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Branched-Chain Amino Acid Supplementation Complements Conventional Treatment for Spontaneous Bacterial Peritonitis

EITARO TANIGUCHI, MD, PhD,† TAKUMI KAWAGUCHI, MD, PhD,* MARI SHIMADA, MD,† REIICHIRO KUWAHARA, MD, PhD,† YUMIKO NAGAO, MD, DDS,* MOMOKA OTSUKA, Dietitian,† SHOKO IWASAKI, Dietitian,† TOKIKO MATSUDA, Nrs,† RYOKO IBI, Nrs,† SATOMI SHIRAISHI, Nrs,† MINORU ITOU, MD,† TETSUHARU ORIISHI, MD,† RYUKICHI KUMASHIRO, MD, PhD,† SHOKO TANAKA, Dietitian,† YUKO SARUWATARI, Nrs,† and MICHIO SATA, MD*

KEY WORDS: nutrition; liver failure; late evening snack; hepatic fibrosis; hepatocyte growth factor.

Spontaneous bacterial peritonitis (SBP) is a life-threatening complication of liver cirrhosis (1). The prevalence of SBP among hospitalized cirrhotic patients with ascites has been estimated to be approximately 15% (2). SBP in cirrhotic patients is thought to occur as a consequence of impaired defensive mechanisms against infection, such as a decrease in the lymphocyte count and an impaired phagocytic function of neutrophils (3).

Mortality rate related to SBP has been improved by the development of new antibiotics (4); however, the rate is still 17–50% (2). Branched-chain amino acids (BCAA) supplementation improves not only the nutritional and metabolic status such as serum albumin concentration and Fisher's ratio, but also defensive mechanisms against infection in cirrhotic patients (5). Strengthening of resistance to infection is believed to be the result of the elevation of the absolute lymphocyte count (6). Moreover, a recent study disclosed that BCAA supplementation improves phagocytic function of neutrophils in cirrhotic patients (7). Although BCAA supplementation seems to be an effective therapy in patients with SBP, the beneficial effects of BCAA supplementation have never been reported in this regard.

In this report, we present the first documented case showing that BCAA supplementation complements conventional treatment for SBP and subsequent liver failure. BCAA supplementation should be considered as a complementary treatment for patients with SBP.

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From the *Department of Digestive Disease Information and Research and †Nutritional Curative Team and Second Department of Medicine, Kurume University School of Medicine, Kurume, Japan.

Address for reprint requests: Eitaro Taniguchi, MD, PhD, Second Department of Medicine, Kurume University School of Medicine, 67 Asahi-machi, Kurume 830-0011, Japan; eitaro@med.kurume-u.ac.jp.

CASE REPORT

A 71-year-old Japanese woman was referred to Kurume University Hospital for abdominal pain. Chronic hepatitis C was diagnosed when the patient was 54 years old. Despite various treatments including interferon, liver cirrhosis developed when the patient was 66 years old. Ascites was evident 2 months before the patient presented with abdominal pain.

Physical examination showed a height of 152 cm and a weight of 51.7 kg. Vital signs were within normal range except for her body temperature (BT) of 37.2 °C. The patient had icteric pigmentation of the sclera and skin, tense ascites, and peripheral edema. Laboratory data on admission are summarized in Table 1. Briefly, liver failure with inflammation was indicated. In the patient's ascitic fluid, the protein concentration was 0.29 g/dL and the polymorphonuclear count was 837/mm^3; few red blood cells and no malignant cells were seen. Abdominal x-ray showed no free air. Abdominal ultrasound examination showed no findings suggesting acute pancreatitis, tumor, or hemorrhage in the abdominal organs. Although no pathogenic organisms could be isolated from the ascites or peripheral blood, the patient exhibited abdominal pain and fever, a polymorphonuclear count >500/mm^3 in the ascitic fluid, and an absence of clinical, laboratory, radiologic, and ultrasound findings suggesting secondary peritonitis. Therefore, a diagnosis of SBP with liver failure was established.

SBP was immediately treated with a third-generation cephalosporin (sulbactam/cefoperaazone; Figure 1). Infusions of albumin were also administered to reduce the risk of renal failure and disseminated intravascular coagulation. At first, the antibiotic treatment seemed to be effective because the serum CRP concentration gradually decreased; however, the patient exhibited persistent fever and abdominal pain. The antibiotic was then

TABLE 1. LABORATORY DATA ON ADMISSION

Hematology		Glucose	84 mg/dL
WBC	5,600/mm^3	BUN	21.7 mg/dL
Neutrocytes	68.5%	Creatinine	0.66 mg/dL
Lymphocytes	23.5%	Na^+	143 mEq/L
RBC	361 × 10^4 mm^3	K^+	4.5 mEq/L
Platelets	3.4 × 10^4 mm^3	Cl^-	107 mEq/L
Biochemical examination		Prothrombin time	55%
AST	142 U/L	Endotoxin	<0.4 pg/mL
ALT	132 U/L	Blood culture	negative
LDH	494 U/L	**Ascitic fluid examination**	
Total protein	5.54 g/dL	Gross appearance	turbid
Albumin	2.7 g/dL	Protein	0.29 g/dL
Total bilirubin	3.74 mg/dL	WBC	1922/ mm^3
Direct bilirubin	1.10 mg/dL	Polymorphonuclear cells	837/ mm^3
CRP	7.15 mg/dL	culture	negative

changed to a carbapenem (imipenem/cilastatin) and infusions of -globulin were also administered. To improve the patient's nutritional status and resistance to infection, 50 g of Aminoleban EN containing abundant BCAA with 210 kcal energy (Table 2) was given as a late evening snack, although no findings of hepatic encephalopathy were seen. We also treated a decayed tooth, because it could have been an origin for elevated CRP concentration. With these treatments, abdominal pain disappeared, and BT and serum CRP concentration gradually decreased. However, BT and CRP concentration were not fully normalized, and a subsequent increase in serum total bilirubin concentration and a decrease in prothrombin activity occurred, indicating development of severe liver failure.

The patient's required energy expenditure was estimated to be 1700–1842 kcal/d based on the Harris-Benedict formula (8). Although the patient seemed to have an adequate dietary caloric intake with the BCAA supplementation, the serum concentration of free fatty acids showed 1531 μEq/L (normal range,

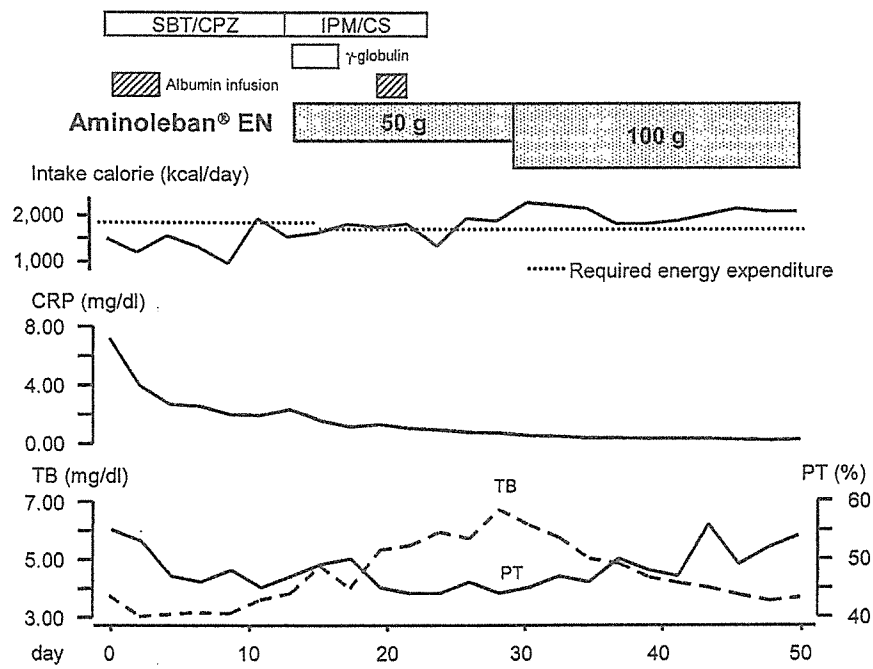

Fig 1. Laboratory indices over the days of admission. By treatment with antibiotics and -globulin infusions, CRP concentration gradually decreased. However, CRP concentration was not fully normalized. Total bilirubin reachd peak value and prothrombin activity reached its nadir on day 29. There was a dramatic response to additional administration of BCAA supplementation (total 100 g), with decrease in total bilirubin concentration and increase in prothrombin activity. CRP concentration returned to normal. *Abbreviations:* SBT/CPZ, sulbactam/cefoperazone; IPM/CS, imipenem/cilastatin; TB, total bilirubin; PT, prothrombin activity.

TABLE 2. COMPOSITION OF AMINOLEBAN EN (PER 50 G)

Total energy	210 kcal	Histidine	0.27 g
Protein	13.5 g	Proline	0.98 g
Amino acids		Serine	0.24 g
(Fischer's ratio = 38)			
Valine	1.79 g	Tyrosine	0.05 g
Leucine	2.25 g	Lysine	0.60 g
Isoleucine	2.04 g	Aspartic acid	0.45 g
Threonine	0.29 g	Glutamic acid	0.85 g
Tryptophan	0.08 g	Glycine	1.74 g
Methionine	0.06 g	Fat (rice oil)	3.50 g
Phenylalanine	0.17 g	Carbohydrates (Dextrin)	31.05 g
Alanine	0.75 g	Vitamins	
Arginine	0.88 g	Minerals	

Note. Vitamins include trace amounts of magnesium sulphate, calcium glycerophosphate, potassium iodide, potassium chloride, sodium dihydrogen phosphate dihydrate, sodium ferrous citrate, cupric sulphate, zinc sulphate, and manganese sulphate. Minerals include retinol palmitate, ergocalciferol, bisbentiamine, riboflavin, pyridoxine HCl, cyanocobalamin, folic acid, sodium l-ascorbate, tocopherol acetate, phytonadione, calcium pantothenate, nicotinamide, and biotin.

100–540 μEq/L), suggesting starvation. Moreover prothrombin activity was only 44% of normal, indicating impaired protein synthesis. We therefore administered additional BCAA supplementation after breakfast to improve the protein–energy malnutrition condition.

This action resulted in a dramatic decrease of total bilirubin concentration and increased prothrombin activity as well as the disappearance of ascites and peripheral edema (see Figure 1). Moreover, BT and CRP concentration were normalized with BCAA supplementation without the use of antibiotics. Simultaneously, increases in total lymphocyte count and serum hepatocyte growth factor (HGF) concentration were seen (Figure 2). Thus, SBP and subsequent liver failure were successfully managed.

We continued the BCAA supplementation. At 3 months' follow-up, the patient did not show any evidence of SBP or liver failure, and the serum concentrations of hyaluronic acid and type IV collagen were decreased, suggesting improvement of hepatic fibrosis (Figure 3).

DISCUSSION

This represent the first description of a case showing BCAA supplementation complementing conventional treatment of SBP. Thus, the use of BCAA supplementation as add-on therapy to antibiotics appears to reduce the risk of mortality in patients with SBP.

Although recent advances in the diagnosis and treatment of bacterial infections have improved the prognosis in patients with SBP (9), SBP accompanying severe liver failure continues to have a poor prognosis. Tito *et al.* reported that predictive factors for poor prognosis of SBP patients were serum bilirubin >4 mg/dL, prothrombin activity 45%, and protein concentration in ascitic fluid 1 g/dL (1). The patient in the current report demonstrated even poorer values for all of the predictive factors; therefore, poor prognosis was anticipated.

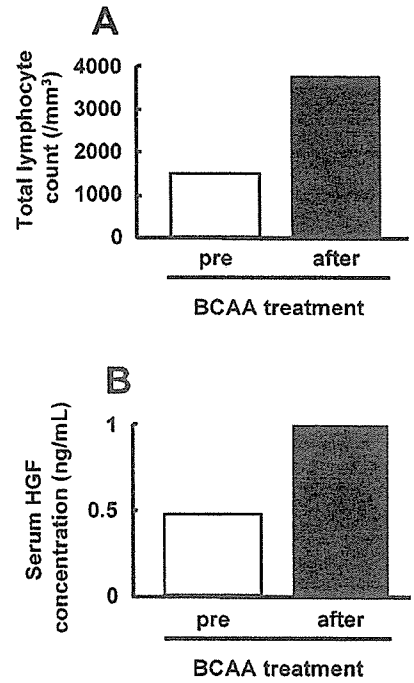

Fig 2. The effects of BCAA supplementation on total lymphocyte count (A) and serum HGF concentration (B). Total lymphocyte count and serum HGF concentration were increased by administration of BCAA supplementation.

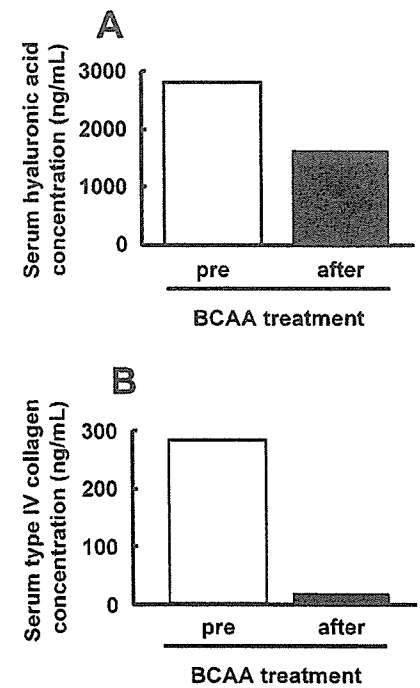

Fig 3. The effects of BCAA supplementation on serum concentrations of hyaluronic acid (A) and type IV collagen (B). Serum concentrations of hyaluronic acid and type IV collagen were decreased by administration of BCAA over 3 months.

After the administration of antibiotics and BCAA supplementation in our patient, abdominal pain disappeared and BT and CRP concentration normalized. Carbapenem and -globulin played a significant role in suppression of the bacterial infection; however, BT and CRP concentration did not revert to the normal range. BCAA administration has been shown to improve immune function parameters, for example, the elevation of total lymphocyte count (6). In our patient, total lymphocyte count was increased from 1450–3740/μL. Furthermore, BCAA supplementation is reported to improve phagocytic function of neutrophils in cirrhotic patients (7). Taken together, these observations indicate that BCAA supplementation the the potential to complement conventional treatment for patients with SBP.

Given a similar situation as that of our patient, one would most likely choose total parenteral nutrition rather than enteral nutrition. However, additional bacterial translocation may occurr because the defensive mechanisms of gut against infection are weakened. This suggests that total parenteral nutrition may worsen the prognosis for patients with SBP. Therefore, it is recommended that these patients be treated via enteral nutrition.

Because our patient appeared to suffer from both of starvation and protein synthesis, additional BCAA supplementation was given to improve protein–energy malnutrition. The administration of additional BCAA supplementation caused a dramatic decrease of total bilirubin concentration and an increase in prothrombin activity. BCAA supplementation has been reported to have various pharmacologic effects besides being an essential substrate for protein synthesis. Tomiya *et al.* reported that BCAA, especially leucine, stimulate the production of HGF by hepatic stellate cells (10). HGF stimulates proliferation of hepatocytes and bile duct epithelial cells (10) and facilitates liver regeneration. Moreover, HGF prevents hepatic fibrogenesis. Therefore, a possible explanation for the recovery from severe liver failure is that BCAA supplementation leads to an increase in HGF production by hepatic stellate cells and subsequently improves liver failure through promotion of liver regeneration and inhibition of fibrogenesis. This possibility is supported by observations in our patient: serum HGF concentration was increased after administration of BCAA supplementation and serum hyaluronic acid and type IV collagen concentrations, both indicators of hepatic fibrosis, were decreased.

In conclusion, we report a case herein, where BCAA supplementation complemented conventional treatment for SBP and subsequent liver failure. BCAA supplementation should be considered as complementary therapy for patients with SBP.

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Case Report

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Appearance-specific Satiety Increases Appetite and Quality of Life in Patients with Metastatic Liver Tumor: A Case Report

TAKUMI KAWAGUCHI*,**, EITARO TANIGUCHI**, MINORU ITOU**, JYUNJI AKIYOSHI**, SATOSHI ITANO**, MOMOKA OTSUKA†, SHOKO IWASAKI†, TOKIKO MATSUDA‡, RYOKO IBI‡, SATOMI SHIRAISHI‡, TETSUHARU ORIISHI**, SHOKO TANAKA†, YUKO SARUWATARI‡ AND MICHIO SATA*,**

Departments of Digestive Disease Information & Research and Medicine**, Kurume University School of Medicine, Departments of Nutrition†, Nursing‡, Kurume University Hospital, Kurume 830-0011, Japan*

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Summary: Loss of appetite is frequently seen and is a main factor affecting quality of life (QOL) in patients with advanced cancer. The etiology for loss of appetite in patients with cancer is multifactorial. The sensory properties of food are factors regulating appetite. Changes in taste, smell and texture of foods influence food intake. The appearance of the food is also a notable factor in sensory-specific satiety. We described a 46-year-old Japanese woman with multiple metastatic liver tumors. Although there was no obvious factor for loss of appetite, she suffered from a loss of appetite and subsequent malnutrition. In order to improve the appearance of food, we reduced the diet to 1000 kcal/day from 1500 kcal/day. On the new diet, the patient's appetite significantly increased and patient's nutritional status was improved. Eating whole diet was an important achievement and increased in mental aspects of QOL. Arrangement for the appearance of food may be a simple and nontoxic therapeutic strategy for patients with cancer suffering a loss of appetite.

Key words advanced cancer, malnutrition, sensory-specific satiety, anorexia, nutritional status, SF-36

INTRODUCTION

Patients with advanced cancer often suffer from a loss of appetite, leading to malnutrition [1]. As many as 20% of patients with cancer die of the effects of malnutrition rather than of the malignancy [2]. Loss of appetite is also a main factor affecting quality of life (QOL). Thus, maintaining appetite is a valuable part of managing patients with advanced cancer.

The etiology for loss of appetite in patients with cancer is multifactorial [3]. Loss of appetite can result from systemic effects of cachexia and altered metabolism by tumor. Local effects of tumor, such as obstruction and pain, can also interfere with dietary intake. Furthermore, treatment for cancer may cause fatigue, taste changes, diarrhea, and nausea, which lead to a subsequent loss of appetite [4,5].

Currently prescribed appetite stimulatory drugs are corticosteroids, megestrol acetate, and metoclopramide. Although corticosteroids may increase appetite, their metabolic, infectious, and psychiatric side effects usually limit their use to the short term. Megestrol acetate has been found to improve appetite [6], but is associated with water retention and an increase in the risk of venous thromboembolism. Metoclopramide has been shown to improve chronic nausea [7], but it has no proven effect on appetite. Clearly, many patients are not helped by or cannot tolerate currently available treatments to stimulate appetite. A simple and nontoxic therapeutic strategy

Corresponding author: Takumi Kawaguchi, MD., PhD, Department of Digestive Disease Information & Research, Kurume University School of Medicine, 67 Asahi-machi, Kurume 830-0011, Japan. Tel: +81-942-31-7902 Fax: +81-942-31-7747 E-mail: takumi@med.kurume-u.ac.jp

Abbreviations: MH, mental health; QOL, quality of life; RE, role-emotional; SCC, squamous cell carcinoma; SF, social functioning; SF-36v2, medical outcomes study 36-item short-form health survey version 2; VT, vitality.

for increasing appetite is needed.

The sensory properties of food regulate appetite. Several studies have been carried out to investigate the impact of food varying in sensory properties. Changes in taste, smell, and texture of foods can influence food intake [8,9]. An increase in the amount of pasta offered affects its' appearance, resulting in a decrease in the pleasure of eating [8]. The appearance of the food seems to be a notable factor in sensory-specific satiety. In other words, the appearance of food may influence appetite through visual information.

Patients with cancer are usually prescribed a high-calorie diet because cancer increases energy expenditure. High-calorie diet is accompanied by heavy appearance, which can provide a visual stress leading to a decrease in appetite. It is possible that the appearance of the food in a high-calorie diet spoils appetite in patients with advanced cancer.

In this report, we describe a case in which the appearance-specific satiety of food increased appetite and improved nutritional status and QOL as well. Arrangement for appearance of food may be a simple and nontoxic therapeutic strategy for patients with cancer suffering a loss of appetite.

CASE REPORT

A 46-year-old Japanese woman was referred to Kurume University Hospital for treatment of multiple metastatic liver tumors and obstructive jaundice. The patient was diagnosed at age 44 with cervical cancer and was treated with radiation and chemotherapy following a radical hysterectomy. Two years later, computed tomography revealed multiple hepatic metastases due to cervical cancer and a rapid growth of metastatic liver tumors. These tumors were treated with a continuous intrahepatic arterial injection of 5-fluorouracil (375 mg/day) and intermittent cisplatin (30 mg/day) via a reservoir system for 5 days [10]. With 2 periods of this regimen, the serum squamous cell carcinoma (SCC) antigen level (normal range $<$ 1.5 ng/ml) decreased from 119.4 ng/ml to 30.1 ng/ml. Metastatic liver tumors were responsive to the chemotherapy.

The patient did not show any troublesome adverse effects of the anticancer drugs administered, such as vomiting and the patient's liver function was preserved, however, she suffered from loss of appetite 2 weeks before admission, which resulted in 4 kg loss of body weight. The patient's body mass index, arm muscle circumference, and triceps skin-fold thickness decreased to 14.6 kg/m^2, 16.3 cm, and 8 mm, respectively. The percentage of the age-adjusted standard value for Japanese women was 65.3%, 80.5%, and 47%, respectively. Laboratory data showed decrease in total lymphocyte count and levels of hemoglobin and albumin (Table 1). Thus, physical examination and laboratory data indicated a state of severe malnutrition.

TABLE 1.
Characteristics of the patient on admission

	Value	% of standard value
Physiological measurements		
Height	158.5 cm	101.9%
Weight	36.5 kg	67.9%
Body mass index	14.5	65.3%
Arm muscle circumference	16.3 cm	80.5%
Triceps skin-fold thickness	8 mm	47.0%
Loss of body weight in 2 weeks	4 kg	
Laboratory data		(Normal range)
Hemoglobin	7.0 g/100ml	(11-15)
Total lymphocyte count	435/μl	(1200-3870)
Albumin	3.2 g/dl	(4.0-5.0)
Total cholesterol	246 mg/dl	(128-256)
Glucose	86 mg/dl	(80-112)
Aspartate aminotransferase (U/l)	22 U/l	(13-33)
Alanine aminotransaminase (U/l)	34 U/l	(6-27)
Total bilirubin (mg/dl)	2.2 mg/dl	(0.3-1.5)
Prothrombin time	96%	(60-130)

Note. Standard values of physiological measurements were referred to Japanese anthropometric reference data

Acute bowel toxicity is one of the common complications of chemotherapy that leads to malnutrition. Oral administration of glutamine reduces this anticancer drug-induced bowel toxicity. The patient was treated with glutamine via oral administration, however, her appetite did not increase. Granisetron is effective against nausea induced by anticancer drugs. Nevertheless, loss of appetite persisted after the patient received an infusion of 3 mg of granisetron. Corticosteroids and megestrol acetate are also known to increase appetite in patients with advanced cancer. On the other hand, the long term use of these agents is associated with decreased efficacy and increasingly unacceptable adverse effects. Therefore, corticosteroids and megestrol acetate were not used in our case.

The patient's energy requirement was estimated to be 1400 to 1600 kcal/day, based on the Harris-Benedict equation [11]. Accordingly, the patient was prescribed a diet of 2000 or 1500 kcal/day. She understood the importance of nutritional therapy, however, her actual intake was only about 600 kcal/day. The sensory properties of the food play important roles in controlling the patient's food intake. Appearance is one of the important sensory properties and heavy appearance of food spoils appetite. In order to improve the appearance of food, we reduced the diet to 1000 kcal/day (Fig. 1). On the new diet, the patient's appetite significantly increased and she ate not only the prescribed diet but also snack. She maintained an intake of more than 1000 kcal/day (Figs 2A and B). Then, we followed her nutritional status until Day 76. Although total lymphocyte count showed no change on the new diet, there was a significant increase in serum albumin level (Figs 3A and B).

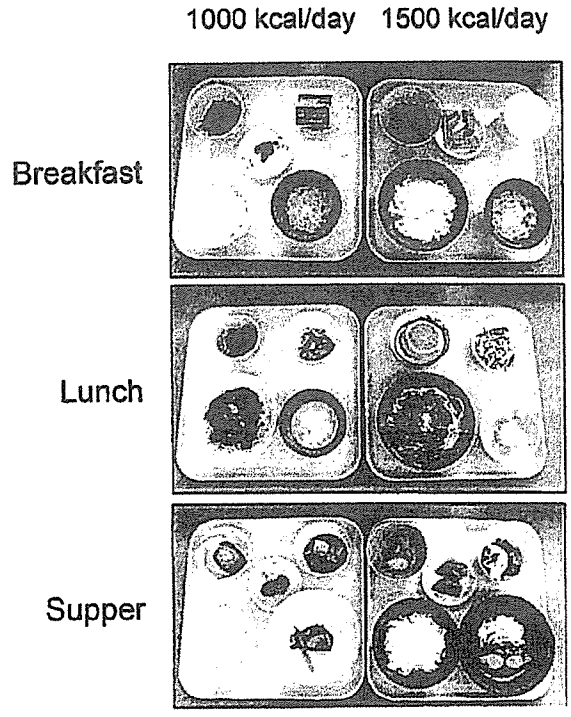


Fig. 1. Appearance of food in the 1000 kcal diet (left) and the 1500 kcal diet (right). A nutrient balance of 1000 kcal diet is similar to that of 1500 kcal diet.

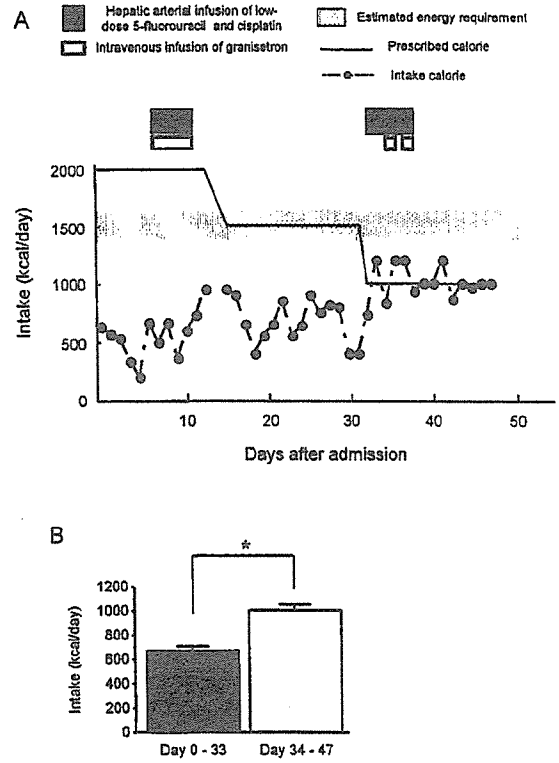
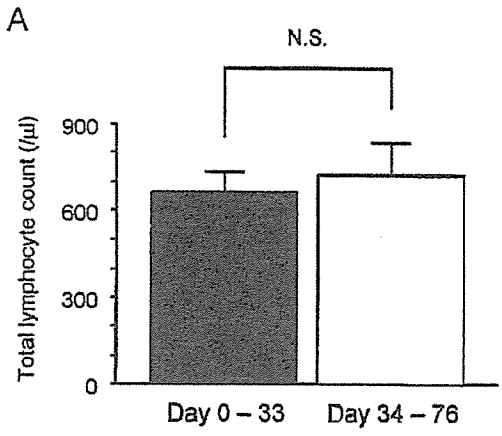


Fig. 2. (A) Time course of daily caloric intake. Metastatic liver tumors were treated with a continuous intrahepatic arterial injection of 5-fluorouracil (375 mg/day) and intermittent cisplatin (30 mg/day) via a reservoir system (■). Granisetron (3 mg) was administrated by intravenous infusion at 30 min before chemotherapy (□). The patient's energy requirement was estimated to be 1400 to 1600 kcal/day, based on the Harris-Benedict equation. (B) Caloric intake after improvement of the appearance of food in the prescribed diet. 2000 or 1500 kcal/day diet was prescribed during Day 0 to 33 (n=34; black bar). 1000 kcal/day diet was prescribed during Day 34 to 47 (n=14; white bar). Caloric intake was evaluated everyday and changes in caloric intake were statistically analyzed by the Mann-Whitney *U* test. *$P<0.01$.



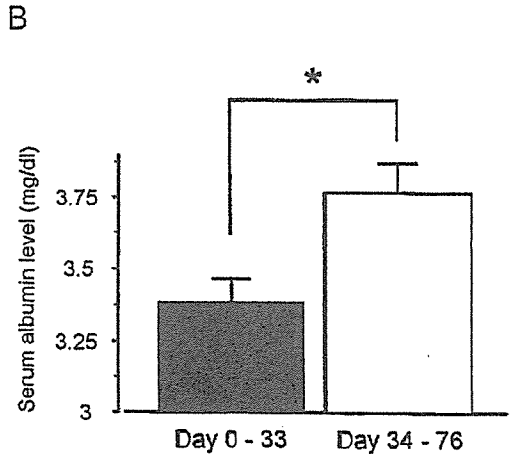
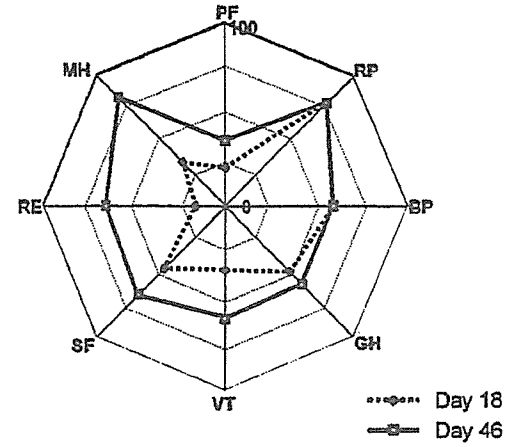


Fig. 3. Long-term effects on (A) total lymphocyte count, and (B) serum albumin levels after improvement of appearance of the food in the prescribed diet. 2000 or 1500 kcal/day diet was prescribed during Day 0 to 33 (black bar). 1000 kcal/day diet was prescribed during Day 34 to 76 (white bar). During Day 0 to 33, total lymphocyte count (n=5) and serum albumin levels (n=5) were measured. During Day 34 to 76, total lymphocyte count (n=4) and serum albumin levels (n=5) were measured. Changes in total lymphocyte count and serum albumin were statistically analyzed by the Mann-Whitney *U* test. N.S.; not significant. *P<0.05.



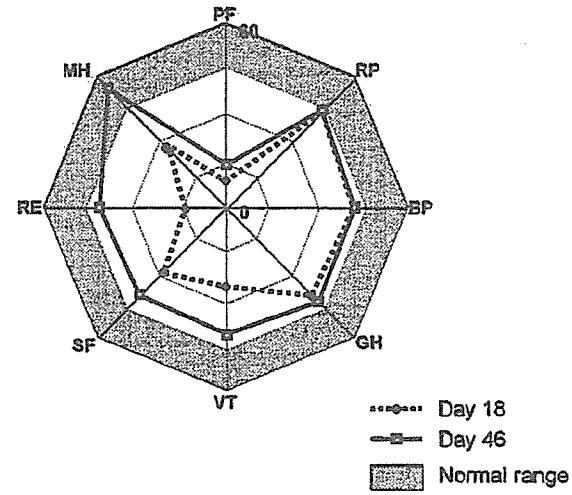


Fig. 4. Changes in SF-36v2 scores after improvement of appearance of the food in the prescribed diet. (A) actual score on a scale of 0 to 100. (B) Norm-based score. PF: physical functioning; RP: role-physical; BP: bodily pain; GH: general health; VT: vitality; SF: social functioning; RE: role-emotional; MH: mental health.

We also evaluated the effects of appearance-specific satiety on the patient's QOL by using the Medical Outcomes Study 36-Item Short-Form Health Survey version 2 (SF-36v2) on Day 18 and 46. By improvement in appearance of food, her scores for vitality (VT), mental health (MH), role-emotional (RE), and social functioning (SF) were markedly increased on a scale of 0 to 100 (Fig. 4A). In particular, the MH score reached to normal range (Fig. 4B). Appearance-specific satiety stimulated appetite and resulted in the improvement of nutritional status as well as in the QOL in this patient with metastatic liver tumors.

DISCUSSION

In this study, we describe a case in which the appearance-specific satiety of food increased appetite

and improved both nutritional status and QOL. Improvement in the sensory properties of food, including appearance, is a simple and nontoxic strategy to increase appetite and QOL in patients with advanced cancer suffering from a loss of appetite.

Loss of appetite is frequently associated with advanced cancer and its treatment. Multiple, interactive factors that are the probable cause of loss of appetite include direct interference of tumors with food intake, malabsorption and poor digestion, and treatment-related complications such as changes in smell and taste. No effective therapy has been established previously for improvement of anorexia in patients with advanced cancer. A simple nontoxic means to improve anorexia would be most benefical in the supportive management of the cancer patient and also could possibly enhance the effectiveness of other therapeutic measures.

Sensory-specific satiety has an important influence on the amount of food eaten [12]. Invariable foods decrease the pleasure of eating even though the food are savory [13-16]. The same phenomenon occurs when eating a sweet food to satiety [13-15] or when drinking a weak-smelling tea compared to a strong-smelling tea [17]. On the other hand, varying the texture of the yogurt in the diet caused a 12.6% elevation of intake compared to the amount of the previously preferred yogurt eaten [14]. Taste, smell, and texture-sepcific satieties are important for regulation of appetite.

Appearance of food is also an important factor involved in sensory-specific satiety. Cancer increases energy expenditure and patients with cancer are normally prescribed a high-calorie diet with heavy appearance of food. In fact, our patient was prescribed a diet of 2000 or 1500 kcal/day, but achieved a caloric intake of only about 600 kcal/day. Since there was no obvious reason for loss of appetite, we assumed that heavy appearance of food spoiled her appetite. Although she was in a state of malnutrition, we reduced her diet to 1000 kcal/day in order to improve appearance of food. In results, the patient's appetite was significantly increased. One would think that chemotherapy itself increased appetite through improvement in cachexia, however, she complained of persistent appetite loss after tumors showed partial remission. On the other hand, Marcelino et al. [18] reported that the desire to eat pizza depended on the visual quality of the pizza. Appearance-specific satiety is also related to areas of the brain that control motivation and the reward value of foods [19-21]. These reports suggests appearance-specific satiety stimulated appetite in our case.

Long-term effects of appearance-specific satiety on the patient's nutritional status was evaluated by measureing total lymphocyte count, an indicator of visceral proteins, and serum albumin levels. We must be cautious in the interpretation of these results because there was no time-couse study in lymphocyte count and albumin levels. Alhough total lymphocyte count did not change, serum albumin levels were significantly increased. The discrepancy between total lymphocyte count and serum albumin levels may be due to adverse effects of chemotherapy. Bone marrow suppression is observed during the chemotherapy which is same regimen used in this case [22] and total lymphocyte count might not reflect the nutritional status adequately in our case. Serum albumin level is a standard marker for evaluation of nutritional status and is not influenced by anti-cancer drug itself. Since serum albumin levels were significantly increased, it is possible that appearance-specific satiety improved long-term status of nutrition in our case.

Appearance-specific satiety increased the scores of SF, ME, RE, and VT of the SF-36 score, which is widely used for evaluating QOL [23,24]. Although it was not clear how appearance-specific satiety increased QOL, following possibilities are exist. Increase in appetite improved the nutritional status. Nutritional status is closely related to liveliness, therefore, improvement of nutritional status may increase in lively aspect of QOL, such as VT. Alternatively, the patient could eat prescribed whole diet by changing the appearance of diet. Eating whole diet was an important achievement and it gave her great pleasure that caused an increase in mental aspects of QOL, such as MH, RE, and SF. Thus, appearance-specific satiety may increase QOL by improvement of lively and mental aspects in our case.

In conclusion, we report here a case in which appearance-specific satiety increased appetite and improved both nutritional status and QOL in patient with advanced cancer. Arrangement for the appearance of food in a diet may be a simple and nontoxic therapeutic strategy for patients who suffered from loss of appetite.

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A Decrease in AFP Level Related to Administration of Interferon in Patients with Chronic Hepatitis C and a High Level of AFP

SHIRO MURASHIMA, MD, PhD, MASATOSHI TANAKA, MD, PhD, MAKOTO HARAMAKI, MD, PhD, SHIGERU YUTANI, MD, PhD, YUTAKA NAKASHIMA, MD, PhD, KAZUNORI HARADA, MD, PhD, TATSUYA IDE, MD, PhD, RYUKICHI KUMASHIRO, MD, PhD, and MICHIO SATA, MD, PhD

It is known that there is a very high incidence of hepatocellular carcinoma (HCC) among patients with type C chronic hepatitis and cirrhosis, and α-fetoprotein (AFP) has been widely used as a diagnostic marker for HCC. However, there are some patients showing continuous high AFP values but no evidence of HCC, and some studies have defined such patients as a high-risk group for HCC. In vitro study has shown that interferon (IFN) inhibits cell proliferation and enhances apoptosis as well as specific cytotoxic T lymphocytes against HCC, resulting in direct anticancer actions. In this study, we investigated the effect of IFN on AFP changes in chronic hepatitis C patients. Of 40 patients with chronic hepatitis C in whom diagnostic imaging confirmed the absence of HCC, 24 patients showed high pretreatment AFP values (high AFP group: AFP level > 10 ng/dl; mean ± SD, 46.3 ± 41.5 ng/dl) and 16 showed low pretreatment AFP values (low AFP group: pretreatment AFP level ≤ 10 ng/dl; mean ± SD, 5.3 ± 2.2 ng/dl). Pretreatment clinical parameters were statistically evaluated in relation to the AFP value. In the high AFP group, the platelet count, albumin level, and prothrombin (%) were significantly lower ($P = 0.047$, $P = 0.0002$, and $P = 0.044$, respectively), suggesting that AFP value increases with advancing liver disease. Subsequently 27 patients were administered IFN (IFN group), and the remaining 13 patients were administered Stronger Neo-minophagen C (SNMC), a glycyrrhizin preparation (SNMC group), as a control group receiving liver-protective therapy. Alanine aminotransferase was reduced in both the IFN and the SNMC group (mean, 132.56 to 60.07 mg/ml [$P < 0001$] and 147.85 to 56.23 mg/ml [$P = 0.0240$], respectively). AFP was significantly reduced in the IFN group (mean, 30.03 to 12.65 ng/ml; $P = 0.0034$), but there was no significant change in AFP in the SNMC group (mean, 29.70 to 39.17 ng/ml). AFP is useful for diagnosing HCC; however, some patients show a persistently high AFP level in the absence of HCC, and these patients have been described as a high-risk group for HCC. In this study, we found that IFN therapy but not SNMC universally reduced the AFP baseline. Since AFP is a significant predictor for HCC, therapeutic strategies for hepatitis C, e.g., long-term low-dose IFN treatment, may reduce hepatocarcinogenesis.

KEY WORDS: hepatitis C; interferons; hepatocellular carcinoma; α-fetoprotein.

Recently, combination therapy with pegylated interferon (IFN) and ribavirin for 48 weeks has achieved viral eradication in 54 to 56% of patients, and the occurrence of hepatocellular carcinoma (HCC) was prevented in these responders (1, 2). For nonresponders to IFN therapy, liver-protective therapy, such as oral administration of

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From the Department of Internal Medicine, Kurume Medical Center, Kurume, Fukuoka, Japan.
Address for reprint requests: Shiro Murashima, MD, PhD, Department of Internal Medicine, Kurume Medical Center, 155-1 Kukubu-machi, Kurume, Fukuoka 839-0863, Japan; muracy@h3.dion.ne.jp.

ursodeoxycholic acid or intravenous injection of Stronger Neo-minophagen C (SNMC), is commonly performed in Japan, and it is considered that these treatments may delay the progression of liver disease (3, 4). SNMC is a glycyrrhizin preparation that exhibits potent anti-inflammatory actions and has been used to treat allergic diseases and hepatitis in Japan for centuries. However, this agent is not considered to have any antiviral or anticancer ability (5), while IFN is considered to have antiviral, anti-inflammatory, and anticancer effects, and is employed in clinical practice to treat certain types of cancer, such as germ cell tumor and RCC (6, 7).

α-Fetoprotein (AFP) has been widely used as a diagnostic marker for HCC. However, there are some patients with a high AFP baseline but no evidence of HCC, although some papers have reported that AFP is a significant predictor of HCC in such patients (8, 9). This study investigated the clinical characteristics of such patients with a high AFP baseline and assessed the effect of IFN administration in terms of AFP changes, since AFP is suggested to be an important risk factor for HCC.

METHODS

Forty patients with type C chronic hepatitis and compensatory liver cirrhosis patients who were being followed at Kurume University Medical Center were retrospectively investigated. All patients were confirmed to be positive for serum hepatitis C virus (HCV)-RNA by polymerase chain reaction (PCR). HBs-Ag-positive, autoimmune, alcoholic, and drug-induced hepatitis patients were excluded from the study. Furthermore, the absence of HCC was confirmed by abdominal ultrasonography (US) or dynamic computed tomography (CT) in all subjects.

According to the pretreatment AFP value, the 40 subjects were divided into two groups: the high AFP group (AFP > 10 ng/dl; $n = 24$) and the low AFP group (AFP ≤ 10 ng/dl; $n = 16$). Then the pretreatment clinical background parameters were statistically investigated using the Mann-Whitney U-test and chi-square test to compare the high and low AFP groups.

These 40 subjects were divided into two groups, the IFN group ($n = 27$) and the SNMC group ($n = 13$). Six million units of recombinant IFNα-2b was injected intramuscularly three times a week or more in the IFN group. SNMC was administered intravenously three times a week at a dose of 40 to 100 ml in the SNMC group. Both alanine aminotransferase (ALT) and AFP values after 4 weeks of treatment were compared with the pretreatment values. Paired t-test was used, and $P < 0.05$ was regarded as significant.

RESULTS

Clinical Characteristics in Patients with High AFP Baseline (High AFP) vs. Low AFP Group. There were no significant differences in age, gender, ALT level, HCV genotype, or HCV-RNA level between the high and the low AFP groups; however, in the high AFP group, the platelet count, albumin level, and prothrombin (PT) value were significantly lower ($P = 0.0014$, $P = 0.0026$, and $P = 0.0041$) (Table 1). These results suggest that the AFP level increases with the progression of liver disease.

Pretreatment Backgrounds in IFN and SNMC Treatment Groups. There were no significant differences in the pretreatment background parameters such as AFP value, age, gender, ALT value, platelet count, albumin level, PT (%), and HCV-RNA level between the two groups (Table 2). Fourteen of the 27 IFN-treated patients (52%) showed a high pretreatment AFP value (>10 ng/ml), and 9 of the 13 SNMC-treated patients (69%) showed a high pretreatment AFP value (>10 ng/ml).

ALT Changes in IFN and SNMC Treatment Groups. With respect to changes in the ALT level, the AFP level was significantly decreased in the IFN group (132.6 ± 72.7 to 61.1 ± 43.3 U/L; $n = 27$; $P < 0.0001$). In the SNMC group, ALT levels were also significantly decreased (149.4 ± 17.2 to 83.0 ± 57.7 U/L; $n = 12$; $P = 0.019$) (Figure 1).

AFP Changes in IFN and SNMC Treatment Groups. As for AFP changes, the AFP value was significantly

TABLE 1. PRETREATMENT CLINICAL CHARACTERISTICS ACCORDING TO AFP VALUE

	High AFP (n = 24) (AFP > 10 ng/ml)	*Low AFP (n = 16) (AFP ≤ 10 ng/ml)*	P *value*
AFP (ng/ml)	46.264 ± 41.534	5.348 ± 2.229	—
Age (yr)	55.875 ± 9.252	52.938 ± 12.179	0.3914
Gender (M/F)	14/10	12/4	0.2790
ALT (U/L)	144.333 ± 88.122	125.813 ± 83.818	0.5108
PLT ($\times 10^4/\mu l$)	11.421 ± 4.997	14.550 ± 4.030	0.0467*
Albumin (g/dl)	3.617 ± 0.444	4.138 ± 0.238	0.0002*
PT (%)	72.368 ± 11.923	80.237 ± 10.796	0.0439*
HCV-RNA (KIU/mL)	472.667 ± 286.404	463.067 ± 323.334	0.9257

Note. Mann-Whitney U-test or chi-square test was used. $P < 0.05$ was considered significant.
Values are expressed as mean ± SD.

TABLE 2. PRETREATMENT PATIENT PROFILES IN THE SNMC AND IFN GROUPS

	SNMC (n = *13*)	*IFN* (n = *27*)	P *value*
AFP (ng/ml)	29.970 ± 35.229	30.030 ± 39.643	0.9798
Age (yr)	54.308 ± 10.427	54.889 ± 10.685	0.8719
Gender (M/F)	9/4	17/10	0.6071
ALT (U/L)	147.846 ± 110.816	132.556 ± 272.702	0.6039
Platelets (×104/μl)	11.015 ± 6.244	13.441 ± 3.870	0.1387
Albumin (g/dl)	3.738 ± 0.568	3.867 ± 0.408	0.4185
PT (%)	72.615 ± 13.775	77.615 ± 10.887	0.2607
HCV-RNA (KIU/mL)	502.900 ± 299.403	455.500 ± 302.124	0.6752

Note. Mann-Whitney *U*-test or chi-square test was used. $P < 0.05$ was considered significant.
Values are expressed as mean ± SD.

decreased in the IFN group (53.0 ± 44.3 to 20.3 ± 26.7 ng/ml; $n = 14$; $P = 0.0023$). Interestingly, all 27 IFN-treated patients showed a decrease in AFP value regardless of response to treatment. However, there was no significant change in the AFP value after SNMC administration (31.1 ± 36.4 to 39.0 ± 46.5 ng/ml; $n = 9$; $P = 0.11$) (Figure 2). Mean AFP value was slightly increased in the SNMC group.

DISCUSSION

AFP is a fetal protein that is not normally present in the serum of adults and is commonly used as a tumor marker for HCC. However, serum AFP is also elevated during pregnancy and in chronic hepatitis patients (10, 11). In this study, a considerable number of type C chronic hepatitis and compensated cirrhosis patients demonstrated persistently elevated AFP levels in the absence of HCC. In addition, the AFP level decreased significantly after IFN administration. Furthermore, the AFP decrement was universally observed regardless of treatment response to IFN therapy. Transient AFP elevation has been observed after a rise in transaminase in acute hepatitis and fulminant hepatitis (12–14). This type of AFP elevation is explained as a result of hepatocyte regeneration accompanied by necroinflammatory change. In this study, AFP was not changed in the SNMC group despite significant improvement in transaminase, suggesting that the AFP elevation was not caused by hepatocyte regeneration in chronic hepatitis patients.

AFP production is supposed to regulate the transcription level of hepatocytes (15). Among HCV-infected patients, the HCV-coding core protein is regarded to be one of the proteins responsible for hepatocarcinogenesis, up-regulating several molecules resulting in activation of the cell cycle and cell proliferation at the transcriptional level in hepatocytes (16). The HCV-coding core protein may also upregulate AFP production at the transcriptional

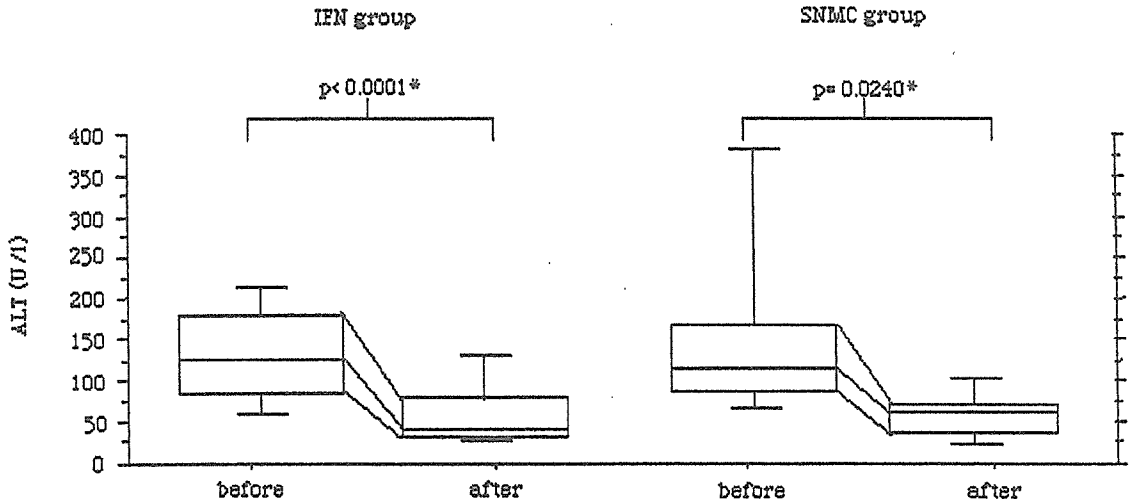

Fig 1. Changes in alanine aminotransferase (ALT) after IFN and SNMC administration. Paired *t*-test was used. *$P < 0.05$ was regarded as significant.

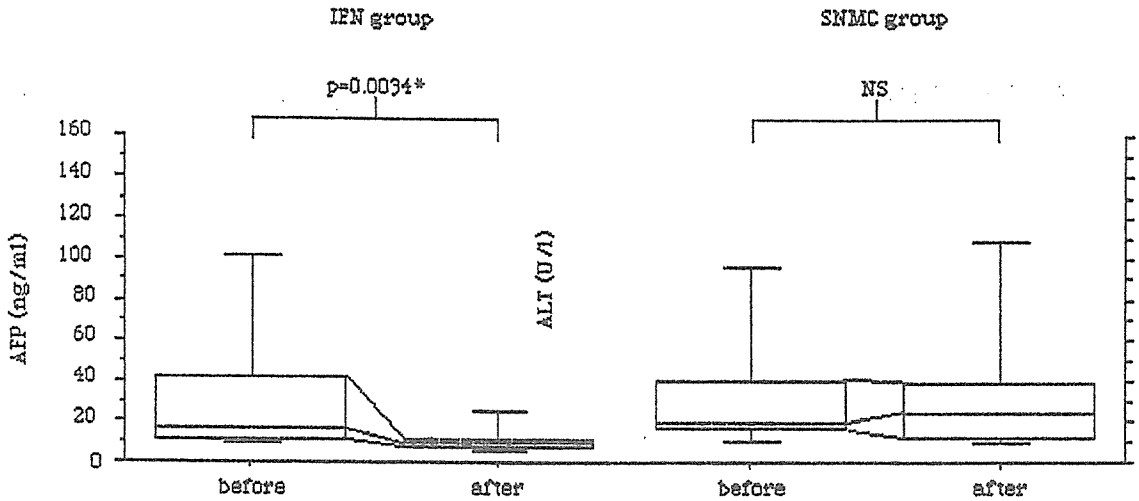

Fig 2. α-Fetoprotein (AFP) changes with IFN and SNMC administration Paired *t*-test was used. $*P < 0.05$ was regarded as significant. NS, not significant.

level. In contrast, IFN is considered to down-regulate cell cycle progression at the transcriptional level and induce apoptosis via the IFN receptor-mediated JAK-STAT signaling pathway (17). This competing action of IFN against HCV-related protein may be a direct anticancer mechanism that inhibits HCC. Actually, a clinical study has demonstrated anticancer effects of IFN administration against intrahepatic recurrence after resection of HCC (18), and IFN has also been used to treat HCC in combination with anticancer agents such as 5-fluorouracil (19).

Many reports have cited elevated AFP baselines as an independent HCC risk factor (8, 9) along with age, gender, liver histology stage, and ethnicity in HCV-infected patients. In the present study, the AFP baseline was decreased in all IFN-treated patients, even IFN nonresponders. This indicates that IFN therapy, rather than liver-protective therapy, universally reduces the risk factors of HCC in HCC high-risk subjects with high AFP values and advanced liver disease. Therefore, therapeutic strategies, such as long-term administration of low-dose IFN, may inhibit HCC in patients who have failed to respond to routine IFN treatment. Further investigation is needed to evaluate IFN effect in relation to AFP production and hepatocarcinogenesis.

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Clinical Studies

Premature telomere shortening and impaired regenerative response in hepatocytes of individuals with NAFLD

Nakajima T, Moriguchi M, Katagishi T, Sekoguchi S, Nishikawa T, Takashima H, Kimura H, Minami M, Itoh Y, Kagawa K, Tani Y, Okanoue T. Premature telomere shortening and impaired regenerative response in hepatocytes of individuals with NAFLD.
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Abstract: *Aims:* The risk factors associated with poor prognosis of nonalcoholic fatty liver disease (NAFLD) are not fully understood. Our aim was to assess the role of progressive hepatocellular telomere shortening in the clinical course of NAFLD. *Methods:* We measured average telomere lengths in liver tissue samples from 44 patients with NAFLD by quantitative fluorescence *in situ* hybridization using a telomere-specific probe. Patients in which telomeres measured at least 80% of the lengths of age-matched controls were categorized as group A. Those patients with telomeres measuring less than 80% of the control lengths formed group B. *Results:* Within group B, some samples showed a remarkable shortening of hepatocyte telomeres in younger patients, whereas some group A patients showed almost normal telomere lengths until their seventies. Among clinicopathological factors, body mass index (BMI), homeostasis model assessment insulin resistance (HOMA-IR), histological degree of steatosis and intensity of 8-hydroxy-2′-deoxyguanosine (8-OHdG) immunostaining were all significantly higher in group B than in group A. Ki-67 immunohistochemistry demonstrated that group B liver tissues were significantly less proliferative than those from group A, despite no significant difference in the necroinflammatory activities of group A and B samples. In group B patients, the ratios of Ki-67 positive index to alanine aminotransferase value were significantly lower than group A. *Conclusions:* Greater insulin resistance can result in more severe hepatic steatosis among group B patients, leading to an overproduction of reactive oxygen species, which may accelerate telomere erosion. Furthermore the regenerative response of hepatocytes with prominent telomere shortening may be impaired, making these cells vulnerable to the effect of a 'second-hit' insult.

Tomoki Nakajima[1], Michihisa Moriguchi[1], Tatsuo Katagishi[1], Satoru Sekoguchi[1], Taichirou Nishikawa[1], Hidetaka Takashima[1], Hiroyuki Kimura[1], Masahito Minami[1], Yoshito Itoh[1], Keizo Kagawa[2], Yoichi Tani[3] and Takeshi Okanoue[1]

[1]Molecular Gastroenterology and Hepatology, Kyoto Prefectural University of Medicine Graduate School of Medical Science, [2]Department of Medicine, Fukuchiyama City Hospital, Fukuchiyama, [3]Department of Medical Science, DakoCytomation, Kyoto, Japan

Key words: nonalcoholic fatty liver disease – quantitative fluorescence *in situ* hybridization – replicative senescence – telomere

Tomoki Nakajima, MD, Molecular Gastroenterology and Hepatology, Kyoto Prefectural University of Medicine Graduate School of Medical Science, Kawaramachi-Hirokoji, Kamigyo-ku, Kyoto 602-8566, Japan.
Tel: +81-75-251-5519
Fax: +81-75-251-0710
e-mail: tomnaka@silver.ocn.ne.jp

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Nonalcoholic fatty liver disease (NAFLD) represents a spectrum of clinicopathologic conditions, ranging from simple steatosis to nonalcoholic steatohepatitis (NASH), with varying risks for progression to cirrhosis. A study by Matteoni et al. (1) revealed that 25% of those initially diagnosed with histologic evidence of hepatocellular necrosis (with or without fibrosis) progressed to cirrhosis in less than 20 years. Fassio and colleagues documented that progression of fibrosis was found by the second liver biopsy from seven out of 22 NASH patients, 4.3 years after the first biopsy (2). One prospective study showed that nine out of 23 patients with NASH-associated cirrhosis died of liver-related causes in less than 10 years (3). To date, limited information on the natural history of NAFLD has hampered discovery of the risk factors associated with poor prognosis of this disease.

Telomeric regions present at the ends of chromosomes consist of hexameric DNA repeat sequences $(TTAGGG)_n$ in association with telomere-binding

Abbreviations: NAFLD, nonalcoholic fatty liver disease; NASH, nonalcoholic steatohepatitis; BMI, body mass index; HOMA-IR, homeostasis model assessment insulin resistance; ROS, reactive oxygen species; Q-FISH, quantitative fluorescence *in situ* hybridization; ALT, alanine aminotransferase.

proteins (4). Telomeric repeat sequences effectively 'cap' the ends of linear chromosomes, thus preventing fusion between chromosome ends. Telomere repeat sequences are subject to shortening with each cell division because they cannot be replicated completely during normal DNA synthesis (5). Normally, telomere shortening places an upper limit on the number of divisions undergone by a somatic cell, since critically short telomeres function as targets for cell-cycle checkpoint systems that induce apoptosis or an irreversible cell-cycle arrest, termed replicative senescence (6, 7).

During the process of chronic liver cell death and regeneration, hepatocyte-specific telomere shortening and replicative senescence are linked to progressive fibrosis and the development of cirrhosis (8–10), and it is argued that process can account for the development of cirrhosis due to hepatitis viruses, autoimmune diseases and alcohol in an age-independent manner (10). For NAFLD, neither the degree of telomere shortening nor the association between telomere shortening and hepatic fibrosis has been studied.

In addition to the shortening of telomeres during cell division, recent reports demonstrate that telomeres also suffer damage by reactive oxygen species (ROS) (11–13), since oxidative damage is not repaired as efficiently in telomeric DNA as elsewhere in the chromosome. Since severe hepatic steatosis can lead to increases in intracellular ROS, it may accelerate telomere loss. The relationship between these processes in NAFLD remains to be determined.

In this study, we assessed the progression of hepatocellular telomere shortening in NAFLD by quantitative fluorescence *in situ* hybridization (Q-FISH) using a telomere-specific probe (14–16). This method differs from conventional Southern blot analysis in that the lengths of telomeres can be measured specifically in hepatocytes from the biopsy specimens used for analysis. We evaluated telomere shortening with respect to hepatic regenerative indices, as well as to oxidative stress measures in patients with NAFLD vs age-matched controls. Our findings contribute to an understanding of the biological significance of telomere shortening in NAFLD, especially its effect on replicative response to cell injury.

Materials and methods

Tissue preparation

The patients diagnosed as NAFLD in 2003–2004 were selected from the files of Department of Surgical Pathology of Kyoto Prefectural University of Medicine. Each liver biopsy specimen was read and diagnosed blindly by two hepatologists (T.N. and T.O.), according to the histopathologic criteria summarized by attendees of the AASLD Single Topic Conference 2002 (17). Patients with a history of alcoholism (consumed more than 20 g/day), showing evidence of hepatitis B or C infection, taking known hepatotoxic drugs, or demonstrating symptoms of another specific liver disease were excluded from the study.

A total of 44 paraffin-embedded liver tissue biopsies were selected. Eleven histologically normal liver tissues obtained by partial hepatectomy for metastatic liver tumors from the patients without NAFLD, and negative for hepatitis B surface antigen and anti-hepatitis C antibody, were selected as controls. Informed consent to our using these tissues for this study was obtained from all patients in a written form.

Of four sections (5 μm each) cut serially from each paraffin block, one each was used for hematoxylin and eosin (HE) staining, Masson trichrome staining, immunohistochemical staining for Ki-67 antigen or Q-FISH for the telomeric region. The degree of steatosis was assessed by percent fat in each tissue section. The degree of fibrosis was graded as stage 1, zone 3 perisinusoidal fibrosis; stage 2, as above with portal fibrosis, stage 3, as above with bridging fibrosis, or stage 4, cirrhosis, based on the standards proposed by Brunt et al. (18). The necroinflammatory activity was graded as none or mild (grade 1), moderate (grade 2), or severe (grade 3), based on the modified standards also proposed by Brunt et al. (18). Activity was graded 1 in 18 cases, graded 2 in 21 cases, and graded 3 in 5 cases; fibrosis stage was F0 in 14 cases, F1 in 15 cases, F2 in 7 cases, F3 in 6 cases and F4 in 2 cases.

The clinicopathological variables analyzed were body mass index (BMI), homeostasis model assessment insulin resistance (HOMA-IR), serum values of alanine aminotransferase (ALT) and ferritin at the time of liver biopsy. (HOMA-IR = fasting immunoreactive insulin (μU/ml) × fasting blood glucose (mg/dl)/405). High HOMA-IR values indicate the presence of insulin resistance, and the upper limit of HOMA-IR is defined to be 2.5 (19, 20). Values for serum ferritin in 8 cases, and HOMA-IR in 14 cases, were not available in archived clinical charts and thus were excluded from analyses of these samples.

Ki-67 immunohistochemistry

Proliferative activity was scored by the frequency of immunohistochemical detection of the Ki-67 antigen. A paraffin section was dewaxed, dehy-

drated, and then immersed in tap water. Endogenous peroxidase was inactivated by incubating the sections in 0.3% H_2O_2. Antigen retrieval was performed by autoclaving the sections at 121 °C for 10 min in Target Retrieval Solution (catalog no. S1700; DakoCytomation, Kyoto, Japan). Nonspecific reactions were blocked by incubating the sections in Tris-buffered saline (TBS) containing 2% fetal bovine serum. The section was incubated overnight at 4 °C following application of one drop of mouse anti-Ki-67 antigen monoclonal primary antibody (MIB-1; DakoCytomation, Carpinteria, CA). After three 5-min washes in TBS, they were incubated with biotin-conjugated anti-mouse immunoglobulins (DakoCytomation, Kyoto, Japan) for 60 min at room temperature. After three 5-min washes in TBS, they were incubated with horseradish peroxidase (HRP)-conjugated streptavidin (DakoCytomation, Kyoto, Japan) for 60 min at room temperature. After three 5-min washes in TBS, immunospecific reactivity was visualized by peroxidase oxidation of diaminobenzidine substrate (DAB; Wako, Osaka, Japan). The section was counterstained with hematoxylin. Negative control slides without the primary antibody were included for each staining. The Ki-67-positive index (Ki-67-PI, the percent cells immunoreactive to anti-Ki-67 antibody) was calculated from a minimum of 1000 scored hepatocytes.

8-Hydroxy-2′-deoxyguanosine (8-OHdG) immunohistochemistry

Hepatic expression of 8-OHdG, a reliable marker of oxidative DNA damage (21), was immunohistochemically investigated in NAFLD following the procedure of Kato et al. (22). After visualizing sites of immunoreactivity with DAB, each section was counterstained using Meyer's hematoxylin. Three grades of staining intensity were defined as follows: Grade 1; most of the hepatocytes were 8-OHdG-negative and only stained by hematoxylin (Fig. 1a), grade 2; most of the hepatocytes were 8-OHdG-positive but faintly stained (Fig. 1b), and grade 3; most of the hepatocytes were 8-OHdG-positive and strongly stained (Fig. 1c).

Q-FISH for telomere length

The combined length of telomeres within single cells was determined by the intensity of fluorescence detected in telomere FISH of each paraffin section, measured according to the procedure of Meeker et al. (14). Paraffin sections were deparaffinized with xylene, hydrated through a graded ethanol series, and then placed in deionized water (DW). Slides were then autoclaved at 121 °C for 20 min in Target Retrieval Solution (catalog no.

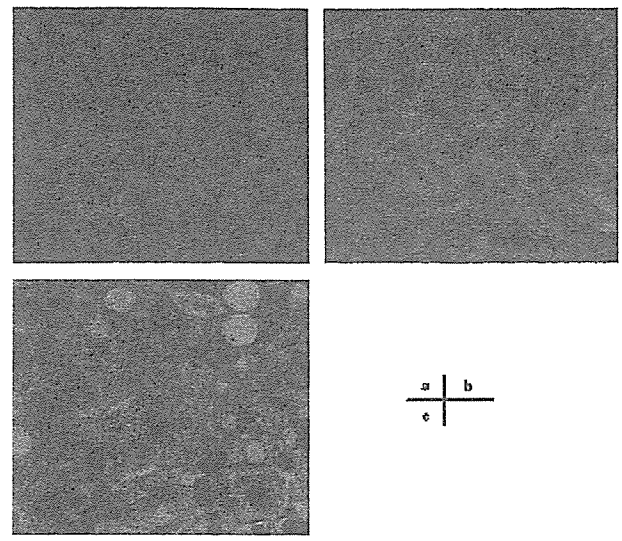

Fig. 1. Representative photographs of 8-hydroxy-2′-deoxyguanosine (8-OHdG) immunostaining. After immunoreactivity was visualized by diaminobenzidine, each section was counterstained by Meyer's hematoxylin. The staining intensity was classified into three grades (a) grade 1; most of the hepatocytes were 8-OHdG-negative and only stained by hematoxylin, (b) grade 2; most of the hepatocytes were 8-OHdG-positive but faintly stained, and (c) grade 3; most of the hepatocytes were 8-OHdG-positive and strongly stained.

S1700; DakoCytomation, Kyoto, Japan). After cooling to room temperature for 20 min, the slides were washed in DW for 1 min three times, and immersed in 0.3% H_2O_2/absolute methanol at room temperature for 20 min to block endogenous peroxidase. Slides were rinsed in DW twice for 3 min, in 70% ethanol for 1 min, in 95% ethanol for 1 min, in 100% ethanol for 1 min, and then air dried. Ten microliters of a fluorescent isothiocyanate (FITC)-labelled telomere-specific peptide nucleic acid (PNA) probe (vial 2 in K 5325, DakoCytomation, Copenhagen, Denmark) was applied to each sample, which was then covered with an 18 × 18 mm coverslip, and denatured on a heat block at 90 °C for 5 min. Slides were then incubated in a dark, moist chamber at 45 °C overnight. Coverslips were then carefully removed in Tris buffered saline with 0.1% Tween 20 (TBST) (item no. 003178 in K0618, DakoCytomation, Kyoto, Japan) and the slides were washed in Wash Solution (vial 4 in K 5325) at a dilution of 1:50 at 52 °C for 20 min, followed by five 3-min washes in TBST. The slides were incubated in anti-FITC-HRP (item no. 004404 in K0618) diluted 1:100 in anti-FITC-HRP Diluent (item no. 004407 in K0618) at room temperature for 30 min, followed by five 3-min washes in TBST. Seventy microliters of Fluorescyl Tyramide (item no. 004409 in K0618) were applied to each slide at room temperature for 15 min. The slides were then

immersed in TBST for five 3-min incubations, counterstained with 4′-6-diamidino-2-phenylindole (DAPI) (1000 ng/ml, VYS-32-804830, Vysis, Downers Grove, IL) and finally coverslipped for image analysis.

Fluorescent microscopy and image analysis of telomeres

Following the protocol of Meeker et al. (14), image-processed telomeric signals were quantified from digitized fluorescence microscopic images using the image analysis software package IP Labs (version 3.54, Scanalytics, Fairfax, VA). Lymphocytes, which maintain relatively stable telomere length, especially in adults 40 years and older, were used as measures for the normal-length telomere fluorescence signal intensity within each tissue sample. Telomere erosion of lymphocytes is reported to be only 1.68 kb from age 40 to 80 years and 2.00 kb from age 20 to 40 years (23). Therefore, we compared the intensities of telomere-specific fluorescence signals from hepatocytes and lymphocytes from groups A and B with those measured from biopsies from two age-matched populations without liver disease, age 20–40 years and 40–80 years.

As reported previously (10), three different cell populations were distinguishable by morphological features following DAPI staining of each Q-FISH section and HE staining of each serial section. Hepatocytes showed round nuclei and a large area of cytoplasmic space. Stellate cells were recognized as elongated cells with elongated nuclei. Lymphocytes were characterized by round nuclei and very little cytoplasm. Telomeric pixel intensities of individual hepatocyte and lymphocyte nuclei were recorded. To control for different amounts of DNA in the sectioned nuclei, telomeric signal intensity was adjusted by dividing each telomere fluorescence sum for a given nucleus by the sum of the pixels of the DAPI signal within that nucleus, as reported previously (14). For each field of view, the adjusted telomeric signal intensities of each hepatocyte nucleus and lymphocyte nucleus were designated as Tel-H and Tel-L, respectively. The ratio of mean Tel-H/mean Tel-L was calculated for each field of view after assessing 15–20 hepatocytes and more than 10 lymphocytes. Relative telomere intensity is defined as the mean of the Tel-H/Tel-L ratios measured from least five different view fields per sample under various histological conditions.

Classification of NAFLD patients into group A or B required comparisons of telomere shortening in these liver biopsies with normal controls. Furthermore, both NAFLD and normal control values needed to reflect reductions in telomere length that occur normally during aging. Towards this end, the ratios of age to relative telomere intensity among control samples were used to calculate a best-fit linear regression, which indicated the decrease in relative telomere intensity relative to age among control samples. Among NAFLD study patients, those with liver biopsies at least 80% of the normal ratio of relative telomere intensity to age were classified into group A, and those showing less than 80% of the normal ratio were designated group B.

Results

Twenty-seven male patients and 17 female patients were studied, with an average age of 50 ± 15 years. Photographs of representative samples of Q-FISH and Ki-67 immunostained tissues are shown in Fig. 2. The slope of the regression line for age v–s relative telomere intensity of hepatocytes in normal individuals was calculated to be $y = -0.0157x + 1.9576$ ($R^2 = 0.9077$, $P < 0.001$, $n = 11$), where x is patient age and y is relative telomere intensity (Fig. 3a). Twenty-two of the 44 liver tissues analyzed met the criterion for classification in group B. The distributions of relative telomere intensities within groups A and B are shown in Fig. 3b and c, respectively. In both groups, relative telomere intensity and age were negatively correlated. The slopes of the regression lines for age vs relative telomere intensity in group A patients and group B patients were calculated to be $y = -0.0127x + 1.872$ ($R^2 = 0.2314$, $P < 0.05$, $n = 22$) and $y = -0.0069x + 1.0694$ ($R^2 = 0.4588$, $P < 0.01$, $n = 22$), respectively.

The average relative telomere intensity of normal control patients from 20 to 40 years old was 1.51 (Fig. 3a). In group B patients in this age range, the average relative telomere intensity was 0.90, which was 60.0% of that of normal controls (Fig. 3c). Thus, the telomere lengths of some NAFLD patients in group B were already shortened remarkably in early age. Without a longitudinal study, the relative telomere intensity of these group B patients after age 40 cannot be predicted. However, the regression slopes of group B and that of control group were significantly different ($df = 27$, $t = 3.551$, $P = 0.001$). Therefore, the rate of age-related telomere shortening within group B was significantly slower than the control group. It appears that a minimum telomere length is achieved at an early age in some group B patients. Alternatively, several group A NAFLD patients of all ages showed normal telomere length (Fig. 3b). At age 70, the average relative telomere intensities in the normal control