fat oxidation rate in cirrhotic patients.[23,27,37] Previous report demonstrated that approximately 70% of total BMR or 80% of non-protein energy requirements is supplied from FFA oxidation in cirrhotic patients after an overnight fast.[27]

In this study, fat oxidation contributed to 36–59% of total energy generation or 42–74% of non-protein energy (Fig. 1), giving a lower contribution than reported value as described above.[27] Such difference could be explained by the patients' characteristics. Cirrhotics in the Owen *et al.*'s study were all alcoholics with advanced stage,[27] while 74% of the cirrhotics in our study were viral and 67% were of Child–Pugh grade A (Table 1). Actually, serum FFA level correlated with the increasing grade of disease severity as defined by the Child–Pugh classification in patients with LC (Fig. 2). Moreover, significant correlation was found between FFA and oxidation rate of fat, and inverse correlation was observed between FFA and oxidation rate of carbohydrate (Fig. 3). Thus, FFA is a significant good marker
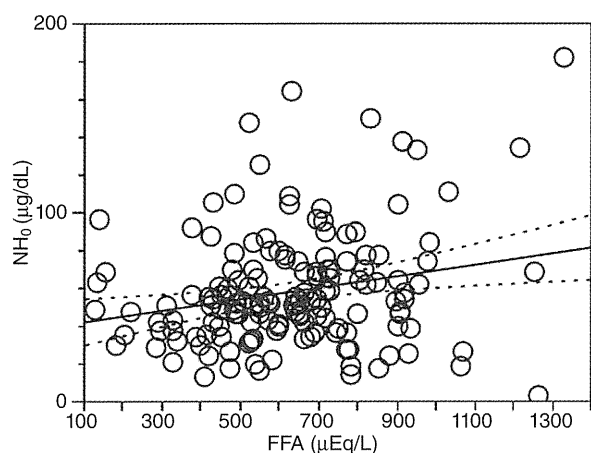
Figure 5 Correlation between blood ammonia and serum (FFA) levels ($r = 0.22$, $P = 0.0055$) in patients with liver cirrhosis. Dotted lines indicate the 90% confidence range of the regression line. NH_3, ammonia.

for cirrhotic patients to evaluate energy metabolism in a fasting state.

Ketone body is synthesized mainly in the liver by β-oxidation of FFA derived from the adipose tissue. In a fasting state, ketone body is also one of energy substrates for total body tissue, especially in the brain.[38] Previous studies demonstrated that the concentration of ketone body is elevated in cirrhotic patients after starvation, in a similar manner to FFA.[26,27] In this study, there was a correlation between npRQ and ketone body in a fasting state (Table 3), but ketone body did not correlate with the Child–Pugh classification (Table 1).

Increase in fat oxidation suggests an increased rate of lipolysis in the fat tissue. We previously reported that tumor necrosis factor-α (TNF-α) is elevated in cirrhosis, and correlates significantly with fat oxidation.[28] IL-6 is a pleiotropic cytokine, such as TNF-α, that is involved in many biological activities. Several reports showed that IL-6 is elevated in patients with LC[39] and also stimulates lipolysis and correlates with fat oxidation.[40] These studies and our findings show that serum IL-6 concentration correlates with the increasing grade of disease severity as defined by the Child–Pugh classification (Table 1), and that inverse correlation was observed between IL-6 and npRQ (Table 3).

Free fatty acid is also reported as a biomarker for hepatic encephalopathy.[41] A significant correlation between serum FFA and blood ammonia level in our study (Fig. 5) supports the previous description as given

Table 6 Effects of LES supplementation in cirrhotic patients†

	Before LES	After LES	P‡
Total bilirubin (mg/dL)	1.16 ± 0.54	1.09 ± 0.47	0.6079
Albumin (g/dL)	3.26 ± 0.36	3.16 ± 0.32	0.1382
Alanine aminotransferase (IU/L)	35.0 ± 18.1	46.4 ± 32.2	0.3084
Prothrombin time (%)	84.1 ± 5.8	82.1 ± 11.1	0.4592
Ketone body (μmol/L)	204.7 ± 159.6	68.9 ± 54.7	0.0184*
Free fatty acid (μEq/L)	914.9 ± 312.3	504.3 ± 224.5	0.0015**
3-methylhistidine (μmoL/day)	123.9 ± 39.2	125.4 ± 52.7	0.1850
BTR	3.15 ± 0.83	4.58 ± 1.47	0.0182*
FPG (mg/dl)	89.5 ± 6.2	97.5 ± 9.8	0.0405*
IRI (μU/ml)	11.5 ± 5.3	12.5 ± 9.5	0.6975
REE (kcal/d)	1242.5 ± 215.1	1343.4 ± 211.0	0.0936
BMR (kcal/d)	1170.0 ± 176.6	1173.8 ± 188.9	0.6477
npRQ	0.770 ± 0.031	0.840 ± 0.048	0.0043**
CHO (%)	19.2 ± 8.1	39.7 ± 12.0	0.0012**
FAT (%)	65.0 ± 12.2	45.5 ± 15.6	0.0104*
PRO (%)	15.8 ± 5.6	14.9 ± 6.4	0.9454

†Values are presented as mean ± SD.
‡Compared between before and after LES supplementation by paired t-test.
*$P < 0.05$, **$P < 0.01$.
BCAA: branched-chain amino acid; BMR: basal metabolic rate; BTR: BCAA to tyrosine ratio; CHO: substrate oxidation rate of carbohydrate; FAT: substrate oxidation rate of fat; FPG: fasting plasma glucose; IRI: immuno-reactive insulin; LES: late evening snack; npRQ: non-protein respiratory quotient; PRO: substrate oxidation rate of protein; REE: resting energy expenditure.
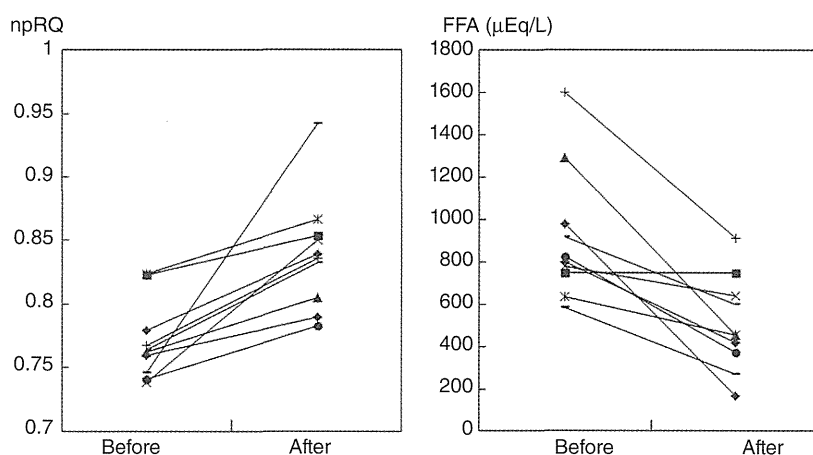
Figure 6 Increase in non-protein respiratory quotient (npRQ; $P < 0.01$) and decrease in free fatty acid (FFA; $P < 0.01$) were observed in cirrhotic patients after 1 week of late-evening snack supplementation.

above. Possible impact of DM on FFA level would be another interest, because FFA interferes insulin action in the peripheral tissues. Actually, in this study, immunoreactive insulin level was significantly higher in cirrhotics with FFA of 660 μEq/L or higher than in those with FFA of less than 660 μEq/L. However, there was no correlation between FFA and FPG or the presence/absence of DM. This concern regarding FFA and DM in cirrhotics should be further addressed.

According to anthropometric parameters, energy malnutrition state is also estimated by TSF and AC. Decrease in TSF and AC predicts a low survival rate in patients with LC.[4,29] However, there was no correlation between anthropometric parameters and neither Child-Pugh grade nor npRQ in this study. In addition, no correlation was observed between FFA and anthropometric parameters (data not shown). These results might have been affected by the presence of peripheral edema in cirrhotic patients.

As described earlier, npRQ of less than 0.85 predicted a significantly lower survival rate in patients with LC than in those with higher scores.[5] In order to avoid such a state of nocturnal starvation, LES is recommended as one of the effective nutritional interventions to shorten catabolic state in current guidelines.[7,8,10] Several reports showed that supplementation of LES increased npRQ and decreased serum FFA level after overnight fasting.[33,42,43] Also, in our validation group, only 1 week of supplementation with LES significantly recovered npRQ and reduced FFA. Thus, FFA could alternately represent npRQ in patients with LC as an indicator of energy malnutrition, and show fair sensitivity and accuracy. In this patient group, serum ketone body and FPG addi-

tionally showed significant changes (Table 6), but these parameters were not significant in the exploration cohort (Tables 3 and 4) and require further evaluation.

An interesting observation in this study is that there was no significant difference in serum albumin concentration between FFA of less than 660 and FFA of 660 μEq/L or higher, suggesting that FFA of 660 μEq/L or higher appears to indicate only the presence of energy malnutrition, independently of protein nutritional state in patients with LC.

However, we could not reach a significant difference in the survival rate of patients with LC according to the FFA cut-off value (660 μEq/L), due to the short observation period in this study. Hence, long-term observation is essential to address this concern in the future. In addition, to further confirm the clinical significance of FFA, an interventional study with LES in a larger patient cohort would be required.

In conclusion, FFA is an alternative marker to represent npRQ measured by indirect calorimetry to evaluate energy malnutrition in LC.

ACKNOWLEDGMENTS

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REVIEW

Pharmaceutical and nutraceutical approaches for preventing liver carcinogenesis: Chemoprevention of hepatocellular carcinoma using acyclic retinoid and branched-chain amino acids

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The poor prognosis for patients with hepatocellular carcinoma (HCC) is associated with its high rate of recurrence in the cirrhotic liver. Therefore, more effective strategies need to be urgently developed for the chemoprevention of this malignancy. The malfunction of retinoid X receptor α, a retinoid receptor, due to phosphorylation by Ras/mitogen-activated protein kinase is closely associated with liver carcinogenesis and may be a promising target for HCC chemoprevention. Acyclic retinoid (ACR), a synthetic retinoid, can prevent HCC development by inhibiting retinoid X receptor α phosphorylation and improve the prognosis for this malignancy. Supplementation with branched-chain amino acids (BCAA), which are used to improve protein malnutrition in patients with liver cirrhosis, can also reduce the risk of HCC in obese cirrhotic patients. In experimental studies, both ACR and BCAA exert suppressive effects on HCC development and the growth of HCC cells. In particular, combined treatment with ACR and BCAA cooperatively inhibits the growth of HCC cells. Furthermore, ACR and BCAA inhibit liver tumorigenesis associated with obesity and diabetes, both of which are critical risk factors for HCC development. These findings suggest that pharmaceutical and nutraceutical approaches using ACR and BCAA may be promising strategies for preventing HCC and improving the prognosis of this malignancy.

1 Introduction

Hepatocellular carcinoma (HCC), which usually develops in the livers of patients with chronic hepatitis and liver cirrhosis, is a serious clinical and social issue worldwide. Annually, the number of new cases is approximately 750 000, with an estimated 700 000 patients dying because of the malignancy [1, 2]. Although effective methods of diagnosis and treatment for HCC have been recently developed, improvement in the prognosis for this cancer is limited; overall survival, 10 years after curative treatment, is only 22–35% [3, 4]. The primary reason for the poor prognosis of HCC is its high frequency of recurrence after curative treatment; the recurrence rate, 5 years after definitive therapy in cirrhotic patients, may exceed 70% [5–7]. These facts indicate that curative treatment for HCC is difficult once this malignancy has developed, and therefore, effective strategies for preventing this cancer are urgently required.

In a previous, prospective, randomized trial, we reported that the oral administration of acyclic retinoid (ACR), a novel synthetic retinoid, significantly suppressed the posttherapeutic recurrence of HCC and improved the survival rate of patients [8–10]. Oral supplementation with branched-chain

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amino acids (BCAA), which is widely used in patients with liver cirrhosis to improve protein energy malnutrition (PEM), also reduced the risk of HCC in obese cirrhotic patients [11]. The effects of ACR and BCAA on the chemoprevention of HCC and the inhibition of HCC cell growth have been reported in several experimental studies [12–16]. In particular, recent rodent studies demonstrated that administration of ACR and BCAA suppresses the liver carcinogenesis associated with obesity and diabetes, both of which are critical risk factors for HCC development [17,18]. The results of these clinical and basic studies strongly suggest that pharmaceutical and nutraceutical approaches, especially using ACR and BCAA, might be effective strategies for preventing liver carcinogenesis. In this article, we provide an overview of the clinical characteristics and molecular pathogenesis of HCC, focusing on the role of retinoid X receptor α (RXRα) phosphorylation in liver carcinogenesis. The detailed effects of ACR and BCAA in the prevention of HCC development are reviewed, based on our clinical and basic research. We also review the possibility of pharmaceutical and nutraceutical approaches for the inhibition of obesity- and diabetes-related liver carcinogenesis through the targeting of the pathophysiological conditions caused by these metabolic abnormalities, concentrating on the effects of ACR and BCAA.

2 Clinical characteristics of HCC

Most cases of HCC, which is the dominant form of primary liver carcinoma, are associated with the chronic inflammation and subsequent cirrhosis of the liver, that is induced by a persistent infection with one of the hepatitis viruses, hepatitis B virus (HBV) or hepatitis C virus (HCV) [19,20]. After development of virus-induced chronic hepatitis and liver cirrhosis, the entire liver enters a precancerous state, possessing multiple, independent, premalignant, or latent malignant clones. Therefore, the typical clinical pattern of liver carcinogenesis is **ulticentric carcinogenesis, which is also described as **ield cancerization. This carcinogenesis pattern contributes to the high frequency of HCC development in patients with viral liver cirrhosis. Significantly, the annual rate for HCC development is approximately 7% in cirrhotic patients, and even after curative treatment, the annual incidence of recurrence is approximately 20–25% [5–7]. These facts highlight the poor prognosis of viral liver cirrhotic patients and suggest the possibility of improved clinical outcomes if effective strategies are developed for preventing HCC.

One of the most effective approaches for preventing the development of HCC is the eradication of the hepatitis viruses. Several meta-analyses have shown the effectiveness of IFN therapy for preventing HCV-related HCC [21–23], indicating that sustained antiviral response to IFN-based therapy is associated with a reduced risk of developing this malignancy. In addition, IFN treatment might be effective for preventing HCC development in HCV patients, even if sustained antiviral response is not achieved [24]. Antiviral treatments,

such as IFN therapy and nucleos(t)ide analog therapy, also prevent the development of HBV-related HCC [25,26]. These clinical evidences strongly suggest that antiviral treatment is effective for reducing the incidence of HCC development in patients with chronic HBV or HCV infections. In addition, two cohort studies of HCV patients demonstrated that hepatic inflammation alleviation therapy, involving glycyrrhizin injection, suppressed HCC development [27,28]. These results also indicate that attenuation of chronic inflammation might be effective for inhibiting liver carcinogenesis.

3 Molecular pathogenesis of HCC

HCC is a heterogeneous tumor because it develops in a complex multistep process in which many signaling cascades are altered. That is, the accumulation of genetic alterations is critically involved in hepatocarcinogenesis [29,30]. Genomic mutations in the *p53* tumor suppressor gene occur in 10–35% of HCC cases [31]. Genomic mutations in the *CTNNB1* gene, which encodes β-catenin, have also identified in approximately 20–40% of liver cancers [31]. Because of these alterations, several signaling pathways related to cell proliferation and survival are activated during liver carcinogenesis. For instance, epithelial growth factor receptor, which is a receptor tyrosine kinase (RTK), is expressed in 68% of HCC cases, and this receptor is associated with the proliferation and clinical stage of this malignancy [32]. Activation of insulin-like growth factor (IGF) 1 receptor (IGF-1R) signaling, which is another RTK, also contributes to the early stages of liver carcinogenesis [30]. The major signaling pathways activated by the RTKs/Ras pathways are the mitogen-activated protein kinase (MAPK)/extracellular signal-regulated kinase (ERK) and the phosphoinositide-3-kinase (PI3K)/Akt pathways, both of which play important roles in the proliferation and survival of cancer cells. These reports, therefore, strongly suggest that targeting specific RTKs and their downstream signaling pathways is a potentially effective strategy for preventing some types of human malignancies, including HCC [33–38].

4 Retinoid abnormalities and HCC

In addition to the pathophysiological mechanisms as mentioned above, recent studies have revealed the magnitude of the abnormalities in the expression and function of retinoids on liver carcinogenesis [12–15, 39]. Retinoids are a group of natural and synthetic molecules that are structurally and/or functionally related to fat-soluble vitamin A. These molecules participate in a broad spectrum of biological activities, including embryogenesis, growth, differentiation, proliferation, apoptosis, and metabolism [40–42]. The fundamental effects of retinoids on cellular activities are largely mediated through the expression of two distinct families of nuclear receptors, the retinoic acid receptors (RARs) and RXRs. The RARs are activated by all-*trans*-retinoic acid and 9-*cis*-retinoic

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acid, with similar affinities, whereas RXRs are only activated by 9-*cis*-retinoic acid [40–42]. Both the RARs and RXRs are composed of three subtypes (α, β, and γ), which are characterized by a modular domain structure, and these nuclear receptors are ligand-dependent transcription factors [40–42]. After ligand binding, the RXRs form homodimers and heterodimers with the RARs and interact with the retinoid X response element or the RAR responsive element, which are located in the promoter region of the target genes, thereby modulating gene expression [40–42]. RXRs can also form heterodimers with other nuclear receptors, such as peroxisome proliferator-activated receptor, indicating that RXRs act as common heterodimerization partners for various types of nuclear receptors [41]. Thus, RXRs are considered the master regulators of nuclear receptors because they are involved in the regulation of fundamental cell activities, including normal cell proliferation, metabolism, and death (regulation of apoptosis). In particular, RXRα plays a critical role in the normal control of hepatocyte lifespan and proliferation [43,44].

These characteristics also suggest that abnormalities in the expression and function of retinoid signaling are closely associated with deviations from normal cell proliferation and death, which are key factors in the development of several types of human cancers, including HCC. For example, retinol, a transport form of retinoid in the plasma, is locally deficient in HCC, but not in the adjacent, normal liver tissue in a rodent model of hepatocarcinogenesis [45]. In a rat model of chemically induced liver carcinogenesis, repression of RXRα occurs even in the early stages of carcinogenesis because its expression is decreased not only in HCC and liver cell adenoma, but also in precancerous HCC lesions [46]. The expression levels of RARβ, which is regarded as a tumor suppressor gene because of its ability to regulate cell growth and apoptosis [47], are markedly decreased in both human [48] and rat HCC [46]. On the other hand, RARγ, which is over-expressed in human HCC tissues and cells, enhances the growth of HCC cells through the activation of the PI3K/Akt signaling pathway [49]. These reports strongly indicate that the restoration of the function and expression of retinoid receptors, via treatment with retinoids, might be effective for the prevention of certain types of human malignancies, including HCC [12–15,50,51].

5   RXRα phosphorylation and HCC

We proposed that RXRα phosphorylation and its malfunction is closely associated with liver carcinogenesis [12–15]. RXRα protein, which is anomalously phosphorylated at its serine and threonine residues, prominently accumulates in both surgically resected human HCC tissues and human HCC-derived cell lines [39,52]. Activation of the RTK/Ras/MAPK signaling frequently occurs in HCC cells [30,32]. The constitutive phosphorylation of serine-260 in RXRα, a MAPK/ERK consensus site, by this signaling pathway is closely associated with retarded degradation of RXRα, lowered transcriptional

activity of this nuclear receptor, and promotion of cancer cell growth [39,53]. In human HCC cells, phosphorylated RXRα is resistant to proteolytic degradation via the ubiquitination-/proteasome-mediated pathway, facilitating the accumulation of this phosphorylated protein within HCC tissues [54]. Furthermore, phosphorylated RXRα abolishes its ability to form heterodimers with RARβ, and this is implicated in uncontrolled cell growth and retinoid resistance [55]. These findings suggest that the accumulation of phosphorylated RXRα, regarded as the nonfunctional form of RXRα, may interfere with the function of normal (unphosphorylated) RXRα in a dominant-negative manner, thus, playing a critical role in liver carcinogenesis (Fig. 1). On the other hand, the abrogation of RXRα phosphorylation by a MAPK inhibitor or transfection with the nonphosphomimetic mutant RXRα restores the degradation of RXRα in a ligand-dependent manner [39,53]. Thus, the targeting of RXRα phosphorylation might be a strategy for preventing HCC, and ACR is a promising agent for this purpose, as discussed in Section 6.

6   Mechanisms of ACR in HCC chemoprevention

ACR, also known as NIK-333 and Peretinoin (Kowa Pharmaceutical, Tokyo, Japan), is a synthetic retinoid that was initially developed as an agonist for both RXR and RAR [56,57]. ACR inhibits growth of human HCC-derived cells by activating the promoter activity of retinoid X response element and RAR responsive element and regulating the expression of retinoid target genes, including *RARβ*, *p21^{CIP1}*, and *cyclin D1*, resulting in the induction of apoptosis and cell cycle arrest in the G_0–G_1 phase [53,58–63]. These findings indicate that ACR exerts growth inhibitory effects in HCC cells, at least in part, by working as a ligand for retinoid receptors and controlling their target genes, especially *RARβ* and *p21^{CIP1}*. The antitumor effects of ACR are also associated with suppression of telomerase activity, attenuation of oxidative stress, and inhibition of angiogenesis [64–66]. Moreover, the suppressive effects of ACR on liver carcinogenesis have been demonstrated in several animal experiments [17,45,67–69].

Furthermore, we have proposed that inhibition of RXRα phosphorylation is a critical mechanism of ACR, allowing it to exert chemopreventive effects in liver carcinogenesis. In human HCC-derived cells, ACR can restore RXRα function by inactivating the Ras/MAPK signaling system and dephosphorylating RXRα, although 9-*cis*-retinoic acid is incapable of suppressing ERK and RXRα phosphorylation [53]. Moreover, recent studies have revealed that ACR suppresses the growth of several types of cancer cells, such as HCC and head and neck squamous cell carcinoma cells, and prevents chemically induced liver carcinogenesis by inhibiting the activation and expression of several types of growth factors and their corresponding RTKs [63,66,68–73]. ACR also inhibits Ras activation, and this is associated with prevention of obesity-related liver tumorigenesis in mice and the
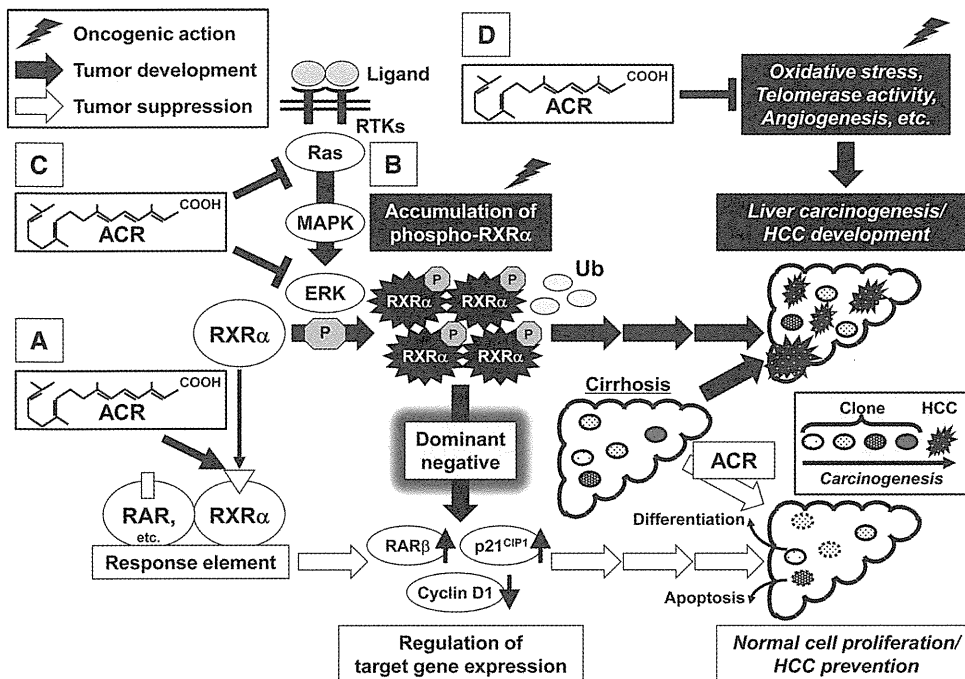
Figure 1. Retinoid refractoriness due to phosphorylation of RXRα, and its restoration by ACR in liver carcinogenesis. When ACR binds to and activates RXRα in normal hepatocytes, the receptor forms homodimers and/or heterodimers with other nuclear receptors, including RARs. This results in the expression of the target genes, such as RARβ, p21^{CIP1}, and cyclin D1, which regulate normal cell proliferation and differentiation and control the induction of apoptosis and cell cycle progression. Therefore, in the cirrhotic liver, ACR can delete and inhibit malignant clones, at least in part, by controlling the expression of these RXRα-target genes (A). In HCC cells, several types of RTKs, such as epidermal growth factor receptor superfamily and IGF-1R and their downstream Ras/MAPK pathway, are highly activated, which results in the phosphorylation of ERK and RXRα and subsequent suppression of dimer formation and transactivation functions of RXRα (refractoriness to retinoid). Furthermore, nonfunctional phosphorylated RXRα, which is sequestered from ubiquitin (Ub)/proteasome-mediated degradation and accumulates in liver cells, interferes with the physiological functions of the remaining nonphosphorylated (i.e., functional) RXRα in a dominant-negative manner, and this is also involved in liver carcinogenesis (B). ACR inhibits phosphorylation of RXRα, restores the function of this receptor, and activates the transcriptional activity of the responsive element associated with this receptor. This is accomplished by inhibiting the Ras/MAPK signaling pathway and the ligand-dependent (growth factor) RTK activities, which contribute to the prevention of liver carcinogenesis and suppression of growth in HCC cells (C). In addition, ACR inhibits growth of HCC cells through the attenuation of oxidative stress, inhibition of telomerase activity, and repression of angiogenesis (D). The pleiotropic effects of ACR to prevent HCC development have also been summarized in recent reviews [12–15].

inhibition of cell growth in human HCC and pancreatic cancer cells [17,58,74]. These findings indicate that activation of the RTK/Ras/MAPK signaling pathway, which is involved in HCC development [30,32], and the subsequent phosphorylation of RXRα are critical targets of ACR for the inhibition of liver carcinogenesis [12–15] (Fig. 1).

7 HCC chemoprevention by ACR: Clinical trial results

Because the results from numerous preclinical experiments indicated that ACR may be an effective agent for HCC chemoprevention, an early-phase, randomized, controlled clinical trial was conducted to determine whether ACR can reduce the incidence and recurrence of second primary HCC in patients who underwent potentially curative treatment for initial

HCC [8–10]. In this trial, oral administration of ACR (44 patients, 600 mg/day) for 12 months significantly reduced the incidence of recurrent or new HCC compared to placebo (45 patients) after a median follow-up period of 38 months; 12 patients (27%) in the ACR group developed HCC as compared with 22 patients (49%) in the placebo group ($p = 0.04$) [8]. After a further follow-up period of 62 months, ACR treatment demonstrated improved recurrence-free survival ($p = 0.002$) and overall survival ($p = 0.04$) [9]. The relative risk for the development of secondary HCC and death were 0.31 (95% confidence interval [CI], 0.12–0.78) and 0.33 (95% CI, 0.11–0.79), respectively [8,9]. Therefore, the estimated 6-year overall survival was 74% in the ACR group and 46% in the placebo group [9].

A multicenter, large-scale ($n = 401$), randomized, placebo-controlled trial also confirmed the effectiveness of ACR in preventing second primary HCCs in HCV-positive patients

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who underwent curative treatment for primary or the first recurrence of HCC, with a median follow-up of 2.5 years. In this trial, oral administration of ACR (600 mg/day) had a strong effect on the prevention of a second primary HCC with a hazard ratio of 0.27 (95% CI, 0.07–0.96), 2 years after treatment, and at 3 years, the cumulative recurrence-free survival rates in the ACR-treated group (43.7%) were higher than those in the placebo group (29.3%) [75]. In addition, a subgroup analysis of this study showed that ACR prevented development of a second primary HCC with a hazard ratio of 0.38 (95% CI, 0.20–0.71) in patients who were Child-Pugh A and had small tumors (size, <20 mm) [76]. These results indicated that ACR administration at an early stage of liver cirrhosis contributes to the prevention of HCC. In addition to the effectiveness of ACR for the prevention of HCC development, the results of these clinical trials [8–10,75,76], together with a phase I pharmacokinetics trial [77], have proven the safety of ACR in a clinical setting. Therefore, the findings of these clinical trials [8–10,75–77] strongly suggest that ACR is a novel first-line therapy for reducing the development of a second primary HCC.

8   HCC chemoprevention by ACR: The concept of "clonal deletion" therapy

Two interesting facts were revealed in an early-phase, ACR clinical trial [8–10]. First, the preventive effects of ACR on HCC development lasted up to 50 months after randomization or 38 months after completion of ACR administration, indicating that a 12-month administration of this agent conferred a long-term effect on the prevention of second primary HCCs [10]. Second, ACR administration for 12 months significantly reduced the serum levels of lectin-reactive α-fetoprotein factor 3, which might be produced from latent (i.e., invisible) malignant clones in the remnant liver [78]. These facts suggest the following two possibilities: (i) ACR can delete the α-fetoprotein factor 3 producing premalignant clones from the remnant liver before they expand into clinically detectable HCC and (ii) after the elimination of the malignant clones from the remnant liver by ACR, several years elapse before the clinical appearance of the next HCC clones. The cirrhotic liver is a precancerous field that possesses multiple, independent premalignant, or latent malignant clones. Therefore, before expanding into clinically detectable tumors, a positive approach for the removal and inhibition of such latent malignant clones from the cirrhotic liver should be conducted to prevent HCC development. We consider that implementation of this approach, termed clonal deletion therapy, is a practical approach for preventing HCC, and that ACR is a consistent and reasonable agent for this purpose [12–15] (Fig. 1).

A recent study by Honda et al. [79] reported that an 8-wk administration of ACR significantly elevated the expression levels of many retinoid target genes and tumor suppressor-related genes, but decreased the expression levels of tumor

progression-related genes in the liver of HCV-positive patients. This report may also provide evidence that ACR can change the hepatic environment to a non-hypercarcinogenic one.

9   BCAA supplementation and chronic liver disease

BCAA (valine, leucine, and isoleucine) is a widely accepted therapy for improving hepatic insufficiency and its related PEM, which is a common manifestation of patients with liver cirrhosis [80, 81]. PEM affects the outcome of the cirrhotic patients by determining both their quality of life and survival [82, 83]. Cirrhotic patients frequently demonstrate a decreased serum ratio of BCAA to aromatic amino acids, reduced serum albumin levels, and decreased skeletal muscle volume [80, 81]. They have also demonstrated that an increased consumption of foods containing high BCAA content does not affect plasma BCAA levels [84]. On the other hand, nutritional intervention with BCAA has been shown to increase the serum albumin concentration and improve patient quality of life and prognosis by preventing severe complications associated with this disease [85–88]. For instance, in a multicenter, large scale ($n = 646$), randomized, and nutrient intake-controlled trial in Japan, the long-term survival study, oral supplementation with BCAA (12 g/day) for 2 years to patients with decompensated cirrhosis significantly decreased the incidence of events associated with progression to hepatic failure (hazard ratio, 0.67; 95% CI, 0.49–0.93; $p = 0.015$; median observation period, 445 days) [85]. The reports of the trial [85–88], therefore, indicated that BCAA supplementation may serve as a first-line therapy for patients with decompensated cirrhosis.

10   HCC chemoprevention by BCAA supplementation

Several experimental studies have revealed the precise mechanisms of BCAA in the suppression of cancer cell growth and chemoprevention of HCC. Hagiwara et al. [89] reported that BCAA directly suppresses HCC cell proliferation by inducing apoptosis and inhibiting the activation of PI3K/Akt and nuclear factor-κB signaling pathways. BCAA treatment also inhibits the proliferation of human HCC-derived cells by increasing cellular levels of $p21^{CIP1}$ and arresting the cell cycle in the G_0/G_1 phase [90]. Both in vitro and in vivo studies have demonstrated the antiangiogenesis activity of BCAA induced by suppressing the expression of vascular endothelial growth factor in HCC cell lines and in the liver of rats bearing neoplasm [91, 92]. BCAA supplementation also reduces oxidative stress in HCV-positive patients with liver cirrhosis as well as in rats with advanced liver cirrhosis [93, 94]. These reports suggest that BCAA exerts chemopreventive effects against HCC, at least in part, by suppressing angiogenesis and

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improving oxidative stress, both of which are critically involved in liver carcinogenesis.

Moreover, recent clinical trials revealed that BCAA supplementation may influence the prevention of HCC development [11, 95–100]. The results of a retrospective analysis showed that BCAA supplementation (12 g/day for >6 months) reduced the incidence of HCC in patients with liver cirrhosis with a hazard ratio of 0.42 (95% CI, 0.22–0.80; $p = 0.009$) [95]. Oral supplementation of BCAA (12 g/day for 6 months) significantly decreased the serum levels of AFP and reduced early recurrence after hepatic resection in patients with HCC [98]. In a subset analysis of the long-term survival study, Muto et al. also showed that long-term oral supplementation with BCAA significantly inhibited the development of HCC in type C cirrhotic patients with BMIs >25 [11]. Moreover, the administration of BCAA granules (12 g/day for 60 months) markedly inhibited the cumulative recurrence of HCC, after curative treatment in patients, with insulin resistance [96]. Therefore, long-term treatment with BCAA is an effective strategy for improving the clinical outcomes in cirrhotic patients by reducing the likelihood of liver failure and in obese and diabetic patients, by suppressing liver carcinogenesis. Pathophysiological conditions involved in the development of obesity-related HCC and in the precise mechanisms of BCAA to inhibit liver carcinogenesis, in particular the mechanisms associated with obesity, are discussed in the following sections.

11 Obesity and HCC

Among patients with liver cirrhosis, the proportion of obese subjects is gradually increasing [101, 102]. This is a serious problem when considering the medical care of chronic liver disease because obesity and its related metabolic abnormalities, especially diabetes mellitus, are major risk factors for the development of HCC [11, 103–106]. Nonalcoholic fatty liver disease, a hepatic manifestation of obesity and metabolic syndrome, is also an important healthcare problem, especially in developed countries, since it can progress to nonalcoholic steatohepatitis), which in turn leads to liver cirrhosis and HCC development [107, 108].

Recent studies have shown several pathophysiological mechanisms linking obesity and liver carcinogenesis, including the emergence of insulin resistance, activation of the IGF/IGF-1R axis, development of a state of chronic inflammation, induction of oxidative stress, and adipokine imbalance [103, 104]. In particular, insulin resistance, which leads to systemic and hepatic inflammation, liver steatosis, and activation of the IGF/IGF-1R axis, is considered to play a critical role in the development of HCC [35, 103, 104, 109]. On the other hand, these reports strongly indicate that targeting such pathophysiological disorders via pharmaceutical and nutraceutical intervention might be an effective strategy to prevent obesity-related liver carcinogenesis [16, 110]. For instance, pitavastatin, a drug widely used for the treatment of

hyperlipidemia, and (−)-epigallocatechin-3-gallate, one of the green tea catechins, significantly inhibit the obesity-related liver tumorigenesis by attenuating the chronic inflammation induced by excess fat deposition [111, 112]. Administration of ACR also suppresses diethylnitrosamine-induced liver tumorigenesis in obese and diabetic mice and this is associated with inhibition of Ras activation and phosphorylation of the ERK and RXRα proteins [17]. Increase in insulin sensitivity and the attenuation of systemic and hepatic inflammation by ACR also contribute to this inhibition [17], indicating that ACR might be useful in the chemoprevention of obesity-related HCC (Fig. 2).

12 Preventive mechanisms of BCAA in obesity-related liver carcinogenesis

Recent experimental studies have revealed that BCAA improves insulin resistance and glucose tolerance via the enhancement of glucose metabolism in skeletal muscle, adipose tissue, and the liver [113–118]. Improvements in insulin resistance and glucose tolerance, by oral BCAA supplementation in chronic liver disease patients, have also been reported in several clinical trials [119–121]. In addition, a recent in vitro study showed that BCAA treatment suppresses insulin-induced proliferation of HCC cells by inhibiting the insulin-induced activation of the PI3K/Akt pathway and the subsequent antiapoptotic pathway [89]. We, therefore, consider that improvements in glucose metabolism and insulin resistance might be a critical mechanism in the reduction of the incidence of HCC development in obese cirrhotic patients [11]. This hypothesis was evaluated using an obesity- and diabetes-related liver carcinogenesis mouse model [18]. In the model, BCAA supplementation significantly inhibited diethylnitrosamine-induced liver tumorigenesis in obese and diabetic *db/db* mice by improving liver steatosis and fibrosis, insulin resistance, and hyperleptinemia [18]. Supplementation with BCAA also inhibited the spontaneous development of hepatic premalignant lesions in *db/db* mice via the attenuation of chronic inflammation in both the liver and white adipose tissue [122]. Moreover, BCAA treatment significantly inhibited the proliferation of human HCC-derived cells induced by visfatin, a serum adipokine that is significantly correlated with stage progression and tumor enlargement of HCC [90]. Yoshiji et al. [92] also reported that, in obese and diabetic rats exhibiting insulin resistance, BCAA treatment significantly exerted a chemopreventive effect against HCC through the suppression of hepatic neovascularization. The results of these reports [18, 89, 90, 92, 122] strongly indicate that BCAA inhibits obesity-related liver carcinogenesis by targeting insulin resistance and subsequently by reducing chronic inflammation and adipokine imbalance (Fig. 2). In addition to the liver, supplementation with BCAA suppressed obesity- and diabetes-related carcinogenesis in the colorectum, and this was also associated with the improvement of insulin resistance and inhibition of the activation of
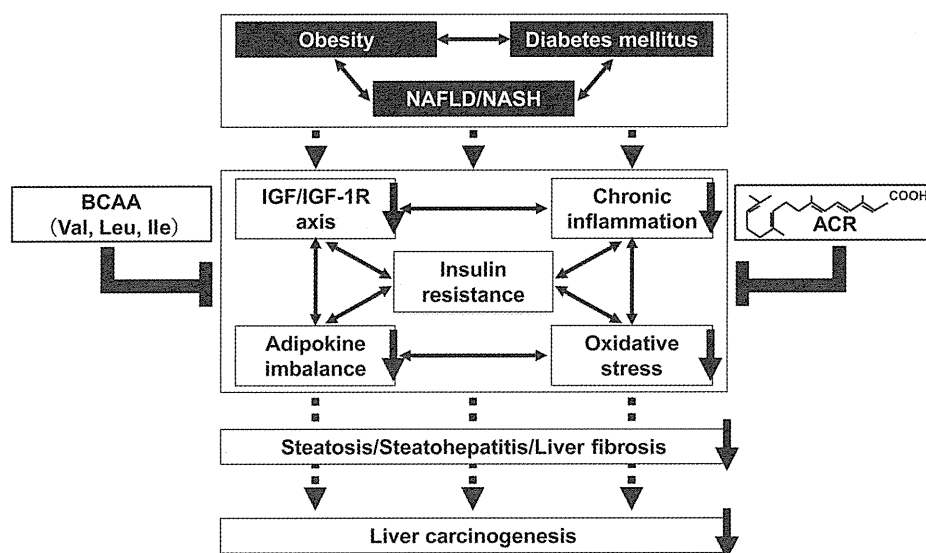
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Figure 2. The mechanisms of action of ACR and BCAA in the inhibition of obesity-related liver carcinogenesis. Obesity and diabetes mellitus significantly increase the risk of HCC. Nonalcoholic fatty liver disease and nonalcoholic steatohepatitis, which are usually associated with obesity and diabetes, also play critical roles in the development of HCC. Several pathophysiological mechanisms link obesity and liver carcinogenesis, including the emergence of insulin resistance, activation of the IGF/IGF-1R axis, a state of chronic inflammation, induction of oxidative stress, and occurrence of adipokine imbalance. Among them, in particular, insulin resistance plays a key role in obesity-related liver carcinogenesis. Oral supplementation with BCAA significantly reduces the risk of HCC development in obese cirrhotic patients, and this might be associated with decreased insulin resistance and hepatic steatosis, inhibition of the activation of the IGF/IGF-1R axis, and attenuation of oxidative stress and hyperleptinemia. ACR administration also prevents obesity- and diabetes-related liver tumorigenesis in mice by improving hepatic steatosis and insulin resistance, while attenuating chronic inflammation.

the IGF/IGF-IR axis [123]. BCAA, therefore, may be a useful chemoprevention modality for HCC and probably colorectal cancer in obese people.

13 Conclusion

Throughout this review, we have indicated that both ACR and BCAA are promising agents for the prevention of liver carcinogenesis. Therefore, we considered that a combination therapy involving both ACR and BCAA may better inhibit HCC cell growth. Interestingly, a combined ACR and BCAA treatment significantly inhibited the growth of human HCC xenografts in nude mice by inhibiting the phosphorylation of the RXRα, ERK, Akt, and IGF-1R proteins in the xenografts [124]. These results indicated that this combination might be effective for the treatment and probably chemoprevention of HCC. The beneficial effects of the combination approach to chemoprevention, using ACR as a key agent for the prevention and treatment of HCC, have been previously reported [58, 59, 125–127]. A clinical trial also demonstrated that the combination of BCAA and perindopril, an antihypertensive drug, inhibited the cumulative recurrence of HCC after curative therapy and this was associated with improved insulin resistance [128]. Therefore, a combination therapy us-

ing ACR and/or BCAA may represent a potential new strategy for chemoprevention of HCC development.

In summary, the poor prognosis of patients with HCC is because of its high incidence and recurrence in cirrhotic livers. Therefore, more effective strategies for the chemoprevention of HCC should be developed to directly improve prognoses for these patients. The results from both experimental and clinical studies strongly suggest that pharmaceutical and nutraceutical approaches, in particular using ACR and BCAA, play a central role in this strategy. These agents may also play a critical role in the prevention of obesity-related liver carcinogenesis, which is a new, serious problem in modern society.

The authors have declared no conflict of interest.

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