Int. J. Radiation Oncology Biol. Phys., Vol. ■, No. ■, pp. 1–8, 2010

0360-3016/$–see front matter

ELSEVIER

doi:10.1016/j.ijrobp.2009.12.048

CLINICAL INVESTIGATION

DOSE–VOLUME HISTOGRAM ANALYSIS OF THE SAFETY OF PROTON BEAM THERAPY FOR UNRESECTABLE HEPATOCELLULAR CARCINOMA

MITSUHIKO KAWASHIMA, M.D.,*† RYOSUKE KOHNO, PH.D.,* KOHEI NAKACHI, M.D.,‡ TEIJI NISHIO, PH.D.,* SHUICHI MITSUNAGA, M.D.,‡ MASAFUMI IKEDA, M.D.,‡ MASARU KONISHI, M.D.,§ SHINICHIRO TAKAHASHI, M.D.,§ NAOTO GOTOHDA, M.D.,§ SATOKO ARAHIRA, M.D.,† SADAMOTO ZENDA, M.D.,* TAKASHI OGINO, M.D.,* AND TAIRA KINOSHITA, M.D.§

Divisions of *Particle Therapy and Radiation Oncology, Research Center for Innovative Oncology, †Radiation Oncology, ‡Hepatobiliary and Pancreatic Medical Oncology, and §Hepatobiliary and Pancreatic Surgery, National Cancer Center Hospital East, Kashiwa, Japan

Purpose: To evaluate the safety and efficacy of radiotherapy using proton beam (PRT) for unresectable hepatocellular carcinoma.

Methods and Materials: Sixty consecutive patients who underwent PRT between May 1999 and July 2007 were analyzed. There were 42 males and 18 females, with a median age of 70 years (48–92 years). All but 1 patient had a single lesion with a median diameter of 45 mm (20–100 mm). Total PRT dose/fractionation was 76–cobalt Gray equivalent (CGE)/20 fractions in 46 patients, 65 CGE/26 fractions in 11 patients, and 60 CGE/10 fractions in 3 patients. The risk of developing proton-induced hepatic insufficiency (PHI) was estimated using dose-volume histograms and an indocyanine-green retention rate at 15 minutes (ICG R15).

Results: None of the 20 patients with ICG R15 of less than 20% developed PHI, whereas 6 of 8 patients with ICG R15 values of 50% or higher developed PHI. Among 32 patients whose ICG R15 ranged from 20% to 49.9%, PHI was observed only in patients who had received 30 CGE (V30) to more than 25% of the noncancerous parts of the liver (n = 5 Local progression-free and overall survival rates at 3 years were 90% (95% confidence interval [CI], 80–99%) and 56% (95% CI, 43–69%), respectively. A gastrointestinal toxicity of Grade ≥2 was observed in 3 patients.

Conclusions: ICG R15 and V30 are recommended as useful predictors for the risk of developing PHI, which should be incorporated into multidisciplinary treatment plans for patients with this disease. © 2010 Elsevier Inc.

Hepatocellular carcinoma, Proton beam radiotherapy, Dose–volume histogram, Radiation tolerance of the liver.

INTRODUCTION

Recent improvements in diagnostic imaging and radiotherapy (RT) techniques have made high-dose radiotherapy a safe and effective treatment for selected patients with unresectable hepatocellular carcinoma (HCC) (1). Charged-particle radiotherapy can potentially deliver considerably larger doses of RT to liver tumors, with greater sparing of normal tissues, and proton beam radiotherapy (PRT) for HCC using aggressively high total and fractional RT doses has been investigated during the last 2 decades. The results have shown local control rates ranging from 75% to 96% and overall survival (OAS) rates exceeding 50% at 2 years in groups of patients that include those who had HCC tumors of ≥5 cm in diameter (2–4). HCC has a high propensity for venous invasion, which is frequently associated with multiple tumors within resected specimens (5–9). In this context, the extent of resection was determined while considering potential tumor spread via portal blood flow and the necessity of preserving a functional liver reserve (5, 7, 10). Even in preselected patients who underwent hepatectomy, more than 50% of tumors with diameters greater than 4 cm demonstrated microscopic vascular invasion (8, 11). Consequently, it will become more crucial to consider the influence of vascular invasion on undetectable tumor dissemination at the periphery of the gross tumor in RT for unresectable HCC.

Given the high probability of obtaining local control by using PRT, an appropriate definition of the clinical target volume (CTV) according to patterns of tumor spread and patients' functional liver reserves is extremely important in order to maximize the therapeutic ratio. Ideally, the entire portal segment that contains HCC nodules should be covered within the CTV when the tumor shows macro- or microscopic vascular invasion. This requires a considerably larger

Reprint requests to: Mitsuhiko Kawashima, 6-5-1, Kashiwanoha, Kashiwa, Chiba, Japan, 277-8577. Tel: 81-4-7133-1111; Fax: 81-4-7171-4724; E-mail: mkawashi@east.ncc.go.jp

Conflict of interest: none.
Received Nov 24, 2009, and in revised form Dec 21, 2009. Accepted for publication Dec 21, 2009.

irradiated volume even with PRT, partly because of unavoidable uncertainty in treatment planning without using intraoperative ultrasonography (7). Another possible way to eradicate satellite HCC nodules, which are disseminated via portal blood flow, is transarterial chemoembolization (TACE). Currently, the standard treatment for patients with unresectable HCC that is not amenable to local ablation therapy is TACE instead of best supportive care (12). The OAS rate at 3 years after TACE ranges from 32% to 47% in patients with stage III cancer and with liver damage A to B, according to the staging system used in a nationwide cohort study conducted by the Liver Cancer Study Group of Japan (13). Considering that the tumoricidal effect of TACE in HCC with vascular invasion is frequently incomplete (13), a significant benefit of adding PRT to TACE would be expected. However, presently, there has been no robust evidence supporting this concept. Before we examine the validity of targeting the entire anatomical portal segment containing HCC in a multidisciplinary approach that includes PRT, practical methods to estimate the safety of PRT according to the dose–volume histogram (DVH) should be established in patients who have various levels of severity of liver dysfunction. Findings from our previous study consisting of 30 patients suggested that the risk of proton-induced hepatic insufficiency (PHI) could be predicted by the indocyanine green clearance test and the retention rate at 15 minutes (ICG R15) in combination with DVH parameters (14) such as percentages of hepatic noncancerous portions receiving doses of >30 cobalt-Gray-equivalent (CGE) (3). We have subsequently accumulated data from additional patients in clinical practice. The clinical results were evaluated, and we have again used the DVH analysis to examine the relationship between probability of PHI and dose–volume parameters.

METHODS AND MATERIALS

Patients

Patient eligibility was reported previously (3); in brief, they were required to have uni- or bidimensional measurable HCC nodules of ≤10 cm in maximum diameter on computed tomography (CT) and/or magnetic resonance imaging (MRI) without evidence of extrahepatic tumor spread. All patients had a white blood cell count of ≥2,000/mm^3; a hemoglobin level of ≥7.5 g/dl; a platelet count of ≥25,000/ mm^3; and adequate hepatic function (total bilirubin, ≤3.0 mg/dl; alkaline phosphatase, aspartate aminotransferase, and alanine aminotransferase of <5.0× normal; no ascites). Patients who had multicentric HCC nodules were not considered as candidates for PRT, except for those who fulfilled the following two conditions: (*1*) multiple nodules could be encompassed within a single clinical target volume; and (*2*) lesions other than those of the targeted tumor were judged to be controlled with prior surgery and/or local ablation therapy. This retrospective study was approved by the institutional ethics committee, and written informed consent was obtained from all patients.

Treatment Planning

ICG R15 was measured in all patients to quantitatively assess the hepatic functional reserve. Serological testing for hepatitis B surface antigen and anti-hepatitis C antibody was done. All patients were judged to be unresectable by expert hepatobiliary surgeons at our institution, based on the patient's serum bilirubin level, ICG R15, and expected volume of resected liver (10). Percutaneous fine-needle biopsies were performed for all patients unless they had radiologically compatible, postsurgical recurrent HCC (3).

Treatment methods were published previously (3). In brief, gross tumor volume (GTV) was defined using a treatment-planning CT scan, and CTV and planning target volume (PTV) were defined as follows in all but 2 patients: CTV = GTV + 5 mm, and PTV = CTV + 3 mm of lateral, craniocaudal, and anteroposterior margins. CTV encompassed the entire volume of the right lobe in 1 patient who had a tumor of 4 cm in diameter that broadly attached to the bifurcation of the right anterior and posterior portal veins. In this patient, right portal vein embolization was done to facilitate compensatory hypertrophy of the left lobe for expected surgery. However, the patient was finally judged to be unresectable, and PRT was selected. Another patient was treated with a CTV encompassing the entire right anterior portal segment because a tumor of 2 cm in diameter had invaded the bifurcation of the right anterosuperior and anteroinferior portal vein associating with daughter HCC at the right anterosuperior portal segment. The beam energy and spread-out Bragg peak (15) were fine-tuned so that a 90% isodose volume of the prescribed dose encompassed the PTV.

Forty-six patients received PRT to a total dose of 76 CGE in 3.8 CGE once-daily fractions, four to five fractions in a week. Another 3 patients underwent 60 CGE /10 fractions/2 weeks, depending on availability of the proton beam. Eleven patients whose PTV encompassed the gastrointestinal wall received 65 CGE in 2.5 CGE /fraction, five fractions per week. All patients were treated using a 150- to 190-MV proton beam. The relative biological effectiveness of our proton beam was defined as 1.1 (16). No concomitant treatment such as TACE, local ablation, or systemic therapy was allowed during or after the PRT, unless a treatment failure was detected. Both scanning of CT images for treatment planning and irradiation by the proton beam were done during the exhalation phase using the respiration-gated irradiation system and intrahepatic fiducial markers as previously reported (3).

Outcomes

Death from any cause was defined as an event in calculation of OAS, whereas tumor recurrences at any site or patient deaths were defined as events in disease-free survival (DFS). An increase of the tumor diameter within the PTV was defined as local progression, and patients who died without evidence of local progression were censored at the time of last radiographic examination. Adverse events were reviewed weekly during the PRT regimen by means of physical examination, complete blood count, liver function tests, and other biochemical profiles as indicated. The severity of adverse events was assessed using the National Cancer Institute common terminology criteria for adverse events, version 3.0. After completion of PRT, reviews that monitored disease status, including CT and/or MRI examinations and long-term toxicity, were done at a minimum frequency of every 3 months in all 60 patients. The percentages of hepatic noncancerous portions (entire liver volume minus gross tumor volume) receiving CGE doses of >0 (V0), ≥10 (V10), ≥20 (V20), ≥30 (V30), ≥40 (V40), and ≥50 (V50) were calculated using PRT planning software (PT-PLAN/NDOSE System, Sumitomo Heavy Industries Ltd., Tokyo, Japan), and their influence on the outcomes were analyzed (3). Time-to-event analyses were done using Kaplan-Meier estimates from the start of PRT. The differences between time-to-event curves were evaluated with the log-rank test. Multivariate analyses were performed with Cox's proportional hazards model.

RESULTS

Patients

A total of 60 patients with HCC underwent PRT in our institution between May 1999 and July 2007. Approximately 1400 patients with HCC were newly presented to our institution during this study period and about 35%, 30%, 25%, and the remainder primarily treated with hepatectomy, TACE, percutaneous local ablation, and other treatments, respectively. Therefore 60 patients in this study corresponded to approximately 4% of overall, or 7% of patients with unresectable HCC. Patient characteristics at the start of PRT are listed in Table 1. All patients had underlying chronic liver disease. One patient had a history of schistosomiasis, and another patient had autoimmune hepatitis as the cause of liver cirrhosis. Five additional patients were diagnosed with liver cirrhosis caused by non-B, non-C hepatitis. A total of 24 patients received PRT as the first treatment for their HCC. Ten patients had postsurgical recurrences, 22 patients received unsuccessful local ablation and/or TACE to the targeted tumor, and 4 patients underwent successful local ablation to a tumor other than the target prior to PRT. Histological confirmation was not obtained in 1 patient who had a tumor with typical radiographic features compatible with HCC (3). Six patients had HCC nodules of ≤3 cm in diameter; however, they were not considered candidates for local ablation therapy because of the tumor locations, which were in close proximity to the great vessels or the lung.

Table 1. Characteristics of patients

Characteristics	No. of patients (%)
Age (years)	
Median	70
Range	48–92
Gender	
Male	42 (70)
Female	18 (30)
ECOG performance status	
0–1	57 (95)
2	3 (5)
Viral markers	
Hepatitis B surface antigen-positive	3 (5)
Hepatitis C antibody-positive	49 (82)
Both positive	1 (2)
Both negative	7 (12)
Child-Pugh classification	
A	47 (78)
B	13 (22)
C	0
% patients with pretreatment ICG R15 values	
<20	20 (20)
20–40	25 (55)
40–50	7 (12)
≥50	8 (13)
Tumor size (mm)	
Median	45
Range	20–90
20–50	42 (70)
>50	18 (30)
Macroscopic vascular invasion	
Yes	42 (70)
No	18 (30)
Morphology of primary tumor	
Single nodular	45 (75)
Multinodular, aggregating	9 (15)
Diffuse	5 (8)
Portal vein tumor thrombosis	1 (2)
Serum alpha-fetoprotein level (IU/mL)	
<300	41 (68)
≥300	19 (32)
Histology	
Well-differentiated	15 (25)
Moderately-differentiated	28 (47)
Poorly-differentiated	7 (12)
Differentiation not specified	9 (15)
Negative (radiological diagnosis only)	1 (2)
Prior treatment	
None	24 (40)
Surgery	10 (17)
Local ablation/TACE	26 (43)

Adverse events during PRT

All patients completed the treatment plan. Prolongation of the overall treatment time for more than 1 week occurred in 4 patients: treatment of 3 patients was extended due to availability of the proton beam machine, and 1 patient's treatment was extended because of fever associated with grade 3 elevation of total bilirubin that spontaneously resolved within a week. A total of 14 patients experienced transient grade 3 leukopenia and/or thrombocytopenia without infection or bleeding that necessitated treatment. In addition, 8 patients experiencing grade 3 elevation of transaminases without clinical manifestation of hepatic insufficiency maintained good performance status. PRT was not discontinued for these patients; nevertheless, these events spontaneously resolved within 1 to 2 weeks.

Estimation of the risk of PHI by DVH analysis

Development of hepatic insufficiency presented with anicteric ascites and/or asterixis within 6 months after completion of PRT in the absence of disease progression was defined as PHI. Eleven patients, all of whom received a total PRT dose of 76 CGE, developed PHI at 1 to 6 months (median, 2 months) after completion of PRT without elevation of serum bilirubin and transaminases of more than threefold above normal levels. DVHs for hepatic noncancerous portions were drawn according to pretreatment ICG R15 values (Fig. 1A–C). Results showed that all 20 patients with ICG R15 of <20% were free of PHI, regardless of the DVH, for 2 to 94 months (median, 44 months). On the other hand, 6 of 8 patients with pretreatment ICG R15 values of ≥50% died of PHI with (n = 3) or without (n = 3) evidence of HCC recurrence at 2 to 15 months (median, 8 months). There was no obvious relationship between DVH and development of PHI in these 8 patients, as shown in Fig. 1C.

Among 32 patients whose ICG R15 values ranged from 20% to 49.9%, 5 patients developed PHI. The V0 to V50 in these 32 patients are shown in Fig. 2. Differences in distributions of these DVH parameters between patients who did

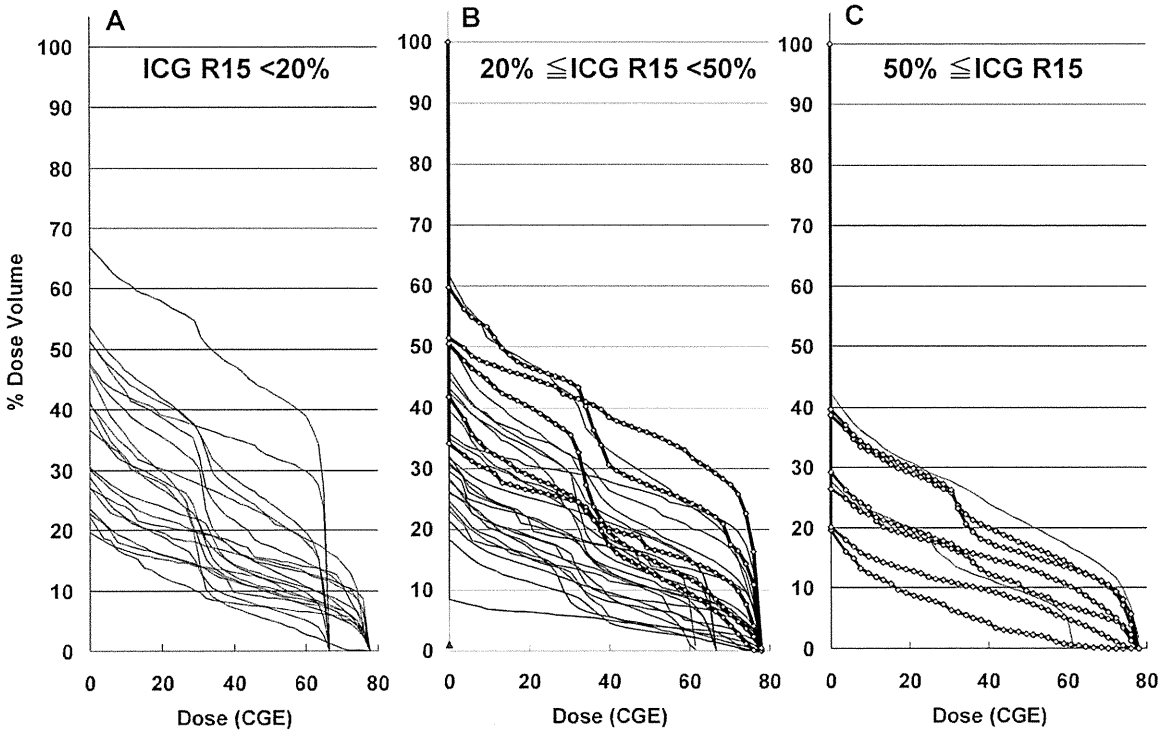

Fig. 1. DVH are shown for all patients according to their pretreatment ICG R15 values, as noted in each panel. Thick lines with rhomboid symbols represent DVHs for patients suffering from hepatic insufficiency within 6 months after completion of PRT.

and did not develop PHI were statistically significant, with p values of 0.012 in V0, 0.009 in V10, 0.012 in V20, 0.006 in V30, 0.016 in V40, and 0.024 in V50 (Mann-Whitney U test). The lowest p value was observed in the difference at V30. Among 32 patients whose ICG R15 values ranged from 20% to 49.9%, none of the 21 patients whose V30 were <25% experienced PHI, whereas 5 of 11 patients (45%) whose V30 was ≥25% developed PHI (p = 0.037, Mann-Whitney U test). The incidence of PHI was 2/25 (8%) in Child-Pugh class A patients, whereas PHI incidence was 3/7 (43%) in class B patients in this group of 32 patients (p = 0.218, Mann-Whitney U test). Of 5 patients who experienced PHI, 1 died at 8 months without evidence of HCC recurrence. PHI spontaneously resolved in 4 patients; 2 patients died of intrahepatic recurrence at 22 and 71 months, respectively; 1 patient died of brain metastasis at 8 months; and 1 patient was alive and disease free at 50 months. In both of the patients who survived for more than 4 years despite development of PHI, the pretreatment functional liver reserve was Child-Pugh class A and ICG R15 was less than 40%. On the other hand, all 3 patients who experienced PHI and died within 2 years had Child-Pugh class B liver functions. Relationships between ICG R15 and V30 according to occurrence of PHI in Child-Pugh class A and B patients are shown in Fig. 3a and b, respectively.

Other serious adverse events

Three patients experienced a gastrointestinal toxicity grade of ≥2. One patient developed hemorrhagic duodenitis associated with anemia at 2 months after completion of 76 CGE/20 fractions/30 days of PRT. The dose administered to the duodenum was estimated to be 50 to 80% of the prescribed dose. Bypass surgery was attempted to alleviate the symptoms; however, this patient died of postoperative hepatic failure at 6 months. Two patients received 65 CGE/26 fractions of PRT, with the entire circumference of the gastrointestinal walls covered within the PTV. One of these 2 patients experienced grade 3 hemorrhagic ulcer at the ascending colon, within the PTV. The patient was managed successfully with right hemicolectomy at 10 months; however, the patient

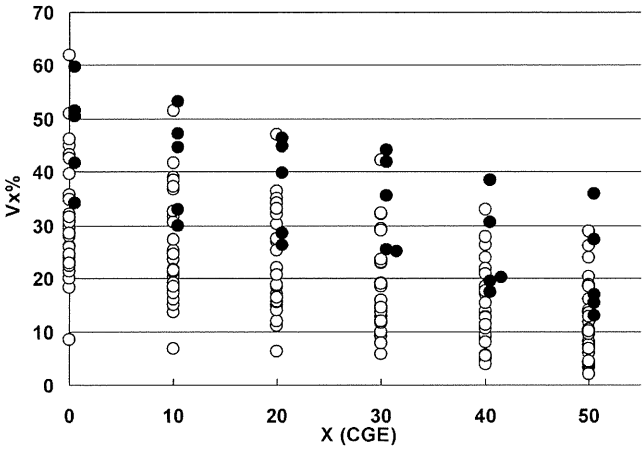

Fig. 2. Distribution of V0 to V50 in DVHs for 32 patients whose pretreatment ICG R15 values ranged from 20% to 49.9%. Open circles represent values for patients who did not experience PHI, whereas closed circles represent those who developed PHI.

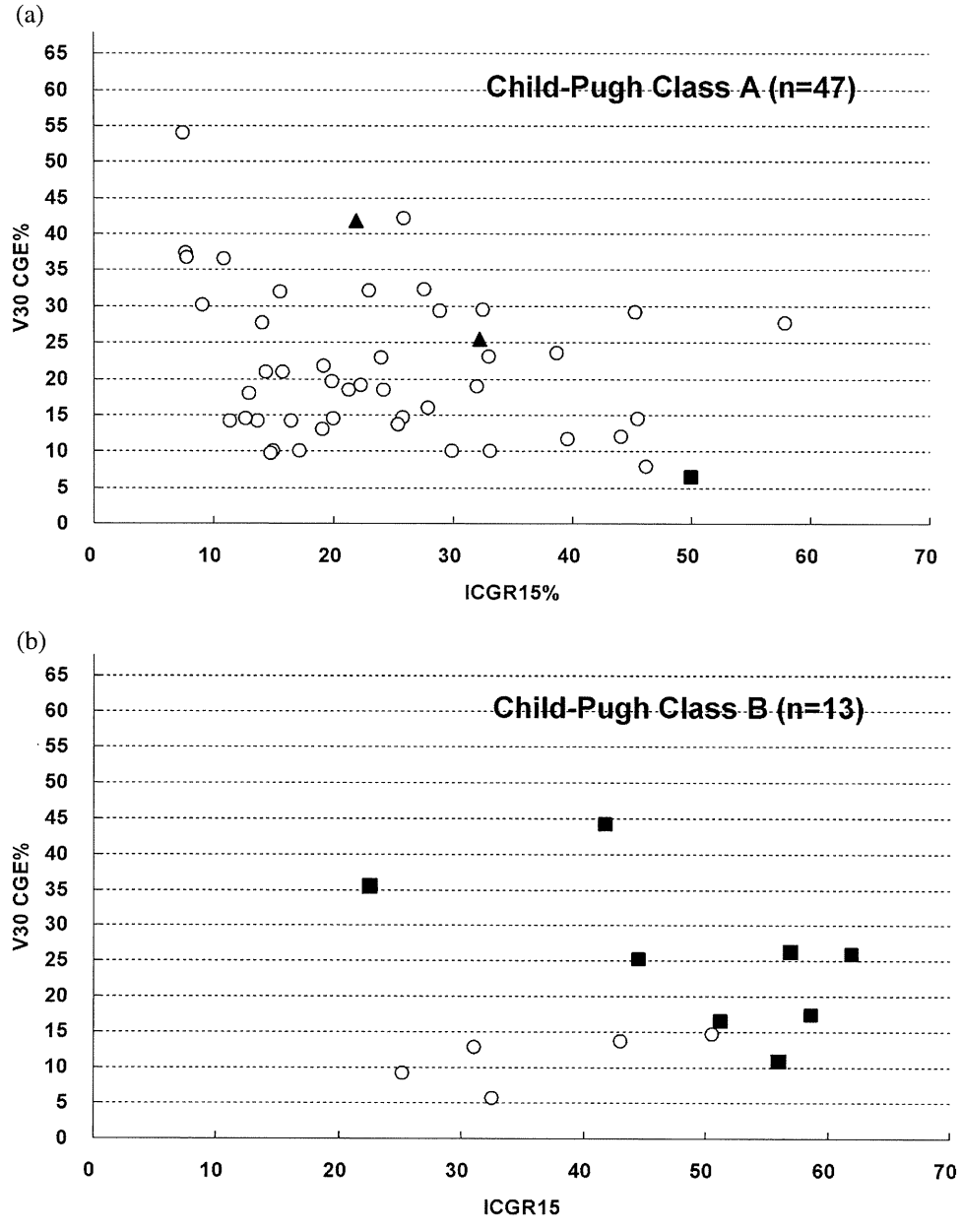

Fig. 3. Scattergram of V30 in each patient who had pretreatment liver functions classified as Child-Pugh class A (a) and class B (b), as shown in each panel, according to the ICG R15 value. Open circles represent values in patients who did not experience PHI. Closed squares represent those who developed PHI and died within 2 years with ($n = 5$) or without ($n = 4$) disease recurrence. Closed triangles represent those who experienced transient PHI and survived for more than 4 years after commencement of PRT.

died of local recurrence and subsequent hepatic failure at 23 months. The other patient developed grade 2 esophagitis within the PTV at 7 months. Repetitive balloon dilatations were required to alleviate the patient's dysphagia; however, the patient was alive without disease and taking a normal diet at 30 months. There were no other observations made of adverse events of Grade ≥3 in any of the patients.

Tumor control and survival

At the time of analysis in August 2009, 42 patients had already died because of intrahepatic recurrence in 27 nodal recurrence in 1 distant metastasis in 3 hepatic insufficiency without recurrence in 9 comorbidity in 1 and senility in 1. Forty of these 42 patients had been free from local progression until death; the durations ranged from 2 to 77 months (median, 20 months). Two patients who experienced local progression died subsequently. A total of 15 patients were alive at 25 to 92 months (median, 43 months) without local progression. Three patients were alive at 49, 53, and 94 months, respectively, after salvage treatment for local progression, using local ablation in 2 and TACE in 1 A total of 37 patients achieved complete disappearance of the primary tumor at 1 to 50 months (median, 10 months) post-PRT. Eighteen patients had residual tumor masses on CT

and/or MRI for 2 to 44 months (median, 21 months) until the time of death or last follow-up visit without local progression. The local progression-free (LPF) rates at 3 and 5 years were 90% (95% confidence interval [CI], 80%–99%) and 86% (95% CI, 74%–98%), respectively.

Of 5 patients who experienced local progression, 3 patients underwent 65 CGE/26 fractions, and 2 patients received 76 CGE/20 fractions of PRT. All 3 patients who received 60 CGE/10 fractions were free from local progression at 6, 30, and 51 months, respectively. LPF rates at 3 and 5 years for 46 patients who received 76 CGE/20 fractions were 97% (95% CI, 92%–100%) and 93% (95% CI, 83%–100%), respectively. LPF rates at 3 years for 11 patients who underwent 65 CGE/26 fractions of PRT were 56% (95% CI, 16%–95%) and was worse than that in patients who received 76 CGE/20 fractions with statistical significance ($p = 0.005$).

A total of 32 patients developed intrahepatic tumor recurrences that were outside of the PTV at 1 to 62 months (median, 20 months). Nine of these tumors occurred within the same segment of the primary tumor. Nodal recurrence at the hepatoduodenal ligament and distant metastasis were observed as the first sites of failure in 2 and 3 patients, respectively. In addition to the above-mentioned five deaths from PHI or postsurgical mortality, 4 patients died of hepatic failure because of underlying liver disease at 17 to 23 months, and 2 patients died from other reasons (comorbidity or senility) without evidence of HCC recurrence. Seven patients remained alive and disease free at 27 to 51 months (median, 30 months). The median survival time for all 60 patients was 41 months, and actuarial OAS rates at 3 and 5 years were 56% (95% CI, 43%–69%) and 25% (12%–39%), respectively. DFS rates at 3 and 5 years were 18% (95% CI, 7%–29%) and 4% (95% CI, 0%–12%), respectively, as shown in Fig. 4. Two Child-Pugh class A patients who underwent PRT with the CTV covering the entire right lobe or right anterior portal segment were alive and disease free at 50 and 26 months, respectively. The former patient had a pre-PRT ICG R15 of 22% and received a V30 of 42% and experienced transient PHI that resolved spontaneously; the latter patient, whose corresponding parameters were 8% and 37%, respectively, did not experience PHI.

Factor analysis

Univariate analyses revealed that factors related to functional liver reserve and occurrence of PHI had significant influence on OAS ($p < 0.05$). Liver function (Child-Pugh class A or B) and prior treatment (none or recurrent) were independent and significant prognostic factors ($p < 0.002$), and occurrence of PHI had marginal significance ($p = 0.011$) by multivariate analysis, as shown in Table 2. The DFS rate at 3 years for 24 patients who had no prior treatment for HCC was 35% (95% CI, 14%–56%), whereas DFS for the remaining 36 patients was 7% (95% CI, 0%–17%) ($p = 0.011$). In Child-Pugh class A patients, OAS at 3 and 5 years for those who had no prior treatment ($n = 17$) was 76% (95% CI, 56%–97%) and 59% (95% CI, 33%–86%), respectively, and 63% (95% CI, 45%–80%) and 25% (95% CI, 7%–42%), respectively, for 30 patients with recurrent tumor ($p = 0.060$). In Child-Pugh class B patients, the 2-year OAS for patients without PHI ($n = 5$) was 80% (95% CI, 45%–100%), while 8 patients who developed PHI died within 2 years with ($n = 5$) or without ($n = 3$) HCC recurrence ($p = 0.009$).

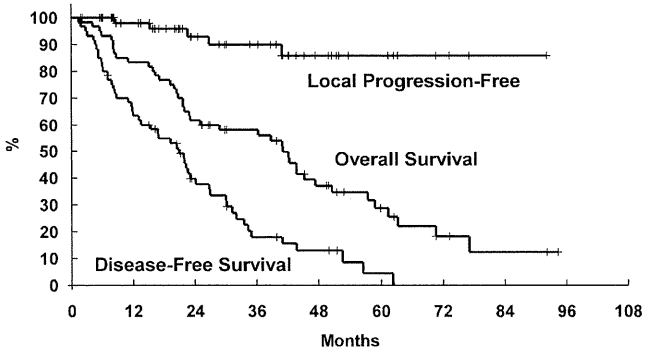

Fig. 4. Kaplan-Meier estimation of local progression-free survival, OAS, and disease-free survival rates for all 60 patients.

DISCUSSION

The promising tumoricidal effect of PRT using aggressive escalation of total and fractional doses, which has been repeatedly reported previously, was reproduced in this study (3, 4). The estimated actuarial local progression-free rate within the PTV in patients receiving 76 CGE/20 fractions exceeded 90% at 3 years. DFS at 3 years for patients who underwent PRT as an initial treatment ($n = 24$) was 35%, and, among them, OAS at 3 years was 76% in Child-Pugh class A patients ($n = 17$). These results are comparable to those observed after surgical treatment (17). Although the number of patients was small, these data indicate that appropriate local control with PRT may provide survival benefit in adequately selected patients with unresectable HCC. The fact that 9 of the 32 intrahepatic HCC recurrences occurred within the same anatomical portal segments showed that it should still be possible to improve the progression-free rate by defining the CTV so it covers undetectable tumor spread via the portal blood flow.

As shown in Fig. 3, no patient who had ICG R15 of less than 20% experienced PHI. In addition, only Child-Pugh class A patients with pre-PRT ICG R15 of less than 40% survived for longer than 4 years despite development of PHI. One of them underwent systematic portal segmental irradiation with the CTV covering the entire right lobe, and the details for this patient will be reported separately. On the other hand, all patients who had pre-PRT liver functions classified as Child-Pugh class B and/or ICG R15 of 40% or higher died within 2 years when they developed PHI. This suggests that the role of systematic portal irradiation requiring a large irradiated volume should be pursued further in Child-Pugh class A patients with favorable ICG R15 values; otherwise, the CTV should be confined to the GTV with adequate margins. Furthermore, in patients who have ICG R15 of 50% or

Table 2. Factors related to overall survival

Factor	No. of patients	% of OAS at 3 years (MST, months)	Univariate *p* value	Multivariate *p* value, hazard ratio (95% CI)
Age				
<70	29	55 (41)	0.660	0.087
≥70	31	61 (42)		0.52 (0.24–1.10)
Gender				
Male	42	62 (41)	0.332	0.194
Female	18	44 (42)		0.62 (0.29–1.30)
Tumor size (mm)				
<50	36	66 (44)	0.178	0.070
≥50	24	46 (23)		0.54 (0.28–1.05)
Pretreatment ICG R15				
<40%	45	67 (44)	0.002	
≥40%	15	33 (15)		
Child-Pugh classification				
A	47	68 (45)	<0.001	<0.001
B	13	23 (15)		0.19 (0.07–0.50)
Serum alfa-fetoprotein level (IU/mL)				
<300	41	61 (42)	0.617	0.618
≥300	19	53 (39)		0.83 (0.39–1.74)
PHI				
No	49	65 (44)	0.001	0.011
Yes	11	18 (9)		0.29 (0.11–0.76)
% of patients receiving V30				
<25%	40	57	0.724	
≥25%	20	60		
Total dose = 65 Gy				
Yes	11	44 (29)	0.646	0.185
No	49	61 (42)		1.88 (0.73–4.76)
Prior treatment				
None	24	67 (47)	0.112	0.002
Recurrence	36	53 (36)		0.32 (0.15–0.66)

Abbreviations: OAS = overall survival; MST = median survival time; CI = confidence interval; PHI = proton-induced hepatic insufficiency.

higher, the indication for PRT should be considered with extreme caution to prevent life-threatening PHI, as shown in Fig. 3.

Results of this retrospective study showed 56% OAS at 3 years in all patients and 68% in 47 Child-Pugh class A patients. All of them were judged strictly as unresectable and not amenable to local ablation. Therefore, a survival benefit of adding PRT to TACE could be expected, which should be tested in randomized trials. Suitable candidates for such a study may be patients who have unresectable HCC of >4 cm in diameter (*i.e.*, a high probability of microscopic vascular invasion) or who show macroscopic vascular invasion, which is amenable to selective segmental TACE as a curative treatment. Nevertheless, before developing that kind of randomized study, data should still be compiled regarding the safety and patterns of failure after PRT combined with TACE while ICG R15 and V30 are taken into account. Preliminary results of hypofractionated stereotactic body radiotherapy for patients with relatively small primary or metastatic liver tumors showed 70% to >90% of objective response rates and 20 or more months of median survival time (1, 18–20). Mature data regarding the relationship between oncological outcomes and tumor characteristics, as well as functional reserve of the liver, are needed to optimize cost-effectiveness of localized, high-dose RT using X-ray or charged particles for treatment of this disease. Nonetheless, RT should have no role in preventing multifocal tumorigenesis, which will be continuously encountered by multidisciplinary approaches (21).

The risk of developing serious gastrointestinal sequela after PRT is another important issue to consider in patients who have HCC located adjacent to the digestive tract. We attempted once-daily fractionation of PRT with 65 CGE/26 fractions. However, 2 of 11 patients who received this treatment developed gastrointestinal toxicity grade of ≥2. Moreover, these 11 patients showed significantly worse LPF rates than those who received 76 CGE/20 fractions of PRT. Three patients who received 60 CGE/10 fractions of PRT were controlled locally. Although our current data are based on a limited number of patients, precluding definitive conclusions, they suggest a low α/β ratio (22) of HCC, and this assumption should be examined further in clinical trials. Based on currently available data, efforts to exclude the gastrointestinal loop from the PTV by using, for example, surgical manipulations, seem to be positively considered in order to expand the role of PRT for HCC.

CONCLUSIONS

In conclusion, PRT achieved excellent local progression-free rates when aggressive, high-dose/fractionation was administered. Child-Pugh class A patients with ICG R15 of less than 40% tolerated PRT of a large irradiated volume well, despite development of transient PHI. However, in Child-Pugh class B patients, it seems reasonable to minimize the irradiated volume to prevent detrimental liver damage induced by PRT and underlying liver diseases. A V30 of less than 25% in the noncancerous portion of the liver is considered an indicator of the safety of PRT in patients who have pre-PRT ICG R15 of 20% to 50%. We believe that there are extremely few indications for PRT in patients who have ICG R15 of 50% or higher. Gastrointestinal toxicity is a major drawback of PRT for tumors adjacent to the gastrointestinal tract, and surgical manipulation to exclude the intestinal loop from the PTV should be positively considered as indicated. If these issues are carefully considered, with special attention to the patterns of tumor spread, when determining the

CTV, aggressive high-dose PRT could become a legitimate treatment for a certain population of patients with unresectable HCC for whom there is no standard treatment available other than TACE or liver transplantation.

REFERENCES

1. Krishnan S, Dawson LA, Seong J, *et al.* Radiotherapy for hepatocellular carcinoma: An overview. *Ann Surg Oncol* 2008;15: 1015–1024.
2. Bush DA, Hillebrand DJ, Slater JM, *et al.* High-dose proton beam radiotherapy of hepatocellular carcinoma: Preliminary results of a phase II trial. *Gastroenterology* 2004;127:S189–S193.
3. Kawashima M, Furuse J, Nishio T, *et al.* Phase II trial of radiotherapy employing proton beam for hepatocellular carcinoma. *J Clin Oncol* 2005;23:1839–1846.
4. Chiba T, Tokuuye K, Matsuzaki Y, *et al.* Proton beam therapy for hepatocellular carcinoma: A retrospective review of 162 patients. *Clin Cancer Res* 2005;11:3799–3805.
5. Kosuge T, Makuuchi M, Takayama T, *et al.* Long term results after resection of hepatocellular carcinoma: experience of 480 cases. *Hepato-Gastroenterol* 1993;40:328–332.
6. The Liver Cancer Study Group of Japan. Predictive factors for long term prognosis after partial hepatectomy for patients with hepatocellular carcinoma in Japan. *Cancer* 1994;74:2772–2780.
7. Makuuchi M, Sano K. The surgical approach to HCC: Our progress and results in Japan. *Liver Transpl* 2004;10:S46–S52.
8. Tsai TJ, Chau GY, Lui WY, *et al.* Clinical significance of microscopic tumor venous invasion in patients with resectable hepatocellular carcinoma. *Surgery* 2000;127:603–608.
9. Vauthey JN, Lauwers GY, Esnaola NF, *et al.* Simplified staging for hepatocellular carcinoma. *J Clin Oncol* 2002;20:1527–1536.
10. Imamura H, Sano K, Sugawara Y, Kokudo N, Makuuchi M. Assessment of hepatic reserve for indication of hepatic resection: Decision tree incorporating indocyanine green test. *J Hepatobiliary Pancreat Surg* 2005;12:16–22.
11. Esnaola NF, Lauwers GY, Mirza NQ, *et al.* Predictors of microvascular invasion in patients with hepatocellular carcinoma who are candidates for orthotopic liver transplantation. *J Gastrointest Surg* 2002;6:224–232.
12. Cammà C, Schepis F, Orlando A, *et al.* Transarterial chemoembolization for unresectable hepatocellular carcinoma: Meta-analysis of randomized controlled trials. *Radiology* 2002;224: 47–54.
13. Takayasu K, Arii S, Ikai I, *et al.* Prospective cohort study of transarterial chemoembolization for unresectable hepatocellular carcinoma in 8510 patients. *Gastroenterology* 2006;131:461–469.
14. Lawrence TS, Tesser RJ, Ten Haken RK. An application of dose volume histograms to the treatment of intrahepatic malignancies with radiation therapy. *Int J Radiat Oncol Biol Phys* 1990;19:1041–1047.
15. Tsujii H, Tsuji H, Inada T, *et al.* Clinical results of fractionated proton therapy. *Int J Radiat Oncol Biol Phys* 1993;25:49–60.
16. Ando K, Furusawa Y, Suzuki M, *et al.* Relative biological effectiveness of the 235 MeV proton beams at the National Cancer Center Hospital East. *J Radiat Res* 2001;42:79–89.
17. Schwartz JD, Schwartz M, Mandeli J, Sung M. Neoadjuvant and adjuvant therapy for resectable hepatocellular carcinoma: review of the randomised clinical trials. *Lancet Oncol* 2002;3: 593–603.
18. Méndez Romero A, Wunderink W, Hussain SM, *et al.* Stereotactic body radiation therapy for primary and metastatic liver tumors: A single institution phase I-II study. *Acta Oncol* 2006;45: 831–837.
19. Choi BO, Jang HS, Kang KM, *et al.* Fractionated stereotactic body radiotherapy in patients with primary hepatocellular carcinoma. *Jpn J Clin Oncol* 2006;36:154–158.
20. Liang SX, Zhu XD, Lu HJ, *et al.* Hypofractionated three-dimensional conformal radiation therapy for primary liver carcinoma. *Cancer* 2005;103:2181–2188.
21. Avila MA, Berasain C, Sangro B, Prieto J. New therapies for hepatocellular carcinoma. *Oncogene* 2006;25:3866–3884.
22. Thames HD, Withers HR, Peters LJ, *et al.* Changes in early and late radiation responses with altered dose fractionation: Implications for dose-survival relationships. *Int J Radiat Oncol Biol Phys* 1982;8:219–226.

JJCO Japanese Journal of Clinical Oncology

Jpn J Clin Oncol 2011;41(3)328–333
doi:10.1093/jjco/hyq219
Advance Access Publication 29 November 2010

A Phase I/II Study of Combined Chemotherapy with Mitoxantrone and Uracil/Tegafur for Advanced Hepatocellular Carcinoma

Eiichiro Suzuki[1,2,*], Junji Furuse[2], Masafumi Ikeda[1], Hiroshi Ishii[3], Takuji Okusaka[4], Kohei Nakachi[1], Shuichi Mitsunaga[1], Hideki Ueno[4] and Chigusa Morizane[4]

[1]Division of Hepatobiliary and Pancreatic Oncology, National Cancer Center Hospital East, Chiba, [2]Department of Internal Medicine, Medical Oncology, Kyorin University School of Medicine, [3]Hepatobiliary and Pancreatic Section, Gastroenterological Division, Cancer Institute Hospital and [4]Hepatobiliary and Pancreatic Oncology Division, National Cancer Center Hospital, Tokyo, Japan

*For reprints and all correspondence: Eiichiro Suzuki, Department of Internal Medicine, Medical Oncology, Kyorin University School of Medicine, 6-20-2, Shinkawa, Mitaka, Tokyo 181-8611, Japan. E-mail: eisuzuki@ks.kyorin-u.ac.jp

Received July 5, 2010; accepted October 28, 2010

Objective: The aim was to determine the recommended dose of combined chemotherapy with mitoxantrone and uracil/tegafur (Phase I part) and to clarify its efficacy and safety in patients with advanced hepatocellular carcinoma at the recommended dose (Phase II part).
Methods: Patients eligible had histologically confirmed, chemo-naive advanced hepatocellular carcinoma and were amenable to established forms of treatment. The therapy consisted of mitoxantrone administered intravenously at one of three dosages (6, 8 and 10 mg/m^2/day) on day 1 and uracil/tegafur administered orally at 300 mg/m^2 from day 1 through day 21. The treatment was repeated every 4 weeks until evidence of tumor progression or unacceptable toxicity.
Results: A total of 25 patients were enrolled. In the Phase I part, dose-limiting toxicities occurred in all three patients, given mitoxantrone at the dosage of 10 mg/m^2/day, and the recommended mitoxantrone dosage was determined to be 8 mg/m^2/day. Among 19 patients administered the drug at the recommended dosage, 1 patient (5.3%) showed partial response, 8 patients (42.1%) showed stable disease and 10 patients (52.6%) showed progressive disease. The median survival and median progression-free survival were 8.4 and 2.5 months, respectively. The most common toxicities were Grade 3–4 leukopenia (63.2%) and neutropenia (68.4%).
Conclusions: Mitoxantrone at 8 mg/m^2 combined with uracil/tegafur at 300 mg/m^2/day was determined to be the recommended regimen. Although this regimen was generally well tolerated, it appeared to have little activity against advanced hepatocellular carcinoma. These findings do not support the use of this combination regimen in practice.

Key words: hepatocellular carcinoma – chemotherapy Phase I/II – mitoxantrone – uracil/tegafur

INTRODUCTION

Hepatocellular carcinoma (HCC) is one of the most commonly occurring cancers worldwide (1,2). Surgical resection, liver transplantation and local ablation therapy, including radiofrequency ablation and ethanol injection, are considered as curative treatment for HCC (3). Transcatheter arterial chemoembolization (TACE) has been applied to patients with advanced incurable HCC (4,5). However, the majority of HCC patients develop recurrence or metastasis, regardless of the treatment modalities employed. Although patients with HCC at this advanced stage are generally treated by systemic therapy, the prognosis remains poor (6,7). Sorafenib is an orally administered molecular-targeted drug that targets tumor cell proliferation and tumor angiogenesis by inhibiting the serine–threonine kinases Raf-1 and B-Raf and the receptor tyrosine kinase activity of vascular endothelial growth factor receptors 1, 2 and 3 and platelet-derived growth factor

receptor β. This drug was reported to confer an overall survival advantage, with manageable toxicity, in comparison with placebo in a Phase III trial, and it has been accepted worldwide as the first-line chemotherapy for advanced HCC (8). But the advantage is modest. There is urgent need to develop more effective regimens.

5-Fluorouracil (5-FU) has been widely used for the treatment of various gastrointestinal malignancies, including advanced HCC (9,10). A high level of efficacy can be expected when the drug is given as a continuous intravenous infusion (11). However, this would necessitate a permanent intravenous access. Uracil/tegafur (UFT) is an orally administered drug which is a mixture of uracil and tegafur at a molar ratio of 4:1. Tegafur is a prodrug of 5-FU that is hydroxylated and converted to 5-FU by hepatic microsomal enzymes, and uracil prevents the degradation of 5-FU by inhibiting the enzyme dihydropyrimidine dehydrogenase, which results in an increased level of 5-FU in the plasma and tumor tissues (12,13). UFT has been reported to be as effective as intravenous 5-FU for the treatment of malignancies (14,15) and to be effective for the treatment of advanced HCC (16,17).

The therapeutic usefulness of doxorubicin in patients with advanced HCC has also been widely explored since the 1970s. A randomized trial in which doxorubicin was compared with supportive care alone for advanced HCC showed a significant survival benefit in the doxorubicin arm. However, treatment with this drug has not been accepted as a standard chemotherapy because of the high rate of fatal complications reported (18). Mitoxantrone, another anthracycline, has shown similar antitumor activity to that of doxorubicin in both human tumor cell lines and animal models of leukemia and has fewer myelotoxic and cardiotoxic effects than doxorubicin (19). Clinical trials of mitoxantrone have also demonstrated moderate activity against HCC, with a low incidence rate of adverse effects (20,21).

Combination chemotherapeutic regimens composed of a fluoropyrimidine and an anthracycline antibiotic have been reported to show moderate efficacy against HCC with tolerable toxicity (22–24), but combined chemotherapy with UFT and mitoxantrone has not yet been examined. We conducted Phase I/II studies to determine the recommended dosage of the combination of UFT with mitoxantrone (UFM regimen) and to clarify the efficacy and safety when administered at the recommended dose in patients with advanced HCC.

PATIENTS AND METHODS

Eligibility Criteria

The eligibility criteria for study enrolment were: (i) patients with histologically confirmed HCC, who were (ii) unsuitable for surgical resection, local ablation therapy or TACE, (iii) were ≥20 years old, (iv) had an Eastern Cooperative Oncology Group (ECOG) performance status (PS) of 0–2, (v) had adequate bone marrow function (white blood cell ≥3000 cells/mm^3, absolute neutrophil count ≥1500 cells/mm^3, platelet count ≥70 000 cells/mm^3 and hemoglobin ≥8.0 g/dl), renal function [serum creatinine concentration ≤upper limit of normal (ULN)] and hepatic function [serum albumin level ≥3.0 mg/dl, total bilirubin level ≤3.0 mg/dl, serum aspartate aminotransferase (AST) and alanine aminotransferase (ALT) levels ≤5.0 × ULN], (vi) had a life expectancy of at least 12 weeks and (vii) provided written informed consent from each patient.

The exclusion criteria were: clinically evident congestive heart failure, serious cardiac arrhythmia, active or symptomatic coronary artery disease or ischemia, clinically serious infection, seizure disorder requiring medication, prior malignancy (any cancer treated curatively was permitted), clinically evident brain or meningeal metastasis, and pregnant/lactating women. This protocol was approved by the Institutional Review Board for clinical investigation of the National Cancer Center, in conformity with the provisions of the Declaration of Helsinki, Good Clinical Practice guidelines, and local laws and regulations.

Study Treatment

UFT was administered orally at the dose of 300 mg/m^2 per day in two divided doses for 21 consecutive days, followed by a rest period of 7 days (400 mg/body per day in patients with a body surface area of <1.50 m^2 and 500 mg/body/day in patients with a body surface area of ≥1.50 m^2). Mitoxantrone was given as a 60 min intravenous infusion on day 1. This cycle was repeated every 28 days. Patients continued to receive additional courses of this regimen until a cumulative dose of mitoxantrone of 100 mg/m^2, evidence of disease progression or the appearance of unacceptable toxicity.

Phase I Part

The objectives of the Phase I study were to investigate the frequency of dose-limiting toxicity (DLT) and to determine the recommended dose of mitoxantrone and UFT. The criteria of DLT included: Grade 4 leukopenia or neutropenia, Grade 3 neutropenia accompanied by fever (≥38°C) or infection (clinically or biologically confirmed), thrombocytopenia <25 000/mm^3 or necessity of transfusion, Grade 3 or 4 non-hematological toxicity (except nausea/vomiting, anorexia, fatigue and hyperglycemia), AST and ALT >10 times the ULN, suspension of UFT administration for over 3 successive weeks, or an over 6-week delay in the commencement of the next treatment cycle.

Three possible dosage levels of mitoxantrone (Level 1: 6 mg/m^2/day, Level 2: 8 mg/m^2/day and Level 3: 10 mg/m^2/day) were assigned for the Phase I part (Table 1). The first patient to enter the study was started at Level 1. At least three patients were treated at this level and observed for DLT. Dose escalation was continued until at least one-third

Table 1. Dose-escalation schedules of mitoxantrone and uracil/tegafur

Dose level	Mitoxantrone (mg/m^2)	UFT (mg/m^2)	Number of patients enrolled
1	6	300	3
2	8	300	6
3	10	300	3

UFT, uracil/tegafur.

of the patients in a given cohort showed DLT. If none of the first three treated patients developed DLT during the first cycle at a specific dose level, the dose escalation was continued. If one of the first three treated patients developed DLT at any dose level, three additional patients were entered at the same dose level; if only one or two of six patients at a given level experienced a DLT, the dose escalation was continued. The maximum tolerated dose (MTD) was defined as the dose level at which one-third or more of the patients experienced a DLT. The recommended dose for the Phase II study was defined as the dose level preceding the attainment of the MTD.

Phase II Part

The primary endpoint of the Phase II part was the objective response rate. The secondary endpoints were the overall survival, progression-free survival and the frequency and severity of adverse events. The Phase II part was begun after determination of the recommended dosage from the Phase I part.

Assessment of the Response and Toxicity

Physical examination including cardiac symptoms, complete blood cell counts, serum chemistries and urinalysis was performed at the baseline and at least once every 2 weeks after the start of the treatment. Dynamic computed tomography or magnetic resonance imaging was undertaken to evaluate the response at 4- to 6-week intervals after the start of treatment. Tumor response was assessed using the Response Evaluation Criteria in Solid Tumors (25). Toxicity was graded according to the National Cancer Institute common toxicity criteria, version 2.0. Progression-free survival was calculated from the first day of treatment to the appearance of evidence of tumor progression, clinical progression or last date of follow-up. The overall survival was calculated from the first day of treatment until death due to any cause or date of last follow-up. Survival data were analyzed using the Kaplan–Meier method.

Statistical Analysis

In the Phase II part, the primary endpoint was the response rate, and data from at least 19 patients were accrued. The threshold response rate was set at 5% and the expected response rate at 15%. If no responses were observed in the 19 patients and the upper limit of the 90% confidence interval (CI) did not exceed the expected rate of 15%, the UFM regimen was judged to have no activity against HCC. If response was confirmed in one or more of the 19 patients, the decision of whether or not to proceed to a further study using the UFM regimen was taken on the basis of other factors, such as the safety and rate of response, overall survival and time to progression in this study.

RESULTS

Patients

From April 2004 to April 2007, 25 patients were registered for the present study: 12 patients completed the Phase I part (Level 1: 3 patients, Level 2: 6 patients and Level 3: 3 patients). Nineteen patients who received the recommended dose (6 patients received this dose during the Phase I part) were analyzed during the Phase II part. Table 2 shows the baseline characteristics of the patients in the Phase I and Phase II parts of the study of the UFM regimen. There were 19 males and 6 females with a median age of 67 years. All the patients had a good ECOG PS score of 0–1. There were 21 (84%) and 4 (16%) patients with the Child–Pugh Stages A and B, respectively. Thirteen (68%) patients had extrahepatic metastasis, and the major sites of metastasis were lymph node [$n = 7$ (28%)] and lung [$n = 6$ (24%)].

Treatments

In the Phase I part, there was no occurrence of DLT at the Level 1 and Level 2 doses, but all of the three patients who received the Level 3 dose experienced DLT; two of these patients developed Grade 4 neutropenia and one patient developed Grade 3 creatinine elevation. The additional three patients at the Level 2 dose did not experience any DLT. Therefore, Level 3 was considered as the MTD and Level 2 (UFT 300 mg/m^2 and mitoxantrone 8 mg/m^2) as the recommended dose for the Phase II part.

At the recommended dosage level, a total of 69 courses of the UFM regimen were administered with a median of three courses to each patient (range, 1–8 courses). The dose intensity was 98.9% of the planned dosage for mitoxantrone and 97.9% for UFT.

The reasons for treatment discontinuation in the Phase I and Phase II parts were disease progression in 19 patients, liver dysfunction in 1 patient, DLT according to this protocol in 3 patients during the Phase I part and an over 6-week delay in the start of the next course because of the development of leukopenia in 2 patients. After abandoning the UFM regimen, 10 patients received the second-line treatment. Five patients received systemic chemotherapy, one patient received UFT alone and four patients received a combined chemotherapy with UFT and doxorubicin. Two

Table 2. Profile of hepatocellular carcinoma patients population

	Phase I	Phase II
No. of patients	12	19
Gender		
Male	9	14
Female	3	5
Age (years)		
Median	63	67
Range	56–78	56–77
Performance status		
0	11	7
1	1	12
Viral marker		
Hepatitis C antibody+	7	7
Hepatitis B antigen+	2	5
Previous treatment		
Surgical resection	4	10
Percutaneous ablation therapy	3	3
Transcatheter arterial chemoembolization	5	8
Transcatheter arterial infusion	3	5
Radiation therapy	1	2
None	3	3
Child–Pugh classification		
A	8	17
B	4	2
UICC tumor stage[a]		
III	4	6
IVa	3	1
IVb	5	12
Portal vein tumor thrombosis		
(+)	5	4
Extrahepatic metastasis		
Lymph node	5	7
Lung	0	6
Bone	0	3
Adrenal gland	0	1
Peritoneum	0	1
None	7	6

[a]The International Union Against Cancer, 6th edition.

patients received transcatheter arterial infusion with cisplatin, one patient received salvage TACE because of HCC rupture during the follow-up period, one patient received salvage radiofrequency ablation because of rapid growth of HCC that needed control and one patient received immnunotherapy.

Table 3. Toxicity

Toxicity grade	Phase I part									Phase II part		
	Level 1 ($n = 3$)			Level 2 ($n = 6$)			Level 3 ($n = 3$)			Level 2 ($n = 19$)		
	1–2	3	4	1–2	3	4	1–2	3	4	1–2	3	4
Hematological toxicity												
Leukopenia	2	1	0	0	2	0	0	1	1	4	9	3
Neutropenia	0	1	0	0	2	0	0	0	2	4	11	2
Thrombocytopenia	1	1	0	0	0	0	1	0	0	4	1	0
Anemia	0	0	0	1	0	0	0	0	0	1	0	0
Non-hematological toxicity												
Nausea	3	0	0	0	0	0	2	0	0	3	0	0
Anorexia	0	0	0	2	0	0	1	0	0	3	0	0
Elevated bilirubin	2	0	0	0	1	0	1	0	0	6	0	0
Hypoalbuminemia	1	0	0	0	0	0	0	0	0	1	0	0
Fatigue	0	0	0	0	0	0	1	0	0	1	0	0
Hyperpigmentation	0	0	0	0	0	0	0	0	0	1	0	0
Constipation	0	0	0	0	0	0	0	0	0	1	0	0
Elevated creatinine	0	0	0	0	0	0	0	1	0	0	0	0
Elevated AST	0	0	0	1	0	0	0	0	0	2	1	1[a]
Elevated ALT	0	0	0	1	0	0	0	0	0	1	2	1[a]
Liver dysfunction	0	0	0	0	0	0	0	0	0	0	0	1[a]

AST, aspartate aminotransferase; ALT, alanine aminotransferase.
[a]Death related to adverse event.

Toxicity

Table 3 summarizes the toxicities observed in the patients. At the recommended dose (level 2), the major Grade 3–4 hematological toxicities were leukopenia (63.2%) and neutropenia (68.4%). The most common non-hematological toxicities were elevated serum total bilirubin level (31.6%), elevated AST level (26.3%), elevated ALP level (26.3%) and anorexia (21.1%); however, no Grade 3–4 non-hematological toxicities were observed. One patient died of hepatic failure due to hepatitis B virus (HBV) reactivation.

Efficacy

Of the 19 patients who were administered the recommended dosage, 18 died during the follow-up period. All of the 19 patients administered the recommended dosage were evaluable for tumor response; of these, 1 patient achieved partial response (PR), with an overall response rate of 5.3% (95% CI, 0.0–26.0%). Eight patients (42.1%) had stable disease and 10 patients (52.6%) had progressive disease. The 1-year survival rate, median overall survival, median progression-free survival and time to progression were 26.3%, 8.4

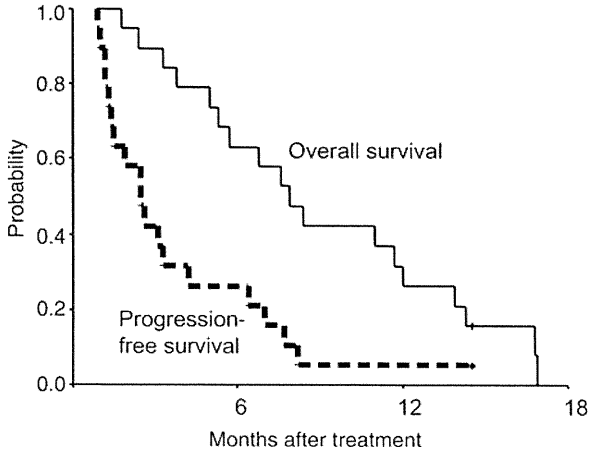

Figure 1. Overall survival and progression-free survival in 19 patients at the recommended dose. Tick marks indicate censored cases.

months (95% CI, 5.4–11.4) and 2.5 months (95% CI, 1.5–3.5), respectively (Fig. 1).

DISCUSSION

Systemic chemotherapy for unresectable HCC is recognized as an important treatment modality, because some patients who have recurrent or very advanced disease are not suitable candidates for effective local treatments such as surgical resection, liver transplantation, local ablation therapy and TACE. Many patients with HCC have underlying chronic liver disease and impaired hepatic function, increasing the toxicity of standard doses of many chemotherapeutic agents and causing difficulty in delivering combination chemotherapies. The results, in terms of the therapeutic efficacy, of investigation of cytotoxic agents for advanced HCC have been disappointing, with few agents have yielded response rates of over 20%, and no cytotoxic agents have produced convincing survival benefits in the Phase III setting (26–28).

In Japan, only five anticancer agents, UFT, adriamycin, cytarabine, mitomycin and 5-FU, had been approved for the systemic chemotherapy of HCC by the Ministry of Health, Labor and Welfare of Japan before sorafenib has been approved. Among these drugs, the results of multiagent regimens containing both a fluoropyrimidine and an anthracycline antibiotic have shown favorable results for advanced HCC (22–24). Thus, it was expected that the combination of mitoxantrone and UFT (UFM regimen) would have effective anticancer activity, and we conducted a Phase I/II study to evaluate this regimen.

In the Phase I part, we determined the recommended dose of mitoxantrone as 8 mg/m^2 on day 1 and of UFT as 300 mg/m^2 from days 1 to 21 of a 28-day cycle. The DLTs observed at Level 3 were Grade 4 neutropenia (two patients) and Grade 3 creatinine elevation (one patient).

Patients with HCC tend to experience more severe myelosuppression and hepatic toxicity than those with other malignant diseases, because most have underlying cirrhosis, which is usually associated with compromised hepatic function, leukopenia and thrombocytopenia (24). In 19 patients treated at the recommended dose level, the most frequently encountered toxicities were leukopenia and neutropenia, which are well-known toxicities of the two drugs. When compared with that in trial of mitoxantrone or UFT for other malignancies, Grade 3 or 4 hematological toxicities occurred more frequently (29–31). However, these toxicities were reversible and generally well tolerated in patients with advanced HCC, except for one case of treatment-related death; this patient developed hepatic failure due to HBV reactivation, because no antiviral drug for HBV infection, such as lamivudine or entecavir, was given. This is a well-recognized complication in patients with HBV infection who received immunosuppressive therapy or chemotherapeutic agents (32,33). Thus, patients with HBV infection should receive prophylactic antiviral treatment before chemotherapy.

In the current study, 1 of the 19 patients showed a PR (response rate, 5.3%). However, the rate of progressive disease was 52.6%. In addition, the result of median time to progression was only 2.5 months. Those results were unfavorable when compared with those reported from other clinical trials (8,21–23). Therefore, this regimen is considered to be ineffective and cannot be recommended for use in clinical practice. There were several reasons for this negative result. One of the reasons was the number of anticancer drugs in the regimen. A regimen containing two drugs may have little activity, and three or more drugs may be needed to obtain activity against HCC, because many of the regimens that have been shown to exert anticancer effect against HCC contain three or more drugs. The other reason was the recommended doses of the drugs in this regimen. We set the criteria of DLT which had included Grade 4 neutropenia or leukopenia. Two patients experienced DLT based on these criteria. However, both recovered soon, with only observation. Therefore, the criteria may be too strict, although the two drugs have been used at these recommended doses for other malignancies. It may be possible to set higher dose levels to obtain higher antitumor effect.

Recently, increasing knowledge of the molecular pathogenesis of HCC as well as the introduction of molecular-targeted therapies has created an encouraging trend in the management of HCC. Combination regimens consisting of molecular-targeted agents such as sorafenib and cytotoxic agents have been reported as promising regimens for patients with advanced HCC and other malignancies (34–37). The UFM regimen itself has little antitumor activity, but the result may be useful in the setting of future clinical trials of cytotoxic agents used in combination with molecular-targeted agents.

In conclusion, the recommended dose was mitoxantrone at 8 mg/m^2 and UFT at 300 mg/m^2/day. A combined chemotherapy with mitoxantrone and UFT appeared to show little activity in patients with advanced HCC, although this regimen was generally well tolerated. These findings do argue against the use of this regimen in clinical practice.

Acknowledgements

The authors thank Ms Kayo Takei and Ms Keiko Kondo for their devoted work and support.

Funding

This study was supported in part by a Grant-in-Aid for Cancer Research from the Ministry of Health and Welfare, Japan.

Conflict of interest statement

None declared.

References

1. Parkin DM, Pisani P, Ferlay J. Estimates of the worldwide incidence of 25 major cancers in 1990. *Int J Cancer* 1999;80:827–41.
2. Parkin DM, Bray F, Ferlay J. Global cancer statistics 2002. *CA Cancer J Clin* 2005;55:74–108.
3. Bruix J, Sherman M, Llovet JM, Beaugrand M, Lencioni R, Burroughs AK, et al. Clinical management of hepatocellular carcinoma. Conclusions of the Barcelona-2000 EASL conference. European Association for the Study of the Liver. *J Hepatol* 2001;35:421–30.
4. Llovet JM, Real MI, Montaña X, Planas R, Coll S, Aponte J, et al. Arterial embolization or chemoembolization versus symptomatic treatment in patients with unresectable hepatocellular carcinoma: a randomized controlled trial. *Lancet* 2002;359:1734–9.
5. Takayasu K, Arii S, Ikai I, Omata M, Okita K, Ichida T, et al. Prospective cohort study of transarterial chemoembolization for unresectable hepatocellular carcinoma in 8510 patients. *Gastroenterology* 2006;131:461–9.
6. Forner A, Hessheimer AJ, Isabel Real M, Bruix J. Treatment of hepatocellular carcinoma. *Crit Rev Oncol Hematol* 2006;60:89–98.
7. Thomas MB, Zhu AX. Hepatocellular carcinoma: the need for progress. *J Clin Oncol* 2005;23:2892–9.
8. Llovet JM, Ricci S, Mazzaferro V, Hilgard P, Gane E, Blanc JF, et al. Sorafenib in advanced hepatocellular carcinoma. *N Engl J Med* 2008;359:378–90.
9. Falkson G, Moertel CG, Lavin P, Pretorius FJ, Carbone PP. Chemotherapy studies in primary liver cancer: a prospective randomized clinical trial. *Cancer* 1978;42:2149–56.
10. Tetef M, Doroshow J, Akman S, Coluzzi P, Leong L, Margolin K, et al. 5-Fluorouracil and high-dose calcium leucovorin for hepatocellular carcinoma: a phase II trial. *Cancer Invest* 1995;13:460–3.
11. Kim SJ, Seo HY, Choi JG, Sul HR, Sung HJ, Park KH, et al. Phase II study with a combination of epirubicin, cisplatin, UFT, and leucovorin in advanced hepatocellular carcinoma. *Cancer Chemother Pharmacol* 2006;57:436–42.
12. Fujii S, Ikenaka K, Fukushima M, Shirasaka T. Effect of uracil and its derivatives on antitumor activity of 5-fluorouracil and 1-(2-tetrahydrofuryl)-5-fluorouracil. *Jpn J Cancer Res* 1978;69:763–72.
13. Pazdur R, Lassere Y, Diaz-Canton E, Bready B, Ho DH. Phase I trials of uracil–tegafur (UFT) using 5 and 28 day administration schedules: demonstration of schedule-dependent toxicities. *Anticancer Drugs* 1996;7:728–33.
14. Baker SD, Diasio RB, O'Reilly S, Lucas VS, Khor SP, Sartorius SE, et al. Phase I and pharmacologic study of oral fluorouracil on a chronic daily schedule in combination with the dihydropyrimidine dehydrogenase inactivator eniluracil. *J Clin Oncol* 2000;18:915–26.
15. Takiuchi H, Ajani JA. Uracil–tegafur in gastric carcinoma: a comprehensive review. *J Clin Oncol* 1998;16:2877–85.
16. Tokyo Liver Cancer Chemotherapy Study Group. Phase II study of co-administration of uracil and tegafur (UFT) in hepatocellular carcinoma. *Jpn J Clin Oncol* 1985;15:559–62.
17. Ishikawa T, Ichida T, Sugitani S, Tsuboi Y, Genda T, Sugahara S, et al. Improved survival with oral administration of enteric-coated tegafur/uracil for advanced stage IV-A hepatocellular carcinoma. *J Gastroenterol Hepatol* 2001;16:452–9.
18. Lai CL, Wu PC, Chan GC, Lok AS, Lin HJ. Doxorubicin versus no antitumor therapy in inoperable hepatocellular carcinoma. A prospective randomized trial. *Cancer* 1988;62:479–83.
19. Durr FE. Biologic and biochemical effects of mitoxantrone. *Semin Oncol* 1984;11:3–10.
20. Colleoni M, Nole F, Di Bartolomeo M, de Braud F, Bajetta E. Mitoxantrone in patients affected by heptatocellular carcinoma with unfavorable prognostic factors. *Oncology* 1992;49:139–42.
21. Yoshida T, Okazaki N, Yoshino M, Ohkura H, Miyamoto K, Shimada Y. Phase II trial of mitoxantrone in patients with hepatocellular carcinoma. *Eur J Cancer Clin Oncol* 1988;24:1897–8.
22. Ellis PA, Norman A, Hill A, O'Brien ME, Nicolson M, Hickish T, et al. Epirubicin, cisplatin and infusional 5-fluorouracil (5-FU) (ECF) in hepatobiliary tumours. *Eur J Cancer* 1995;31:1594–8.
23. Leung TW, Tang AM, Zee B, Lau WY, Lai PB, Leung KL, et al. Factors predicting response and survival in 149 patients with unresectable hepatocellular carcinoma treated by combination cisplatin, interferon-alpha, doxorubicin and 5-fluorouracil chemotherapy. *Cancer* 2002;94:421–7.
24. Ikeda M, Okusaka T, Ueno H, Tekezako Y, Morizane C. A phase II trial of continuous infusion of 5-fluorouracil, mitoxantrone, and cisplatin for metastatic hepatocellular carcinoma. *Cancer* 2005;103:756–62.
25. Therasse P, Arbuck SG, Eisenhauer EA, Wanders J, Kaplan RS, Rubinstein L, et al. New guidelines to evaluate the response to treatment in solid tumors. *J Natl Cancer Inst* 2000;92:205–16.
26. Johnson PJ. Hepatocellular carcinoma: is current therapy really altering outcome? *Gut* 2002;51:459–62.
27. Palmer DH, Hussain SA, Johnson PJ. Systemic therapies for hepatocellular carcinoma. *Expert Opin Investig Drugs* 2004;13:1555–68.
28. Nowak AK, Chow PK, Findlay M. Systemic therapy for advanced hepatocellular carcinoma. *Eur J Cancer* 2004;40:1474–84.
29. Onyenadum A, Gogas H, Kosmidis P, Aravantinos G, Bafaloukos D, Bacoyiannis H. Mitoxantrone plus gemcitabine in pretreated patients with metastatic breast cancer. *J Chemother* 2006;18:192–8.
30. Onyenadum A, Gogas H, Markopoulos C, Bafaloukos D, Aravantinos G, Mantzourani M, et al. Mitoxantrone plus vinorelbine in pretreated patients with metastatic breast cancer. *J Chemother* 2007;19:582–9.
31. Furuse J, Okusaka T, Ohkawa S, Nagase M, Funakoshi A, Boku N, et al. Early phase II study of uracil–tegafur plus doxorubicin in patients with unresectable advanced biliary tract cancer. *Jpn J Clin Oncol* 2006;36:552–6.
32. Foont JA, Schiff ER. Avoid the tragedy of hepatitis B reactivation in immunosuppressed patients. *Nat Clin Pract Gastroenterol Hepatol* 2007;4:128–9.
33. Hoofnagle JH, Doo E, Liang TJ, Fleischer R, Lok AS. Management of hepatitis B: summary of a clinical research workshop. *Hepatology* 2007;45:1056–75.
34. Richly H, Schultheis B, Adamietz IA, Kupsch P, Grubert M, Hilger RA, et al. Combination of sorafenib and doxorubicin in patients with advanced hepatocellular carcinoma: Results from a phase I extension trial. *Eur J Cancer* 2009;45:579–87.
35. Zhu AX. Development of sorafenib and other molecularly targeted agents in hepatocellular carcinoma. *Cancer* 2008;112:250–9.
36. Richly H, Kupsch P, Passage K, Grubert M, Hilger RA, Voigtmann R. Results of a phase I trial of BAY 43-9006 in combination with doxorubicin in patients with primary hepatic cancer. *Int J Clin Pharmacol Ther* 2004;42:650–1.
37. Dal Lago L, D'Hondt V, Awada A. Selected combination therapy with sorafenib: a review of clinical data and perspectives in advanced solid tumors. *Oncologist* 2008;13:845–58.

Biochemical and Biophysical Research Communications 414 (2011) 675–680

Contents lists available at SciVerse ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

miR-146a suppresses the sensitivity to interferon-α in hepatocellular carcinoma cells

Akira Tomokuni, Hidetoshi Eguchi, Yoshito Tomimaru, Hiroshi Wada, Koichi Kawamoto, Shogo Kobayashi, Shigeru Marubashi, Masahiro Tanemura, Hiroaki Nagano *, Masaki Mori, Yuichiro Doki

Department of Gastroenterological Surgery, Osaka University Graduate School of Medicine, 2-2 E2 Yamadaoka, Suita-shi, Osaka 565-0871, Japan

ARTICLE INFO

Article history:
Received 20 September 2011
Available online 1 October 2011

Keywords:
MicroRNA
miR-146a
Hepatocellular carcinoma
Interferon
SMAD4

ABSTRACT

Background: Interferon-based (IFN-based) therapy is effective in the treatment of advanced hepatocellular carcinoma (HCC). However, the issue of resistance to this therapy remains to be solved. The aim of this study was to identify microRNAs (miRNAs) that govern the sensitivity to IFN-α in HCC cells

Methods: miRNA microarray analysis using IFN-α-resistant clones of PLC/PRF/5 (PLC-Rs) and their parental cells (PLC-P) was conducted. Changes in the anti-cancer effects of IFN-α were studied after gain-of-function and loss-of-function of the candidate miRNA.

Results: miR-146a expression was significantly higher in PLC-Rs than in PLC-P. miR-146a decreased the sensitivity to IFN-α through the suppression of apoptosis. Further experiments showed that miR-146a-related resistance to IFN-α was mediated through SMAD4.

Conclusions: The results indicated that miR-146a regulated the sensitivity of HCC cells to the cytotoxic effects of IFN-α through SMAD4, suggesting that this miRNA could be suitable for prediction of the clinical response and potential therapeutic target in HCC patients on IFN-based therapy.

1. Introduction

Hepatocellular carcinoma (HCC) is one of the leading causes of cancer-related deaths in the world. Treatment modalities for HCC include ablative therapy, surgery, transarterial chemoembolization (TACE) and drug therapy. Local ablation and surgical resection, including transplantation, are suitable for local control of HCC, however several limits exist in the application of each modality (e.g., size, number, and location of tumor, liver function, extrahepatic metastasis, portal invasion) [1,2]. TACE has also limitations; in principle, it can be applied only in patients free of tumor thrombi in the major trunk of the portal vein [1,2]. New drug therapies for HCC are therefore desirable.

Several reports have demonstrated that interferon-based (IFN-based) combination chemotherapy is effective in various cancers [3–5]. We and others have reported a striking effect of IFN-α and 5-fluorouracil combination therapy in HCC patients, including those with tumor thrombi in the major trunk of the portal vein [5–11]. Although this IFN-based therapy improves the prognosis of patients with HCC, its effect is modest, at least in a proportion of patients [9]. In a series of studies, we focused on the mechanisms of resistance to IFN-based therapy in HCC patients and have since reported the involvement of several molecules in the IFN-α-sensitivity [8,12–14]. However, such molecules account for only part of the resistance to IFN-based therapy, and each could not predict the sensitivity completely, suggesting the involvement of yet unknown mechanisms in IFN-α-resistance.

MicroRNA (miRNA), a modulator of gene expression in post-transcriptional phase, has attracted the attention of oncologists because its aberrant expression correlates with carcinogenesis or progression of cancers [15,16]. Many studies showed that miRNAs also regulate the sensitivity to several chemotherapeutic agents in various types of cancer. For example, miR-221/222 potentiated the resistance to tamoxifen in breast cancer cells by targeting $p27^{Kip1}$ [17]. miR-214 suppressed the expression level of PTEN and enhanced the resistance to cisplatin in ovarian cancer cells [18]. In addition, we have recently reported that miR-21 plays an important role in resistance of HCC cells to IFN-α, and that the expression level of miR-21 correlated significantly with the clinical efficiency of IFN-based chemotherapy and overall survival in patients with HCC [19]. However, there is a need for systematic assessment of the miRNA expression and its roles in HCC.

In this study, comprehensive expression profiling of miRNAs was performed in HCC cells and their IFN-α-resistant clones. The results

Abbreviations: HCC, hepatocellular carcinoma; IFN, interferon; PLC-P, parental PLC/PRF/5; PLC-R, interferon-α-resistant clone of PLC/PRF/5.

* Corresponding author. Fax: +81 6 6879 3259.
E-mail address: hnagano@gesurg.med.osaka-u.ac.jp (H. Nagano).

0006-291X/$ - see front matter © 2011 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2011.09.124

indicated that miR-146a confers resistance to IFN-α in HCC cells by inhibiting apoptosis through SMAD4.

2. Materials and methods

2.1. Cell lines

Human HCC cell lines, PLC/PRF/5, were obtained from the Japan Cancer Research Resources Bank (Tokyo, Japan). Parental PLC/PRF/5 (PLC-P) cells were exposed to progressively higher concentrations of IFN-α for 10 weeks, until becoming IFN-α-resistant. By limiting dilution of these cells, IFN-α-resistant clones (PLC-Rs; PLC-R1, PLC-R2, and PLC-R3) were established. There were no significant differences between PLC-P and PLC-Rs in cell morphology, cell proliferation in the absence of IFN-α, and the expression levels of type I IFN receptor type 2 (IFNAR2), signal transducer and activator of transcription factor 1 (STAT1), and STAT2. However, the cell viability of PLC-Rs was significantly higher than that of PLC-P under treatment with IFN-α [14]. These cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 100 U ml^{-1} penicillin and 100 mg ml^{-1} streptomycin at 37 °C in a humidified incubator with 5% CO_2 in air.

2.2. Drugs and reagents

Purified human IFN-α was kindly supplied by Otsuka Phar ma ceutical Co. (Tokyo, Japan). The primary antibodies used for Western blot analysis were polyclonal rabbit anti-human SMAD4 antibody (Cell Signaling Technology, Beverly, MA) and monoclonal rabbit anti-human ACTIN (Sigma–Aldrich Co., St. Louis, MO).

2.3. Transfection

The precursory oligonucleotide of hsa-miR-146a (pre-miR-146a), antisense oligonucleotide inhibitor of hsa-miR-146a (anti-miR-146a), *SMAD4* siRNA oligonucleotides (si*SMAD4*), and their scrambled oligonucleotides were obtained from Ambion Inc. (Austin, TX). Pre/anti-miR-146a and si*SMAD4* were transfected using Lipofectamine RNAiMAX (Invitrogen, Carlsbad, CA) according to the protocol provided by the manufacturer. Each scrambled oligonucleotide was transfected in the same way as a matched negative control.

2.4. RNA extraction

Total RNA including the small RNA fraction was isolated from cell lines by miRNeasy Mini Kit (Qiagen, Hiden, Germany). The quality of the RNA was assessed with a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE) at 260 and 280 nm ($A_{260/280}$) wavelengths.

2.5. Real-time quantitative reverse transcription-PCR (qRT-PCR) for microRNA expression

Reverse transcription (RT) reaction was performed with TaqMan MicroRNA RT Kit (Applied Biosystems, Foster City, CA), and real-time quantitative PCR was performed with TaqMan MicroRNA Assays (Applied Biosystems) using the ABI7900HT system (Applied Biosystems). The expression of the target miRNA was normalized relative to that of the endogenous control, RNU48. Data were analyzed according to the comparative Ct method [21].

2.6. Real-time qRT-PCR for messenger RNA expression

RNA was reverse transcribed using High Capacity RNA-to-cDNA Master Mix (Applied Biosystems) according to the instructions supplied by the manufacturer. Real-time quantitative PCR was performed using designed oligonucleotide primers and LightCycler 480 Real-Time PCR system (Roche Diagnostics, Mannheim, Germany). For detection of the amplification products, the LightCycler-DNA master SYBR green I (Boehringer Mannheim, Mannheim, Germany) was used as described previously [22], and the amount of target gene expression was calculated. The expression of the target gene was normalized relative to the expression of GAPDH, which was used as an endogenous control. The designed PCR primers were as follows: *GAPDH* forward primer 5′-GTCGGAGTCAACGGATTTGGT-3′, *GAPDH* reverse primer 5′-GCCATGGGTGGAATCATATTGG-3′; *SMAD4* forward primer 5′-CAGCCTCCCATTTCCAATC-3′, *SMAD4* reverse primer 5′-CAACTGCACACCTTTGCCTA-3′.

2.7. MicroRNA microarray experiments

The purified RNAs obtained from PLC-P, PLC-R1, PLC-R2, and PLC-R3 were used as samples, and assessed as being of high quality by Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA) and NanoDrop (NanoDrop Technologies). Next, 500 ng of extracted total RNA was labeled with Hy5 using the miRCURY LNA Array microRNA Power Labeling kit (Exiqon, Vedbaek, Denmark). The labeled RNAs were hybridized onto 3D-Gene Human miRNA Oligo chips containing 1011 anti-sense probes printed in duplicate spots (Toray, Kamakura, Japan). The annotation and oligonucleotide sequences of the probes conformed to the miRBase miRNA database (http://microrna.sanger.ac.uk/sequences/). After stringent washes, the fluorescent signals were scanned with the ScanArray Express Scanner (Perkin Elmer, Waltham, MA) and analyzed using GenePix Pro version 5.0 (Molecular Devices, Sunnyvale, CA). The raw data of each spot were normalized by substitution with the mean intensity of the background signal determined by the signal intensities of all blank spots with 95% confidence intervals. Measurements of both duplicate spots with signal intensities greater than 2 standard deviations (SD) of the background signal intensity were considered to be valid. The relative expression level of a given miRNA was calculated by comparing the signal intensities of the averaged valid spots with their mean value throughout the microarray experiments after normalization by their median values adjusted equivalently.

2.8. Western blot analysis

Cells grown to semiconfluence were lysed in RIPA buffer [25 mM Tris (pH 7.5), 50 mM NaCl, 0.5% sodium deoxycholate, 2% Nonidet P-40, 0.2% sodium dodecyl sulfate, 1 mM phenylmethylsulphonyl fluoride, and 1.6 mg ml^{-1} aprotinin]. Western blot analysis was carried out as described previously [23].

2.9. Growth inhibitory assay

The growth inhibitory assay was assessed by the3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) (Sigma–Aldrich Co.) assay, as described previously [24]. In brief, cells were incubated for 72 h under several concentrations of IFN-α. After reincubation for 4 h with MTT solution, acid–isopropanol mixture was added to dissolve the resultant formazan crystals. The absorbance of the plate was measured in a microplate reader at a wavelength of 550 nm with a 650 nm reference, and the results were expressed as a percentage of absorbance relative to that of untreated controls.

2.10. Annexin V assay

Annexin V assay was conducted for measuring apoptosis. At 24 h after exposure to IFN-α at concentration of 500 IU ml^{-1}, cells

were stained by annexin V-FITC and propidium iodide (PI) (Bio Vision Research Products, Mountain View, CA) according to the protocol recommended by the manufacturer. The analysis was performed by FACS Calibur (BD Biosciences, Franklin Lakes, NJ). Three different cell populations could be distinguished: (1) viable, double-negative cells; (2) early apoptotic, annexin V-positive/PI-negative cells; (3) secondary necrotic, double-positive cells. Apoptotic cells were defined as the sum of cells from (2) and (3) quadrants, as described previously [25]. Each sample contained approximately 100,000 cells, and the experiment was repeated three times.

2.11. Statistical analysis

Data are expressed as means ± SD. Continuous variables were compared using the Student's *t*-test. A *P*-value <0.05 denoted the presence of a statistically significant difference. Statistical analysis was performed using JMP software version 8.0.2 (SAS Institute Inc. Cary, NC).

3. Results

3.1. miR-146a expression was significantly higher in PLC-Rs than in PLC-P

In order to identify the candidate miRNAs related to the sensitivity to IFN-α, miRNA microarray analysis was performed using PLC-P and PLC-Rs. The analysis showed that, among the 1011 miRNAs, the miRNAs expression levels of 376 (37.2%), 334 (33.0%), 349 (34.5%) in PLC-R1, -R2, and -R3, respectively, were altered by more than 1.2-fold. Furthermore, 115 miRNAs were commonly identified among the three clones (Fig. 1A). These miRNAs are listed in the order of mean fold change of PLC-Rs relative to PLC-P in Supplementary Table 1. Among them, miR-146a showed the highest alteration (3.04-fold increase). The results of real-time qRT-PCR for miR-146a confirmed the up-regulation in PLC-Rs (Fig. 1B). Therefore, miR-146a was selected for further analysis.

3.2. Gain-of-function and loss-of-function of miR-146a alters the sensitivity of PLC/PRF/5 cells to IFN-α

To evaluate the effect of miR-146a on the response to IFN-α in PLC/PRF/5, pre-miR-146a was first transfected into PLC-P. Real-time qRT-PCR showed that transfection of pre-miR-146a markedly increased the miR-146a expression level for over 60 h (Fig. 2A). MTT assay demonstrated that transfection of pre-miR-146a into PLC-P cells induced resistance to IFN-α treatment (Fig. 2C). To further assess the effect of miR-146a on the IFN-α-sensitivity, anti-miR-146a was transfected into PLC-R1. Real-time qRT-PCR showed sufficient inhibition of miR-146a expression for over 60 h (Fig. 2B), and MTT assay demonstrated significant reduction of viability of anti-miR-146a-transfected cells, compared with the control cells (Fig. 2D). In order to clarify whether the altered IFN-α-sensitivity is due to the amount variability of apoptotic cells, we next performed annexin V assay. The results showed that IFN-α-induced apoptosis was reduced in pre-miR-146a-transfected PLC-P cells, and conversely, increased in anti-miR-146a-transfected PLC-R1 cells (Fig. 2E, F, and Supplementary Fig. 1A, B). These results indicate that, at least partially, inhibition of apoptosis contributes to miR-146a-induced IFN-α-resistance in PLC/PRF/5 cells.

3.3. miR-146a inhibits response to IFN-α by targeting SMAD4

Several genes have been identified to be the direct targets of miR-146a [26–29]. Among them, *SMAD4* is a potent tumor suppressor in various cancers and its activation promotes malignant potential, including chemoresistance, of colorectal cancer cells [30,31]. Based on this background, we further investigated the relevance of *SMAD4* in PLC/PRF/5 cells. Pre-miR-146a transfection decreased, and anti-miR-146a transfection increased SMAD4 protein level (Fig. 3A), suggesting that *SMAD4* is one of the target genes of miR-146a in PLC/PRF/5 cells. Next, we carried out siRNA for *SMAD4* to validate its involvement in the sensitivity to IFN-α. Knock down of *SMAD4* was confirmed by real-time qRT-PCR (Fig. 3B). MTT assay demonstrated that transfection of si*SMAD4* attenuated the sensitivity of PLC-P to IFN-α (Fig. 3C). Finally, we performed co-transfection of anti-miR-146a and si*SMAD4* to confirm the involvement of SMAD4 in miR-146a-related resistance to IFN-α. MTT assay using PLC-R3 cells showed that the growth-inhibitory effect of anti-miR-146a transfection only was weakened by co-transfection with si*SMAD4* (Fig. 3D). These results suggest that SMAD4 mediates, at least in part, the miR-146a-related resistance to IFN-α.

4. Discussion

In this study, we focused on miR-146a as a candidate regulator of IFN-α-resistance. Several studies have examined the involvement of miR-146a in various types of cancer. Most of these studies investigated the clinical significance of C/G polymorphism in *miR-146a* gene [32–34]. The cellular phenotypes regulated by miR-146a have also been analyzed, and the results of such studies have demonstrated that the impact of miR-146a on malignant potential differs according to the cell type. For examples, Li and colleagues [35] reported the tumor suppressive activity of miR-146a. Re-expression of miR-146a inhibited the invasive capacity of pancreatic carcinoma cells with concomitant downregulation of epidermal

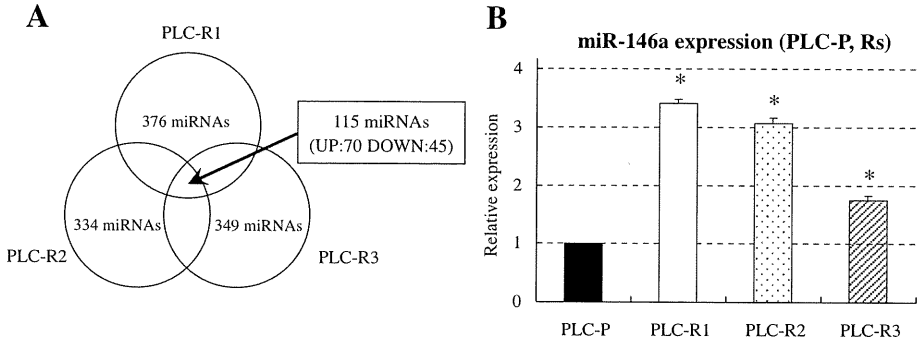

Fig. 1. Analysis of microRNA (miRNA) expression in parental PLC/PRF/5 (PLC-P) and their interferon (IFN)-α resistant clones (PLC-Rs). (A) Schematic diagram of the results of microarray analysis. The protocol identified 115 miRNAs with more than 1.2-fold up- or downregulated gene expression in the three types of cells. (B) Real-time quantitative reverse transcription-PCR (qRT-PCR) demonstrated significantly higher miR-146a expression in PLC-Rs than in PLC-P. Data are mean ± SD of triplicate experiments. *$P < 0.05$.

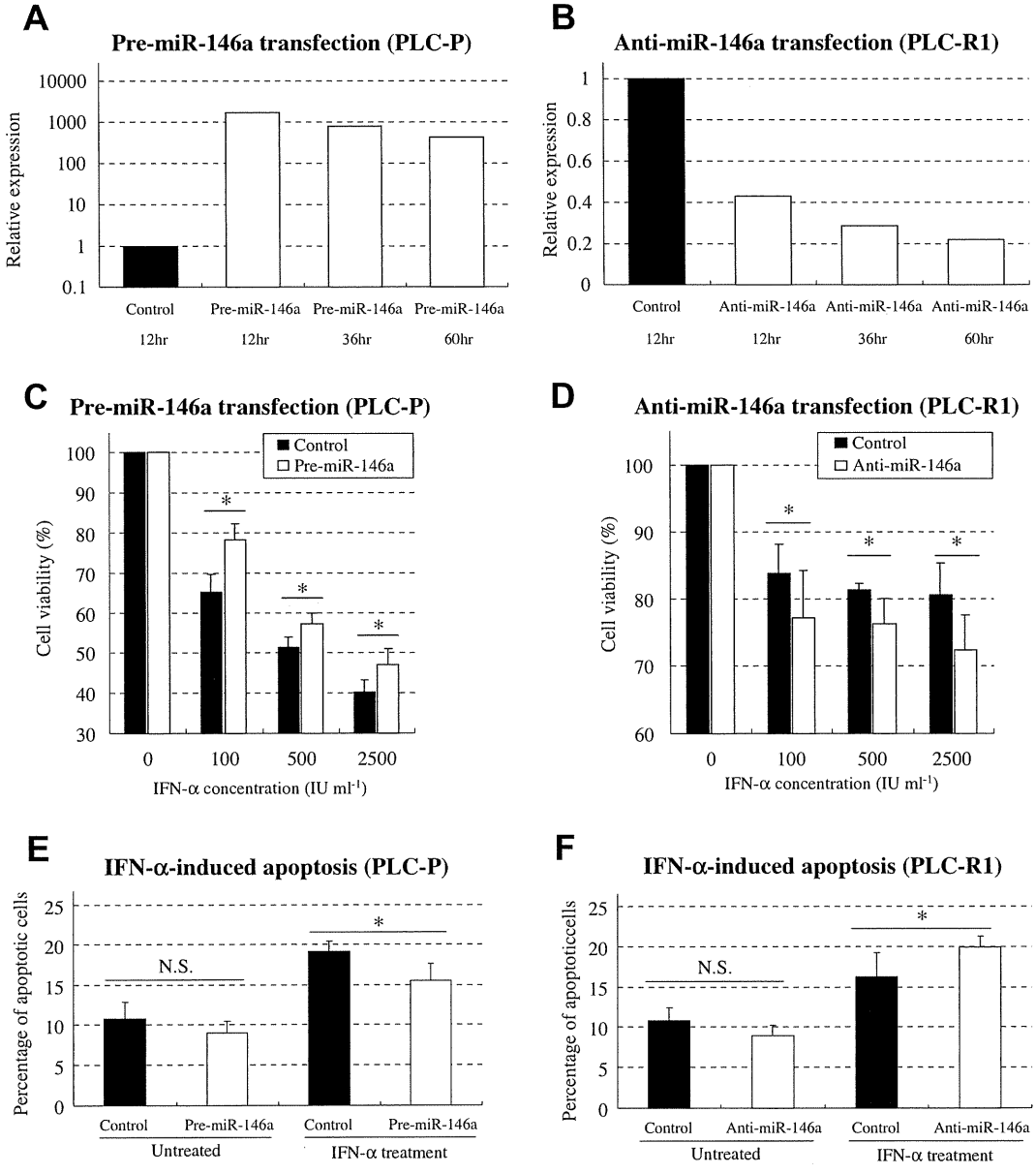

Fig. 2. miR-146a attenuated the IFN-α-sensitivity in PLC/PRF/5 cells. (A, B) Real-time qRT-PCR confirmed overexpression (PLC-P cells transfected with pre-miR-146a), and suppression (PLC-R1 cells transfected with anti-miR-146a) of miR-146a for more than 60 h. (C, D) MTT assay showed significant changes in the sensitivity to IFN-α by gain-of-function of miR-146a in PLC-P and loss-of-function in PLC-R1. (E, F) Pre-miR-146a transfection reduced, while anti-miR-146a transfection increased, IFN-α-induced apoptosis significantly. Apoptotic cells were defined as annexin V-positive cells [i.e., the sum of annexin V-positive/propidium iodide-positive (annexin V-positive/PI-positive) and annexin V-positive/PI-negative cells]. Data are mean ± SD of triplicate independent experiments. $^*P < 0.05$.

growth factor receptor (EGFR) and IL-1 receptor-associated kinase 1 (IRAK1) [35]. Lin et al. [27] reported that miR-146a targeted Rho-activated protein kinase 1 (ROCK1) and reduced the proliferation, invasion and metastasis potential of hormone-refractory prostate cancer cells. Conversely, Perske and co-workers [36] showed that miR-146a suppressed macrophage-induced death of mouse renal cell carcinoma cells by targeting inducible nitric oxide synthase (iNOS). Although many investigators have examined the significance of miR-146a in malignancies, only a few analyzed its role in resistance to anti-cancer drug. Pogribny et al. [37] performed miRNA microarray analysis using human breast cancer cell line and its cisplatin-resistant variant, and their results showed that miR-146a was most up-regulated in cisplatin-resistant cells.

On the other hand, the functional significance of miR-146a was first identified in innate immune response [26]. Furthermore, dysregulation of miR-146a was initially observed in autoimmune diseases [38]. Because miR-146a inhibits type I IFN pathway by targeting STAT1 [38], we first performed experiments of gain-of-function or loss-of-function of miR-146a, but these showed no changes in STAT1 protein levels (data not shown). It was concluded that STAT1 does not seem to be essential in miR-146a-related resistance to IFN-α in HCC cells.

Among the different members of the SMAD family of proteins, SMAD4 is considered as an important tumor suppressor in the transforming growth factor -β(TGF-β) signaling pathway [31]. Previous studies reported that changes in *SMAD4* gene correlated with various types of human cancer. For example, deletion and somatic alterations were most frequently observed in patients with pancreatic carcinoma and colorectal carcinoma [39]. However, somatic alteration of *SMAD4* was observed less commonly in HCC patients

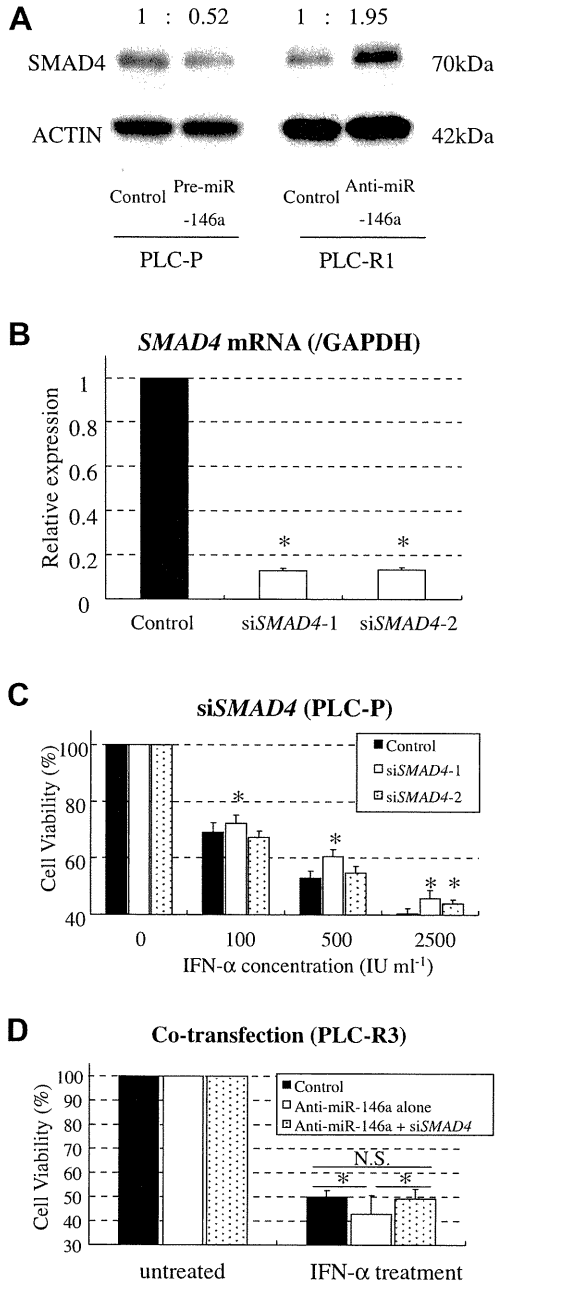

Fig. 3. The miR-146a-related resistance to IFN-α treatment is mediated through SMAD4. (A) Western blot analysis showed suppression of SMAD4 protein expression level by pre-miR-146a transfection, and enhancement by anti-miR-146a transfection. (B) Knock down of *SMAD4* in PLC-P was confirmed by real-time qRT-PCR. (C) MTT assay showed that knock down of *SMAD4* induced resistance to IFN-α treatment. (D) IFN-α-sensitivity provided by anti-miR-146a transfection was attenuated by co-transfection of si*SMAD4* in PLC-R3 cells (IFN-α treatment was performed at a concentration of 2500 IU ml^{-1}).

(6%) [40]. Functional analyses of SMAD4 in HCC cells were performed by Ji and colleagues [41,42], who showed that *SMAD4*-RNAi suppressed proliferation but promoted apoptosis and migration of TGF-β-treated SMMC-7721 cells. In the present study, we used PLC-P and PLC-Rs and showed that knock down of *SMAD4* induced IFN-α-resistance, and both gain-of-function and loss-of-function of miR-146a inversely altered the expression level of SMAD4 protein. Moreover, direct binding of miR-146a to 3′UTR of *SMAD4* gene was recently confirmed in NB4 cells, acute promyelocytic leukemia cell line [29]. Taken together, we propose that miR-146a inhibits IFN-α-induced apoptosis of HCC cells by targeting *SMAD4*. However, annexin V assay showed that the amount variability of apoptotic cells influenced by miR-146a was subtle albeit significant (Fig. 2E, F, and Supplementary Fig. 1A, B). Undiscovered miR-146a-mediated cellular mechanisms other than inhibition of apoptosis have to be determined hereafter.

We have reported the clinical efficiency of IFN-based therapy in HCC patients [5,9–11]. The mechanism of the anti-cancer effect of IFN-α in HCC was also analyzed. For example, IFNAR2 plays a crucial role in IFN-α-induced apoptosis of HCC cells [12]. The Wnt/β-catenin signaling pathway contributes to resistance to the anti-proliferative effect of IFN-α [13]. Furthermore, assessment of the expression levels of IFNAR2 and epithelial cell adhesion molecule (EpCAM), the target molecule of the Wnt/β-catenin signaling pathway, allowed the prediction of the clinical efficiency of IFN-based therapy [8,13]. IGFBP7, which was identified by cDNA microarray analysis using PLC-P and PLC-Rs, was also a useful predictor [14]. In spite of these perceptions, the prediction method established by our group remains imperfect, and the efficient modality for non-responders to IFN-based therapy has not yet been achieved.

Recent evidence suggests that miRNA is a suitable target for the treatment of cancer, based on the numerous advantages of miRNA-based therapeutics compared with conventional drug approach. For example, they regulate multiple components of the same cellular/physiological pathway and their effects are usually long-term [43]. Although several problems remain to be overcome, e.g., drug delivery system, off-target effects, or possible toxicity, a few studies employing animal models of liver diseases have already demonstrated the effectiveness of miRNA-based therapeutics [44,45]. The results of the present study demonstrated the role of miR-146a in the response to IFN-α in HCC cells. Although further assessment of the clinical significance is necessary, clinical application of therapeutics related to this miRNA seems feasible in future.

In conclusion, we demonstrated in the present study that miR-146a inhibited the anti-cancer effect of IFN-α in HCC cells, and that this effect was mediated by SMAD4. In addition, the response to IFN-α in PLC/PRF/5 cells was controlled by genetic manipulation of miR-146a and *SMAD4*. Considered together, the results suggest that miR-146a/SMAD4-mediated IFN-resistance is a potential legitimate target for the treatment of HCC.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2011.09.124.

References

[1] M. Kudo, N. Izumi, N. Kokudo, et al., Management of hepatocellular carcinoma in Japan: Consensus-based clinical practice guidelines proposed by the Japan society of hepatology (JSH) 2010 Updated Version, Dig. Dis. 29 (2011) 339–364.
[2] J. Bruix, M. Shermanm, Management of hepatocellular carcinoma: an update, Hepatology 53 (2011) 1020–1022.
[3] H.M. Kantarjian, A. Deisseroth, R. Kurzrock, et al., Chronic myelogenous leukemia: a concise update, Blood 82 (1993) 691–703.
[4] Medical Research Council Renal Cancer Collaborators. Interferon-alpha and survival in metastatic renal carcinoma: early results of a randomised controlled trial. Medical Research Council Renal Cancer Collaborators, Lancet 353 (1999) 14–17.
[5] M. Sakon, H. Nagano, K. Dono, et al., Combined intraarterial 5-fluorouracil and subcutaneous interferon-alpha therapy for advanced hepatocellular carcinoma with tumor thrombi in the major portal branches, Cancer 94 (2002) 435–442.
[6] Y.Z. Patt, B. Yoffe, C. Charnsangavej, et al., Low serum alpha-fetoprotein level in patients with hepatocellular carcinoma as a predictor of response to 5-FU and interferon-alpha-2b, Cancer 72 (1993) 2574–2582.
[7] T. Urabe, S. Kaneko, E. Matsushita, et al., Clinical pilot study of intrahepatic arterial chemotherapy with methotrexate, 5-fluorouracil, cisplatin and subcutaneous interferon-alpha-2b for patients with locally advanced hepatocellular carcinoma, Oncology 55 (1998) 39–47.
[8] H. Ota, H. Nagano, M. Sakon, et al., Treatment of hepatocellular carcinoma with major portal vein thrombosis by combined therapy with subcutaneous

interferon-alpha and intra-arterial 5-fluorouracil; role of type 1 interferon receptor expression, Br. J. Cancer 93 (2005) 557–564.
[9] H. Nagano, A. Miyamoto, H. Wada, et al., Interferon-alpha and 5-fluorouracil combination therapy after palliative hepatic resection in patients with advanced hepatocellular carcinoma, portal venous tumor thrombus in the major trunk, and multiple nodules, Cancer 110 (2007) 2493–2501.
[10] H. Nagano, M. Sakon, H. Eguchi, et al., Hepatic resection followed by IFN-alpha and 5-FU for advanced hepatocellular carcinoma with tumor thrombus in the major portal branch, Hepatogastroenterology 54 (2007) 172–179.
[11] H. Nagano, H. Wada, S. Kobayashi, et al., Long-term outcome of combined interferon-alpha and 5-fluorouracil treatment for advanced hepatocellular carcinoma with major portal vein thrombosis, Oncology 80 (2011) 63–69.
[12] B. Damdinsuren, H. Nagano, H. Wada, et al., Interferon alpha receptors are important for antiproliferative effect of interferon-alpha against human hepatocellular carcinoma cells, Hepatol. Res. 37 (2007) 77–83.
[13] T. Noda, H. Nagano, I. Takemasa, et al., Activation of Wnt/beta-catenin signalling pathway induces chemoresistance to interferon-alpha/5-fluorouracil combination therapy for hepatocellular carcinoma, Br. J. Cancer 100 (2009) 1647–1658.
[14] Y. Tomimaru, H. Eguchi, H. Wada, et al., Insulin-like growth factor-binding protein 7 alters the sensitivity to interferon-based anticancer therapy in hepatocellular carcinoma cells, Br. J. Cancer 102 (2010) 1483–1490.
[15] C.M. Croce, G.A. Calin, miRNAs, cancer, and stem cell division, Cell 122 (2005) 6–7.
[16] G.A. Calin, C.M. Croce, MicroRNA signatures in human cancers, Nat. Rev. Cancer 6 (2006) 857–866.
[17] T.E. Miller, K. Ghoshal, B. Ramaswamy, et al., MicroRNA-221/222 confers tamoxifen resistance in breast cancer by targeting p27Kip1, J. Biol. Chem. 283 (2008) 29897–29903.
[18] H. Yang, W. Kong, L. He, et al., MicroRNA expression profiling in human ovarian cancer: miR-214 induces cell survival and cisplatin resistance by targeting PTEN, Cancer Res. 68 (2008) 425–433.
[19] Y. Tomimaru, H. Eguchi, H. Nagano, et al., MicroRNA-21 induces resistance to the anti-tumour effect of interferon-alpha/5-fluorouracil in hepatocellular carcinoma cells, Br. J. Cancer 103 (2010) 1617–1626.
[21] T.D. Schmittgen, J. Jiang, Q. Liu, et al., A high-throughput method to monitor the expression of microRNA precursors, Nucleic Acids Res. 32 (2004) e43.
[22] T. Yamamoto, H. Nagano, M. Sakon, et al., Partial contribution of tumor necrosis factor-related apoptosis-inducing ligand (TRAIL)/TRAIL receptor pathway to antitumor effects of interferon-alpha/5-fluorouracil against hepatocellular carcinoma, Clin. Cancer Res. 10 (2004) 7884–7895.
[23] M. Kondo, H. Nagano, H. Wada, et al., Combination of IFN-alpha and 5-fluorouracil induces apoptosis through IFN-alpha/beta receptor in human hepatocellular carcinoma cells, Clin. Cancer Res. 11 (2005) 1277–1286.
[24] H. Eguchi, H. Nagano, H. Yamamoto, et al., Augmentation of antitumor activity of 5-fluorouracil by interferon alpha is associated with up-regulation of p27Kip1 in human hepatocellular carcinoma cells, Clin. Cancer Res. 6 (2000) 2881–2890.
[25] C. Berthou, L. Michel, A. Soulie, et al., Acquisition of granzime B and Fas ligand proteins by human keratiocytes to epidermal cell defence, J. Immunol. 159 (1997) 5293–5300.
[26] K.D. Taganov, M.P. Boldin, K.J. Chang, et al., NF-kappaB-dependent induction of microRNA miR-146, an inhibitor targeted to signaling proteins of innate immune responses, Proc. Natl. Acad. Sci. USA 103 (2006) 12481–12486.
[27] S.L. Lin, A. Chiang, D. Chang, et al., Loss of miR-146a function in hormone-refractory prostate cancer, RNA 14 (2008) 417–424.
[28] A.I. Garcia, M. Buisson, P. Bertrand, et al., Down-regulation of BRCA1 expression by miR-146a and miR-146b-5p in triple negative sporadic breast cancers, EMBO Mol. Med. 3 (2011) 279–290.
[29] H. Zhong, H.R. Wang, S. Yang, et al., Targeting Smad4 links microRNA-146a to the TGF-beta pathway during retinoid acid induction in acute promyelocytic leukemia cell line, Int. J. Hematol. 92 (2010) 129–135.
[30] P. Papageorgis, K. Cheng, S. Ozturk, et al., Smad4 inactivation promotes malignancy and drug resistance of colon cancer, Cancer Res. 71 (2011) 998–1008.
[31] G. Yang, X. Yang, Smad4-mediated TGF-beta signaling in tumorigenesis, Int. J. Biol. Sci. 6 (2010) 1–8.
[32] K. Jazdzewski, E.L. Murray, K. Franssila, et al., Common SNP in pre-miR-146a decreases mature miR expression and predisposes to papillary thyroid carcinoma, Proc. Natl. Acad. Sci. USA 105 (2008) 7269–7274.
[33] T. Xu, Y. Zhu, Q.K. Wei, et al., A functional polymorphism in the miR-146a gene is associated with the risk for hepatocellular carcinoma, Carcinogenesis 29 (2008) 2126–2131.
[34] R. Kogo, K. Mimori, F. Tanaka, et al., Clinical significance of miR-146a in gastric cancer cases, Clin. Cancer Res. 17 (2011) 4277–4284.
[35] Y. Li, T.G. Vandenboom 2nd, Z. Wang, et al., miR-146a suppresses invasion of pancreatic cancer cells, Cancer Res. 70 (2010) 1486–1495.
[36] C. Perske, N. Lahat, S. Sheffy Levin, et al., Loss of inducible nitric oxide synthase expression in the mouse renal cell carcinoma cell line RENCA is mediated by microRNA miR-146a, Am. J. Pathol. 177 (2010) 2046–2054.
[37] I.P. Pogribny, J.N. Filkowski, V.P. Tryndyak, et al., Alterations of microRNAs and their targets are associated with acquired resistance of MCF-7 breast cancer cells to cisplatin, Int. J. Cancer 127 (2010) 1785–1794.
[38] Y. Tang, X. Luo, H. Cui, et al., MicroRNA-146A contributes to abnormal activation of the type I interferon pathway in human lupus by targeting the key signaling proteins, Arthritis Rheum. 60 (2009) 1065–1075.
[39] M. Miyaki, T. Kuroki, Role of Smad4 (DPC4) inactivation in human cancer, Biochem. Biophys. Res. Commun. 306 (2003) 799–804.
[40] M.C. Yakicier, M.B. Irmak, A. Romano, et al., Smad2 and Smad4 gene mutations in hepatocellular carcinoma, Oncogene 18 (1999) 4879–4883.
[41] S. Huang, F. Zhang, L. Miao, et al., Lentiviral-mediated Smad4 RNAi induced anti-proliferation by p16 up-regulation and apoptosis by caspase 3 down-regulation in hepatoma SMMC-7721 cells, Oncol. Rep. 20 (2008) 1053–1059.
[42] A. Huang, S. Huang, F. Zhang, et al., Lentiviral-mediated Smad4 RNAi promotes SMMC-7721 cell migration by regulation of MMP-2, VEGF and MAPK signaling, Mol. Med. Rep. 3 (2010) 295–299.
[43] E. van Rooij, W.S. Marshall, E.N. Olson, Toward microRNA-based therapeutics for heart disease: the sense in antisense, Circ. Res. 103 (2008) 919–928.
[44] J. Kota, R.R. Chivukula, K.A. O'Donnell, et al., Therapeutic microRNA delivery suppresses tumorigenesis in a murine liver cancer model, Cell 137 (2009) 1005–1017.
[45] R.E. Lanford, E.S. Hildebrandt Eriksen, A. Petri, et al., Therapeutic silencing of microRNA-122 in primates with chronic hepatitis C virus infection, Science 327 (2010) 198–201.