**Table 3. Factors Associated with HCC After IFN Therapy**

| | Univariate Analysis | | Multivariate Analysis | |
|---|---|---|---|---|
| Risk Factor Value | Risk Ratio (95% CI) | P Value | Risk Ratio (95% CI) | P Value |
| Age (by every 10 year) | 2.2 (1.8-2.7) | <0.001 | 3.0 (1.9-4.8) | <0.001 |
| Sex | | | | |
| Female | 1 | | 1 | |
| Male | 1.2 (0.9-1.6) | 0.2 | 2.0 (1.0-3.8) | 0.04 |
| BMI (by every 10 kg/m$^2$) | 2.0 (1.2-1.3) | 0.005 | 1.1 (0.4-3.5) | 0.8 |
| Fibrosis stage | | | | |
| F0/F1/F2 | 1 | | 1 | |
| F3/F4 | 5.4 (3.9-7.5) | <0.001 | 2.5 (1.2-4.9) | 0.01 |
| Degree of steatosis | | | | |
| <10% | 1 | | 1 | |
| ≥10% | 4.5 (3.0-6.9) | <0.001 | 3.5 (1.9-6.4) | <0.001 |
| Esophagogastric varices | | | | |
| No | 1 | | 1 | |
| Yes | 3.3 (2.0-5.3) | <0.001 | 1.6 (0.6-4.4) | 0.3 |
| Virological response | | | | |
| SVR | 1 | | 1 | |
| Non-SVR | 3.3 (2.1-5.2) | <0.001 | 2.6 (1.2-5.5) | 0.001 |
| Genotype | | | | |
| Non-1 | 1 | | 1 | |
| 1 | 1.7 (1.2-2.5) | 0.006 | 1.0 (0.5-2.3) | 0.9 |
| Albumin (by every 1 g/dL) | 0.2 (0.1-0.3) | <0.001 | 0.6 (0.2-2.2) | 0.3 |
| ALT (by every 100 IU/L) | 1.0 (0.9-1.0) | 0.8 | 0.4 (0.1-1.8) | 0.6 |
| AST (by every 100 IU/L) | 1.2 (1.1-1.3) | 0.001 | 1.1 (0.6-1.8) | 0.8 |
| γ-GTP (by every 100 IU/L) | 1.3 (1.1-1.6) | 0.009 | 0.6 (0.3-1.6) | 0.3 |
| ALP (by every 100 IU/L) | 1.3 (1.2-1.5) | <0.001 | 0.6 (0.3-1.2) | 0.2 |
| Total bilirubin (by every 1 mg/dL) | 1.6 (1.3-2.1) | <0.001 | 1.2 (0.6-2.7) | 0.6 |
| Total cholesterol (by every 100 mg/dL) | 0.3 (0.2-0.6) | <0.001 | 0.2 (0.1-0.6) | 0.006 |
| Triglyceride (by every 100 mg/dL) | 0.8 (0.5-1.1) | 0.2 | 0.1 (0.02-1.1) | 0.08 |
| Fasting blood sugar (by every 100 mg/dL) | 1.8 (1.5-2.2) | <0.001 | 1.1 (1.0-1.1) | 0.04 |
| WBC (by every 100/μL) | 0.1 (0.03-0.3) | <0.001 | 0.1 (0.01-2.2) | 0.2 |
| RBC (by every 10$^6$/μL) | 0.5 (0.4-0.7) | <0.001 | 1.8 (0.7-4.4) | 0.2 |
| Platelet counts (by every 10$^6$/μL) | 0.3 (0.2-0.4) | <0.001 | 0.6 (0.3-1.5) | 0.3 |
| Baseline AFP (by every 10 ng/mL) | 1.0 (0.9-1.1) | 0.2 | 1.3 (1.0-1.7) | 0.04 |
| Post IFN AFP (by every 10 ng/mL) | 1.2 (1.1-1.3) | <0.001 | 1.9 (1.5-2.4) | <0.001 |
| HCV load (by every 100 KIU/mL) | 1.0 (0.9-1.0) | 0.4 | 1.0 (1.0-1.1) | 0.06 |
| IFN regimen | | | | |
| IFN monotherapy | 1 | | 1 | |
| IFN + RBV (24 W) | 1.2 (0.8-1.8) | 0.4 | 1.5 (0.7-3.2) | 0.3 |
| PEG-IFN monotherapy (48 W) | 1.1 (0.6-1.9) | 0.8 | 1.5 (0.4-5.5) | 0.6 |
| PEG-IFN + RBV | 0.4 (0.2-0.9) | 0.03 | 1.0 (0.3-3.1) | 0.9 |

Risk ratios for development of HCC were calculated by Cox proportional hazards regression analysis. AFP, alpha fetoprotein; ALP, alkaline phosphatase; ALT, alanine aminotransferase; AST, aspirate aminotransferase; BMI, body mass index; γ-GTP, gamma-glutamyltranspeptidase; HCC, hepatocellular carcinoma; IFN, interferon; PEG, pegylated; RBC, red blood cell counts; RBV, ribavirin; SVR, sustained virological response; WBC, white blood cell count.

may be at a higher risk of developing HCC. Further study is necessary to confirm this association in a clinical situation. Because several developed countries are in the midst of a growing obesity epidemic, the risk related to obesity cannot be ignored in patients with chronic hepatitis C who are treated with interferon.

Several retrospective cohort studies have been conducted to evaluate the effect of interferon on the incidence of HCC among patients with chronic hepatitis C.[8-11] Our results, obtained from one of the largest cohort studies, confirm the efficacy of viral eradication in preventing HCC. In one study conducted in a Western population, no statistically significant reduction was found in the development of HCC among patients with SVR compared with those without SVR (adjusted hazard ratio, 0.46; 95% CI, 0.12-1.70; $P = 0.25$).[12] Because relatively few occurrences of HCC were observed in this cohort, and the duration of follow-up was shorter, the differences in HCC development between patients with and without SVR might be less pronounced.

Interestingly, our results demonstrated that the risk for HCC remains even after achieving SVR in older patients, confirming the findings of previous studies conducted with a smaller number of patients.[22,23] The cumulative incidence of HCC during the first 5 years

**Table 4. Factors Associated with Development of HCC After Achieving SVR**

| Risk Factor | Odds Ratio (95% CI) | *P*-value |
|---|---|---|
| Univariate analysis | | |
| Age (by every 10 year) | 3.2 (1.8-5.5) | <0.001 |
| Sex | | |
| Female | 1 | |
| Male | 3.0 (1.0-8.8) | 0.04 |
| Fibrosis stage | | |
| F0/F1/F2 | 1 | |
| F3/F4 | 5.9 (2.5-14.0) | <0.001 |
| Degree of steatosis | | |
| <10% | 1 | |
| ≥10% | 5.5 (2.0-15.2) | 0.001 |
| BMI (by every 10 kg/m$^2$) | 3.2 (0.8-12.6) | 0.09 |
| ALT (by every 10 IU/L) | 0.9 (0.7-1.3) | 0.7 |
| AST (by every 10 IU/L) | 1.1 (0.9-1.4) | 0.3 |
| Genotype | | |
| Non-1 | 1 | |
| 1 | 1.2 (0.6-3.0) | 0.5 |
| HCV load (by every 100 KIU/mL) | 0.9 (0.8-1.0) | 0.2 |
| IFN regimen | | |
| IFN monotherapy | 1 | |
| IFN + RBV (24 W) | 0.7 (0.2-2.3) | 0.5 |
| PEG-IFN monotherapy (48 W) | 0.8 (0.2-3.6) | 0.8 |
| PEG-IFN + RBV | 0.3 (0.03-2.0) | 0.2 |
| Multivariate analysis | | |
| Age (by every 10 year) | 2.7 (1.5-5.1) | 0.002 |
| Sex | | |
| Female | 1 | |
| Male | 4.1 (0.9-18.9) | 0.06 |
| Fibrosis stage | | |
| F0/F1/F2 | 1 | |
| F3/F4 | 2.6 (0.9-7.5) | 0.08 |
| Degree of steatosis | | |
| <10% | 1 | |
| ≥10% | 5.6 (1.9-16.5) | 0.002 |

Odds ratios for SVR were calculated by logistic regression analysis.

ALT, alanine aminotransferase; AST, aspartate aminotransferase; BMI, body mass index; HCV, hepatitis C virus; IFN, interferon; HCC, hepatocellular carcinoma; PEG, pegylated; RBV, ribavirin; SVR, sustained virological response.

after completion of interferon therapy was similar between SVR and non-SVR patients in the older age group, and the risk for HCC remained for 9 years after eradication of HCV in our patients. Therefore, HCC patients with SVR who have a risk factor should be screened for at least 5-10 years after the completion of interferon therapy.

It has been reported that coffee consumption has a protective effect against hepatocarcinogenesis[24,25] and liver disease progression in patients with chronic HCV infection.[26] Because we could not review coffee consumption in all the patients and fewer data were available in the previous literature as to whether a habitual change of reducing coffee consumption occurs in older patients, it is unclear whether increased risk for HCC in older patients is an effect of this habitual change in older patients. However, the majority (68%) of Japanese patients who have HCV (n = 1058) drink less than 1 cup of coffee per day, and only 7.6% consume more than 3 cups of coffee per day.[27] Therefore, it is unlikely that a habitual change in older patients affects the increased risk for hepatocarcinogenesis in older patients.

Recently, it was reported that interferon therapy might be less effective in preventing HCC among patients with chronic hepatitis C who are positive for anti-HBc antibody,[28] but this finding is still controversial.[29,30] In the present study, anti-HBc was only detected in 4 of 22 patients in whom HCC developed after viral eradication, and age distribution was similar among anti-HBc-positive and anti-HBc-negative patients. Because no significant difference in mean age was found between anti-HBc-positive and anti-HBc-negative patients in the recent study conducted in Japan,[28] it is unlikely that previous exposure to hepatitis B virus or occult hepatitis B virus infection is responsible for the difference in risk for HCC between younger and elderly patients found in the present study.

In conclusion, aging has become one of the most important risk factors for HCC. Even after stratification by stage of fibrosis, the risk for HCC after antiviral treatment was significantly higher in older patients, and HCV eradication had a smaller effect on HCC-free survival in older patients. Patients with HCV should therefore be identified at an earlier age and antiviral treatment should be initiated. The present results have potentially important clinical implications for physicians that may influence their decisions about the treatment strategy in individual patients.

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ORIGINAL ARTICLE—LIVER, PANCREAS, AND BILIARY TRACT

# Systemic combination therapy of intravenous continuous 5-fluorouracil and subcutaneous pegylated interferon alfa-2a for advanced hepatocellular carcinoma

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**Abstract**
*Background* In Japan, sorafenib is now the first-line therapy for individuals with advanced hepatocellular carcinoma (HCC), but no other treatment is available for such patients. The aim of this study was to assess the efficacy and safety of combination therapy with systemic continuous intravenous infusion of 5-fluorouracil (5-FU) and subcutaneous peginterferon alfa-2a, which was used before sorafenib was introduced to Japan.
*Methods* Two hundred and twenty-three HCC patients, who were not amenable to curative surgery, percutaneous ablation, or transarterial chemoembolization (TACE), and for whom intraarterial chemotherapy was not indicated because of the presence of extrahepatic metastasis or stenosis of the common hepatic artery, received peginterferon alfa-2a (90 μg subcutaneously on days 1, 8, 15, and 22) and 5-FU (500 mg/day intravenously given continuously on days 1–5 and 8–12). We assessed their response to treatment and survival, and treatment safety.
*Results* The response rate was 9.4 % (including six patients with complete response) and the disease-control rate was 32.7 %. The median time to progression was 2.0 months. The overall median survival time was 6.5 months (Child–Pugh class A: 9.2 months vs. Child–Pugh class B: 2.8 months). In a multivariate analysis, Eastern Cooperative Oncology Group (ECOG) performance status >0, Child–Pugh class B, and the presence of macroscopic vascular invasion were independent predictors of poor prognosis. The major grade 3–4 adverse events were leucopenia (13.9 %) and thrombocytopenia (5.8 %). No treatment-related deaths occurred.
*Conclusions* This combination therapy was well tolerated and showed promising efficacy. Further studies are needed to establish the usefulness of this treatment.

**Keywords** Hepatocellular carcinoma · Systemic chemotherapy · Survival analysis · Time to progression

**Abbreviations**

| | |
|---|---|
| AIC | Akaike information criterion |
| ALT | Alanine aminotransferase |
| AST | Aspartate aminotransferase |
| CR | Complete response |
| CT | Computed tomography |
| DCP | Des-gamma-carboxy prothrombin |
| ECOG | Eastern Cooperative Oncology Group |
| HBV | Hepatitis B virus |
| HCC | Hepatocellular carcinoma |
| HCV | Hepatitis C virus |
| MRI | Magnetic resonance imaging |
| MST | Median survival time |
| NA | Not assessable |
| PD | Progressive disease |
| PR | Partial response |

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| | |
|---|---|
| RECIST | Response to treatment in solid tumors |
| SD | Stable disease |
| TACE | Transcatheter arterial chemoembolization |
| TTP | Time to progression |
| 5-FU | 5-Fluorouracil |

## Introduction

Hepatocellular carcinoma (HCC) is a leading cause of cancer-related death, with a particularly high incidence in Asian countries, including Japan [1, 2]. HCC usually develops in a liver already suffering from chronic disease, most notably due to hepatitis B virus (HBV) or hepatitis C virus (HCV) infection [3]. In the past, HCC was diagnosed often only at a very advanced stage, which was associated with a very poor prognosis [4]. Close surveillance of designated high-risk patients, using advanced diagnostic modalities, has now facilitated HCC detection at a much earlier stage. Together with the considerable advances in HCC treatment, such as surgical resection, percutaneous ablation, transcatheter arterial chemoembolization (TACE), and liver transplantation, the survival time of HCC patients has been much prolonged in recent years [5–10].

However, the potentially curative treatment modalities described above are not indicated for patients with advanced HCC with extrahepatic metastasis or macroscopic vascular invasion , and their prognosis remains poor. In two recent large randomized controlled trials, sorafenib, a multi-kinase inhibitor, significantly prolonged survival in patients with advanced HCC, even when the primary lesion was associated with vascular invasion or extrahepatic metastases, and this agent is now widely regarded as the standard treatment for such patients [11, 12]. However, even with sorafenib, the median survival time (MST) of such patients is rather short, ranging from 6.5 to 10.7 months. Thus, the development of new drugs or new regimens that include cytotoxic and molecular-targeted agents still remains necessary.

Previously, we reported the efficacy of therapy using a combination of intrahepatic arterial 5-fluorouracil (5-FU) and subcutaneous interferon alfa for patients with advanced HCC with portal venous invasion [13]. Because most intraarterially administered 5-FU is taken up by the liver during the first pass, this combination chemotherapy would not be effective against extrahepatic metastasis. Nevertheless, the mechanism underlying the chemotherapy with intraarterial 5-FU would function if 5-FU could reach extrahepatic lesions via systemic administration. Therefore, we expected that a combination of systemic intravenous 5-FU and subcutaneous interferon would be effective against extrahepatic metastasis of HCC. We report the efficacy and safety of this treatment for advanced HCC, which we performed before sorafenib was introduced to Japan.

## Patients, materials, and methods

### Patients

The present study was conducted as a retrospective cohort study. We analyzed 223 consecutive patients who received combination therapy comprised of continuous intravenous infusion of 5-FU and subcutaneous pegylated interferon-alfa for advanced HCC at Kyoundo Hospital from January 1, 2004, to May 31, 2009, when sorafenib was licensed in Japan. The study population consisted of patients with advanced HCC who were not amenable to curative surgery, percutaneous ablation, or TACE, and for whom intraarterial chemotherapy was not indicated because of the presence of extrahepatic metastasis or stenosis of the common hepatic artery. Patients with a previous history of treatment, including systemic chemotherapy, were included. The eligibility criteria also included an Eastern Cooperative Oncology Group (ECOG) performance status score of 2 or less [14], Child–Pugh liver function class A or B, adequate hematologic function (white blood cell count, $\geq$3000/μL; hemoglobin, $\geq$8.5 g/dL; platelet count >30000/μL; and prothrombin time international normalized ratio, $\leq$2.3), adequate hepatic function (albumin, $\geq$2.8 g/dL; total bilirubin, $\leq$3 mg/dL; and alanine aminotransferase [ALT] and aspartate aminotransferase [AST], $\leq$5 times the upper limit of the normal range), and adequate renal function (serum creatinine, $\leq$1.5 times the upper limit of the normal range). Patients were required to have at least one measurable target lesion according to the response to treatment in solid tumors (RECIST) guidelines ver. 1.0 [15]. All patients provided written informed consent before treatment. The treatment protocol was approved by the ethics committee of the institution.

### Diagnosis of HCC

Intrahepatic lesions, vascular invasion, and extrahepatic metastasis of HCC were diagnosed with contrast-enhanced computed tomography (CT) or magnetic resonance imaging (MRI), considering hyperattenuation in the arterial phase with washout in the late phase as the definitive sign of HCC [16, 17]. Ultrasound-guided tumor biopsy was also performed when radiological findings were atypical. Bone scintigraphy was added when bone metastasis was suspected because of symptoms but was not confirmed on CT or MRI.

### Treatment

One cycle of this treatment consisted of 4 weeks (days 1–28). Peginterferon alfa-2a (90 μg) was administered subcutaneously on days 1, 8, 15, and 22, and 5-FU (500 mg/day) was systemically administered via continuous intravenous infusion, using a portable infusion pump, on days 1–5 and 8–12. Treatment was continued until disease progression, unacceptable toxicity, or patient refusal occurred. This protocol had no treatment interval, and the next cycle started on the day after day 28 of the previous cycle. The first one or two treatment cycles were provided during hospitalization and 5-FU was administered through a peripheral intravenous catheter. Patients who could be expected to survive for a relatively long period underwent implantation of an indwelling central intravenous catheter and were treated on an outpatient basis thereafter. Indwelling central intravenous catheters were inserted by ultrasound-guided subclavian vein puncture and the catheter tip was placed into the superior vena cava using a guidewire under fluoroscopic guidance. When adverse events caused by 5-FU became clinically important, the dose of 5-FU was reduced by 50 %. As prevention and treatment for stomatitis, sodium gualenate hydrate and sodium bicarbonate were used as a gargle. Dexamethasone ointment was also used for stomatitis. Antidiarrheal agents such as loperamide hydrochloride were used for diarrhea.

### Response and toxicity assessment

To assess the response to treatment, contrast-enhanced CT or MRI was performed at the end of the first and second cycles and every two cycles thereafter. In principle, treatment responses were evaluated according to the RECIST guidelines ver.1.0 [15]. The best overall response was adopted in the analysis. Complete response (CR) was defined as the disappearance of both intrahepatic lesions and extrahepatic metastasis. CR was confirmed by repeat assessments performed 4 weeks or more after the criteria for response were first met. Patients who had not completed the first cycle were regarded as having progressive disease (PD) if radiological disease progression was confirmed at the time, and as "not assessable (NA)" if imaging was not performed at the time. Toxicity was evaluated using the National Cancer Institute Common Toxicity Criteria version 3.0. During hospitalization, patients were interviewed about their symptoms and underwent a daily physical examination. Blood tests were performed every week. When treated as outpatients, they were required to visit the outpatient department at least once every 2 weeks.

### Statistical analysis

We included in the analysis those patients who could not complete the first cycle. The categorical variables were compared by $\chi^2$tests, whereas continuous variables were compared with an unpaired Student's *t*-test (parametric) or Mann–Whitney *U*-test (nonparametric). A $P$ value of $<0.05$ was considered statistically significant. Overall survival and time to progression (TTP) were calculated using the Kaplan–Meier method. Patients were censored at the time of the last visit, when lost to follow up, or at the end of the study period. Follow up ended on June 30, 2010. The clinical data at baseline were assessed as predictors of survival using univariate and multivariate Cox proportional hazard regression analysis. The following variables were included in this analysis: age, sex, ECOG performance status, hepatitis B surface antigen (HBsAg), hepatitis C virus antibody (HCVAb), Child–Pugh classification, platelet count, Barcelona-Clinic Liver Cancer (BCLC) staging classification [18], presence of viable intrahepatic lesions, macroscopic vascular invasion, extrahepatic metastasis, and a history of previous treatment. Stepwise variable selection with the Akaike information criterion (AIC) was used to find the best model in multivariate analysis. All analytical procedures were performed with S-plus Ver. 7.0 (Insightful, Seattle, WA, USA).

## Results

### Patients

A total of 223 patients, 176 male and 47 female, with an average age of 64.3 years, received this treatment. Patient characteristics are listed in Table 1. Child–Pugh classification was A in 166 patients (74.4 %) and B in 57 (25.6 %). Macroscopic vascular invasion was present in 103 patients (46.2 %). Extrahepatic metastasis was present in 166 (74.4 %) patients. Those patients without extrahepatic metastasis who were treated with this regimen had contraindications to intraarterial chemotherapy because of stenosis of the common hepatic artery, mainly due to repeated TACE. Two hundred and ten (94.2 %) patients had previously received some other treatment. The median number of cycles of the combination treatment was two (range 1–13). Four patients did not complete the first cycle because of deterioration of performance status, unacceptable toxicity, or patient refusal.

### Response to treatment

Six patients had CR (2.7 %), 15 (6.7 %) had a partial response (PR), 52 (23.3 %) had stable disease (SD), and

**Table 1** Demographic and baseline characteristics of patients ($n = 223$)

| Variable, *n* (%) | |
|---|---|
| Age (years)[a] | 64.3 ± 10.6 |
| Male sex | 176 (78.9) |
| ECOG performance status | |
| 0 | 159 (71.3) |
| 1 | 57 (25.6) |
| 2 | 7 (3.1) |
| Viral infection | |
| HBsAg, positive | 58 (26.0) |
| Anti HCVAb, positive | 125 (56.1) |
| Both positive | 4 (1.8) |
| Both negative | 36 (16.1) |
| Child–Pugh classification | |
| Class A | 166 (74.4) |
| Class B | 57 (25.6) |
| Platelet count ($10^3/\mu l$)[b] | 127 (34–840) |
| BCLC stage | |
| B | 22 (9.9) |
| C | 201 (90.1) |
| Viable intrahepatic lesion, present | 213 (95.5) |
| Macroscopic vascular invasion, present[c] | 103 (46.2) |
| Portal vein | 73 |
| Hepatic vein or vena cava | 51 |
| Maximum tumor size (cm)[b] | 5.2 (1.0–20.0) |
| AFP >100 ng/mL | 143 (64.1) |
| AFP-L3 >15.0 %[d] | 147 (66.2) |
| DCP >100 mAU/mL[e] | 152 (68.8) |
| Extrahepatic metastasis, present[c] | 166 (74.4) |
| Lung | 91 |
| Lymph node | 52 |
| Bone | 33 |
| Adrenal gland | 11 |
| Dissemination | 20 |
| Others | 5 |
| Previous therapy[c] | |
| None | 13 (5.8) |
| Surgical resection | 78 (35.0) |
| Percutaneous ablation | 95 (42.6) |
| Transarterial chemoembolization | 150 (67.3) |
| Radiotherapy | 32 (14.3) |
| Transarterial chemotherapy | 65 (29.1) |
| Systemic chemotherapy | 46 (20.6) |
| Cycles of systemic 5-FU + IFN therapy[b] | 2 (1–13) |

*ECOG* Eastern Cooperative Oncology Group, *HBsAg* hepatitis B surface antigen, *HCVAb* hepatitis C virus antibody , *BCLC* Barcelona-Clinic Liver Cancer, *AFP* alpha fetoprotein, *DCP* des-gamma-carboxy prothrombin, *5-FU* 5-fluorouracil, *IFN* interferon

[a] Mean ± SD

[b] Median (range)

[c] Including overlap

[d] Missing in one case

[e] Missing in two cases

**Table 2** Summary of efficacy measures ($n = 223$)

| | |
|---|---|
| Level of response, *n* (%) | |
| Complete response | 6 (2.7) |
| Partial response | 15 (6.7) |
| Stable disease | 52 (23.3) |
| Progressive disease | 132 (59.2) |
| Not assessable | 18 (8.1) |
| Response rate (%) | 9.4 |
| Disease-control rate (%) | 32.7 |
| Time to progression (months) | |
| Median | 2.0 |
| 95 % confidence interval (CI) | 2.0–3.1 |
| Overall survival (months) | |
| Median | 6.5 |
| 95 % CI | 5.13–9.13 |
| 1-year survival rate (%) | 31.2 |
| 2-year survival rate (%) | 12.7 |
| 3-year survival rate (%) | 7.1 |

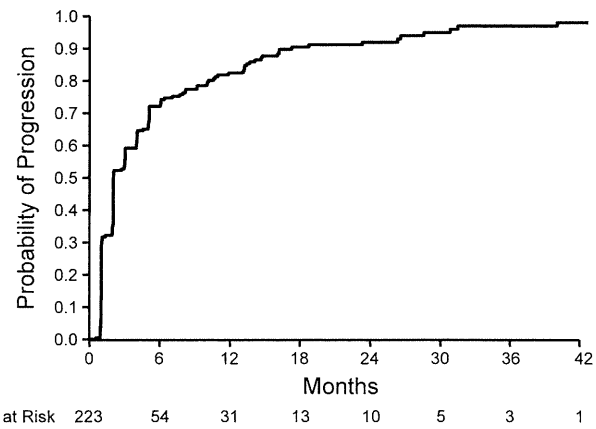


**Fig. 1** Kaplan–Meier analysis of time to progression

132 (59.2 %) had PD. Treatment response was not assessable in the remaining 18 (8.1 %) patients due to symptomatic PD or their being lost to follow up before evaluation. The response rate was 9.4 % and the disease-control rate was 32.7 % (Table 2). The median TTP was 2.0 months (Fig. 1). There was no statistically significant difference in TTP between Child–Pugh class A and class B patients (median 3.0 vs. 2.0 months, $P = 0.19$).

## Survival

The overall MST was 6.5 months (Fig. 2a). The survival rates at 1, 2, and 3 years were 31.2, 12.7, and 7.1 %, respectively (Table 2). MST was significantly longer in Child–Pugh class A as compared with class B patients (9.2 vs. 2.8 months, $P < 0.001$) (Fig. 2b). The MSTs of patients

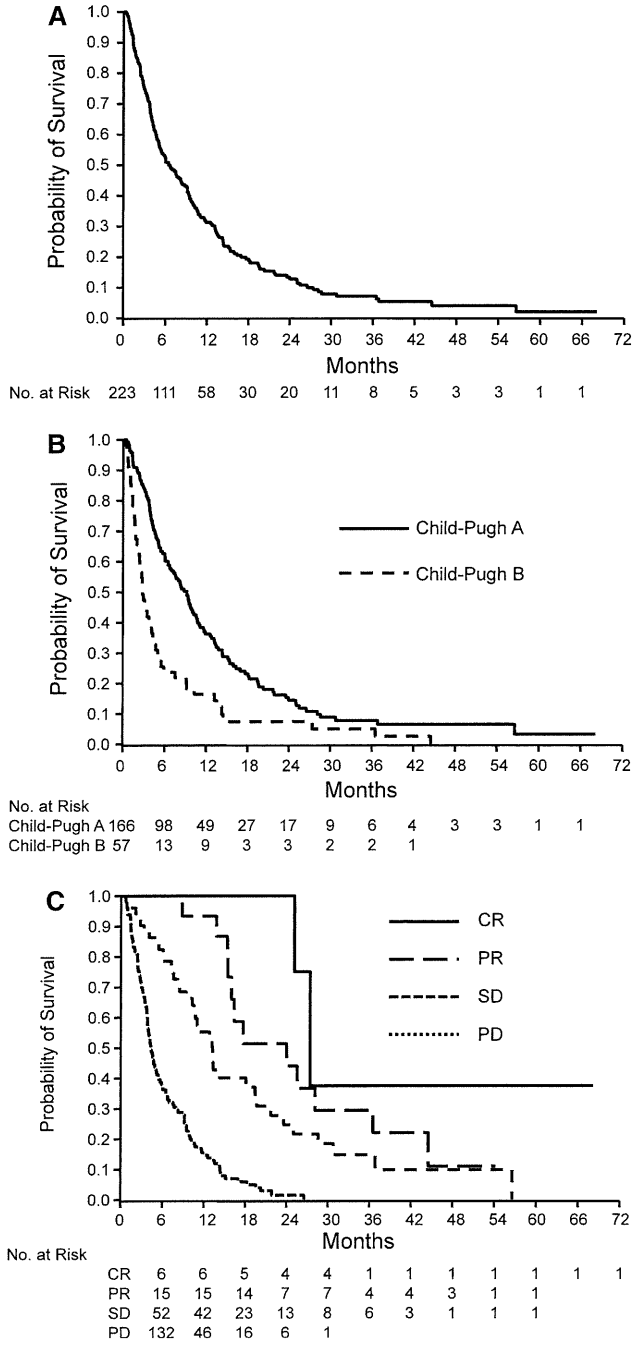


**Fig. 2** Kaplan–Meier analysis of overall survival (**a**); stratified based on Child–Pugh classification (**b**) and response to treatment (**c**). *CR* complete response, *PR* partial response, *SD* stable disease, *PD* progressive disease

with CR, PR, SD, and PD were 27.4, 24.0, 13.2, and 4.4 months, respectively (Fig. 2c, $P < 0.001$). Based on a univariate analysis, the following factors were significantly associated with shorter survival time: ECOG performance status >0, Child–Pugh class B, and presence of macroscopic vascular invasion (Table 3). A multivariate analysis showed that all of these factors were also independent prognostic factors (Table 4).

**Table 3** Predictors of overall survival: univariate analysis ($n = 223$)

| Variable | Hazard ratio (95 % CI) | *P* |
|---|---|---|
| Age (years) >65 | 1.01 (0.76–1.35) | 0.94 |
| Male sex | 1.03 (0.73–1.45) | 0.87 |
| ECOG performance status >0 | 1.73 (1.25–2.39) | <0.001 |
| HBsAg, positive | 0.87 (0.63–1.20) | 0.38 |
| Anti HCVAb, positive | 1.06 (0.80–1.42) | 0.68 |
| Child–Pugh class B versus A | 2.12 (1.54–2.92) | <0.001 |
| Platelet count >127,000/μL | 1.25 (0.94–1.67) | 0.13 |
| BCLC stage C | 1.46 (0.89–2.41) | 0.14 |
| Viable intrahepatic lesion, present | 1.85 (0.76–4.49) | 0.17 |
| Macroscopic vascular invasion, present | 1.37 (1.03–1.83) | 0.03 |
| Extrahepatic metastasis, present | 1.35 (0.97–1.87) | 0.08 |
| Previous chemotherapy, present | 1.16 (0.86–1.55) | 0.34 |

**Table 4** Predictors of overall survival: multivariate analysis ($n = 223$)

| Variable | Hazard ratio (95 % CI) | *P* |
|---|---|---|
| ECOG performance status >0 | 1.46 (1.04–2.05) | 0.03 |
| Child–Pugh class B | 1.83 (1.31–2.55) | <0.001 |
| Macroscopic vascular invasion, present | 1.39 (1.03–1.88) | 0.03 |
| Extrahepatic metastasis, present | 1.35 (0.96–1.92) | 0.09 |

**Table 5** Safety profile

| | Grade 1–2, *n* (%) | Grade 3–4, *n* (%) |
|---|---|---|
| Leukopenia | 25 (11.2) | 31 (13.9) |
| Anemia | 0 (0) | 1 (0.4) |
| Thrombocytopenia | 20 (9.0) | 13 (5.8) |
| Stomatitis | 11 (4.9) | 3 (1.3) |
| Anorexia | 2 (0.9) | 1 (0.4) |
| Diarrhea | 2 (0.9) | 0 (0) |
| Skin rash | 2 (0.9) | 1 (0.4) |

## Safety

Adverse events graded as 3 or 4 were observed in 28 (12.6 %) patients. The incidence of major adverse events is presented in Table 5. The major grade 3–4 adverse events were leucopenia (13.9 %) and thrombocytopenia (5.8 %). A common non-hematological toxicity was stomatitis (6.2 %, any grade). Fever, which was mostly low-grade, occurred in about 90 % of the patients, usually after the first administration of peginterferon, and was gradually

attenuated during subsequent administrations. Elevations in bilirubin, AST, and ALT levels from baseline occurred in 7.6 % of patients, although most cases of such elevation occurred due to progression of the intrahepatic lesion, and not due to the treatment itself. There were no catheter-related problems, including infection or occlusion. No treatment-related deaths occurred.

## Discussion

Wadler et al. first reported combination therapy with intravenous 5-FU and subcutaneous interferon for a malignant neoplasm. They treated 30 patients with advanced colorectal cancer using this protocol [19]. However, the following phase III trial failed to establish the efficacy of the treatment [20]. Subsequently, Patt et al. [21] reported systemic combination therapy for HCC patients, reporting that the treatment induced a decrease of more than 50 % in the size of each measurable lesion in 18 % of the treated patients. Since then, several studies have demonstrated the efficacy of combination therapy of intraarterial 5-FU and subcutaneous interferon for patients with advanced HCC with portal venous invasion, reporting response rates of 44–63 % [13, 22, 23]. Furthermore, other studies have revealed the mechanism underlying the antitumor effects of this combination therapy [24–31]. However, only a case series of a small number of patients has reported on this systemic combination therapy in HCC patients [32]. The present study is the first report of this therapy in a large number of patients ($n = 223$).

In the past, systemic chemotherapy for advanced HCC using various cytotoxic agents, such as doxorubicin, 5-FU, cisplatin, and etoposide, has been investigated. However, few agents showed response rates above 20 %, and the number of patients included in those studies was small. Furthermore, no regimens demonstrated convincing survival benefits in phase III trials [33, 34]. Single-agent 5-FU [35–37] and related drugs such as eniluracil/5-FU [38, 39] and uracil/tegafur [40, 41] showed low response rates. An impressive result came from phase II and phase III studies of PIAF (combination of cisplatin, interferon alfa, doxorubicin, and 5-FU). The response rates of these studies were 26 and 20.9 %, respectively [42, 43], which were actually better than that of the present study, although the number of patients was small and the characteristics of the patients differed from those in our study.

At present, sorafenib is the standard treatment for advanced HCC with extrahepatic metastasis or vascular invasion. Before the availability of sorafenib, we treated such patients with a combination of systemic intravenous 5-FU and subcutaneous interferon. The MSTs in the SHARP study and the Asian-Pacific study of sorafenib (both randomized controlled trials) were 10.7 and 6.5 months, respectively, whereas the MST in the present study was 6.5 months. However, both these trials of sorafenib consisted only of Child–Pugh class A patients, and the MSTs in these two studies were comparable to the MST of the Child–Pugh class A patients in our study (9.2 months). The disease-control rate in our study was 32.7 %, which was comparable to that of sorafenib (43 % in the SHARP study; 35.3 % in the Asian-Pacific study). Moreover, there were no complete responders in either of these randomized controlled trials, and the response rates were also low (2 % in the SHARP study; 3.3 % in the Asian-Pacific study). On the other hand, in the present study, six (2.7 %) patients achieved a complete response, and the response rate of 9.4 % was higher than that in these two studies. Thus, the combination of intravenous 5-FU and subcutaneous interferon is worth consideration as a choice of treatment for advanced HCC.

The response rate of 52.6 % that we observed in our previous study where we treated HCC patients with portal venous invasion with a combination of intraarterial 5-FU and subcutaneous interferon [13] was much better than that observed here. This may be partly because the local concentration of 5-FU in the liver is higher after intraarterial infusion than after systemic administration. However, systemic rather than intraarterial administration is appropriate for patients with extrahepatic metastases because intraarterially administered 5-FU is substantially removed by the liver in the first pass [44, 45].

In our previous study [13], we combined interferon alfa, not pegylated, with the intraarterial administration of 5-FU. Here, we combined pegylated interferon alfa with the systemic administration of 5-FU mainly because of the convenience in an outpatient setting. Whereas non-pegylated interferon needs to be administered three times a week, pegylated interferon requires only once-a-week administration.

Cirrhotic patients have lower clearance rates of 5-FU than non-cirrhotic patients [46]. Thus, such patients with poor liver function may have more severe adverse events. However, there were few serious adverse events in the present study, although as many as 25.6 % of the patients were Child–Pugh class B. Although grade 3 or 4 leucopenia and thrombocytopenia were observed, the baseline white blood cell and platelet counts in the patients with these events were almost always low because of background cirrhosis, and they were able to continue to receive treatment. In addition, we did not observe any serious adverse events in relation to infection.

According to our data, ECOG performance status, Child–Pugh classification, and the presence of vascular invasion were independent prognostic factors. This is consistent with our previous study findings on the prognosis of patients with

extrahepatic metastasis of HCC [47]. In the present study, we also analyzed prognosis as stratified by treatment response, and better treatment response resulted in better prognosis. This point is to be confirmed in future prospective studies.

The combination therapy described in the present study was performed before the advent of sorafenib. It will now be important to evaluate the efficacy of this combination therapy in cases of sorafenib failure. It is also necessary to assess the efficacy and safety of this treatment, as well as that of sorafenib, for patients with poor liver function [48, 49].

In conclusion, the combination of continuous intravenous infusion of 5-FU and subcutaneous peginterferon alfa-2a was well tolerated and showed promising efficacy in a subset of patients with advanced HCC. Further studies; for example validating the efficacy of this treatment in patients with sorafenib failure and conducting a randomized controlled trial comparing this treatment with sorafenib, are needed to definitively establish the usefulness of this treatment.

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HEPATOLOGY

# Genetic risk of hepatocellular carcinoma in patients with hepatitis C virus: A case control study

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**Key words**
chronic hepatitis C, hepatocellular carcinoma, single nucleotide polymorphism.

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## Abstract

**Backgroud and Aim:** Chronic hepatitis C virus (HCV) infection is a well known risk factor for hepatocellular carcinoma (HCC). The aim of this study is to elucidate the genetic risk of development and recurrence of HCC in patients with HCV.

**Methods:** A total of 468 patients with HCV, including 265 with HCC were enrolled. We genotyped 88 single nucleotide polymorphisms (SNPs) in 81 genes expected to influence hepatocarcinogenesis using the iPLEX assay. Risk of HCC was clarified by stratifying patients into risk groups based on the multiplied odds ratio (MOR) for SNPs associated with HCC, and the cumulative effects on the development and recurrence of HCC were analyzed.

**Results:** Six SNPs associated with risk of HCC were identified (OR range: 0.29–1.76). These included novel SNPs for hepatocarcinogenesis with HCV CCND2 rs1049606, RAD23B rs1805329, CEP164 rs573455, and GRP78rs430397 in addition to the known SNPs MDM2 rs2279744 and ALDH2 rs671. MOR analysis revealed that the highest risk group exerted about a 19-fold higher relative OR compared with the lowest risk group ($P = 1.08 \times 10^{-5}$). Predicted 10-year HCC risk ranged from 1.7% to 96% depending on the risk group and the extent of fibrosis. Recurrence-free survival of radiofrequency ablation-treated HCC in the high risk group ($n = 53$) was lower than that of low risk group ($n = 58$, $P = 0.038$).

**Conclusion:** Single nucleotide polymorphisms of CCND2, RAD23B, GRP78, CEP164, MDM2, and ALDH2 genes were significantly associated with development and recurrence of HCC in Japanese patients with HCV.

## Introduction

More than 170 million people worldwide are estimated to have chronic hepatitis C virus (HCV) infection. The most important sequelae of chronic HCV infection are progressive liver fibrosis leading to cirrhosis and hepatocellular carcinoma (HCC), the latter responsible for significant morbidity and mortality throughout the world.[1–3] Alcohol intake, older age at time of infection, male sex, and co-infection with hepatitis B virus accelerate disease progression in HCV-infected patients,[1,4,5] but do not fully account for the development of HCC.

As with many cancers, variants of genes involved in multistage carcinogenesis may determine an individual's susceptibility to developing HCC. Single nucleotide polymorphisms (SNPs) are the most common type of genomic sequence variation and are thought to be associated with population diversity, susceptibility to disease, and individual response to drug treatment.[6] Many SNPs are silent, with no direct effect on gene products, but by virtue of linkage disequilibrium existing across the human genome they can be used as genetic markers to locate nearby functional variants that contribute to disease. SNPs may also have functional consequences if they affect coding or regulatory (usually promoter) regions of genes. Information accumulated from numerous studies on the association between cancer risk and SNPs in selected candidate genes may shed light on the molecular and genetic basis of the polygenic nature of cancer.

We performed a search for SNPs in candidate genes associated with susceptibility to the development of HCC. A total of 88 SNPs in 81 genes were examined in Japanese patients with chronic HCV infection. We identified two previously-reported and four novel variant SNPs as significant risk factors for incident HCC among patients with chronic HCV infection. The six SNPs were also associated with recurrence of HCC.

## Methods

### Study subjects

This case-control study included 468 Japanese patients with chronic HCV infection who were admitted to Okayama University Hospital or Kagawa Prefectural Central Hospital in Japan between January 2004 and December 2009. Patients comprised 265 with HCC and 203 without HCC. Chronic HCV infection was judged by a positive test for HCV antibody. We excluded patients who tested positive for the hepatitis B surface antigen. Patients with HCC were newly diagnosed and the HCC were previously untreated. Diagnosis of HCC was made by several imaging modalities, including angiography, computed tomography, and magnetic resonance imaging, or by tumor biopsy. Diagnostic criteria for HCC via imaging were based on previous reports of hyper-attenuation at the arterial phase, hypo-attenuation at the portal phase in dynamic computed tomography (CT) or magnetic resonance imaging (MRI), and tumor staining on angiography. According to guidelines of the American Association for the Study of Liver Disease, we confirmed HCC diagnosis using at least two dynamic imaging modalities.[7] Nodules without positive imaging were histologically confirmed as HCC via ultrasound-guided, fine-needle biopsy. Any patients imbibed over 80 g/day alcohol for longer than 10 years were considered to have a positive history of alcohol abuse. Age, gender, and information on clinical status were obtained for each patient at the time of whole-blood collection. Interferon had been administered to 61 (23%) cases and 49 (24%) controls, with 12 (5%) and 11 (5%) sustained virologic responders. Informed consent was obtained from all subjects. The study protocol conformed to the ethical guidelines of the World Medical Association Declaration of Helsinki and was approved by The Bioethics Committee of Okayama University Medical School.

### Gene selection

We selected 88 SNPs in 81 candidate genes considered to play a possible role in carcinogenesis by virtue of their ability to modify cell growth, hepatic inflammation, and/or hepatocyte apoptosis. (Table 1) The candidate sites include genes related to growth factors, growth factor receptors, cytokines/chemokines, cytokine/chemokine receptors, apoptosis, tumor suppression, DNA repair, cell-cycle regulation, metabolism, cell–cell interaction, and chromosome segregation. Most of them have been reported to be associated with carcinogenesis as well as HCC susceptibility.[8–30] SNPs of the selected genes were extracted from the Japanese Single Nucleotide Polymorphisms database (http://snp.ims.u-tokyo.ac.jp), a database for SNPs found in the Japanese population, and from National Center for Biotechnology Information.

### Genotyping

Genomic DNA was extracted from peripheral blood leukocytes using a QIAamp DNA blood mini kit (Qiagen, Hilden, Germany) according to the instructions of the manufacturer. SNPs were genotyped using Sequenom® MassARRAY technology (Sequenom®, San Diego, CA, USA). The iPLEX™ assay was conducted according to the manufacturer's instructions using 20 ng of genomic DNA after classification into four groups of multiplex analyses. The primers for amplification and extension were designed using Mass ARRAY Assay Design v.3.1 software; primer sequences are available from the authors upon request. Briefly, DNA was amplified using PCR and the unincorporated nucleotide triphosphates were deactivated by phosphatase treatment shrimp alkaline phosphatase. A single base primer extension step was performed and allele-specific extension products of different masses were quantitatively analyzed using matrix-assisted laser desorption/ionization time of flight mass spectrometry (MALDI-TOF MS).

### Treatment and follow-up for HCC

Among 265 HCC patients, 137 received radiofrequency ablation (RFA) as their first treatment modality, of whom 111 met the following inclusion criteria: HCC with size $\leq$ 3 cm and without extrahepatic metastasis, tumor number 3 or less, and Child–Pugh grade A or B. After RFA, all patients underwent dynamic computed tomography (CT) or magnetic resonance imaging (MRI) and complete ablation was confirmed. The patients were followed regularly using abdominal ultrasound examination, CT, or MRI every 3 months. All patients were followed until death or their last hospital visit.

### Statistical analysis

Differences between cases and controls in the distributions of demographic characteristics were tested using Student's t-test or the $\chi^2$ test. Tests for Hardy–Weinberg equilibrium were performed for each SNP separately among control subjects using Fisher's exact test. Differences in allele frequencies between patients with and without HCC were tested for each SNP using the $\chi^2$ test. Odds ratios (OR) and 95% confidence intervals (CI) were calculated using the major allele as reference. Differences in genotype frequencies were tested using dominant, recessive, and overdominant genetic models for each SNP. OR and 95% CI were estimated via unconditional logistic regression with a multiplicative model adjusting for age, gender, drinking history, and Child–Pugh grades (SPSS Ver.12.0, SPSS Inc, Tokyo, Japan). Permutation tests with 10 000 reiterations were performed using Haploview and declared statistically significant with $P < 0.05$.

We tested the combined effects of the six SNPs associated with HCC development by calculating a multiplied odds ratio (MOR) separately for each individual patient. The MOR is the product of ORs for all risk genotypes detected among the six SNPs in a patient, with adjusted ORs based on the best-fitting genetic models from single-SNP analyses. When calculating the MOR, we multiplied the risk ORs of six SNPs. The MOR was then categorized into five groups based on quintiles: very low, low, moderate, high, and very high risk. The performance of each cutoff was determined in terms of discriminatory ability. ORs, CIs, and *P*-values for each MOR risk category were estimated using the very low risk group (lowest quintile of MOR) as the reference category.

It has been reported that among untreated patients the annual incidence of HCC increases with degree of liver fibrosis, being 0.45% among patients with liver fibrosis stage 0 or 1, 1.99% with stage 2, 5.34% with stage 3, and 7.88% with stage 4 (cirrhosis).[31] Based on the data and likelihood ratio (LR) of each MOR quintile

**Table 1** List of 88 single nucleotide polymorphisms (SNPs) analyzed in this study

| Gene symbol | NCBI SNP ID | Gene symbol | NCBI SNP ID |
|---|---|---|---|
| ADH1B | rs1229984 | IRF3 | rs7251 |
| ADH1C | rs698 | ITGAV | rs2290083 |
| ALDH2 | rs671† | JUP | rs1126821 |
| ATR | rs2227928 | LPL | rs328 |
| AURKA | rs2273535 | MAD1L1 | rs1801368 |
| AXIN2 | rs2240308 | MDM2 | rs2279744† |
| BARD1 | rs2070094 | miR-146a | rs2910164† |
| CASP9 | rs1052571 | miR-34b/c | rs4938723 |
| CCND1 | rs9344 | MMP27 | rs1276286 |
| CCND2 | rs1049606† | MMP9 | rs17577 |
| CDC6 | rs4134994 | MTHFR | rs1801133 |
| CDH17 | rs2514813 rs3214050 | MTRR | rs10380 |
| CEP110 | rs10818504 | NIN | rs2236316 |
| CEP152 | rs2289181 | NOB1 | rs3811348 |
| CEP164 | rs573455† | NSL1 | rs15702 |
| CEP192 | rs578208 | PCNT | rs2070425 |
| CEP250 | rs3748433 | PKCI | rs481781 |
| CEP55 | rs3740370 rs2293277 | POL I | rs8305 |
| CEP57 | rs644799 | PTPN13 | rs2230600 rs989902 |
| CEP68 | rs12611491 | PTPRJ | rs1566734 rs1503185 |
| CEP72 | rs868649 | RAD18 | rs373572 |
| CRHR2 | rs2267716 | RAD23B | rs1805329† |
| CTLA4 | rs231775 | RAG1 | rs3740955 |
| CYP1B1 | rs1056836 | RAPGEF6 | rs1291602 |
| CYP2C19 | rs4986893 | RASSF1 | rs2073498 |
| DLC1 | rs621554 | RASSF6 | rs12507775 |
| DUSP6 | rs2279574 | SCYB14 | rs2237062 |
| EGF | rs4444903 | SNAI 1 | rs4647958 |
| EGFR | rs2293347 rs763317 | SNM1B | rs11552449 |
| ERBIN | rs36303 | SPARC | rs2304052 |
| ESR1 | rs2077647 | SRD5A2 | rs523349 |
| ETL1 | rs7439869 | TDG | rs4135113 |
| EXO1 | rs4149963 rs1047840 | TGFB1 | rs1800469 |
| FSHR | rs6165 | TP53 | rs1042522† |
| GFRA1 | rs12762746 | TRAP1 | rs2074805 |
| GRP78 | rs430397† | WISP3 | rs1230345 |
| GSTP1 | rs1695 | WRN | rs1801195† |
| HER2 | rs1136201 | XPC | rs2228000† |
| IGF2 | rs11541372 | XPG | rs17655 |
| IL10 | rs1800872 | XRCC | rs25487 |
| IL1B | rs1143627 rs16944 | | |

†SNPs with significantly different frequencies between cases and controls.
NCBI, National Center for Biotechnology Information.

risk category, we calculated the predicted 10-year absolute risk for HCC incidence (%) in each group according to stage of liver fibrosis as following.

$$10\text{-year risk} = \frac{\mathrm{LR} \times \mathrm{Odds}}{1 + \mathrm{LR} \times \mathrm{Odds}} \times 100$$

In this formula, odds were converted from 10-year risk of HCC that was calculated from the reported annual incidence of HCC at each fibrosis stage.

In the analysis of HCC recurrence after RFA, we combined the five MOR risk categories into two groups: one including very low, low, and moderate risk ($n = 58$), the other combining high and very high risk ($n = 53$). Cumulative recurrence rates were estimated using the Kaplan–Meier method and compared using the log rank test. Because early development of HCC is thought to involve pre-existing intra-hepatic metastasis, we defined recurrence of HCC as a new legion that developed more than 3 months after treatment, with the starting date of follow-up for tumor recurrence being the day when all tumors were ablated by RFA.

## Results

Patient characteristics are shown in Table 2. There was no significant difference between HCC cases and controls in terms of

**Table 2** Characteristics of the patients' background

| Patients number | Cases | Controls | *P*-value* |
|---|---|---|---|
| | 265 | 203 | |
| Age (years) | 68.4 (40–87) | 57.7 (21–86) | < 0.001 |
| Sex (male) | 182 (68.7%) | 100 (49.3%) | < 0.001 |
| Alcohol > 80 g/day | 22 (8.3%) | 13 (6.4%) | 0.440 |
| TB (mg/dL) | 1.1 (0.3–27.9) | 1.3 (0.3–13) | 0.423 |
| Albumin (g/dL) | 3.6 (2.1–4.7) | 3.9 (2.1–5.1) | < 0.001 |
| PT (%) | 96 (54–146) | 99 (23–152) | 0.072 |
| ALT (IU/L) | 54 (10–269) | 61 (9–368) | 0.087 |
| Platelet ($\times 10^4/\mu L$) | 13 (2.1–41.9) | 16 (1.5–34.7) | 0.016 |
| Child-Pugh grade | | | |
| A | 217 (81.9%) | 173 (85.2%) | 0.003 |
| B | 43 (16.2%) | 17 (8.4%) | |
| C | 5 (1.9%) | 13 (6.4%) | |
| IFN therapy | 61 (23%) | 49 (24%) | 0.777 |

Values are median (range) or number (%).
**P* values were derived from the Pearson $\chi 2$ test or student's *t*-test.
ALT, alanine aminotransferase; IFN, interferon; PT, prothrombin time; TB, total bilirubin.

alcohol intake, total bilirubin, prothrombin time, alanine aminotransferase (ALT) and IFN therapy. Age, proportion of males, and proportion of Child–Pugh grade B were higher in patients with HCC than in patients without HCC, whereas serum albumin level and platelet count were lower in patients with HCC.

### SNPs related to HCC

Among the 88 SNPs, we selected ones with minor allele frequency $\geq 10\%$, genotyping success rate $\geq 95\%$, and no evidence of deviation from Hardy–Weinberg equilibrium ($P < 0.05$) in control subjects. Seventy-five SNPs satisfied these criteria, of which 10 showed a positive association with HCC incidence (significant crude OR): CCND2 rs1049606, RAD23B rs1805329, miR-146a rs2910164, GRP78 rs430397, MDM2 rs2279744, ALDH2 rs671, TP53 rs1042522, WRN rs1801195, XPC rs2228000, and CEP164 rs573455. To evaluate the effects of these polymorphisms on the occurrence of HCC, unconditional logistic regression analysis was performed with adjustment for age, gender, drinking history, and Child–Pugh grade. As further validation, permutation tests for allele frequencies demonstrated significant differences with six SNPs: CCND2 rs1049606, RAD23B rs1805329, MDM2 rs2279744, ALDH2 rs671, CEP164 rs573455, and GRP78 rs430397 (Table 3). After determining the best fitting genetic model for each variant (Table 4), CEP164 rs573455 had the strongest association with HCC; adjusted OR for the CC genotype was 0.29 (95% CI: 0.15–0.56, $P = 1.9 \times 10^{-4}$) using TT+TC as the reference.

### Prediction of HCC development by MOR

Multiplied odds ratio was calculated for the abovementioned six SNPs to assess the cumulative effects on HCC incidence after inverting protective ORs (OR < 1) (equivalently, using the OR for the reference genotype compared with risk genotype as reference). The risk genotype and risk OR for each SNP is shown in Table 4. Calculated MOR ranged from 1.0 to 71.7 and was categorized into five groups using cutpoints 6, 13, 24, and 70. The difference in the relative OR of HCC incidence among the risk categories was highly significant. Compared with the very low risk group, relative OR of HCC was high in the very high risk group (OR = 19.1, 95% CI: 3.95–92.6, $P = 1.08 \times 10^{-5}$) (Table 5). Table 5 also presents predicted cumulative incidence of HCC over 10 years according to stage of liver fibrosis in each MOR category. Ten-year predicted cumulative incidence ranged widely from 1.67 in the very low risk group of F0 or 1 to 96.2% in the very high risk group of F4. Within any particular fibrosis stage, the predicted incidence differed by anywhere from 1.7- to 15-fold among MOR categories, with the widest range in the lowest fibrosis stages. Even with fibrosis stage 0 or 1, predicted 10-year cumulative HCC incidence is 24.5% in the very high risk MOR group. Interferon (IFN) therapy was conducted in 61 cases (23%) before development of HCC. Twelve (5%) were sustained virologic responders and 49 (18%) were non-responders. No difference in MOR was observed between sustained virological responders and non-responders.

### Impact of MOR on recurrence-free survival of RFA-treated HCC patients

We compared the recurrence-free survival of RFA-treated patients between high risk and low risk groups. Characteristics of the 111 patients are shown in Table 6. The high risk group was younger, but there was no significant difference in sex, tumor size, tumor number, alanine aminotransferase (ALT), total bilirubin, albumin, platelet, prothrombin time, or α-fetoprotein. The median follow-up period was 1043 days (range, 176–2533 days). The 3-year cumulative recurrence rates were 77% in the high risk group and 59% in the low risk group. Patients in the high risk group had higher recurrence rates than those in the low risk group ($P = 0.038$) (Fig. 1).

## Discussion

We studied 88 SNPs in 81 genes that were expected to be associated with hepatocarcinogenesis. Putative genetic markers for susceptibility to hepatocarcinogenesis were identified in patients with chronic HCV infection. Six SNPs in six different genes were identified as being associated with HCC: CCND2 rs1049606, RAD23B rs1805329, MDM2 rs2279744, ALDH2 rs671, GRP78 rs430397, and CEP164 rs573455. To our knowledge, the findings of associations linking CCND2 rs1049606, RAD23B rs1805329, and CEP164 rs573455 with HCC are novel. The relationships of polymorphisms MDM2 rs2279744 and ALDH2 rs671 with HCC confirm previous results independently reported by other groups in Japan.[29,30,32,33]

Single nucleotide polymorphism rs1805329 (Ala249Val) is located in the RAD23B gene. The RAD23B protein is crucial in recognition and initiation of global genomic repair (GGR). SNP rs573455 (Gln1119Arg) is located in the CEP164 gene, which encodes a centriole appendage protein and is also associated with DNA repair.[34,35] SNP rs1049606 (T-171C) is located in the 5′-UTR of the CCND2 gene. The protein encoded by this gene is involved in phosphorylation of the tumor-suppressor protein Rb; abnormal levels of CCND2 are associated with poor prognosis in gastric

**Table 3** Alleles and genotype frequencies of six single nucleotide polymorphisms (SNPs) demonstrating a significant difference between cases and controls

| Gene symbol Chromosomal Region | SNP ID | Alleles | MAF | | OR | 95% CI | Permutation *P*-value† | Genotype | Case | Control | *P*-value‡ |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | Case (%) | Control (%) | | | | | (*n*) | (*n*) | |
| CCND2 | rs1049606 | T / C | 37.5 | 46.6 | 0.69 | 0.53–0.90 | 0.0051 | TT | 99 | 64 | |
| 2p13.321 | | | | | | | | TC | 133 | 89 | 0.869 |
| | | | | | | | | CC | 33 | 50 | 0.001 |
| RAD23B | rs1805329 | C / T | 17.2 | 25.6 | 0.60 | 0.44–0.83 | 0.003 | CC | 180 | 117 | |
| 9q31.2 | | | | | | | | CT | 79 | 68 | 0.167 |
| | | | | | | | | TT | 6 | 18 | $6.8 \times 10^{-4}$ |
| GRP78 | rs430397 | G / A | 15.1 | 10.0 | 1.58 | 1.06–2.36 | 0.034 | GG | 192 | 164 | |
| 9q33.3 | | | | | | | | GA | 66 | 37 | 0.068 |
| | | | | | | | | AA | 7 | 2 | 0.155 |
| MDM2 | rs2279744 | G / T | 40.9 | 47.7 | 0.76 | 0.58–0.99 | 0.03t8 | GG | 88 | 56 | |
| 12q15 | | | | | | | | GT | 129 | 96 | 0.471 |
| | | | | | | | | TT | 41 | 47 | 0.031 |
| ALDH2 | rs671 | G / A | 29 | 21.6 | 1.48 | 1.09–2.01 | 0.012 | GG | 132 | 126 | |
| 12q24.12 | | | | | | | | GA | 111 | 60 | 0.004 |
| | | | | | | | | AA | 21 | 13 | 0.244 |
| CEP164 | rs573455 | T / C | 38.7 | 45.4 | 0.76 | 0.58–0.99 | 0.035 | TT | 88 | 61 | |
| 11q23.3 | | | | | | | | TC | 138 | 93 | 0.895 |
| | | | | | | | | CC | 30 | 43 | 0.012 |

†*Permutation tests with 10 000 reiterations were performed by the Haploview program.*
‡$\chi^2$ test for $2 \times 2$ contingency table.
CI, confidence interval; MAF, minor allele frequency; OR, odds ratio.

**Table 4** Best-fitting model fit to data on six single nucleotide polymorphisms (SNPs) with significant case-control frequency differences

| Gene symbol | SNPID | Best-fitting model† | | | Adjusted OR (95%CI) | *P*-value‡ | Risk genotype | Risk OR |
|---|---|---|---|---|---|---|---|---|
| | | Model | Genotype | | | | | |
| | | | Reference | Associated | | | | |
| CCND2 | rs1049606 | Recessive | TC+TT | CC | 0.50 (0.28–0.88) | 0.016 | TC+TT | 2.00 |
| RAD23B | rs1805329 | Dominant | CC | CT+TT | 0.56 (0.36–0.89) | 0.013 | CC | 1.79 |
| GRP78 | rs430397 | Dominant | GG | GA+AA | 1.76 (1.04–2.96) | 0.035 | GA+AA | 1.76 |
| MDM2 | rs2279744 | Recessive | GT+GG | TT | 0.50 (0.28–0.87) | 0.014 | GT+GG | 2.00 |
| ALDH2 | rs671 | Overdominant | GG+AA | GA | 1.64 (1.03–2.60) | 0.037 | GA | 1.64 |
| CEP164 | rs573455 | Recessive | TC+TT | CC | 0.29 (0.15–0.55) | $1.9 \times 10^{-4}$ | TC+TT | 3.45 |

†The best-fitting model for each SNP was determined after testing association in a series of genetic models, including dominant, recessive and overdominant models.
‡Odds ratios (ORs) of genotypes and minor alleles were calculated by logistic regression adjusted for age, sex, alcohol drinking, and Child–Pugh grade and 95% confidence intervals (CIs) and *P*-values were derived from the Wald test.

cancer and intrahepatic recurrence in HCC.[36,37] SNP rs430397 (G/A) is located in intron 5 of the GRP78 gene and adjoins the 3′-end of the intron. The GRP78 pathway, one of the most important responders to disease-associated stresses,[38] demonstrates high correlation between expression and cancer progression, has anti-apoptotic function, and leads to drug resistance in HCC.[39,40] MDM2 (rs2279744) in the promoter region of the MDM2 gene, a negative regulator of p53, is associated with accelerated tumor formation in both hereditary and sporadic cancers in humans.[41] SNP rs671 (Glu504Lys) is located in the ALDH2 gene. ALDH2 is a key enzyme in the elimination of acetaldehyde.[42] Our analysis revealed that heterozygotes for rs671 were associated with increased risk of HCC development; a similar result has been reported for esophageal cancer.[43] All six genes are presumed to be associated with carcinogenesis. However, functional effects of the four SNPs excluding ALDH2 and MDM2 have yet to be fully elucidated.

We calculated joint effects on HCC development for the six polymorphisms by multiplying their separate odds ratios for each patient. Although gene–gene interaction is ignored using MOR, even if interaction exists the effects should influence cases and controls similarly. Furthermore, risk can be evaluated in greater detail by calculating the MOR of two or more genes compared with a single gene. Previously, many studies demonstrated cumulative effects of SNPs in detecting the high risk group.[44,45] Moreover, the high risk group may be refined by combining MOR with

**Table 5** Predicted hepatocellular carcinoma (HCC) incidence calculated according to the joint effects of six single nucleotide polymorphisms (SNPs)

| Category | MOR range | Case | Control | OR (95% CI) | *P*-value* | LR | 10-year predicted HCC incidence (%) | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | *n* = 265 *n* (%) | *n* = 203 *n* (%) | | | | F0 or 1 mean = 4.5 | F2 19.9 | F3 53.4 | F4 78.8 |
| Very low risk | 1–6 | 16 (6.0) | 34 (16.7) | 1.00 (Ref.) | 1 | 0.36 | 1.67 | 8.20 | 29.2 | 57.2 |
| Low risk | 6–13 | 60 (22.6) | 62 (30.5) | 2.06 (1.03–4.11) | 0.043 | 0.74 | 3.37 | 15.5 | 45.9 | 73.3 |
| Moderate risk | 13–24 | 62 (23.4) | 48 (23.6) | 2.74 (1.36–5.55) | 0.006 | 0.99 | 4.45 | 19.7 | 53.2 | 78.6 |
| High risk | 24–70 | 109 (41.1) | 57 (28.1) | 4.06 (2.07–7.98) | $3.46 \times 10^{-5}$ | 1.46 | 6.44 | 26.6 | 62.6 | 84.4 |
| Very high risk | 70 < | 18 (6.8) | 2 (1.0) | 19.1 (3.95–92.6) | $1.08 \times 10^{-5}$ | 6.89 | 24.5 | 63.1 | 88.8 | 96.2 |

*$P$ values were calculated using Fisher's exact test (two-sided).

CI, confidence interval; F, stage of liver fibrosis; LR, likelihood ratio; MOR, multiplied odds ratio; OR, odds ratio; Ref, reference.

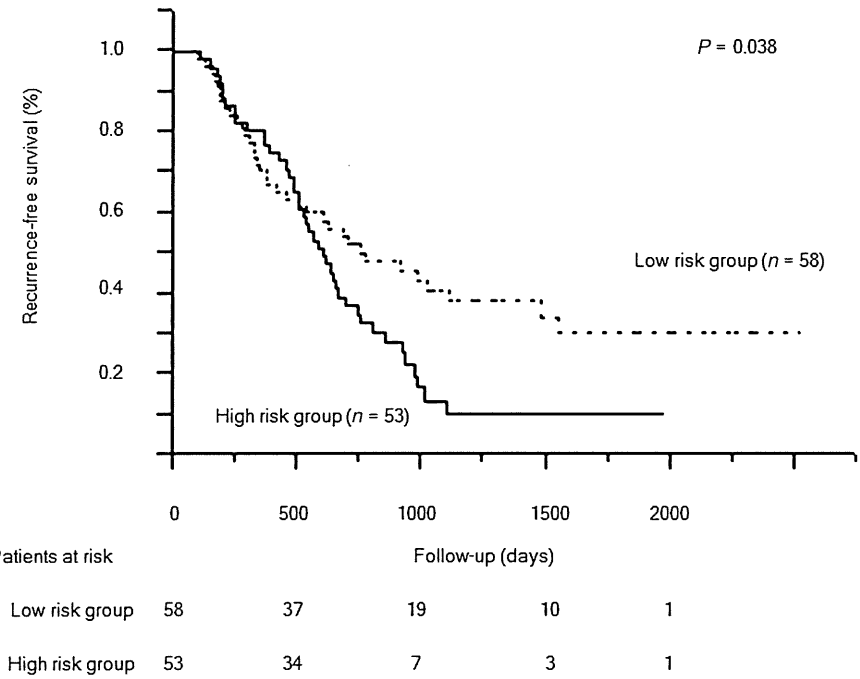


**Figure 1** Recurrence free survival of high risk group and low risk group. Solid line, high risk group ($n$ = 53); dotted line, low risk group ($n$ = 58). High risk group patients had higher recurrence rate than those in low risk group ($P$ = 0.038).

fibrosis stage, an indicator of carcinogenesis. Yet even in the same fibrosis stage, the variation in predicted incidence of HCC can range anywhere from 1.7- to 15-fold. If a patient has mild fibrosis (F0/F1/F2) but is in the high risk group based on MOR, predicted HCC incidence may be higher than for a patient in an advanced stage of fibrosis (F3/F4) with lower risk based on MOR. According to the evidence-based clinical practice guidelines for HCC in Japan,[46] screening is recommended once every 6 months for the high risk group (patients with chronic hepatitis B, C or with cirrhosis) and once every 3–4 months for the very high risk group (patients with cirrhosis type B or C). By adding the concept of MOR, we can further refine the risk group and predict the incidence risk of HCC for each stage of fibrosis according to the risk categories stratified by MOR. Our data suggest that MOR may occasionally be high even in patients whose fibrosis stage is 0 or 1. Such patients are nevertheless at high risk of HCC, which indicates that SNP analysis complements other laboratory tests in identifying high-risk patients.

Moreover, we found that the tumor recurrence rates following RFA therapy differed between patients in the high risk and low risk groups based on MOR category. Those data increased the reliability of MOR in this study and suggest that we could predict not only risk of HCC development but also risk of recurrence. By analyzing the SNPs, we could pay more attention during the surveillance of these high risk patients, and might achieve an early diagnosis of HCC.

Several limitations of the present study should be noted. Histological examination, which is required for precise evaluation of liver fibrosis stage, was not performed on all of the non-cancerous tissues, although we used Child–Pugh grade as a parameter of liver function. This study was lacking the data of HCV viral load or genotype, which might be the risk factors of HCV-related HCC. In addition, the study design was retrospective with a small number of patients. A prospective study with larger sample is needed to confirm our results. Moreover, validation study is needed to confirm the conclusion.

**Table 6** Characteristics of radiofrequency ablation (RFA)-treated patients

| | High risk group ($n = 53$) | Low risk group ($n = 58$) | *P*-value* |
|---|---|---|---|
| Age (years) | 67.3 (40–83) | 70.9 (52–85) | 0.027 |
| Sex (male) | 28 (53%) | 33 (57%) | 0.670 |
| Tumor size (mm) | 16 (9–29) | 16 (6–30) | 0.606 |
| Tumor number (single) | 34 (64%) | 46 (79%) | 0.156 |
| ALT (IU/L) | 55 (17–235) | 57 (21–164) | 0.696 |
| TB (mg/dL) | 0.9 (0.4–2.2) | 0.9 (0.4–1.9) | 0.540 |
| Albumin (g/dL) | 3.6 (2.7–4.6) | 3.7 (2.8–4.6) | 0.215 |
| Platelet count (x 104/μL) | 11.2 (3.1–21.5) | 11.1 (4.4–30.7) | 0.911 |
| PT (%) | 97.8 (71–146) | 97.8 (56–140) | 0.990 |
| AFP (μg/L) | 71.3 (1.9–925) | 97.9 (3.2–2818) | 0.635 |

Values are median (range) or number (%).
*$P$ values were derived from either the Pearson $\chi 2$ test or student's t test.
AFP, α-fetoprotein; ALT, alanine aminotransferase; PT, prothrombin time; TB, total bilirubin.

Six SNPs: CCND2 rs1049606, RAD23B rs1805329, MDM2 rs2279744, ALDH2 rs671, CEP164 rs573455, and GRP78 rs430397, are associated with risk of HCC among Japanese patients with chronic HCV infection. We could predict the absolute risk of HCC among HCV-related hepatitis patients by analyzing cumulative effects of these six SNPs using multiplied OR. The data were also effective for predicting patient's prognosis.

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# Hypovascular Nodules in Patients with Chronic Liver Disease: Risk Factors for Development of Hypervascular Hepatocellular Carcinoma[1]

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**Purpose:** To identify patient characteristics and magnetic resonance (MR) imaging findings associated with subsequent hypervascularization in hypovascular nodules that show hypointensity on hepatobiliary phase gadoxetic acid–enhanced MR images in patients with chronic liver diseases.

**Materials and Methods:** Institutional review board approval was obtained, and informed consent was waived. At multiple follow-up gadoxetic acid–enhanced MR imaging examinations of 68 patients, 160 hypovascular nodules were retrospectively reviewed. A Cox regression model for hypervascularization was developed to explore the association of baseline characteristics, including patient factors (Child-Pugh classification, etiology of liver disease, history of local therapy for hepatocellular carcinoma [HCC], and coexistence of hypervascular HCC) and MR imaging findings (fat content, signal intensity on T2-weighted images, and nodule size). In addition, the growth rate was calculated as the reciprocal of tumor volume doubling time to investigate its relationship with subsequent hypervascularization by using receiver operating characteristic and Kaplan-Meier analyses.

**Results:** The prevalence of subsequent hypervascularization was 31% (50 of 160 nodules). Independent Cox multivariable predictors of increased risk of hypervascularization were hyperintensity on T2-weighted images (hazard ratio [HR] = 8.7; 95% confidence interval [CI]: 3.6, 20.8), previous local therapy for hypervascular HCC (HR = 5.0; 95% CI: 1.8, 13.6), Child-Pugh B cirrhosis (HR = 3.6; 95% CI: 1.4, 9.5) and coexistence of hypervascular HCC (HR = 2.0; 95% CI: 1.0, 3.8). The mean growth rate was significantly higher in nodules that showed subsequent hypervascularization than in those without hypervascularization. Kaplan-Meier analysis based on the receiver operating characteristic cutoff level ($1.8 \times 10^{-3}$/day [tumor volume doubling time, 542 days]) showed that nodules with a higher growth rate had a significantly higher incidence of hypervascularization ($P = 5.2 \times 10^{-8}$, log-rank test).

**Conclusion:** Hyperintensity on T2-weighted images is an independent and strong risk factor at baseline for subsequent hypervascularization in hypovascular nodules in patients with chronic liver disease. Tumor volume doubling time of less than 542 days was associated with a high rate of subsequent hypervascularization.



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