Study protocol

The intervention schedule is presented in Figure 1. Before this study, nutritional education was presented to all patients by dieticians. Daily nutritional intake for each group was calculated as 25–30 kcal with 1.2–1.3 g of protein per kilogram of ideal body weight per day. In the LES group, actual daily nutritional intake from meals was determined by subtracting the calorie content of LES (210 kcal) and protein (13.5 g) from the aforementioned calculated nutritional intake. One pack of the BCAA-enriched mixture (Aminoleban EN; Otsuka, Tokyo, Japan) used as LES food (at 22:00) contains 210 kcal of energy, 31.05 g of carbohydrate, 13.5 g of protein, 3.5 g of fat, and trace amounts of minerals and vitamins.[31] In the control group, patients received ordinary food with the same calorie content as the LES group.

After insertion of a 5-Fr heparin-coated catheter (Anthron P-U Catheter; Toray Medical, Tokyo, Japan) connected to a subcutaneously implanted reservoir, as described in a previous report,[19] patients received repeated arterial infusion of chemotherapeutic agents via the injection port.[20] One course of chemotherapy comprised 5 consecutive days of daily administration of cisplatin (10 mg/body/day on days 1–5; Randa; Nippon Kayaku, Tokyo, Japan) and isovorin (6.25 mg/body/day on days 1–5; Wyeth, Tokyo, Japan), followed by 5-fluorouracil (250 mg/body/day on days 1–5; Kyowa Hakko, Tokyo, Japan). Days 6 and 7 were rest days. This course was repeated for 2 weeks, followed by a 1-week suspension of chemotherapy. The course was then repeated for 2 weeks.

Nutritional parameters

Energy metabolism was analyzed by indirect calorimetry, and npRQ was calculated. A multi-frequency bioelectrical impedance analysis method (InBody 3.2; BIOSPACE, Tokyo, Japan) was used for anthropometric measurements.

Before and after 1 cycle of treatment, nutritional evaluation by indirect calorimetry and InBody, and changes in laboratory examinations, BTR, natural killer (NK) activity of lymphocytes,[32] plasma glucose and insulin level after 75-g oral glucose tolerance test (OGTT) were measured. The 75-g OGTT was performed at 4 time points: before administration, and at 30, 60 and 120 min. Area under the concentration curve for glucose (AUC glucose) and area under the concentration curve for insulin (AUC insulin) were determined using the above-mentioned 4 points and compared between before and after 1 cycle of treatment. We also divided patients into 3 groups according to blood glucose level 120 min after 75-g OGTT. A normal pattern (normal glucose tolerance [NGT]) was defined as blood glucose level <140 mg/mL at 120 min after 75-g OGTT. In comparison, a diabetic pattern (diabetes mellitus[DM]) was defined as glucose level >200 mg/mL and a borderline pattern (impaired glucose tolerance [IGT]) was defined as 140–200 mg/mL at 120 min after 75-g OGTT. CHI reflects skeletal muscle volume,[33] and was calculated using the following formula: CHI = (urinary creatinine excretion per day (mg))/(ideal body weight × A), where A is 23 for males and 18 for females.

Assessment of therapeutic efficacy

Dynamic computed tomography (CT) was performed before and after treatment. Tumor response was assessed on completion of 1 cycle of treatment. Response was classified according to ECOG criteria.[30] Complete response (CR) was defined as disappearance of all measurable lesions with no remaining signs, symptoms, or biochemical changes related to the tumor, which must have existed for >4 weeks, and appearance of no new lesions. Partial response (PR) was defined as a reduction of >50% in the sum of the products of the greatest perpendicular diameters of all measurable lesions, and appearance of no new lesions. Stable disease (SD) was defined as a reduction of <50% or an increase of <25% in the sum of the products of the greatest perpendicular diameters of all measurable lesions, and appearance of no new lesions. Progressive disease (PD) was defined as an increase of >25% in the sum of the products of the greatest perpendicular diameters of all measurable lesions, or appearance of new lesions.

Statistical analysis

Data are expressed as mean±standard deviation. Statistical analyses were performed using the unpaired *t*-test and the Mann-Whitney *U*-test, as appropriate. Survival period was calculated using the Kaplan-Meier method[34] from the date on which chemotherapy was started until death, and significance was determined by the log-rank test. Survival was confirmed up to 31 October, 2009. Values of $P < 0.05$ were considered statistically significant.

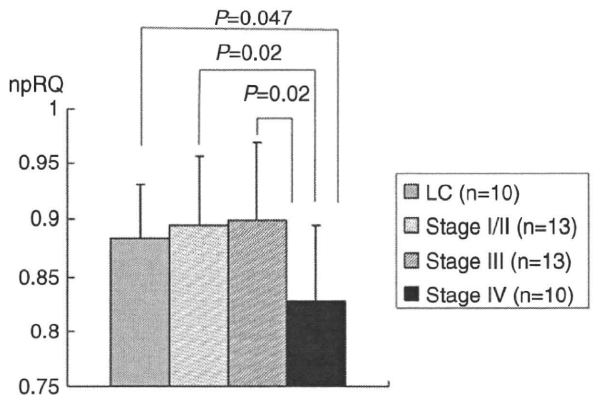

Figure 2 Value of non-protein respiratory quotient (npRQ) in cirrhotic patients without hepatocellular carcinoma (HCC) and with various stages of HCC. No significant difference in npRQ was seen among 3 groups (LC group, HCC stage I/II group, and HCC stage III group). However, npRQ was significantly lower in patients with stage IV HCC than in cirrhotic patients without HCC, or in patients with stage I/II or stage III HCC (LC group vs. HCC stage IV group, $P = 0.047$; HCC stage I/II group vs. HCC stage IV group, $P = 0.02$; HCC stage III group vs. HCC stage IV group, $P = 0.02$).

RESULTS

Energy metabolism in patients with HCC

FIGURE 2 SHOWS npRQ in cirrhotic patients without HCC and with various stages of HCC. Values of npRQ in cirrhotic patients without HCC, with stage I/II HCC, with stage III HCC, and with stage IV HCC were 0.88 ± 0.05, 0.89 ± 0.06, 0.90 ± 0.07, and 0.83 ± 0.07, respectively. No significant differences in npRQ were identified among the 3 groups (LC group, HCC stage I/II group, and HCC stage III group). However, npRQ was significantly lower in patients with stage IV HCC than in cirrhotic patients without HCC, or in patients with stage I/II or stage III HCC (LC group vs. HCC stage IV group, $P = 0.047$; HCC stage I/II group vs. HCC stage IV group, $P = 0.02$; HCC stage III group vs. HCC stage IV group, $P = 0.02$).

Effect of LES for advanced HCC during HAIC

Response to therapy

In the LES group ($n = 13$), 0 (0%), 3 (23%), 8 (62%), and 2 (15%) patients exhibited CR, PR, SD, and PD, respectively (response rate [patients with CR+PR/all patients], 23%). In the control group ($n = 10$), 1 (10%), 2 (20%), 4 (40%), and 3 (30%) patients exhibited CR, PR, SD, and PD, respectively (response rate, 30%). No significant differences in response rates were seen between groups ($P = 0.90$; Mann-Whitney *U*-test). As a result, no significant differences between groups were seen in relation to background.

Energy metabolism

Table 3 shows changes in npRQ before and after 1 cycle of treatment. The value of npRQ increased significantly after 1 cycle of treatment in the LES group (0.81 ± 0.08 vs. 0.88 ± 0.08, $P = 0.01$). However, npRQ did not differ in the control group (0.85 ± 0.08 vs. 0.86 ± 0.06,

Table 3 Changes in energy metabolism and laboratory parameters

	LES group (n = 13)			Control group (n = 10)		
	Before	After	*P*-value	Before	After	*P*-value
npRQ	0.81 ± 0.08	0.88 ± 0.08	0.01	0.85 ± 0.08	0.86 ± 0.06	0.69
BTR	3.76 ± 0.90	4.55 ± 1.38	0.008	4.16 ± 1.03	3.97 ± 1.22	0.47
Total Protein (g/dL)	7.25 ± 0.75	7.46 ± 0.97	0.29	7.32 ± 0.68	7.01 ± 0.59	0.17
Albumin (g/dL)	3.06 ± 0.51	3.08 ± 0.53	0.70	3.26 ± 0.48	3.24 ± 0.42	0.83
Prealbumin (mg/dL)	8.64 ± 4.49	10.17 ± 4.56	0.049	10.42 ± 4.76	11.43 ± 5.24	0.27
Total bilirubin (mg/dL)	0.98 ± 0.55	1.05 ± 0.75	0.57	1.05 ± 0.35	1.00 ± 0.37	0.75
ALT (IU/L)	38.6 ± 31.3	31.3 ± 12.9	0.04	50.9 ± 35.14	36.9 ± 23.22	0.67
PT (%)	76.0 ± 12.5	76.9 ± 7.9	0.72	81.5 ± 9.86	82.6 ± 10.8	0.76
Total cholesterol (mg/dL)	153.2 ± 31.1	153.2 ± 31.1	0.79	162.0 ± 57.4	167.6 ± 68.4	0.58
ChE (IU/L)	140.2 ± 70.4	134.2 ± 73.1	0.20	163.7 ± 68.6	131.1 ± 52.2	0.01
NH3 (μ mol/dL)	58.5 ± 23.1	69.3 ± 27.6	0.07	57.3 ± 23.2	66.0 ± 35.0	0.26
Natural killer cell activity (%)	24.8 ± 11.9	18.2 ± 13.8	0.14	25.5 ± 12.8	18.1 ± 10.9	0.16

ALT, alanine aminotransferase; BTR, branched-chain amino acid/tyrosine ratio; ChE, cholinesterase; LES, late evening snack; NH3, ammonia; PT, prothrombin time; npRQ, non-protein respiratory quotient.

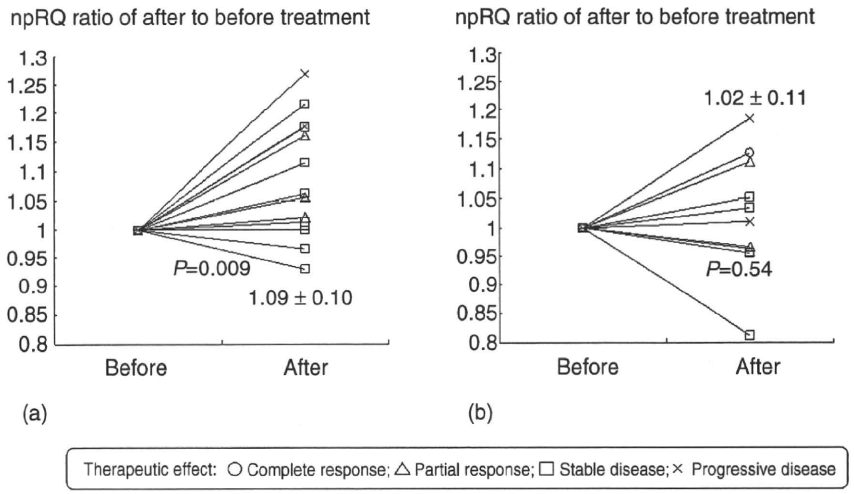

Figure 3 The npRQ ratio after compared to before 1 cycle of HAIC. In the LES group, npRQ improved in 10 patients, was stable in 1 patient, and worsened in 2 patients, regardless of response to therapy ($P = 0.009$) (a). In the control group, npRQ improved in 6 patients and worsened in 4 patients ($P = 0.54$) (b).

$P = 0.69$). Figure 3 shows the npRQ ratio of after compared to before 1 cycle of HAIC. In the LES group, npRQ improved in 10 patients, was stable in 1 patient, and worsened in 2 patients, regardless of response to therapy ($P = 0.009$). In the control group, npRQ improved in 6 patients and worsened in 4 patients ($P = 0.54$).

Blood biochemistry

Significant improvements in BTR, prealbumin and ALT levels were observed after 1 cycle of treatment in the LES group, but not in the control group. Cholinesterase levels were significantly decreased after 1 cycle of treatment in the control group, but did not differ in the LES group (Table 3). Figure 4 shows the BTR ratio of after to before 1 cycle of HAIC. In the LES group, BTR improved in 10 patients and worsened in 3 patients ($P = 0.005$). Conversely, BTR worsened in 7 patients in the control group ($P = 0.46$).

Anthropometry

No significant differences in anthropometric measurements (weight; skeletal muscle mass; body fat mass; fat-free mass; mid-upper arm muscle circumference (AMC); midarm circumference (AC); and body cell mass (BCM)) as measured using InBody were observed between groups (data not shown).

Changes in glucose tolerance

We examined the effects of LES using a BCAA-enriched nutrient on glucose tolerance using the 75-g OGTT in 21

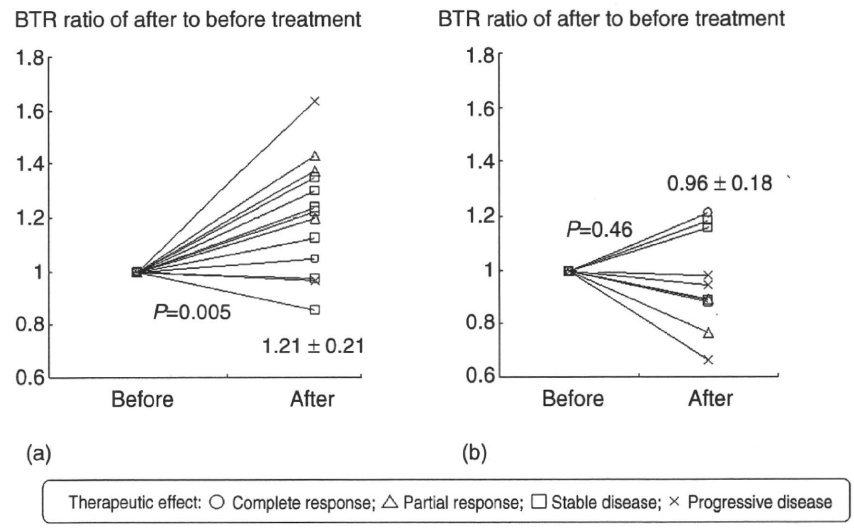

Figure 4 Branched-chain amino acid/tyrosine ratio (BTR) after compared to before 1 cycle of HAIC. In the LES group, BTR improved in 10 patients and worsened in 3 patients ($P = 0.005$) (a). Conversely, BTR worsened in 7 patients in the control group ($P = 0.46$) (b).

Table 4 Changes in glucose tolerance

	LES group (n = 12)			Control group (n = 9)		
	Before	After	*P*-value	Before	After	*P*-value
AUC glucose	396.1 ± 117.0	363.2 ± 135.6	0.055	355.3 ± 62.9	321.1 ± 108.6	0.17
AUC insulin	181.8 ± 123.3	223.2 ± 169.0	0.10	155.4 ± 85.3	117.7 ± 71.4	0.15
Fasting glucose (mg/dL)	99.5 ± 27.9	98.5 ± 32.6	0.71	100.7 ± 13.9	99.6 ± 18.7	0.89
Fasting insulin (μ U/mL)	11.3 ± 10.2	12.8 ± 7.1	0.38	14.5 ± 15.6	8.2 ± 1.8	0.28
HOMA-IR	2.9 ± 2.7	3.3 ± 2.5	0.47	4.0 ± 5.2	2.1 ± 0.8	0.32

AUC, area under the concentration curve; HOMA-IR, homeostasis model assessment method for insulin resistance; LES, late evening snack.

of 23 patients. One patient with DM in the LES group did not undergo the 75-g OGTT after treatment due to markedly high glucose levels, while the remaining patient in the control group declined to undergo the 75-g OGTT after treatment.

Table 4 shows changes in glucose tolerance before and after 1 cycle of treatment. In the LES group ($n = 12$), 1, 2, and 9 patients exhibited NGT, IGT, and DM, respectively. In the control group, 3, 2, and 4 patients exhibited NGT, IGT, and DM, respectively. No significant differences at baseline were seen between groups with regard to NGT, IGT, or DM using the 75-g OGTT, fasting glucose, fasting insulin, homeostasis model assessment method for insulin resistance (HOMA-IR), AUC glucose, and AUC insulin. AUC glucose tended to improve after 1 cycle of treatment in the LES group ($P = 0.055$). However, no significant differences in other parameters were apparent.

Prognosis

No significant differences in survival rates were seen between groups ($P = 0.667$; log-rank test) (Fig. 5a). On the other hand, survival in patients assessed as SD or PD according to the response criteria[30] tended to improve in the LES group ($n = 10$ in the LES group, $n = 7$ in the control group; $P = 0.156$; log-rank test) (Fig. 5b). In addition, no significant differences between groups assessed as SD or PD were seen with relation to background (data not shown).

By final follow-up, 2 patients remained alive (LES group, $n = 1$; control group, $n = 1$), while the other 21 patients had died. In the LES group, cause of death was cancer progression in 12 patients. In the control group, cause of death was cancer progression in 8 patients and hepatic failure in 1 patient.

Case presentation

Figure 6 shows a patient from the LES group. This 49-year-old man showed multiple HCCs in both lobes (stage III).[27,28] Mild ascites was identified, but no hepatic encephalopathy was present. On admission, hepatic reserve function was defined as Child-Pugh B (9 points). Prior to starting LES, npRQ was 0.71, and BTR value was low, at 2.2. After 1 cycle of HAIC, laboratory investigations were improved, and no ascites was apparent. Hepatic reserve function had improved to Child-Pugh A (6 points). Values of npRQ and BTR increased to 0.75 and 2.68, respectively, after 1 cycle of treatment. The patient exhibited DM on the 75-g OGTT. AUC glucose improved after 1 cycle of treatment (before, 414.75;

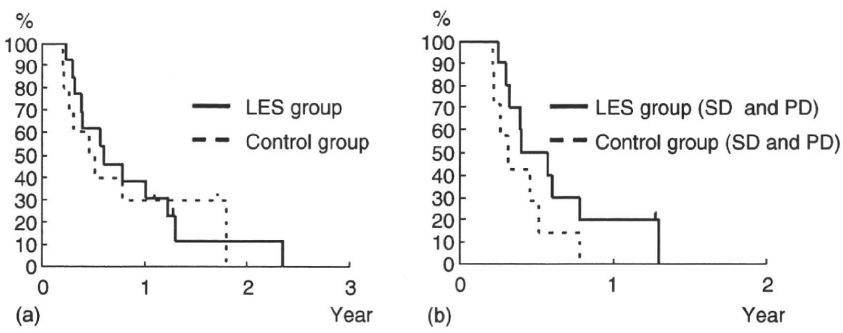

Figure 5 No significant differences in survival rates were seen between groups ($P = 0.667$; log-rank test) (a). On the other hand, survival in patients assessed as stable disease (SD) or progressive disease (PD) according to the response criteria tended to improve in the LES group ($n = 10$ in the LES group, $n = 7$ in the control group; $P = 0.156$; log-rank test) (b).

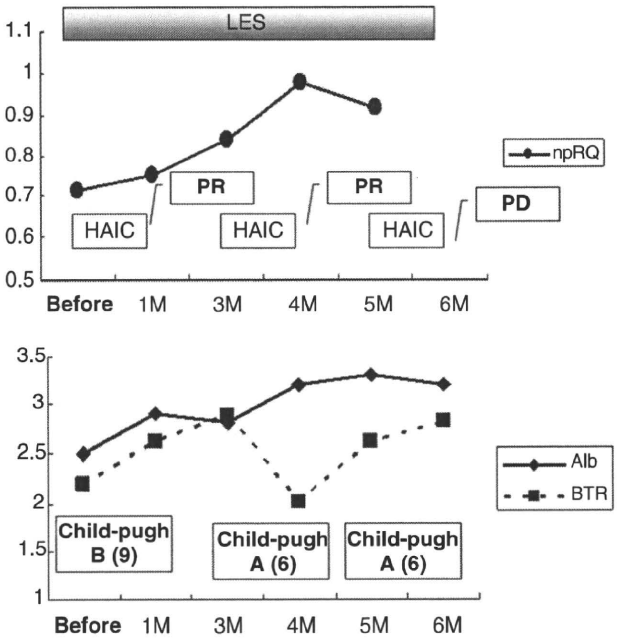

Figure 6 A case in the LES group. This 49-year-old man had multiple HCCs in both lobes (stage III). On admission, hepatic reserve function was Child-Pugh B (9 points). Prior to starting LES, npRQ was 0.71, and BTR was low at 2.2. After 1 cycle of HAIC, hepatic reserve function was improved to Child-Pugh A (6 points) from Child-Pugh B (9 points). Values of npRQ and BTR increased to 0.75 and 2.68, respectively, after 1 cycle of the treatment. The patient exhibited PR according to the response criteria. Thereafter, he received 3 courses of HAIC, and npRQ, serum albumin and BTR values improved. After the third course of HAIC, he exhibited PD. One year after the first course of HAIC, he died of tumor progression.

after, 369.75). PR was exhibited according to the response criteria.[30] Thereafter, the patient received 3 courses of HAIC, and npRQ, serum albumin and BTR value improved. After the third course of HAIC, he exhibited PD. One year after the first course of HAIC, he died of tumor progression.

Figure 7 shows a patient from the control group. This 73-year-old woman presented with massive HCC in the right lobe with tumor thrombus in the main trunk of the portal vein (Vp4) (stage IV A).[27,28] No ascites or hepatic encephalopathy was identified. On admission, hepatic reserve function was defined as Child-Pugh A (6 points). Prior to starting HAIC, npRQ was 0.91. However, BTR was low, at 2.34. After 1 cycle of HAIC, laboratory investigations showed slight deterioration. Values for npRQ and BTR decreased to 0.87 and 2.06, respectively, after 1 treatment cycle. The patient exhibited DM on the 75-g OGTT. AUC glucose remained almost unchanged (before, 446.25; after, 437.75). She exhibited SD according to the response criteria.[30] Although she received a second course of HAIC, npRQ, serum albumin and BTR values worsened. In addition, she showed moderate ascites during the second course of treatment. After the second course of treatment, hepatic reserve function was classified as Child-Pugh C (10 points). Thereafter, she developed hepatic encephalopathy and died of hepatic failure 6 months after the first course of HAIC.

DISCUSSION

PEM IS OFTEN observed in cirrhotic patients,[2,3] and this malnutrition adversely affects prognosis.[3] When energy metabolism of cirrhotic patients is measured using indirect calorimetry, npRQ decreases as the severity of liver cirrhosis increases.[3] However, no clinical studies have evaluated energy metabolism in patients with HCC. We therefore investigated energy metabolism

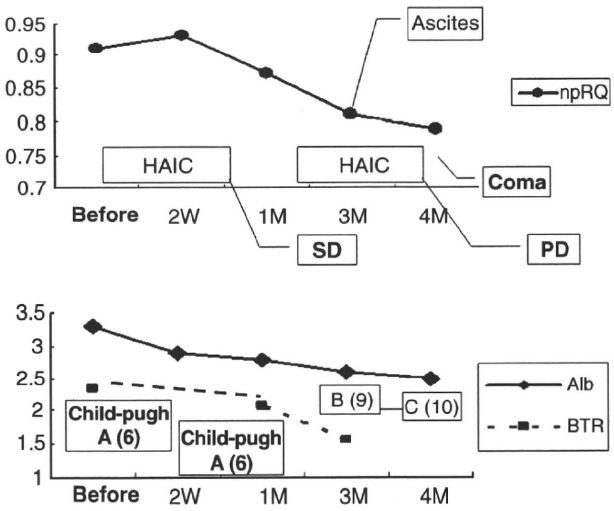

Figure 7 A case in the control group. This 73-year-old woman presented with massive HCC in the right lobe with tumor thrombus in the main trunk of the portal vein (Vp4) (stage IV A). On admission, hepatic reserve function was Child-Pugh A (6 points). Prior to starting HAIC, npRQ was 0.91. However, BTR was low at 2.34. After 1 cycle of HAIC, npRQ and the BTR value decreased to 0.87 and 2.06, respectively. She exhibited SD according to the response criteria. Although she received a second course of HAIC, npRQ, serum albumin and BTR values all worsened. After the second course of treatment, hepatic reserve function was Child-Pugh C (10 points). Thereafter, she developed hepatic encephalopathy and died of hepatic failure 6 months after the first course of HAIC.

using indirect calorimetry in cirrhotic patients without HCC and with various stages of HCC under the same conditions of liver capacity, namely Child-Pugh A. In our study, no significant differences were seen among 3 groups (LC group, HCC stage I/II group, and HCC stage III group), but npRQ was significantly lower in patients with stage IV HCC than in cirrhotic patients without HCC, or in patients with stage I/II or stage III HCC. Although there was a significant difference in age between the HCC stage III group and the HCC stage IV group, there was no significant correlation between the value of npRQ before treatment and age (data not shown). These findings suggest that patients with advanced HCC remained under conditions of severe energy malnutrition even if liver capacity was good. A hypermetabolic rate in patients with gastrointestinal malignancy is reportedly usually associated with the most advanced stage of the disease.[24] The p53 tumor suppressor gene regulates glucose metabolism, and loss of p53 upregulates energy metabolism.[35] Mutation of the p53 gene is associated with poor tumor differentiation and advanced stage of HCC.[36] We speculate that reductions in npRQ among patients with advanced HCC may be related to the upregulation of glucose metabolism in cancer cells, but the underlying mechanisms remain unclear. Our results suggest that nutritional support is warranted for cirrhotic patients with advanced HCC, even if liver capacity is good.

In an attempt to improve the state of energy malnutrition, LES has been developed and improved energy metabolism has been reported.[5–8,11–13] However, those studies focused on the effects of LES in patients with cirrhosis.

Poon *et al.* reported that nutritional supplementation with oral BCAAs is beneficial for increasing serum albumin level, reducing morbidity and improving quality of life in patients undergoing transarterial chemoembolization for HCC,[25] but the nutritional supplementation did not use LES. Takeshita *et al.* only reported that LES using BCAA-enriched nutrients prevents suppression of liver function in patients with HCC undergoing transarterial chemoembolization.[26] That study did not evaluate energy metabolism before and after LES. We therefore investigated the efficacy of nutritional support using LES in patients with advanced HCC undergoing HAIC using indirect calorimetry.

The present findings showed that LES using BCAA-enriched nutrients improves npRQ, BTR, ALT, and prealbumin significantly before and after 1 cycle of HAIC compared with the control group. Nakaya *et al.* reported that LES using BCAA-enriched nutrients improved npRQ, BTR, and serum albumin before and 3 months after in cirrhotic patients compared with LES using ordinary food.[8] Although no significant difference in serum albumin was identified in our study, prealbumin (a rapid turnover protein with a half-life in plasma of 2 days) was significantly increased by LES. Prealbumin is more sensitive to changes in protein-energy status than albumin.[37] We thus consider that improvement of npRQ and prealbumin reflects energy metabolism in cirrhotic patients with advanced HCC undergoing HAIC. Unfortunately, we could not evaluate nutritional parameters in the long term, as some patients died in the short term. Despite the small sample size, of the 12 patients for whom npRQ was evaluated at 3 months after HAIC, patients treated with LES ($n = 6$) tended to show improved npRQ ($P = 0.10$), and npRQ was not significantly difference before and 3 months after HAIC in control patients ($n = 6$; $P = 0.91$; data not shown).

BCAAs reportedly improve glucose intolerance.[11–13,38] The present study evaluated glucose tolerance before and at the end of 1 cycle of treatment. AUC glucose in the LES group tended to improve ($P = 0.055$). In addition, AUC glucose in patients who showed glucose intolerance (IGT and DM; $n = 11$) in the LES group tended to improve (before, 412.3 ± 107.7; after, 376.1 ± 134.3; $P = 0.052$) and AUC glucose in patients who had glucose intolerance ($n = 6$) in the control group showed no significant difference (before, 376.5 ± 67.8; after, 338.3 ± 132.7; $P = 0.30$). One reason might be the effect of LES itself. A LES improves postprandial hyperglycemia, because the glucose load per meal is decreased by fractionated meals including a LES, and glucose is properly oxidized in the tissues. Another reason might be the effects of the leucine and isoleucine contained among the BCAAs. Leucine and isoleucine promote glucose uptake in skeletal muscle under insulin-free conditions.[39] Leucine also increases the activity of p70S6 kinase via the mammalian target of rapamycine pathway, and the ability to synthesize glycogen is improved.[39] However, we have previously reported that glucose tolerance worsened after 3 months of LES administration in cirrhotic patients with DM according to the 75-g OGTT.[40] In 12 patients (LES group, $n = 6$; control group, $n = 6$) for whom glucose tolerance was evaluated using the 75-g OGTT at 3 months after HAIC, AUC glucose was significantly worsened in both groups in this study (data not shown). We reported that LES combined with an alpha-glucosidase inhibitor to slow glucose absorption into the blood and ameliorate postprandial hyperglycemia improved glucose tolerance (AUC glucose) over the long term (3 months) in

patients with liver cirrhosis,[41] and concomitant use of an alpha-glucosidase inhibitor with LES might be a useful nutritional therapy in patients with advanced HCC who show glucose intolerance.

Unfortunately, no significant differences in survival rate were identified between groups ($P = 0.667$; log-rank test). Poon *et al.* also reported no difference in survival between patients who received BCAAs and those receiving ordinary food.[25] However, survival in patients assessed as SD or PD tended to improve in the LES group ($P = 0.156$; log-rank test), although no significant differences between groups assessed as SD or PD were seen with relation to background. Significant improvement in npRQ was observed in the LES group, and significant reductions in cholinesterase and natural killer cell activity were observed in the control group, for groups assessed as SD or PD (data not shown). In addition, the frequency of HCC treatment tended to be increased in the LES group (data not shown). We speculate that life prolongation in patients assessed as SD or PD may be related to improvements in energy metabolism and immune defense,[32] and the continuation of HCC treatment, by means of LES using BCAA-enriched nutrients. Our previous study identified therapeutic effect as an independent prognostic factor in cirrhotic patients with advanced HCC treated using HAIC.[20] In this study, patients exhibited CR or PR showed good prognosis without relation to LES (data not shown). Therefore, we consider that patients exhibited SD or PD may be suitable candidates for LES using BCAA-enriched nutrients because of life prolongation. As our study examined only a small population, further investigations are necessary.

In conclusion, LES using BCAA-enriched nutrients offers the possibility of improving energy metabolism and glucose tolerance in cirrhotic patients with advanced HCC undergoing HAIC. Although our study design shows limitations in the comparison between ordinary food and both LES and BCAA, we speculate that these results are caused by effects from both LES and BCAA. We consider that tailored nutritional support, such as tumor staging, is required in patients with HCC.

ACKNOWLEDGEMENTS

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Methylated *cyclin D2* gene circulating in the blood as a prognosis predictor of hepatocellular carcinoma

Masahito Tsutsui [a], Norio Iizuka [a,b], Toyoki Moribe [c], Toshiaki Miura [c], Naoki Kimura [c], Shigeru Tamatsukuri [c], Hideo Ishitsuka [c], Yusuke Fujita [d], Yoshihiko Hamamoto [d], Ryouichi Tsunedomi [a], Michihisa Iida [a], Yoshihiro Tokuhisa [a], Kazuhiko Sakamoto [a], Takao Tamesa [a], Isao Sakaida [e], Masaaki Oka [a,*]

[a] *Department of Digestive Surgery and Surgical Oncology (Department of Surgery II), Yamaguchi University Graduate School of Medicine, 1-1-1 Minami-Kogushi, Ube, Yamaguchi 755-8505, Japan*
[b] *Department of Complementary Medicine, Yamaguchi University Graduate School of Medicine, 1-1-1 Minami-Kogushi, Ube, Yamaguchi 755-8505, Japan*
[c] *Molecular Diagnostics R&D Department, Molecular Diagnostics Division, Roche Diagnostics K.K., 6-1, Shiba 2-chome, Minato-ku, Tokyo 105-0014, Japan*
[d] *Department of Computer Science and Systems Engineering, Faculty of Engineering, Yamaguchi University, 2-16-1 Tokiwadai, Ube, Yamaguchi 755-8611, Japan*
[e] *Department of Gastroenterology and Hepatology, Yamaguchi University Graduate School of Medicine, 1-1-1 Minami-Kogushi, Ube, Yamaguchi 755-8505, Japan*

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ABSTRACT

Background: Prognosis of hepatocellular carcinoma (HCC) remains poor because of high recurrence rate. We examined preoperatively the methylated *CCND2* gene levels present in the serum following release from HCC cells as a prognosis predictor in patients undergoing curative hepatectomy.
Methods: Quantitative real-time RT-PCR and quantitative methylation-specific PCR were used to measure methylated *CCND2* gene and its mRNA levels.
Results: The *CCND2* mRNA levels were down-regulated in HCC with early intrahepatic recurrence (IHR) within 1 year of curative hepatectomy. We also identified that this down-regulation was due to promoter hypermethylation. In 70 HCC patients who underwent curative hepatectomy, 39 patients sero-positive for the methylated *CCND2* gene (>70 pg/ml serum) exhibited a significantly shorter disease-free survival (DFS) period ($P=0.02$) than the 31 patients who were sero-negative for the methylated *CCND2* gene. None of the sero-negative patients demonstrated early IHR, and this method of serum testing did not produce any false-negative predictions for early IHR. Multivariate analysis showed that the serum level of methylated *CCND2* was an independent risk factor for DFS (hazard ratio of 1.866, 95% CI: 1.106–3.149).
Conclusion: Methylated *CCND2* gene in the serum serves as a prognosis predictor of HCC after curative hepatectomy.

1. Introduction

Hepatocellular carcinoma (HCC) mainly develops from chronic liver diseases with persistent infection of hepatitis B virus (HBV) or hepatitis C virus (HCV), and represents a major international health problem due to its increasing incidence in many countries [1–3]. Although resection provides the best chance for cure in HCC patients, data from the International Cooperative Study Group for HCC shows a 5-year survival rate of only 31–41%, regardless of ethnic background [4]. This poor prognosis has been attributed to a high rate of recurrence, especially intrahepatic recurrence (IHR) via intrahepatic spread of cancer cells, and not *de novo* multicentric hepatocarcinogenesis [1,5–7]. Numerous novel molecular agents are currently available as adjuvant therapies for this disease [8]. It is therefore essential to identify a robust predictive marker for HCC.

Our genome-wide studies revealed that a reduction in *cyclin D2* (*CCND2*) mRNA levels in HCC was associated with IHR developing within 1 year of curative surgery [9–11]. We and others have shown that the promoter region of the *CCND2* gene is highly methylated in HCC, but not in non-HCC liver tissues [12,13], suggesting that *CCND2* mRNA in HCC cells is down-regulated largely via promoter hypermethylation. More recently, several investigators have reported the diagnostic efficacy of the measurement of methylated genes circulating in the blood of HCC patients [14,15]. It has also been shown that the concentration of cell-free DNA in the blood of HCC patients is sufficient for the analysis of the methylation status of cancer-specific genes [16,17]. The current study aimed to measure the methylated *CCND2* gene levels obtained preoperatively from serum samples in the hope that this measurement may serve as a prognostic marker in HCC patients undergoing curative hepatectomy.

* Corresponding author. Department of Digestive Surgery and Surgical Oncology (Department of Surgery II), 1-1-1 Minami-Kogushi, Ube, Yamaguchi 755-8505, Japan. Fax: +81 836 22 2262.
E-mail address: 2geka-1@po.cc.yamaguchi-u.ac.jp (M. Oka).

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2. Materials and methods

2.1. Tissue samples

We used 38 HCC and 6 non-HCC liver tissue samples isolated from 38 HCC patients (Table 1) who underwent curative hepatectomy at our institute between April 2001 and Oct 2005. According to the criteria described previously, our screening program revealed that 6 (15.8%) of the 38 patients had early IHR within 1 year of surgery [9]. Resected specimens were immediately divided into several blocks, frozen and stored at −80 °C until required. The 38 HCC and 6 non-HCC liver tissue samples were then subjected to quantitative real-time PCR (qRT-PCR) and quantitative methylation-specific PCR (QMSP) assays. This study protocol was pre-approved by the Institutional Review Board for the Use of Human Subjects at the Yamaguchi University School of Medicine, and written informed consent was obtained from all patients prior to their entry into this study.

2.2. Serum samples

We collected serum samples from 70 patients who were positive for HCV antibody, all of whom underwent hepatectomy in our institute between May 1998 and April 2006 after pathological diagnosis of HCC according to previously described methods [17]. Twelve (17.1%) of the 70 patients exhibited early IHR within 1 year of surgery. Our surveillance program [9] also revealed that 59 (84.3%) of the 70 patients exhibited recurring HCC during a mean follow-up period of 58 months. The tumor-node-metastasis (TNM) staging system revised by the Liver Cancer Study Group of Japan (LCSGJ) [18] was used in this study. Serum samples obtained from 36 HCV-infected patients without HCC with a mean age of 64.3 years (male: female = 22:14) served as control DNA for the determination of cutoff values.

Table 1
Tissue levels of *CCND2* mRNA and clinicopathologic features.

	mRNA levels[a] (mean ± SE)	*P* values
Sex		P=NS[b]
Male (n=26)	0.028 ± 0.049	
Female (n=12)	0.027 ± 0.049	
Age (year)		P=NS[b]
≤60 (n=9)	0.022 ± 0.003	
>60 (n=29)	0.029 ± 0.005	
Hepatitis C virus antibody		P=NS[b]
Negative (n=13)	0.022 ± 0.003	
Positive (n=25)	0.030 ± 0.005	
Tumor size		P=NS[b]
≤3.0 cm (n=16)	0.031 ± 0.006	
>3.0 cm (n=22)	0.025 ± 0.004	
Histological grading		P=NS[c]
Well (n=13)	0.024 ± 0.004	
Moderately (n=22)	0.028 ± 0.006	
Poorly (n=3)	0.036 ± 0.016	
Venous invasion		P=NS[b]
Negative (n=25)	0.031 ± 0.005	
Positive (n=13)	0.022 ± 0.004	
Stage		P=NS[c]
I (n=14)	0.031 ± 0.005	
II (n=18)	0.023 ± 0.004	
III (n=6)	0.031 ± 0.016	
Early intrahepatic recurrence		P=0.044[b]
Absence (n=32)	0.031 ± 0.004	
Presence (n=6)	0.011 ± 0.003	

[a] Relative levels of *CCND2* mRNA to *GAPDH*.
[b] Student's *t* test.
[c] ANOVA test.

2.3. qRT-PCR

Relative levels of *CCND2* mRNA to *glyceraldehyde phosphate dehydrogenase* (*GAPDH*) were determined by qRT-PCR as described previously [19]. Briefly, total RNA was extracted from frozen tissues using TRIzol (Life Technologies, Grand Island, NY, USA) and PCR amplification of cDNA (from 10 ng of initial RNA) was performed on the LightCycler II (Roche Diagnostics GmbH, Mannheim, Germany) using the Roche Universal Probe Library (https://www.roche-applied-science.com/sis/rtpcr/upl/index.jsp). PCR primers used were: sense (5′-tcaccaacacagacgtgga-3′) and antisense (5′-tgtaggggtgctggcttg-3′) for the *CCND2* gene (NM_001759) and sense (5′-agccacatcgctcagacac-3′) and antisense (5′-gcccaatacgaccaaatcc-3′) for the *GAPDH* gene (NM_002046). PCR products for *CCND2* and *GAPDH* were 143 base pairs (bp) and 66 bp, respectively. Universal Probe Library probes #88 (Roche Diagnostics GmbH, Mannheim, Germany) and #60 were used for the measurement of *CCND2* and *GAPDH* levels, respectively.

2.4. DNA extraction and QMSP

DNA was extracted from resected specimens using the DNA Isolation Kit for Cells and Tissues (Roche Diagnostics GmbH), and from 1 ml of sera using the DNA Extractor SP Kit for Serum and Plasma (Wako Pure Chemical Industries Ltd., Osaka, Japan) according to the manufacturer's instructions. The extracted DNA was then treated with bisulfite (BIS) and quantified as described previously [13,16,20]. We used the QMSP assay to measure methylated levels of *CCND2* in the DNA extracted from tissues and serum as described previously [13,20]. We have confirmed previously that the results achieved using QMSP are consistent with those achieved by pyro-sequencing after BIS treatment [13].

For the determination of the methylation levels of *CCND2* in tissues, the amount of methylated DNA in 5 μl (1 ng) of BIS-treated DNA solution was quantified using a standard curve constructed from simultaneously measured standards made from a dilution series (1000, 200, 40 and 4 pg μl^{-1}) of artificially methylated DNA (CpGenome™ Universal Methylated DNA, Chemicon International Inc., CA, Temecula CA). Tissue methylation levels were then calculated as pg per μl of BIS-treated DNA solution. Serum methylation levels of *CCND2* were calculated as the relative methylated DNA amount (pg) per ml serum.

3. Statistical analysis

Pearson correlation coefficients were calculated to evaluate correlations between methylation and mRNA levels of *CCND2* in tissue. Student's *t* test, Mann–Whitney *U* test, and ANOVA tests were used to evaluate differences between 2 or more variables. Fisher's exact test or χ^2 test was used to evaluate the differences between factors with discontinuous variables. We carried out multivariate analysis to assess independent factors for DFS using the Cox proportional hazard model. Survival curves were drawn using the Kaplan–Meier method and the statistical significance was determined using the log-rank test. All statistical analyses were performed using SPSS 11.0J (SPSS, Inc., Chicago, IL) software. A $P<0.05$ was considered significant.

4. Results

To evaluate the reproducibility of our previous study that used DNA microarray technology [9], we measured the levels of *CCND2* mRNA in a new sample set of 38 HCC tissues using qRT-PCR analysis. We found that *CCND2* mRNA levels were significantly lower in HCC with early IHR (n=6) than in HCC without IHR (P=0.044 by Student's *t* test; n=32. Table 1). There appeared to be no association

between tumor *CCND2* mRNA levels and other clinicopathologic factors (Table 1).

Our qRT-PCR and QMSP revealed that there was an inverse association ($r=-0.585$, $P=0.046$) between *CCND2* methylation levels and its mRNA levels in 6 HCC and 6 non-HCC liver tissues (Fig. 1). In 3 serum samples from 4 HCC patients with *CCND2* methylation in primary tumors, the methylated form was detected (data not shown). Serum levels of methylated *CCND2* gene were 435 ± 890 and 29 ± 40 pg/ml serum (mean ± SD) in 70 HCC patients and 36 HCV-infected patients without HCC, respectively. Those were significantly higher in HCC patients than in HCV-infected patients without HCC ($P=0.0001$ by Mann–Whitney *U* test) (Fig. 2). These results prompted us to test the hypothesis that methylated DNA fragments of the *CCND2* gene may be released from HCC cells with high metastatic potential into the bloodstream. We found that serum levels of methylated *CCND2* gene were significantly higher in HCC patients with early IHR than in HCC patients without IHR ($P=0.0011$ by Mann–Whitney *U* test). Serum levels were not found to be associated with other clinicopathologic factors (Table 2).

Serum levels of methylated *CCND2* gene were 27 ± 27 pg/ml serum in 4 healthy people (data not shown). There was no significant difference in serum levels of methylated *CCND2* gene between healthy people and HCV-infected patients without HCC. We defined 70 pg/ml serum (equal to mean + 1 SD of HCV-infected patient without HCC) as the cutoff value in distinguishing HCC from non-HCC. Among the 70 HCC patients, 39 (55.7%) were positive for serum *CCND2* methylation, while the remaining 31 (44.3%) were negative (Table 3). The incidence of early IHR in patients sero-positive for methylated *CCND2* was significantly higher than patients who were sero-negative for methylated *CCND2* ($P=0.001$; Table 3). None of the 31 patients who were sero-negative for methylated *CCND2* exhibited early IHR. That is, this serum test did not result in false-negative predictions. In our cohort consisting of 70 HCC patients, primary tumor number ($P=0.026$) and tumor stage ($P=0.051$) were also linked to early IHR (Table 3).

Patients sero-positive for methylated *CCND2* exhibited significantly shorter DFS than those sero-negative for methylated *CCND2* ($P=0.02$ by log-rank test; Fig. 3A). However, the serum *CCND2* test was not associated with overall survival (OS) ($P=0.103$; Fig. 3B).

To identify independent predictors of DFS, 6 factors including primary tumor number, tumor size, tumor differentiation, venous invasion, stage and serum methylation levels of *CCND2* were entered into a Cox proportional hazard model using stepwise selection. The Cox regression analysis selected only 2 factors that were associated with DFS. These were serum levels of methylated *CCND2* (hazard ratio 1.866, 95% CI: 1.106–3.149) and venous invasion (hazard ratio 1.981, 95% CI: 1.049–3.740) (Table 4).

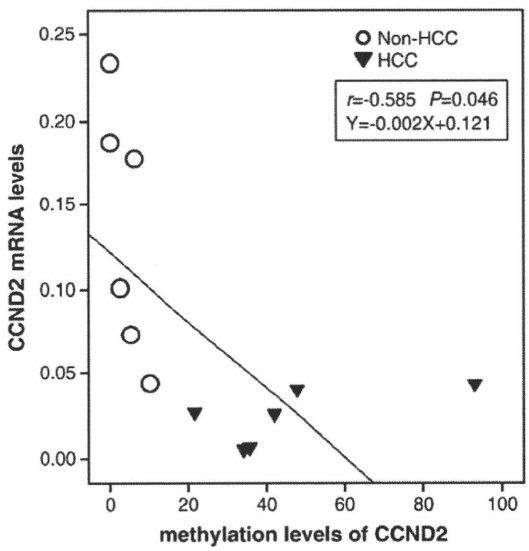

Fig. 1. Association between mRNA levels and tissue levels of methylated *CCND2* gene. Note the inverse association between mRNA levels and methylation levels of *CCND2* in tissues ($r=-0.585$; $P=0.046$). Black triangle: HCC tissues. White circle: non-HCC liver tissues.

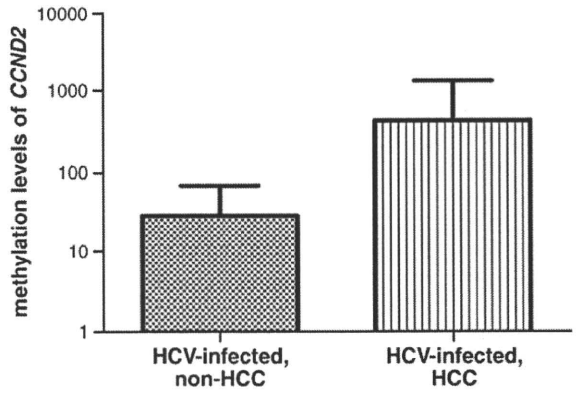

Fig. 2. Serum levels of methylated *CCND2* gene in sera from 36 HCV-infected patients without HCC and 70 HCC patients. Note that serum levels of methylated *CCND2* gene were significantly higher in HCC patients (435 ± 890 pg/ml serum) than in HCV-infected patients without HCC (29 ± 40 pg/ml serum) ($P=0.0001$). Error bars, SD.

5. Discussion

The most striking finding arising from the present study was that the serum epigenetic marker gene *CCND2* was closely related to early IHR and DFS in HCC patients who had undergone curative hepatectomy. The possible detection and measurement of tumor-derived cell-free DNA (cfDNA) has opened a new avenue in predictive oncology [21–23]. On the basis of genome-wide information, our current study focused on a unique gene *CCND2*, as it has been shown previously that its mRNA levels are decreased at the primary site of HCC in patients with an increased risk of early IHR [9,11], and that the *CCND2* gene is

Table 2
Serum levels of methylated *CCND2* DNA and clinicopathologic features.

	Methylated DNA levels[a] (mean ± SE)	Median (range)	*P* values
Sex			$P=$NS[b]
Male ($n=49$)	455.5 ± 138.0	102.0 (0.2–5155.0)	
Female ($n=21$)	388.4 ± 152.8	26.0 (0.2–2561.0)	
Age (year)			$P=$NS[b]
≤60 ($n=12$)	514.9 ± 423.4	13.1 (0.2–5155.0)	
>60 ($n=58$)	418.9 ± 96.71	106.5 (0.2–3635.0)	
Tumor size			$P=$NS[b]
≤3.0 cm ($n=33$)	206.8 ± 50.41	80.0 (0.2–1064.0)	
>3.0 cm ($n=37$)	639.3 ± 191.3	146.0 (0.2–5155.0)	
Histological grading			$P=$NS[c]
Well ($n=13$)	323.0 ± 194.0	93.0 (0.2–2595.0)	
Moderately ($n=50$)	488.5 ± 137.3	145.5 (0.2–5155.0)	
Poorly ($n=7$)	264.8 ± 224.1	42.0 (0.2–1607.0)	
Venous invasion			$P=$NS[b]
Negative ($n=56$)	320.9 ± 75.8	97.5 (0.2–2595.0)	
Positive ($n=14$)	893.4 ± 427.8	102.0 (0.2–5155.0)	
Stage			$P=$NS[b]
I/II ($n=39$)	320.1 ± 88.86	73.0 (0.2–2561)	
III/IV ($n=31$)	580.4 ± 212.0	146.0 (0.2–5155)	
Early intrahepatic recurrence			$P=0.0011$[b]
Absence ($n=58$)	261.6 ± 64.81	52.0 (0.2–2595.0)	
Presence ($n=12$)	1276.0 ± 481.2	499.0 (73.0–5155.0)	

[a] Relative methylated DNA amount (pg) per ml serum.
[b] Mann–Whitney *U* test.
[c] ANOVA test.

Table 3
Factors related to early intrahepatic recurrence.

	Early intrahepatic recurrence		*P* values
	Absence ($n=58$)	Presence ($n=12$)	
Sex			$P=NS^a$
Male	40	9	
Female	18	3	
Liver cirrhosis			$P=NS^a$
Negative	22	7	
Positive	36	5	
Primary tumor number			$P=0.026^a$
Single	36	3	
Multiple	22	9	
Tumor size			$P=NS^b$
≤3.0 cm	28	5	
>3.0 cm	30	7	
Histological grading			$P=NS^a$
Well	12	0	
Moderately	41	10	
Poorly	5	2	
Venous invasion			$P=NS^a$
Negative	48	8	
Positive	10	4	
Stage			$P=0.051^a$
I	10	0	
II	24	5	
III	20	3	
IV	4	4	
CCND2 methylation levels			$P=0.001^b$
Negative (≤70 pg per 1-ml serum)	31	0	
Positive (>70 pg per 1-ml serum)	27	12	

[a] Fisher exact test.
[b] Chi-square test.

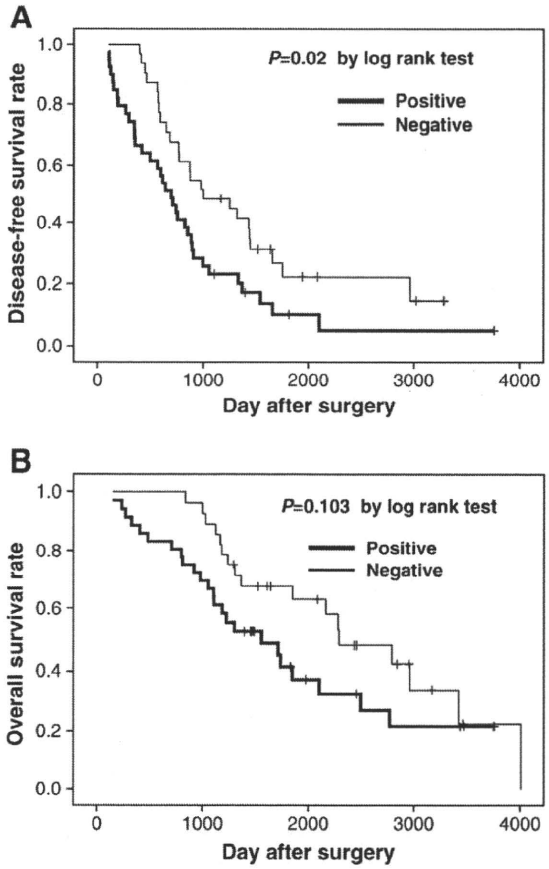

Fig. 3. Disease-free (A) and overall (B) survivals in HCC patients according to serum levels of methylated *CCND2* gene.

highly methylated in HCC [12,13]. In fact, *CCND2* mRNA levels were inversely associated with methylation levels in HCC and non-HCC liver tissues, indicating that its decrease may be due to promoter methylation. We were able to quantitatively detect the epigenetic signature in serum DNA from HCC patients with methylated *CCND2*.

The current study reports a remarkable advancement in the treatment and diagnosis of HCC [8]. That is, HCC patients may have a generally poorer prognosis due to their high rate of early IHR caused by intrahepatic spread of cancer cells, even when curative hepatectomy is performed [1,4–7,9]. A great deal of effort has been devoted to the development of predictive systems for early IHR. Many genome-wide studies using high-tech array systems [9,10,24,25] have raised the possibility of accurately predicting early IHR in HCC patients. However, a shortcoming of this approach is that such DNA array systems are high in cost, generate unstable information and require a tissue sample as a source for the molecular profiling, raising the issues of preoperative and daily risk assessment in early IHR. In this regard, our serological epigenetic marker *CCND2* may allow risk evaluation of early IHR or recurrence preoperatively in the setting of the daily clinical practice.

CCND2 encodes *cyclin D2*, an important cell cycle regulator. However, it remains unclear how *CCND2* functions during cell cycle progression in malignant cells. It has been reported that numerous types of malignancies exhibit hypermethylation in the promoter region of *CCND2* [26–29], suggesting that this molecule may play an important role in a common pathway of malignancy transformation. Further examination is required to fully elucidate the molecular basis underlying *CCND2* activity, and to evaluate whether *CCND2* serves as a molecular target for the treatment of this disease.

HCC recurrence is a complicated process. There are at least 3 modes of postoperative recurrence [5], including early and late IHR that appear in the remnant liver, and extrahepatic recurrence that appears in distant tissues and organs such as bone and lung. Of the 3 modes of recurrence, the most clinically important is early IHR, as it imparts a significant impact on patient prognosis [1,6,7]. In the current study, we defined IHR within 1 year of surgery as early IHR, a definition that was consistent with previously described reports [9,10]. As proposed by Sakon et al. [6], it appears reasonable to define IHR within 2 years of surgery as early IHR from the standpoint of an accurate classification of prognosis. However, prediction of either the presence or absence of IHR at a shorter interval may allow for a more accurate diagnosis due to less bias between samples. In this regard, it may be more desirable to predict IHR within 1 year of surgery rather than 2 years.

In the current study, samples subjected to serum analysis for the examination of *CCND2* gene methylation levels were all isolated from HCV-infected patients. A previous molecular profiling study [30] revealed that HBV and HCV cause HCC via different carcinogenetic pathways. It was also reported that there was a significant difference in postoperative clinical course (i.e., outcome) between HBV- and HCV-related HCCs [31]. In evaluating a new prognostic marker, it is crucial to minimize a bias caused by distinct agents such as hepatitis viruses. Thus, we investigated whether hypermethylation of *CCND2* in DNA isolated from serum was associated with early IHR or the metastatic potential of HCC in a cohort consisting of only HCV-infected patients.

Given that analysis of methylated *CCND2* gene in sera could serve as a robust predictor for recurrence or early IHR of HCV-related HCC,

Table 4
Independent risk factors for disease-free survival.

	Regression coefficient	Standard error	Risk ratio (95%CI)	*P* values
CCND2	0.624	0.267	1.866 (1.106–3.149)	0.019
Venous invasion	0.683	0.324	1.981 (1.049–3.740)	0.035

further studies are needed to examine it in sera from patients with HCV-unrelated HCC. From the standpoint of daily clinical use, the development of predictive systems must enable the accurate detection of patient risk for early IHR. There are limitations in the use of a single marker in the present setting. We previously developed an accurate predictor for early IHR by combining expression data of 12 marker genes in HCC tissues [9]. Therefore, the identification of numerous serum epigenetic marker genes is required to develop a combined predictor with high accuracy for early IHR of HCC.

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ORIGINAL ARTICLE

Safety and tolerance of sorafenib in Japanese patients with advanced hepatocellular carcinoma

Sadahisa Ogasawara · Fumihiko Kanai · Shuntaro Obi · Shinpei Sato · Taketo Yamaguchi · Ryosaku Azemoto · Hideaki Mizumoto · Youhei Koushima · Naoki Morimoto · Nobuto Hirata · Takeshi Toriyabe · Yusuke Shinozaki · Yoshihiko Ooka · Rintaro Mikata · Tetsuhiro Chiba · Shinichiro Okabe · Fumio Imazeki · Masaharu Yoshikawa · Osamu Yokosuka

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Abstract

Purpose Sorafenib provides a survival benefit for patients with advanced hepatocellular carcinoma (HCC). However, there has been little experience with it in Japan. This study evaluated the safety and tolerance of sorafenib in Japanese patients with HCC.

Methods Clinical data for patients given sorafenib for advanced HCC were captured from eight institutions. All patients were classified as Child-Pugh A and the treatment was started at 400 mg twice daily. We recorded adverse events, treatment duration, and survival retrospectively. Adverse events were graded using Common Terminology Criteria, version 3.0; tumor response was assessed according to Response Evaluation Criteria in Solid Tumor, version 1.1.

Results Of the 54 patients treated, their median age was 69 years (range 48–82), 91% were males, 52% had HCV infection, and 22% had HBV infection. The most common drug-related adverse events were hand–foot skin reactions (HFSR) (72%), aspartate transaminase elevation (55%), alanine aminotransferase elevation (52%), rash (50%), fatigue (41%), and diarrhea (32%). Liver failure occurred in 19%. The median time to treatment failure was 2 months. Dose reduction was required in 83% of the patients, and this occurred within 2 weeks in 44%. The median overall survival was 6.9 months.

Conclusions These data suggest that sorafenib is generally tolerated in Japanese patients with HCC. Nevertheless, the majority needed a dose reduction. Adverse events including HFSR, rash, and liver failure occurred more frequently in our patients than those reported elsewhere. Careful attention must be paid to these adverse events during sorafenib administration.

S. Ogasawara · F. Kanai (✉) · T. Toriyabe · Y. Shinozaki · Y. Ooka · R. Mikata · T. Chiba · S. Okabe · F. Imazeki · M. Yoshikawa · O. Yokosuka
Department of Medicine and Clinical Oncology, Graduate School of Medicine, University of Chiba, 1-8-1 Inohana, Chuo-ku, 260-8670 Chiba, Japan
e-mail: kanaif@faculty.chiba-u.jp

S. Obi · S. Sato
Department of Gastroenterology and Hepatology, Kyoundo Hospital, 1-8 Kandasurugadai, Chiyoda-ku, 101-0062 Tokyo, Japan

T. Yamaguchi
Department of Gastroenterology, Chiba Cancer Centre, 666-2 Nitonacho, Chuo-ku, 260-8717 Chiba, Japan

R. Azemoto
Department of Gastroenterology, Kimitsu-Chuo Hospital, 1010 Sakurai, 292-8535 Kisarazu, Japan

H. Mizumoto
Department of Gastroenterology, Funabashi Municipal Medical Centre, 1-21-1 Kanasugi, 273-8588 Funabashi, Japan

Y. Koushima
Department of Gastroenterology, Saitama Red Cross Hospital, 8-3-33 Kamiochiai, Chuo-Ku, 333-8533 Saitama, Japan

N. Morimoto
Department of Gastroenterology, Shimotsuga-Sogo Hospital, 5-32 Fujimi, 328-8505 Tochigi, Japan

N. Hirata
Department of Gastroenterology, Kameda Medical Centre, 929 Higashicho, 296-0042 Kamogawa, Japan

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Introduction

Hepatocellular carcinoma (HCC) is the fifth most common cancer worldwide [1]. HCC develops mostly in patients with liver cirrhosis, which is typically caused by hepatitis C virus (HCV) infection, hepatitis B virus (HBV) infection, or alcohol [2]. The annual incidence of HCC in HCV-positive liver cirrhosis and chronic hepatitis is 6–7% and 1–2%, respectively [2]. The risk of cancer developing from chronic hepatitis or cirrhosis depends on the degree of fibrosis [3]. The hepatocarcinogenesis in the patients with hepatitis viruses differs between HCV and HBV. HCC occurs frequently in the cirrhotic livers of patients with HCV-positive liver disease. By contrast, HCC often develops in chronic HBV infection in the absence of cirrhosis. HCC developing from HBV infection has a lower cirrhosis complication rate than does HCC developing from HCV infection.

The etiology of HCC varies regionally [4]. In the Asia-Pacific region, except Japan, 70% of HCC is HBV-related and 20% is HCV-related [5]. In contrast, in Japan, 71–75% of HCC is HCV-related [2, 6]. The incidence of HCV infection is also increasing in the USA and Europe, as is the incidence of HCC [7].

Both surgical resection and local ablation therapy, including radiofrequency ablation, are considered curative for HCC [8–10]. Transarterial chemoembolization (TACE) has been applied to patients with advanced incurable HCC [11, 12]. However, the majority of patients experience recurrence or metastasis after these treatments. Although systemic therapy is available for advanced HCC, the prognosis remains poor. No standard systemic therapy that prolongs survival had been indentified before sorafenib was approved.

Sorafenib, an oral multikinase inhibitor, blocks tumor cell proliferation by targeting Raf/MEK/ERK signaling at the level of Raf kinase, and exerts an antiangiogenic effect by targeting vascular endothelial growth factor receptor-beta (VEGFR-β, PDGF-β) tyrosine kinases [13]. The Sorafenib HCC Assessment Randomized Protocol (SHARP) and Asia-Pacific studies demonstrated a significant survival benefit and good tolerance in patients with advanced HCC, making sorafenib the new reference standard for systemic therapy of patients with advanced HCC [14, 15]. In the SHARP study, approximately 90% of the patients were enrolled from Europe [14], and the Asia-Pacific study was conducted in China, Taiwan, and South Korea [15], but not Japan. The sorafenib groups in the SHARP and Asia-Pacific studies reflected the geographic patient pools, including HCV infection (29 vs. 10.7%) and HBV infection (19 vs. 70.7%) [14, 15]. In both studies, baseline disease characters differed from those of Japanese HCC patients. HCV-related HCC is most common in Japan, as mentioned above, and most of these patients have hepatitis or cirrhosis due to HCV.

In Japan, a phase I study evaluated the pharmacokinetics, safety, and preliminary efficacy of sorafenib in HCC patients [16]. Then, based on the results of the SHARP and Asia-Pacific studies, together with the phase I study in Japanese HCC, the use of sorafenib to treat HCC patients was approved by the Japanese Ministry of Health, Labour, and Welfare in May 2009 [14–16]. However, the phase I study included few patients (six Child-Pugh A patients and eight Child-Pugh B patients receiving 400 mg twice daily) [16]. Thus, little is, in fact, known about the safety and tolerance profile of sorafenib in Japanese HCC patients. In this study, we evaluated the safety and tolerance of sorafenib in Japanese HCC patients.

Materials and methods

HCC patients treated with sorafenib between May 2009 and December 2009 at eight medical centers in Japan were analyzed retrospectively. Patients were required to meet the following criteria at baseline: (1) diagnosis of HCC based on the European Association for the Study of Liver Disease/American Association for Liver Disease criteria or liver histology [8]; (2) Eastern Cooperative Oncology Group Performance Status (ECOG-PS) 0, 1, or 2; (3) classified as Child-Pugh A; (4) required to have adequate renal, hematological, and hepatic function (platelet count $\geq 50 \times 10^9$/L, hemoglobin concentration $\geq$8.5 g/L, albumin concentration $\geq$2.8 g/L, total bilirubin concentration $\leq$3.0 mg/dL, alanine aminotransferase (ALT) concentration $\leq$5 times the upper limit of normal (ULN), serum creatinine concentration $\leq$1.5 times the ULN, and prothrombin time-international normalized rate (INR) $\leq$2.3. Patients who received 400 mg sorafenib twice daily as an initial dose were selected, and treatment interruptions and dose reductions (first to 400 mg once daily, and then to 400 mg once every other day) were allowed for the toxicity study. Dose reduction and treatment discontinuation were based on the package insert and were required for drug-related toxicities. For grade 3/4 toxicities, patients received a lower dose when the toxicity improved to grade 2 or better, but therapy was discontinued if the recovery time was 30 days or longer. Dose reduction was introduced for grade 3 non-hematologic toxicities until the toxicity was grade 2 or better; patients were then treated at one dose

level lower, and therapy was discontinued if the recovery time was 30 days or longer. Treatment was discontinued for patients with drug-related grade 4 non-hematologic toxicities. However, a modified scale resulting from a phase II trial was used for skin toxicity [17].

We recorded demographics, prior therapy, plasma α-fetoprotein (AFP) level, existence of microvascular invasion, or extrahepatic spread of HCC, Barcelona Clinic Liver Cancer (BCLC) score, tumor response, survival data, and relevant toxicities.

Adverse events were recorded according to the Common Terminology Criteria for Adverse Events, version 3.0 (CTCAE v3.0). Based on contrast-enhanced computed tomography (CT) or contrast-enhanced magnetic resonance imaging (MRI), performed at baseline and 1–3 months after treatment, the tumor response was evaluated using the Response Evaluation Criteria in Solid Tumors criteria version 1.1 (RECIST v1.1). The duration of treatment and survival were estimated using the Kaplan–Meier method.

Results

Patient baseline characteristics

In total, 54 patients were included in this retrospective study. Their median age was 69 years (range 48–82), and 49 patients (91%) were males. Most had good performance status (ECOG-PS was 0 in 81% and 1 in 15% of patients). At baseline, 28 patients (52%) had HCV infection and 12 patients (22%) had HBV infection. Of the patients, 38 (70%) were classified as BCLC stage C and 28 patients (52%) had extrahepatic metastases. Before receiving sorafenib therapy, 50 patients (93%) had been treated with surgery, local ablation, or TACE (Table 1).

Table 1 Baseline demographics and disease characteristics of the enrolled patients

Number of patients	54
Sex, no. (%)	
Male	49 (91)
Female	5 (9)
Age (years)	
Median (range)	69 (48–82)
Body weight (kg)	
Median (range)	60.8 (43.6–81.3)
Body surface area ($m^{2)}$	
Median (range)	1.66 (1.32–1.93)
ECOG PS, no. (%)	
0	44 (81)
1	8 (15)
2	2 (4)
Child-Pugh score, no. (%)	
5	36 (67)
6	18 (33)
Hepatitis virus status, no. (%)	
HCV infection	28 (52)
HBV infection	12 (22)
Alcohol	8 (15)
Other	6 (11)
BCLC stage, no. (%)	
B (intermediate)	16 (30)
C (advanced)	38 (70)
Macroscopic vascular invasion, no. (%)	12 (22)
Extrahepatic spread, no. (%)	
Any	28 (52)
Lymph nodes	8 (15)
Lung	14 (26)
Bone	6 (11)
Prior treatment, no. (%)	
Any	50 (93)
Surgery	27 (50)
Local ablation	25 (46)
Transarterial chemoembolization	43 (80)
Biochemical analysis, median (range)	
Platelets/mm^3	133,500 (50,000–296,000)
Albumin (g/dL)	3.7 (2.8–4.9)
Total bilirubin (mg/dL)	0.8 (0.2–1.9)
Aspartate aminotransferase (AST) (IU/L)	51 (18–176)
Alanine aminotransferase (ALT) (IU/L)	40 (11–162)
Alpha fetoprotein (AFP) (ng/mL)	246.6 (2.8–184,100.0)

Safety and tolerability

The overall incidence of drug-related adverse events of any grade was 98% and 36 patients (68%) experienced grade 3/4 adverse events (Table 2). HFSR occurred in 39 patients (72%) and was grade 3/4 in 14 patients (26%). Rash occurred in 27 patients (50%) and was grade 3/4 in 7 patients (13%). Fatigue, diarrhea, and hypertension occurred in 22 (41%), 17 (32%), and 14 patients (26%), respectively; none of these toxicities was grade 3/4. Liver failure under treatment, defined as encephalopathy, massive ascites, or jaundice, occurred in ten patients (19%). The median average daily dose was 450 mg (range 182–800 mg). Dose reduction was required in 45 patients (83%) (Table 3). The most common adverse events leading to dose reduction were HFSR ($n = 21$, 38%), aspartate transaminase (AST)/ALT elevation ($n = 8$, 15%), rash ($n = 7$, 13%), and liver failure ($n = 4$, 7%). Treatment was discontinued in 17 patients (31%) for sorafenib intolerance (Table 4). The most frequent adverse events leading to

Table 2 Drug-related adverse events

	Any	Grade 3/4
Overall incidence	53 (98)	36 (68)
Hematological		
Hemoglobin	1 (2)	0
Leukocytes	4 (8)	0
Platelets	14 (26)	3 (6)
Dermatologic events		
Hand–foot skin reaction	39 (72)	14 (26)
Rash	27 (50)	7 (13)
Alopecia	9 (17)	
Gastrointestinal events		
Anorexia	12 (22)	4 (7)
Diarrhea	17 (32)	0
Vomiting	3 (6)	1 (2)
Fatigue	22 (41)	0
Voice changes	2 (4)	0
Hypertension	14 (26)	0
Abdominal pain not otherwise specified	5 (9)	0
Bleeding	4 (8)	2 (4)
Laboratory		
AST	30 (55)	13 (24)
ALT	28 (52)	8 (15)
Bilirubin	15 (28)	6 (11)
Amylase	15 (28)	3 (6)
Liver failure	10 (19)	

Liver failure is defined as encephalopathy, massive ascites, or jaundice

Table 3 Adverse events causing dose reduction

	Number of patients (%)
Patients requiring dose reduction	45 (83)
Hand–foot skin reaction	21 (38)
AST/ALT	8 (15)
Rash	7 (13)
Liver failure	4 (7)
Anorexia	2 (4)
Bleeding	2 (4)
Vomiting	1 (2)
Time to dose reduction	
<2 weeks	24 (44)
≥2 weeks to <4 weeks	12 (22)
≥4 weeks	9 (17)

treatment discontinuation were liver failure ($n = 4$, 7%), HFSR ($n = 4$, 6%), fatigue ($n = 3$, 6%), and abdominal pain not otherwise specified ($n = 3$, 6%). The median time to treatment failure (TTF; defined as the period from first treatment to discontinuation of sorafenib treatment, progression, or death) was 2 months (Fig. 1).

Table 4 Adverse events leading to treatment discontinuation

	Number of patients (%)
Any adverse events	17 (31)
Liver failure	4 (7)
Hand–foot skin reaction	3 (6)
Fatigue	3 (6)
Abdominal pain not otherwise specified	3 (6)
Anorexia	2 (4)
Rash	2 (4)

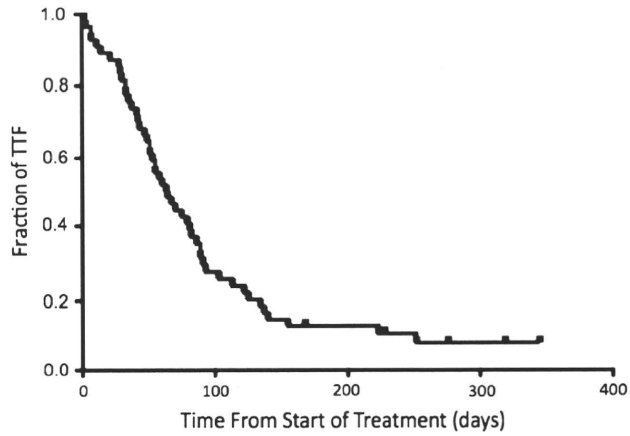

Fig. 1 Kaplan–Meier analysis of time to treatment failure (TTF). The median TTF was 2 months

Efficacy

According to RECIST version 1.1, one patient (2%) had a partial response, 25 patients had stable disease (57%), and the disease control rate (DCR; defined as no disease progression for ≥4 weeks) was 34% (Table 5).

At the time of analysis, with a median follow-up of 5.7 months (range 0.5–13.3), 49 patients had discontinued treatment (92%) and 28 patients were dead (52%). The overall median survival was 6.9 months (Fig. 2)

Discussion

The SHARP and Asia-Pacific studies, large, multicentre, phase III, randomized, double-blind, placebo-controlled trials of sorafenib, revealed a survival benefit and the tolerability of sorafenib in advanced HCC patients. However, considering the varying etiologies and treatment strategies for HCC in different regions [4], it is unclear whether these results apply to Japanese HCC patients. In Japan, high-risk groups for HCC, such as cirrhosis or hepatitis patients, undergo ultrasonography every 3–4 months and CT or MRI every 6–12 months for the early detection of HCC. Because we find HCC when it is earlier, Japanese HCC

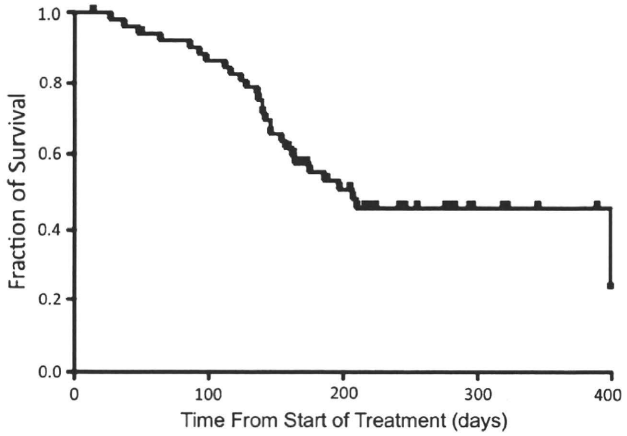

Fig. 2 Kaplan-Meier analysis of overall survival (OS). The median OS was 6.9 months

Table 5 Response rates using the response evaluation criteria in solid tumors

Response ($n = 44$)	Number of patients (%)
Complete response	0
Partial response	1 (2)
Stable disease	25 (57)
Progressive disease	18 (41)
DCR	15 (34)

DCR is the disease control rate, defined as the proportion of patients who had a best response rating of a complete response, partial response, or stable disease that was maintained for ≥4 weeks from the first manifestation of the rating

patients are often able to undergo surgery, local ablation, and TACE. Despite the efficacy of these procedures, patients frequently develop recurrence or disease progression after these treatments. In contrast, in much of the rest of Asia, the majority of patients are present with advanced disease, with large tumors, multiple tumors, and portal tumor thrombosis. These patients are less likely to receive curative treatment [18]. Furthermore, the liver function of HBV-related HCC patients tends to be better than that of HCV-related HCC patients. Shiratori et al. [2] reported that 38.6, 39.3, and 22.1% of cases presented as Child-Pugh A, B, and C when the severity of cirrhosis was classified in Japanese HCV-related HCC patients. By contrast, among the HBV-related HCC patients, 65.2, 26.1, and 8.7% cases presented as Child-Pugh A, B, and C. Additionally, liver function might worsen with the repetition of local therapies because sorafenib was only given to Child-Pugh A patients. Fewer HCV-related HCC patients (52%) were included in the present analysis compared with the general HCC prevalence in Japan (71–75%) [2, 6].

In the SHARP study, common drug-related adverse events were diarrhea (39%), fatigue (22%), HFSR (21%), rash (16%), alopecia (14%), anorexia (14%), and nausea (11%) [14]. Dose reduction due to adverse events was needed in 26% of subjects. The most common adverse events leading to dose reduction were diarrhea (8%), HFSR (5%), and rash (3%) [14]. Treatment was discontinued because of adverse events in 38%. The most frequent adverse events leading to sorafenib discontinuation were gastrointestinal events (6%), fatigue (5%), and liver dysfunction (5%) [14]. In comparison, in the Asia-Pacific study, the common drug-related adverse events were HFSR (45.0%), diarrhea (25.5%), alopecia (24.8%), fatigue (20.1%), rash (18.8%), hypertension (18.8%), and anorexia (12.8%) [15]. Dose reduction due to adverse events was needed in 30.9%, and treatment was discontinued due to adverse events in 19.5% [15]. The most common drug-related adverse events resulting in dose reduction were HFSR (11.4%) and diarrhea (7.4%) [15]. Compared with these studies, we observed a higher incidence of adverse events, especially HFSR, rash, hypertension, and liver failure.

The incidence of HFSR and rash in the Asia-Pacific study was higher than in the SHARP study [14, 15]. In a phase I study of a small population of Japanese patients with HCC, five of the six patients experienced HFSR and four experienced rash; these patients were Child-Pugh A receiving 400 mg twice daily [16]. In a phase II study of Japanese patients with advanced renal cell carcinoma [19], HFSR occurred in 55% and rash occurred in 37.4%. Asian patients, particularly Japanese, frequently develop HFSR. Although it is possible that the physiological difference is partly associated with race, prevention and management of HFSR are required in Japanese patients.

Regarding hypertension, Wu et al. [20] reported a 23.4% (95% CI 16.0–32.9%) overall incidence from a systemic review and meta-analysis of nine studies of renal cell cancer or other solid tumor. Hypertension was experienced by 14 patients (26%) in our study; no case was grade 3/4. Varying rates of hypertension have been reported, with a 5% incidence in the SHARP study and an 18.8% incidence in the Asia-Pacific study. In our study, the incidence of hypertension was comparable with that reported by Wu et al., although it was slightly higher compared with that reported in the SHARP and Asia-Pacific studies.

Liver failure occurred in ten patients (19%), while it was uncommon in the SHARP and Asia-Pacific studies. Nevertheless, Ozenne et al. [21] reported that seven (21%) French patients with Child-Pugh A experienced liver failure. The SHARP and Asia-Pacific studies showed the efficacy of sorafenib in carefully selected patients with advanced HCC. Liver failure may occur with the use of sorafenib in an unselected cirrhotic population. In our study, the median time to experience liver failure was 33 days (range 7–115); liver failure can happen in the

early days of treatment. Furthermore, a common adverse event leading to treatment discontinuation was liver failure (7%).

In our study, 43 patients required dose reduction due to adverse events (83%). This was more frequent than in either the SHARP or Asia-Pacific studies. The most common adverse event leading to dose reduction was HFSR (43%) [12, 13]. Our patients suffered more HFSR than those in the SHARP and Asia-Pacific studies [12, 13]. The cause may be differences, such as age or race. Nevertheless, treatment discontinuation due to HFSR was required in only 6% of the patients; in the majority of the patients, it could be controlled by dose reduction. This concurred with the finding that two of seven patients with Child-Pugh A experienced HFSR when they took 400 mg daily in the Japanese phase I study [16].

In our series, 44% of the patients required dose reduction within 2 weeks and the median daily dose was 450 mg (range 182–800), demonstrating that it is difficult for Japanese patients to continue sorafenib treatment at 400 mg twice daily. Treatment was discontinued because of adverse events in 31% of our patients, which was similar to the rate in the SHARP study, but higher than in the Asia-Pacific study. Adverse events could be managed by dose reduction in the majority of patients. Therefore, careful follow-up is recommended.

The median overall survival was 10.7 months in the SHARP trial and 6.5 months in the Asia-Pacific trial. The differences in survival time might have been caused by differences in patient background. Patients in the Asia-Pacific study displayed more extrahepatic spread, more hepatic tumors, a worse ECOG-PS, and increased concentrations of AFP compared with patients in the SHARP study [14, 15]. The median survival time was 9.2 months in a phase II study [17] and 15.6 months in a Japanese phase I study [16], although Child-Pugh B patients were included in both of these studies. More recently, two retrospective studies from Europe showed that the median survival times for Child-Pugh A patients were 8.9 [21] and 8.3 months [22]. The median overall survival in our series was 6.9 months, although the survival benefits cannot be directly compared, as this was a retrospective study. Our study included many patients with higher serum AFP levels, suggesting the inclusion of highly advanced cases in the present study.

In summary, the present study demonstrated that sorafenib was generally tolerated in Japanese HCC patients because the probability of treatment discontinuation due to adverse events was acceptable, although most patients needed dose reduction. The overall safety profile of sorafenib was similar to that seen in previous studies in patients with HCC, except for the higher rates of HFSR, rash, and liver failure.

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