Table 2 Factors contributing to hepatocellular carcinoma (all patients) Cox regression analysis (multivariate)

		Hazard ratio	95% confidence interval	*P*-value
Therapeutic efficacy	SVR	1		
	TR	2.055	0.709–5.955	0.1845
	NVR	2.985	1.036–8.601	0.0428
Sex	Male	1		
	Female	0.486	0.243–0.969	0.0405
Age	<60	1		
	≥60	2.005	1.035–3.883	0.0391
ALT at 24 weeks after end of treatment (IU/L)	≤40	1		
	>40	3.940	1.754–8.850	0.0009
Platelet count (×10 000/mm^3)	<10	1		
	≥10	0363	0.169–0.779	0.0093
Serum albumin (g/dL)	<4	1		
	≥4	0.594	0.310–1.140	0.1175

Factors examined: Of the 15 factors exhibiting $P < 0.2$ by log-rank test (therapeutic efficacy [1: SVR, 2: TR, 3: NVR], genotype [1: 1, 2: 2 or 3], sex [1: male, 2: female], age [1: <60, 2: ≥60], pre ALT [1: ≤40, 2: >40], +24 w ALT [1: ≤40, 2: >40], pre PLT [1: <10, 2: ≥10], pre ALB [1 <4, 2: ≥4], pre AFP [1: <20, 2: ≥20], grade [1: A0–1, 2: A2–3], stage [1: F0–1, 2: F2–4], hypertension [1: absent, 2: present], diabetes [1: absent, 2: present], heavy drinking [1: absent, 2: present], and treatment duration [1: ≤48 W, 2: >48 W]), nine factors were examined. Excluded were factors for which approximately 30% of values were missing (AFP, grade, stage, diabetes, hypertension, and heavy drinking).
AFP, alpha fetoprotein; ALB, albumin; ALT, alanine aminotransferase; NVR, non-virological response; PLT, platelet count; SVR, sustained virological response; TR, transient response.

(Fig. 4). This tendency is also observed with the 280 patients having pretreatment ALT levels of less or equal to 30 IU/L.

Onset of HCC in SVR patients

Hepatocellular carcinoma developed in 10 patients who achieved SVR. Multivariate analysis indicated that in SVR patients, the ALT level at 24 weeks after the end of treatment was the only significant factor contributing to HCC ($P = 0.0007$) (Table 4). In SVR patients with an ALT level of more than 40 IU/L at 24 weeks after the end of treatment, the 5-year cumulative incidence of HCC was 5.6% while the incidence in patients with an ALT level of less or equal to 40 IU/L was 0.7%, indicating a significant difference ($P = 0.0004$) between the groups (Fig. 5).

DISCUSSION

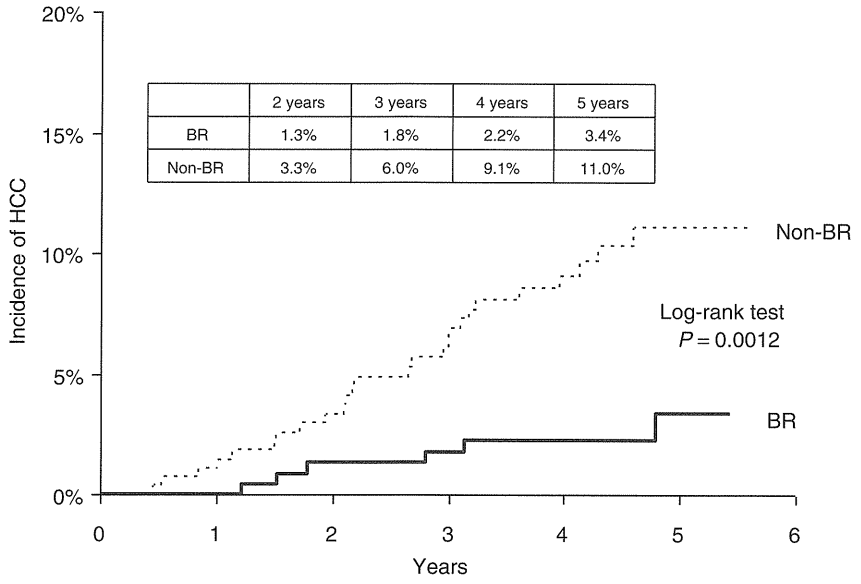
THIS STUDY INDICATED that the risk factors for HCC after PEG-IFN α-2b plus RBV combination therapy are NVR, male sex, older age, low platelet count, and an ALT level of more than 40 IU/L at 24 weeks after the end of treatment.

Kurokawa *et al.*[16] tracked 403 patients receiving PEG-IFN α-2b plus RBV combination therapy for a median

Table 3 Factors contributing to biochemical response in non-sustained virological response patients Logistic regression analysis (multivariate)

		Odds ratio	95% confidence interval	*P*-value
Virological response	NVR	1	1.480–3.203	0.0001
	TR	2.177		
Treatment duration	per week	1	1.000–1.022	0.0424
		1.011		
Platelet count	per 10 000/mm^3	1	1.018–1.099	0.0043
		1.058		

Factors examined were those exhibiting $P < 0.2$ by log-rank test: Genotype, virological response (TR/NVR), treatment duration, pre platelet count, diabetes, stage, and alanine aminotransferase (ALT).

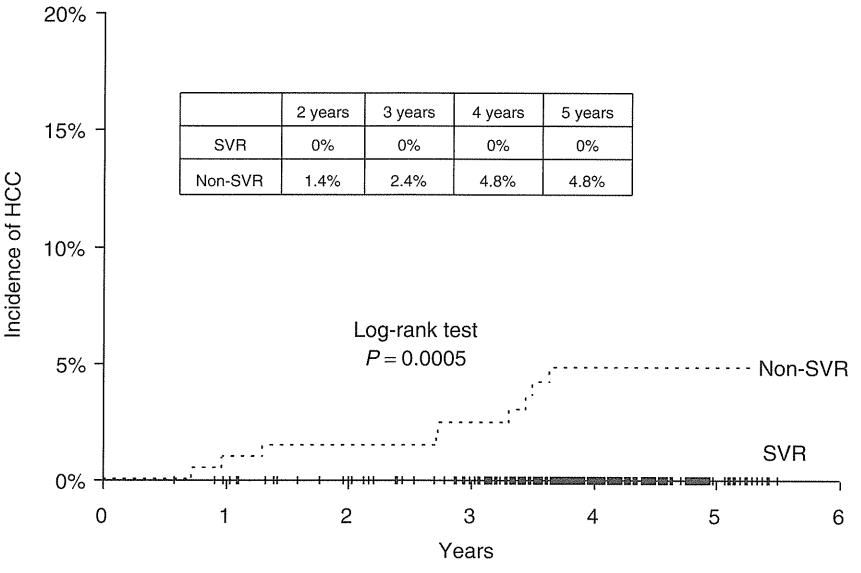


	2 years	3 years	4 years	5 years
BR	1.3%	1.8%	2.2%	3.4%
Non-BR	3.3%	6.0%	9.1%	11.0%

Figure 3 Alanine aminotransferase (ALT) normalization and hepatocellular carcinoma (HCC) in non-virological response [NVR] patients. The cumulative incidence of HCC was calculated by the Kaplan–Meier method. Log-rank test was used to study the difference between biochemical response (BR) and non-BR.

duration of 36.5 months and reported that in multivariate analysis, virological efficacy (SVR vs. non-SVR), age, and hepatic fibrosis were selected as the factors contributing to HCC. Arase *et al.*[15] tracked 500 patients 60 years of age and older receiving IFN alone or in combination with RBV for an average duration of 7.4 years and also reported that the factors contributing to HCC are virological efficacy (SVR vs. non-SVR), age, and hepatic fibrosis. In our study, hepatic fibrosis was not tested with multivariate analysis because more than 30% of values were missing, but it was selected as a significant factor in the univariate analysis. Platelet count was selected in multivariate analysis, and the results in our study are therefore considered to be generally consistent with these reports.

The results of the present study indicated no significant difference between TR and NVR in non-SVR in stratified cumulative incidence of HCC, and although there was a significant difference between SVR and both TR and NVR, TR was not significant against SVR in multivariate analysis, and NVR was the only significant factor. Kurokawa *et al.*[16] reported the same results by



	2 years	3 years	4 years	5 years
SVR	0%	0%	0%	0%
Non-SVR	1.4%	2.4%	4.8%	4.8%

Figure 4 Therapeutic efficacy and hepatocellular carcinoma (HCC) in patients with pretreatment alanine aminotransferase (ALT) of ≤40. The cumulative incidence of HCC was calculated by the Kaplan–Meier method. Log-rank test was used to study the difference between sustained virological response (SVR) and non-virological response (NVR).

Table 4 Factors contributing to hepatocellular carcinoma (sustained virological response [SVR] patients) Cox regression analysis (multivariate)

		Hazard ratio	95% confidence interval	*P*-value
ALT at 24 weeks after end of treatment (IU/L)	≤40	1		
	>40	16.054	3.235–79.681	$P = 0.0007$
Serum albumin (g/dL)	<4	1		
	≥4	0.196	0.036–1.073	$P = 0.0603$

Factors examined: Of the 10 factors exhibiting $P < 0.2$ by log-rank test (Genotype [1: 1, 2: 2 or 3], age [1: <60, 2: ≥60], pre ALT [1: ≤40, 2: >40], +24 w ALT [1: ≤40, 2: >40], pre PLT [1: <10, 2: ≥10], pre ALB [1: <4, 2: ≥4], pre AFP [1: <20, 2: ≥20], grade [1: A0–1, 2: A2–3], stage [1: F0–1, 2: F2–4], and diabetes [1: absent, 2: present]), 5 factors were examined. Excluded were pre ALT, with which HCC did not occur in the ≤40 group, and AFP, grade, stage, and diabetes, the factors for which approximately 30% of values were missing.
ALB, albumin; ALT, alanine aminotransferase; PLT, platelet count;

comparing cumulative incidences of HCC among SVR, TR and NVR (the results of multivariate analysis are not known). On the other hand, Morgan *et al.*,[19] in their follow-up study of the HALT-C Trial, reported that there was no difference between TR and NVR in the incidence of HCC or death related to hepatic disease/liver transplantation, but when all hepatic-related outcomes were examined, a significantly superior inhibition was observed with TR compared to NVR. Our results also demonstrate that although the difference is not significant, the cumulative incidence of HCC is lower in TR patients than in NVR patients, especially in male patients (5-year cumulative incidence of HCC: 6.0% vs. 10.7%). It is therefore necessary to continue to observe this for an extended number of years.

Our results study indicated that in non-SVR patients, whether or not ALT level is normalized after treatment is a greater contributing factor for the onset of HCC than virological response. Normalization of ALT has already been reported to contribute to the inhibition of the onset of HCC even under HCV-positive conditions,[13,20] and this was found to apply also to non-SVR patients receiving PEG-IFN α plus RBV combination therapy.

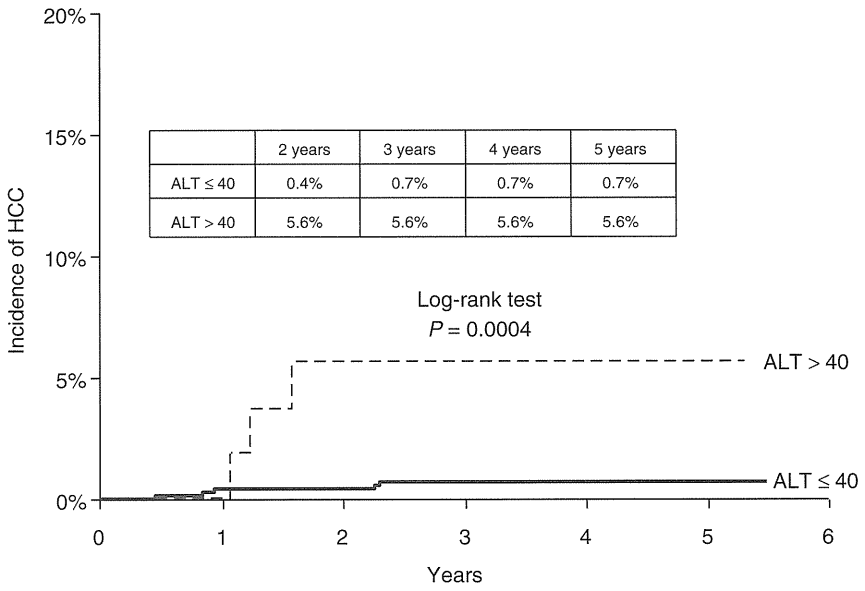


Figure 5 Alanine aminotransferase (ALT) levels at 24 weeks after end of treatment and hepatocellular carcinoma (HCC) in patients with sustained virological response (SVR). The cumulative incidence of HCC was calculated by the Kaplan–Meier method. Log-rank test was used to study the difference between SVR patients with an ALT level of more than 40 IU/L at 24 weeks after the end of treatment and those with an ALT level of less or equal to 40 IU/L.

Our investigation also indicated that abnormal ALT levels also contribute to the onset of HCC in SVR patients. In multivariate analysis, the only contributing factor to the development of HCC in SVR patients was ALT levels at 24 weeks after the end of treatment. However, the onset of HCC is also observed in patients who achieve ALT normalization after treatment, and it is therefore difficult to conclude that ALT is the only risk factor for the onset of HCC in SVR patients. The potential involvement of hepatic fibrosis as well as hepatic steatosis, which persists after viral clearance[21] and small amounts of virus remaining in the liver[22] have also been suggested as risk factors for the onset of HCC in SVR patients. Further detailed investigation is therefore necessary. Nevertheless, regardless of whether or not SVR is achieved, it is clear that abnormal ALT is a factor affecting the onset of HCC. Careful monitoring of changes in ALT and instituting measures to normalize ALT are therefore important regardless of whether or not SVR is achieved.

With the administration of PEG-IFN α plus RBV combination therapy tailored for individual patients and the addition of direct-acting antivirals to current combination therapy, the therapeutic outcomes for CHC will continue to further improve, and the number of patients who develop hepatic cirrhosis and HCC from hepatitis C can be expected to decrease in the future. HCC can occur even in patients achieving SVR, and even if SVR is not achieved, as long as the possibility to inhibit the onset of HCC remains, there will be a need for various treatment innovations to achieve the prevention of HCC, the ultimate goal of treatment of CHC.

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APPENDIX I

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Original Article

Hepatocellular Carcinoma With Extrahepatic Metastasis

Clinical Features and Prognostic Factors

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BACKGROUND: Despite significant advances in the treatment of intrahepatic lesions, the prognosis for patients with hepatocellular carcinoma (HCC) who have extrahepatic metastasis remains poor. The objective of this study was to further elucidate the clinical course and prognostic determinants of patients with this disease. **METHODS:** In total, 342 patients who had HCC with extrahepatic metastasis were enrolled. The metastases were diagnosed at initial presentation with HCC in 28 patients and during follow-up in the remaining patients. The authors analyzed clinical features, prognoses, and treatments and established a scoring system to predict prognosis using a split-sample method with a testing set and a training set. **RESULTS:** The most frequent site of extrahepatic metastasis was the lung followed by lymph nodes, bone, and adrenal glands. These metastases were related directly to death in only 23 patients (7.6%). The median survival after diagnosis of extrahepatic metastasis was 8.1 months (range, 0.03-108.7 months). In univariate analysis of the training set (n = 171), performance status, Child-Pugh classification, the number and size of intrahepatic lesions, macroscopic vascular invasion, symptomatic extrahepatic metastases, α-fetoprotein levels, and complete responses to treatment were associated significantly with prognosis. On the basis of multivariate analysis, a scoring system was developed to predict prognosis that assessed uncontrollable intrahepatic lesions, extent of vascular invasion, and performance status. This scoring system was validated in the testing set (n = 171) and produced a concordance index of 0.73. **CONCLUSIONS:** The controllability of intrahepatic lesions and performance status were identified as important prognostic factors in patients with advanced HCC who had extrahepatic metastasis. ***Cancer* 2011;117:4475–83.** © *2011 American Cancer Society.*

KEYWORDS: hepatocellular carcinoma, extrahepatic metastasis, clinical course, prognosis.

Hepatocellular carcinoma (HCC) is a leading cause of cancer death, and its incidence is particularly high in Asian countries, including Japan.[1,2] HCC usually develops in a liver that already suffers from chronic disease, most notably because of hepatitis B virus (HBV) or hepatitis C virus (HCV) infection.[3] In the past, HCC often was diagnosed only at a far advanced stage, and this was accompanied by a very poor prognosis.[4] However, today, close surveillance with advanced diagnostic modalities on designated high-risk patients has facilitated the detection of HCC at a much early stage. Together with the considerable advances in treatment for HCC, such as surgical resection, percutaneous ablation, transcatheter arterial chemoembolization (TACE), and liver transplantation, the survival of HCC patients has improved much in recent years.[5-9]

Primary HCC lesions often can be removed completely when they are detected at an early stage. Although intrahepatic recurrence of HCC is very frequent, recurrent intrahepatic lesions can be treated successfully using modalities applicable to primary lesions. In particular, percutaneous ablation can be performed repeatedly on recurrent intrahepatic lesions even in patients with moderately impaired liver function. Thus, intrahepatic lesions can be kept under control, but extrahepatic metastasis still may arise.[10,11] Extrahepatic metastasis of HCC were once regarded as a terminal event,[12] and coexisting intrahepatic lesions usually are not treated by locoregional therapies like surgical resection or medical ablation.[13] Although systemic chemotherapies sometimes have been attempted, no standard protocols were established until

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recently.[14,15] In 2 recent, large, randomized controlled trials, it was demonstrated that the multikinase inhibitor sorafenib significantly prolonged survival in patients with advanced HCC, even when the primary lesion was accompanied by extrahepatic metastases; now, sorafenib is widely regarded as the standard treatment for such patients.[16,17] However, the clinical course for patients with extrahepatic metastasis has not yet been fully elucidated, and the prognostic factors remain unclear. This information will be vital when determining whether treatment with sorafenib or other such agents is indicated.

The prognosis for patients with HCC who had extrahepatic metastasis before the availability of sorafenib may represent the natural clinical course for affected patients, because no previous treatments had proven effective. In the current study, we retrospectively analyzed a cohort of these patients to further investigate the clinical features and prognostic factors for HCC with extrahepatic metastasis.

MATERIALS AND METHODS

Patients

This study was conducted according to the ethical guidelines for epidemiologic research designed by the Ministry of Education, Culture, Sports, Science, and Technology and the Ministry of Health, Labor and Welfare, Japan. The study design was approved by the ethics committee of the host institution. Between 1990 and 2006, a total of 2386 patients with HCC were admitted to the University of Tokyo Hospital. A diagnosis of HCC was confirmed radiologically by hyperattenuation in the arterial phase and washout in the late phase using either contrast-enhanced computed tomography (CT) or magnetic resonance imaging (MRI).[18] Ultrasound-guided tumor biopsies were performed when the diagnostic imaging results were inconclusive. In the current analysis, the follow-up period ended on the date of death or on December 31, 2008. Among the 2386 patients in the total HCC cohort in our hospital, extrahepatic metastases were noted in 28 patients at first hospitalization. In addition, extrahepatic metastases were detected in other 314 patients during follow-up observation. Therefore, we retrospectively analyzed 342 patients in our current study.

Diagnosis of Extrahepatic Metastasis and Evaluation of Intrahepatic Lesions

Screenings for extrahepatic metastases were not performed as part of the routine check-up. Most intra-abdominal metastases were detected on abdominal ultrasonography, CT, or MRI studies that were obtained every 3 to 4 months to evaluate intrahepatic lesions. Pulmonary lesions often were noted on chest x-rays, which were obtained routinely at each admission. Additional examinations, such as bone x-ray, bone scintigraphy, and brain CT or MRI studies, were indicated when symptoms attributable to extrahepatic metastasis appeared. These examinations also were undertaken when the HCC-specific tumor markers α-fetoprotein (AFP), lens culinaris agglutinin-reactive fraction of AFP (AFP-L3), or des-γ-carboxy prothrombin (DCP) were elevated and the elevation could not be accounted for by status of the intrahepatic lesion. A diagnosis of extrahepatic metastasis from HCC was based on the enhancement pattern observed on contrast-enhanced CT/MRI studies. Positron emission tomography/CT studies were not obtained routinely, because they were not covered by insurance in Japan. When tumor resections were performed, pathologic investigations also were undertaken. Extrahepatic metastasis detected only at autopsy was not considered an event in this study, because we focused primarily on the diagnosis and treatment of this condition in living patients.

We also evaluated viable intrahepatic lesions at the diagnosis of extrahepatic metastasis by using contrast-enhanced CT/MRI. Post-treatment lesions were not considered viable if they were not enhanced by contrast medium. In the current study, vascular invasion was diagnosed radiologically, indicating *macroscopic vascular invasion*. Vascular invasion included invasion to the portal vein, hepatic vein, inferior vena cava, and bile duct.

Treatment Responses in Patients With Extrahepatic Metastasis

In principle, treatment responses were evaluated according to Response Evaluation Criteria in Solid Tumors (RECIST) guidelines.[19] A complete response (CR) was defined as the disappearance of both intrahepatic lesion and extrahepatic metastasis. In addition, we defined a CR as the disappearance of all intratumoral arterial enhancement according to a recently proposed, modified RECIST assessment for HCC.[20] The evaluation was based on imaging results that were obtained at 2 months after the initiation of treatment. CR was confirmed by repeat assessments performed $\geq$4 weeks after the criteria for response first were met.

Statistical Procedures

Survival after diagnosis of extrahepatic metastases was defined as the interval from the date of diagnosis to the date of death from any cause or to the last visit before

December 31, 2008. The cumulative survival probability was calculated using the Kaplan-Meier method. The cause of death was investigated meticulously using medical records. To develop a scoring system as a prognostic predictor for patients with extrahepatic metastasis, a split-sample method was applied. Our 342 patient cohort was divided randomly into 2 groups: a training set (n = 171) and a testing set (n = 171). The clinical data obtained at the diagnosis of extrahepatic metastasis were assessed as predictors of survival using a Cox proportional hazards model in the training set. The following variables were included in this analysis: age, sex, Eastern Cooperative Oncology Group (ECOG) performance status,[21] hepatitis B surface antigen (HBsAg), HCV antibody, Child-Pugh classification, the size and number of intrahepatic lesion(s), the presence of macroscopic vascular invasion, the presence of symptoms of extrahepatic metastasis, HCC-specific tumor marker levels (AFP, AFP-L3, and DCP), and response to treatment. Each variable was assessed first in a univariate analysis, and the variables that reached a P value $< .05$ were evaluated in a multivariate analysis with stepwise variable selection using Akaike information criterion (AIC). Then, the ratio of regression coefficients of the final model was determined and was rounded to whole digits for convenience. This scoring system was validated in the test group using the chi-square trend test and the Harrell concordance index (c-index).[22] Data were expressed as the mean ± standard deviation unless specified otherwise. All P values $< .05$ were considered statistically significant. All analytical procedures were performed with S-plus (version 7.0; Insightful Corp., Seattle, Wash).

Table 1. Patient Characteristics at the Diagnosis of Extrahepatic Metastasis (n = 342)

Variable	No. of Patients (%)
Age: Mean ± SD, y	66.9 ± 9.0
Men	270 (78.9)
Performance status	
0-1	314 (91.8)
≥2	28 (8.2)
Viral infection	
HBsAg, positive	62 (18.1)
Anti HCVAb, positive	268 (78.4)
Both positive	15 (4.4)
Both negative	27 (7.9)
Child-Pugh class	
A	167 (48.8)
B	153 (44.7)
C	22 (6.4)
Status of intrahepatic lesions	
None	61 (17.8)
≤3 cm and 1-3 lesions	110 (32.2)
>3 cm or ≥4 lesions	171 (50)
Macroscopic vascular invasion, present	65 (19)
Site of extrahepatic metastasis[a]	
Lung	135 (39.5)
Lymph node	117 (34.2)
Bone	87 (25.4)
Adrenal gland	30 (8.8)
Brain	4 (1.2)
Spleen	2 (0.6)
Breast	1 (0.3)
Symptoms of extrahepatic metastasis, present	80 (23.4)
AFP>400 ng/mL	158 (46.2)
AFP-L3>15%[b]	169 (64.8)
DCP>100 mAU/mL	196 (57.3)

SD indicates standard deviation; HCVAb, hepatitis C virus antibody; AFP, alpha-fetoprotein; AFP-L3, lens culinaris agglutinin-reactive fraction of AFP; DCP, des-gamma-carboxy prothrombin.
[a] Including overlap.
[b] Missing in 81 patients.

RESULTS

Patient Background Data

Table 1 indicates that the average age at diagnosis for patients with primary extrahepatic metastasis from HCC was 66.9 ± 9.0 years, and ratio of men to women was 4:1. The distribution of the metastases among patients was the lung in 135 patients (39.5%), lymph node in 117 patients (34.2%), bone in 87 patients (25.4%), adrenal in 30 patients (8.8%), brain in 4 patients (1.2%), spleen in 2 patients (0.6%), and breast in 1 patient (0.3%), for a total of 376 extrahepatic occurrences in 342 patients. Metastases that were detected within 2 weeks after diagnosis of the first metastasis were considered synchronous. Viable, coexisting intrahepatic HCC lesions were identified in 281 patients (82.2%) when the extrahepatic metastasis was diagnosed. Intrahepatic vascular tumor invasion was evident in 65 patients (19%) patients: Portal vein invasion was evident in 57 patients, hepatic vein and inferior vena cava invasion was evident in 13 patients, and invasion into the bile duct was evident in 4 patients. The ECOG performance status was 0 in 229 patients, 1 in 85 patients, 2 in 19 patients, 3 in 5 patients, and 4 in 4 patients. Eighty patients (23.4%) had symptoms caused by extrahepatic metastasis, including dyspnea caused by multiple lung metastases; bone fracture, nerve paralysis, and pain caused by bone metastasis; abdominal pain and obstructive jaundice caused by abdominal lymph node metastasis; and disturbance of consciousness caused by bleeding from brain metastasis.

Table 2. Treatments Received for Extrahepatic Metastasis in the Study Cohort[a]

		No. of Patients (%)					
Organ	Total No.	Resection	Ablation	TACE	Radiation	Chemotherapy	No Treatment
Lung	135	19 (14.1)	–	1 (0.7)	4 (3)	42 (31.1)	69 (51.1)
Lymph nodes	117	8 (6.8)	5 (4.3)	2 (1.7)	26 (22.2)	27 (23.1)	49 (41.9)
Bone	87	–	3 (3.4)	–	68 (78.2)	2 (2.3)	14 (16.1)
Adrenal gland	30	5 (16.7)	7 (23.3)	11 (36.7)	1 (3.3)	–	6 (20)
Brain	4	–	–	–	2 (50)	–	2 (50)
Spleen	2	1 (50)	–	–	–	–	1 (50)
Breast	1	–	–	–	1 (100)	–	–

TACE indicates transarterial chemoembolization
[a] Including overlap.

Treatment of Patients With Extrahepatic Metastasis

Retrospectively reviewed, the treatments for extrahepatic metastatic lesions in our study cohort were considered only in those patients who had Child-Pugh Class B or better liver function and an ECOG performance status ≥ 2 and when intrahepatic lesions, if any, generally were controlled or controllable. Patients also received treatment when they were suffering from symptoms caused by extrahepatic metastasis. Table 2 indicates that these treatments included resection, chemotherapy, irradiation, TACE, and percutaneous ablation.

Surgical resection was undergone by 19 patients who had a lung metastasis (including 13 patients who underwent video-assisted thoracoscopic surgery), 8 patients who had lymph node metastasis, 5 patients who had adrenal metastasis, and 1 patient who has a spleen metastasis. Percutaneous ablation, using either ethanol or radiofrequency, was undergone by 7 patients with adrenal metastasis, 5 patients with lymph node metastasis, and 3 patients with bone metastasis, and TACE was undergone by 11 patients, 2 patients, and 1 patient with of adrenal, lymph node, and lung metastasis, respectively. Irradiation was received by other patients with metastasis as follows: 68 patients with bone metastasis, 26 patients with lymph node metastasis, 4 patients with lung metastasis, 2 patients with brain metastasis, 1 patient with an adrenal metastasis, and 1 patient with a breast metastasis. Systemic chemotherapy was received by an additional 42 patients with lung metastasis, 27 patients with lymph node metastasis, and 2 patients with bone metastasis in our cohort. The most often used chemotherapeutic regimen was cis-diamminedichloroplatinum (CDDP) monotherapy (29 patients) followed by 5-fluorouracil (5-FU) plus interferon (IFN) (24 patients), TS-1 alone (7 patients), CDDP plus 5-FU (6 patients), etoposide alone (6 patients), and TSU-68 (5 patients).

Percutaneous ablation of the intrahepatic lesions, which was indicated only when any extrahepatic lesions had been completely resected or ablated or controlled by irradiation, was performed in 60 patients. TACE treatment of intrahepatic lesions was indicated for patients who had Child-Pugh Class A or B liver function and when the vast majority of the total tumor volume was located in the liver. By using a combination of systemic chemotherapy and/or locoregional therapy to treat intrahepatic lesions, 22 of the patients in our study group achieved a CR as evaluated by the overall response according to RECIST.

Prognosis After the Diagnosis of Extrahepatic Metastasis

In the current study, during the observation period, 301 patients died. The cause of death was related to HCC in 273 patients (90.7%) patients and to liver dysfunction in 15 patients (5%), and death was unrelated to the liver in another 13 patients (4.3%). Extrahepatic metastasis of HCC was related directly to death in 23 patients (7.6%) patients, including 17 deaths from respiratory failure because of a lung metastasis, 5 incidents of cerebral hemorrhage from a brain metastasis, and death in 1 patient who had a bone metastasis and suffered liver failure that caused by hemorrhaging from a bone fracture that was the result of this lesion.

Gastroesophageal varices rupture sometimes became a critical event at the terminal phase of advanced HCC. In the current study, gastroesophageal varices rupture occurred in 25 patients at the end of life. Portal hypertension in these patients was caused either by portal vein tumor thrombus or cirrhosis, which may often coexist and are difficult to discriminate accurately.

The cumulative survival rates at 1 year, 2 years, 3 years, and 5 years after the diagnosis of extrahepatic metastasis in our cohort were 39.3%, 15.3%, 7.4%, and 4%,

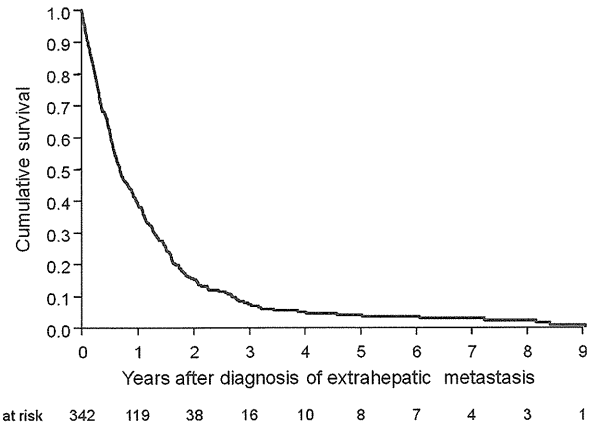

Figure 1. Cumulative survival is illustrated for patients with hepatocellular carcinoma who had a diagnosis of extrahepatic metastasis.

respectively (Fig. 1), and the median survival was 8.1 months (range, from 1 day to 108.7 months). The cumulative survival rates at 1 year, 2 year, and 3 years were 48.9%, 21.2%, and 10.6%, respectively, when the patients had received some treatment for extrahepatic metastasis; and the rates were 19%, 2.3%, and 0%, respectively, when no treatment had been indicated.

Predictors of Prognosis

Prognostic predictors after the diagnosis of extrahepatic metastasis were analyzed in the training set of 171 patients using a Cox proportional hazards model. These predictors were based on clinical factors that were recorded at diagnosis. In univariate analysis, the following factors were associated significantly with a poor prognosis: performance status, Child-Pugh classification, number and size of intrahepatic lesions, the presence of macroscopic vascular invasion, a symptomatic extrahepatic metastasis, AFP level, and CR to therapy (Table 3). Clinical factors that were statistically significant in univariate analysis were analyzed further in multivariate analysis with a stepwise selection of variables to minimize the AIC. To simplify the scoring system using multivariate analysis, intrahepatic tumor extension was categorized as none, a viable lesion without vascular invasion, or a viable lesion with vascular invasion. Only intrahepatic tumor extension at the diagnosis of extrahepatic metastasis and performance status were selected by a stepwise selection as factors in the final model (Table 4). Scores were assigned to each factor according to the estimated regression coefficient in the final model, and the prognosis score was defined as the sum of each score (Table 5). Our scoring system was validated using the testing set of 171 patients. A Kaplan-Meier plot was used to illustrate distinct survival curves according to the prognosis score (chi-square linear trend test: P thinsp;< .001) (Fig. 2). The c-index for the scoring system in the testing set was 0.73, thus reflecting good prognostic discrimination (Table 6).

Table 3. Predictors of Survival After a Diagnosis of Extrahepatic Metastasis: Univariate Analysis (n = 171)

Variable	β	HR (95% CI)	P
Age	0.02	1.02 (1.00-1.03)	.12
Men	0.07	1.08 (0.72-1.61)	.72
Performance status			
0		1.00	
1	0.36	1.44 (1.00-2.07)	.05
2	1.08	2.96 (1.29-6.79)	.01
3	2.61	13.5 (3.90-47.04)	<.0001
4	1.07	2.93 (0.40-21.26)	.29
HBsAg positive	−0.17	0.84 (0.53-1.33)	.46
Anti-HCVAb-positive	−0.27	0.76 (0.51-1.15)	.19
Child-Pugh class			
A		1.00	
B	0.37	1.44 (1.03-2.02)	.03
C	0.64	1.90 (0.97-3.69)	.06
Size of intrahepatic lesion, cm			
Absent		1.00	
≤3.0	0.71	2.04 (1.18-3.51)	.01
>3.0	1.41	4.12 (2.31-7.32)	<.0001
No. of intrahepatic lesion			
Absent		1.00	
1-3	0.67	1.96 (1.16-3.30)	.01
>3	0.93	2.52 (1.55-4.11)	.0002
Macroscopic vascular invasion, present	0.78	2.18 (1.46-3.25)	.0001
Symptom of extrahepatic metastasis, present	0.37	1.45 (1.01-2.09)	.047
AFP>400 ng/mL	0.54	1.71 (1.23-2.39)	.002
AFP-L3>15.0%	0.30	1.34 (0.92-1.96)	.12
DCP>100 mAU/mL	0.08	1.09 (0.78-1.51)	.62
Response to treatment, CR[a]	−0.77	0.46 (0.21-1.00)	.049

HR indicates hazard ratio; CI, confidence interval; HBsAg, hepatitis B surface antigen; HCVAb, hepatitis C virus antibody; AFP, alpha-fetoprotein; AFP-L3, lens culinaris agglutinin-reactive fraction of AFP; DCP, des-gamma-carboxy prothrombin; CR, complete response

[a] Response was evaluated using overall responses according to Response Evaluation Criteria in Solid Tumors (RECIST); treatments included locoregional therapy and systemic chemotherapy for both intrahepatic lesions and extrahepatic lesions.

DISCUSSION

The prognosis for patients with extrahepatic metastasis of HCC was poor in the current study, consistent with previous reports that the 1-year survival rate is approximately 40% for patients with this disease.[23-27] However, from our current analyses, we observed that extrahepatic

Table 4. Predictors of Survival After a Diagnosis of Extrahepatic Metastasis: Multivariate Analysis (n = 171)

Variable	β	HR (95% CI)	P
Intrahepatic viable lesion			
None		1.00	
Without macroscopic vascular invasion	0.67	1.96 (1.21-3.18)	.006
With macroscopic vascular invasion	1.31	3.70 (2.08-6.57)	<.0001
Performance status			
0		1.00	
1	0.30	1.36 (0.94-1.96)	.11
2	1.11	3.05 (1.32-7.06)	.009
3-4	1.78	5.94 (2.09-16.9)	.0008

HR indicates hazard ratio; CI, confidence interval.

Table 5. Scoring System to Predict Survival in Patients With HCC and Extrahepatic Metastasis

Variable	Score
Intrahepatic viable lesion	
None	0
Present without macroscopic vascular invasion	1
Present with macroscopic vascular invasion	2
Performance status	
0-1	0
2	2
3-4	3

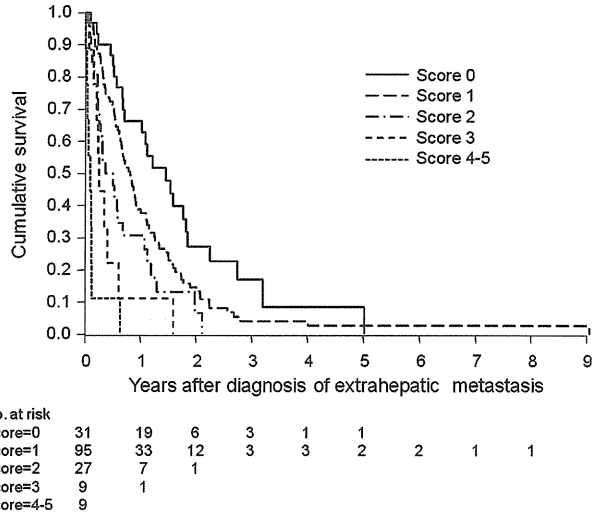

Figure 2. Stratified cumulative survival is illustrated for patients with hepatocellular carcinoma who had a diagnosis of extrahepatic metastasis based on prognostic scores. The prognosis for patients in the testing set could be stratified clearly by the scoring system based on an analysis of patients in the training set.

Table 6. Median Survival According to Prognostic Scores (n = 171)

Score	No. of Patients	Median Survival, mo
0	31	17.5
1	95	9.7
2	27	6.1
3	9	3.0
4-5	9	1.2

metastasis was not the direct cause of death in the majority of affected patients: the exceptions included respiratory failure from a bilateral lung metastasis and cerebral hemorrhage as a result of a brain metastasis, which accords with a previous report.[28] Hence, the presence of extrahepatic metastasis is an indicator of the aggressiveness of the primary HCC as a whole rather than an independent prognostic determinant.

In contrast to extrahepatic metastases, the progression of intrahepatic lesions was identified as the cause of death in 81% of patients in our current cohort, indicating the importance of controlling intrahepatic tumors in patients with HCC whenever possible. Repeated percutaneous ablations or TACE generally are considered for patients with HCC who develop an intrahepatic recurrence.[29,30] Intrahepatic arterial chemotherapy also reportedly is effective against advanced HCC with portal venous tumor invasion.[31] Thus, these locoregional treatments should be considered for intrahepatic lesions in selected patients who have extrahepatic metastasis, although the liver function reservoir should be evaluated cautiously in these patients.

In the current study cohort, patients received treatment for extrahepatic metastasis when their intrahepatic tumor was under control and liver function was maintained. Extrahepatic metastases also were treated when metastasis-related symptoms were strong or when further progression of the metastatic lesions was considered life-threatening. The prognosis was better among the current patients with HCC who received some treatment for their extrahepatic metastasis compared with those who were untreated. However, the contribution of these treatments to the overall prognosis remains unknown, because the patients who received them generally were in better condition. Nevertheless, our current findings indicate that treatments for extrahepatic metastases can be considered in patients who have hepatic lesions under control, because long-term survival was achieved only in those who had received such therapies.

Our current analyses indicated that resection of metastatic lesions produced a satisfactory local response, consistent with previous reports.[32-36] Locoregional therapy for extrahepatic metastasis also was discussed in earlier studies, including irradiation for bone,[37] lymph node,[38] brain,[39] and adrenal[40] metastases; TACE for adrenal metastasis[41]; and percutaneous ablation for adrenal[42] and bone metastases.[43] We also used these methods to treat some patients in our cohort. According to the conventional treatment strategy for solid tumors, the presence of metastatic disease is a contraindication for locoregional therapy, because it is believed that these tumor cells already have spread systemically. However, from the viewpoint of reducing tumor burden, locoregional therapy may be an adequate strategy when the target lesions account for the major portion of the total tumor volume. When resection and other locoregional therapies were contraindicated for extrahepatic metastasis, we sometimes used systemic chemotherapy. However, the overall response rate to conventional chemotherapy in the current study was only 25.4%. The establishment of an effective chemotherapeutic regimen still is needed for these patients, and molecular targeted agents, such as sorafenib,[16,17] are expected to improve their prognosis.

The scoring system we propose in the current study incorporates the presence of intrahepatic lesions, the extent of vascular invasion, and performance status. The progression of an intrahepatic lesion was the major cause of death among our patients, as described above. In patients who had extrahepatic metastases, evaluation of the size and number of intrahepatic lesions often is difficult because of disease progression. From the standpoint of these patients, the proposed scoring system is both simple and convenient. Vascular invasion is 1 of the most important prognostic factors for HCC.[12] Our current results demonstrated that macroscopic vascular invasion is significant even in patients who have extrahepatic metastasis. Performance status, which is an important biologic factor in clinical oncology, also is included in our scoring system.[13] Liver function no doubt is a prognostic determinant for patients with HCC; however, the Child-Pugh classification did not retain significance in our multivariate analysis. This may be because the Child-Pugh class is strongly correlated with performance status, which also includes other significant aspects of cancer biology.

Our current results indicate that the median survival of patients with HCC who have extrahepatic metastases varies widely from within 1 month to 1.5 years and can be discerned using the prognosis factors that were evaluated in this study. Patients who have a prognostic score ≥ 2, which indicates an estimated median survival ≥ 6 months, can be considered for intensive treatment, including surgical procedures. In addition, our scoring system may be used for the enrollment of patients into clinical trials of newly developed agents for which patients with extrahepatic metastasis or vascular invasion may be candidates, although further detailed research will be required to establish such use. We compared the prognosis of patients who were treated in the 1990s and the 2000s and observed no statistical difference between the 2 decades (data not shown). During the study period, newly developed agents, such as sorafenib and drug-eluting beads, were not available in Japan.

There were some limitations in this retrospective cohort study. First, a variety of treatments was provided for various intrahepatic and extrahepatic lesions. Substantial heterogeneity existed in patient background. Second, the proportion of patients who had vascular invasion in our cohort was relatively small despite the presence of extrahepatic metastasis, and this may indicate that the total tumor burden also was relatively small. This may have been because most extrahepatic metastasis in our cohort emerged while treatment for intrahepatic lesions was being repeated. Moreover, the proportion of patients with vascular invasion was not very high, even among the patients who had extrahepatic metastasis at initial presentation. Supposedly, this is because our hospital is a tertiary care center, and patients with an apparent indication for percutaneous ablation were referred to us selectively. Third, the number of patients who had prognostic scores of 3, 4, 5 was not large enough for confirmation, although the linearity of median survival (Table 6) suggests the relevance of the scoring system.

In conclusion, the major cause of death in patients with HCC who have extrahepatic metastases is progression of the intrahepatic HCC lesion. We contend that treatment of intrahepatic lesions should not be contraindicated merely because of the presence of an extrahepatic metastasis. Moreover, radical treatments for extrahepatic metastases may be considered when hepatic lesions are under reasonable control or if the metastasis is accompanied by severe symptoms.

CONFLICT OF INTEREST DISCLOSURES

The authors made no disclosures.

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Apoptosis Signal-Regulating Kinase 1 Inhibits Hepatocarcinogenesis by Controlling the Tumor-Suppressing Function of Stress-Activated Mitogen-Activated Protein Kinase

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The stress-activated mitogen-activated protein kinases (MAPKs), c-Jun NH2-terminal kinase (JNK), and p38 have been implicated in hepatocarcinogenesis. Although the many interrelated functions of JNK and p38 are precisely regulated by upstream signaling molecules, little is known about upstream regulators. We investigated the role of apoptosis signal-regulating kinase 1 (ASK1), a major player in the regulation of JNK and p38 activities, in hepatocarcinogenesis using a mouse hepatocellular carcinoma (HCC) model. ASK1-deficient ($ASK1^{-/-}$) and wildtype (WT) mice were treated with diethylnitrosamine on postnatal day 14. Strikingly, after 7 months, approximately three times as many tumors developed in $ASK1^{-/-}$ mice as in WT mice. Although JNK and p38 activation were attenuated in $ASK1^{-/-}$ HCCs relative to WT HCCs, cell proliferation was comparable in HCCs from both types of mice. On the other hand, both cancer cell apoptosis and hyperphosphorylation of BimEL, a proapoptotic Bcl-2 family member, were suppressed in the $ASK1^{-/-}$ HCCs. $ASK1^{-/-}$ mice showed remarkable resistance to Fas-induced hepatocyte apoptosis *in vivo*, probably because of attenuated JNK-mediated BimEL phosphorylation and mitochondrial apoptotic pathway activation. The reintroduction of ASK1 to $ASK1^{-/-}$ mouse liver using an adenoviral vector restored Fas-induced hepatocyte death and phosphorylation of JNK and BimEL. Similar findings were obtained in tumor necrosis factor alpha-induced hepatocyte apoptosis. Furthermore, ASK1 was involved in DNA damage-induced p21 up-regulation through a p38 pathway. *Conclusion:* ASK1 is involved in death receptor-mediated apoptosis and DNA-damage response by way of stress-activated MAPK in the liver, and thus acts as a tumor suppressor in hepatocarcinogenesis. This study provides new insight into the regulation of stress-activated MAPK signaling in hepatocarcinogenesis. (HEPATOLOGY 2011;54:185-195)

Hepatocellular carcinoma (HCC) is the third most common cause of cancer mortality; thus, understanding the molecular carcinogenic mechanism is an important issue.[1] Several molecular pathways have been reported to play important roles in hepatocarcinogenesis. In particular, clinical and experimental studies have implicated the stress-activated mitogen-activated protein kinase (MAPK) cascades that converge on c-Jun NH_2-

Abbreviations: ALT, alanine aminotransferase; ASK1, apoptosis signal-regulating kinase 1; DEN, diethylnitrosamine; GalN, galactosamine; HCC, hepatocellular carcinoma; JNK, c-Jun NH2-terminal kinase; LPS, lipopolysaccharide; MAPK, mitogen-activated protein kinase; MAP3K, mitogen-activated protein kinase kinase kinase; TNF-α, tumor necrosis factor-α.

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terminal kinase (JNK) and p38 as key regulators of hepatocarcinogenesis.[2-6]

JNK and p38 have complex functions and modulate a wide range of cellular effects, including apoptosis, proliferation, differentiation, migration, and inflammation.[7] Evidence implicating the JNK and p38 signaling pathways in the development of various types of cancer is strong, although certain cells use these signaling pathways to combat cancer development, whereas others use these pathways as cancer promoters.[8,9] Crosstalk between the JNK and p38 pathways further complicates the roles of these pathways in carcinogenesis.[7] Although determining the mechanisms regulating these complex and multifunctional signaling pathways is essential for the development of new therapeutic approaches, these mechanisms are not yet well understood.

The activities of JNK and p38 are tightly regulated by upstream MAPK kinases and MAPK kinase kinases (MAP3Ks). Acting far upstream in the intracellular MAPK signaling cascade, MAP3Ks respond to intracellular and extracellular stimuli and determine cell fate.[10] Apoptosis signal-regulating kinase 1 (ASK1), a ubiquitously expressed MAP3K, selectively activates the JNK and p38 signaling pathways in response to a variety of stimuli, including reactive oxygen species and cytokines, and has been widely accepted as a major player in the modulation of JNK and p38 activities regulating cell death.[11] In liver disease, ASK1 is involved in acetaminophen-induced acute liver injury.[12] Furthermore, recent reports revealed that ASK1 participates in colon and skin cancer development through the regulation of apoptosis and inflammation.[13,14] However, involvement of ASK1 in hepatocarcinogenesis has not been reported.

In this study we examined whether ASK1 plays a role in hepatocarcinogenesis using a diethylnitrosamine (DEN)-induced mouse HCC model. We found that ASK1 deficiency promoted the development of HCC, and ASK1 inhibited hepatocarcinogenesis by controlling the tumor-suppressing function of stress-activated MAPK.

Materials and Methods

Animals. Male ASK1-deficient ($ASK1^{-/-}$), $JNK1^{-/-}$, $JNK2^{-/-}$, and C57BL/6 wildtype (WT) mice (Clea Japan, Tokyo, Japan) were used in the experiments. $ASK1^{-/-}$, $JNK1^{-/-}$, and $JNK2^{-/-}$ mice were generated as described[12,15] and backcrossed into the C57BL/6 strain at least 14 times. Mice were maintained under conventional conditions under a light/dark cycle.

All of the experimental protocols were approved by the Ethics Committee for Animal Experimentation and conducted in accordance with the National Institutes of Health (NIH) *Guidelines for the Care and Use of Laboratory Animals.*

Drug Administration and Experimental Design in an* In Vivo *Model. In the DEN-induced HCC model, DEN (Sigma, St. Louis, MO) dissolved in phosphate-buffered saline (PBS) was injected intraperitoneally into mice (25 mg/kg) on postnatal day 14. Mice were sacrificed after 7 months and their livers were removed and examined for visible tumors. In the DEN-induced acute liver injury model, mice were injected intraperitoneally with 100 mg/kg DEN. In the Fas-induced liver injury model, mice (8-10 weeks old) were injected intraperitoneally with the agonistic anti-Fas antibody Jo2 (0.4 μg/g body weight; BD Pharmingen, CA) dissolved in PBS. In the lipopolysaccharide (LPS)/D-galactosamine (GalN)-induced liver injury model, mice were injected intraperitoneally with LPS (20 μg/kg; Sigma) and GalN (1,000 mg/kg; Wako). Some mice were pretreated with JNK inhibitor SP600125 (25 mg/kg; Biomol, PA) or p38 inhibitor SB203580 (25 mg/kg; Wako, Osaka, Japan) dissolved in PBS containing 10% dimethyl sulfoxide. Inhibitors were administered intraperitoneally 1 hour before Jo2 or DEN injection. Histological analyses, RNA extraction, real-time polymerase chain reaction (PCR), and generation of bone marrow chimeric mice were performed as described in the Supporting Information.

Cells and RNA Interference. The human HCC cell lines HuH7 (Human Science, Tokyo, Japan) and PLC/PRF/5 (Riken, Tsukuba, Japan) and a human normal hepatocyte line (ACBRI, Kirkland, WA) were cultured in Dulbecco's modified Eagle medium supplemented with 10% fetal bovine serum. Cell numbers were determined using a Cell Counting Kit-8 (Dojindo Laboratories, Kumamoto, Japan). RNA oligonucleotides were synthesized by Qiagen (Hilden, Germany), and small interfering RNA (siRNA) transfections were performed using RNAiMAX (Invitrogen, Carlsbad, CA). Ultraviolet (UV) irradiation was performed using a UVB lamp (UVP, Upland, CA).

Immunoblotting and Coimmunoprecipitation Analysis. Details are described in the Supporting Information. Anti-ASK1 and anti-phospho-ASK1 antibodies were as described.[16,17]

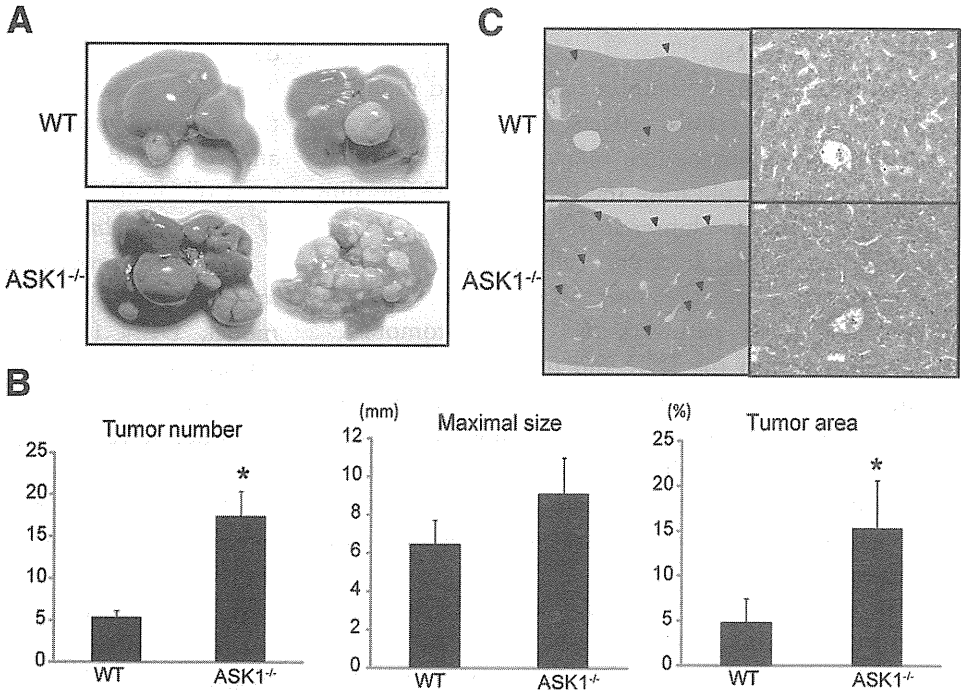

Fig. 1. Increased HCC development in ASK1$^{-/-}$ mice. (A) Livers from 7-month-old DEN-treated WT and ASK1$^{-/-}$ mice. (B) Tumor numbers, maximal tumor sizes, and tumor-occupied areas in WT and ASK1$^{-/-}$ mouse livers. Data are expressed as means ± standard error of the mean (SEM) (WT, n = 14; ASK1$^{-/-}$, n = 12). *P < 0.05, compared with WT mice. (C) Typical histological appearance of livers (left) and HCCs (right) from DEN-treated WT and ASK1$^{-/-}$ mice (hematoxylin and eosin [H&E] staining).

Infection of Recombinant Adenovirus. Recombinant adenoviruses encoding β-galactosidase (LacZ) and HA-tagged ASK1 (Ad-ASK1) were constructed as described.[18] Adenoviruses were diluted in PBS and injected into the tail vein 48 hours before Jo2 administration (1×10^8 plaque-forming units [PFU]/mouse).

Statistical Analyses. Statistical analyses were performed using Student's t test or analysis of variance (ANOVA), followed by Dunnett's test where appropriate. $P < 0.05$ was considered statistically significant.

Results

Loss of ASK1 Accelerates Chemically Induced Hepatocarcinogenesis. To determine the role of ASK1 in hepatocarcinogenesis, male WT and ASK1$^{-/-}$ mice were injected with 25 mg/kg DEN on postnatal day 14. After 7 months, untreated WT and ASK1$^{-/-}$ mice revealed no spontaneous liver dysfunction or tumor formation, whereas all mice given DEN developed typical HCCs. Strikingly, the number of detectable tumors was approximately three times higher in ASK1$^{-/-}$ mice than in WT mice, and the tumor-occupied areas were also more extensive in ASK1$^{-/-}$ mice than in WT mice (Fig. 1B,C). The maximum tumor size tended to be larger in ASK1$^{-/-}$ mice, but the difference was not statistically significant (Fig. 1B). DEN-induced liver tumors were histologically similar to well-to-moderately differentiated human HCCs, and the pathological characteristics of the tumors from WT and ASK1$^{-/-}$ mice were similar (Fig. 1C). Thus, loss of ASK1 accelerated DEN-induced HCC development.

Role of ASK1 in Cancer Cell Proliferation and Apoptosis. We compared the characteristics of DEN-induced HCCs in WT and ASK1$^{-/-}$ mouse livers. The phosphorylation level of JNK, but not of p38, was higher in HCCs than in nontumor tissues, and JNK and p38 phosphorylation levels were lower in ASK1$^{-/-}$ HCCs than in WT HCCs (Fig. 2A). However, important downstream substrates of stress-activated MAPK involved in cell-cycle and tumor promotion, such as c-Jun and cyclin D1, were expressed at comparable levels in WT and ASK1$^{-/-}$ mice (Fig. 2A). Additionally, the frequency of cells positive for proliferating cell nuclear antigen (PCNA), a marker of cell proliferation, was similar for the WT and ASK1$^{-/-}$ HCCs (Fig. 2B). Because ASK1 appeared to be expressed at slightly higher levels in HCCs than in nontumor tissues (Fig. 3A), we examined whether ASK1 affects cancer cell proliferation *in vitro* by treating the HCC cell line HuH7 with ASK1-specific siRNA. ASK1-silencing decreased JNK phosphorylation (but not p38 phosphorylation) and c-Jun expression, decreased cyclin D1 expression slightly, and inhibited cell proliferation slightly (Fig. 3C,D), suggesting that the ASK1–JNK pathway weakly enhances HCC cell proliferation. A similar result was also observed in the PLC/PRF/5 HCC cell line (Fig. 3D). However, as discussed above, the WT

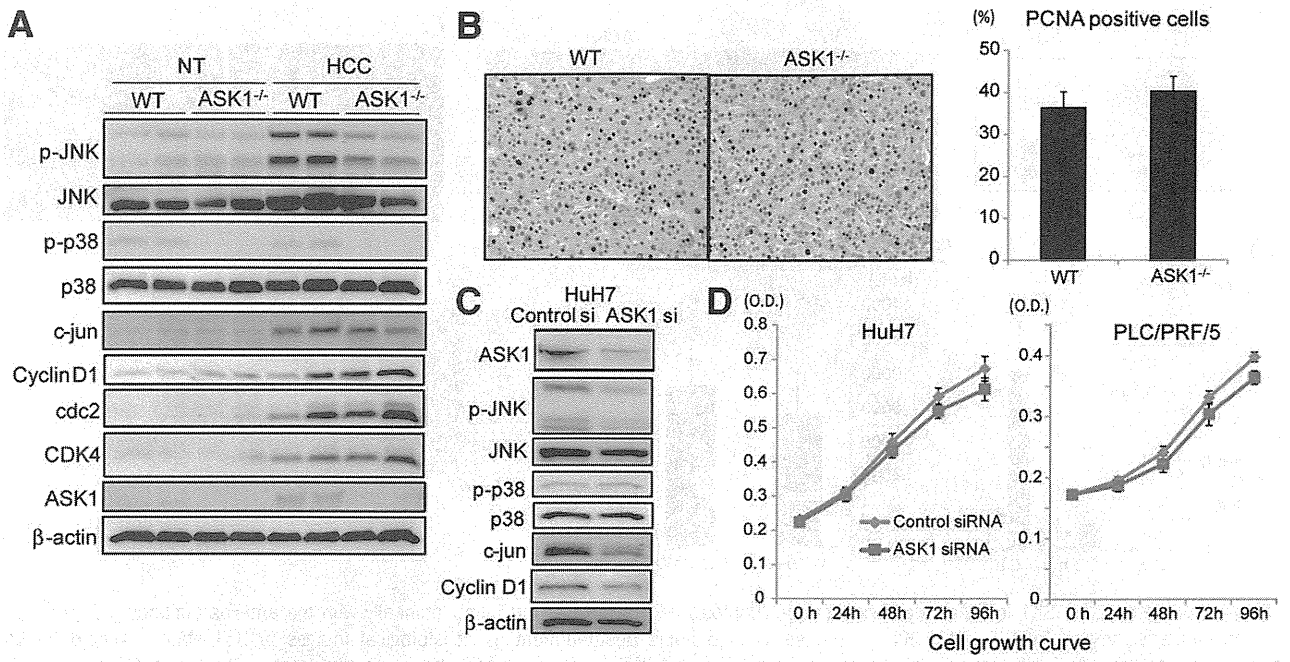

Fig. 2. Role of ASK1 in HCC cell proliferation. (A) Western blot analysis of the indicated proteins in nontumor (NT) and HCC liver tissues from WT and ASK1$^{-/-}$ mice. β-Actin was used as a loading control. (B) Representative PCNA staining of WT and ASK1$^{-/-}$ HCC tissue sections. Bar graph showing frequencies of PCNA-positive cells. Data are expressed as means ± SEM (n = 9 per group). (C) Expression levels of indicated proteins in HuH7 cells 48 hours after transfection with ASK1 or control siRNA. (D) Numbers of HuH7 and PLC/PRF/5 cells counted after transfection. Data are plotted as means ± standard deviation (SD).

and ASK1$^{-/-}$ HCCs exhibited similar c-Jun expression and cell proliferation rates *in vivo*, suggesting that other compensatory pathways promote c-Jun expression and cell proliferation in ASK1$^{-/-}$ HCCs. Based on these results, we conclude that the loss of ASK1 does not promote cancer cell proliferation and that there are other reasons for accelerated hepatocarcinogenesis in ASK1$^{-/-}$ mice.

Next, we compared the numbers of apoptotic cells in the WT and ASK1$^{-/-}$ mice livers using the

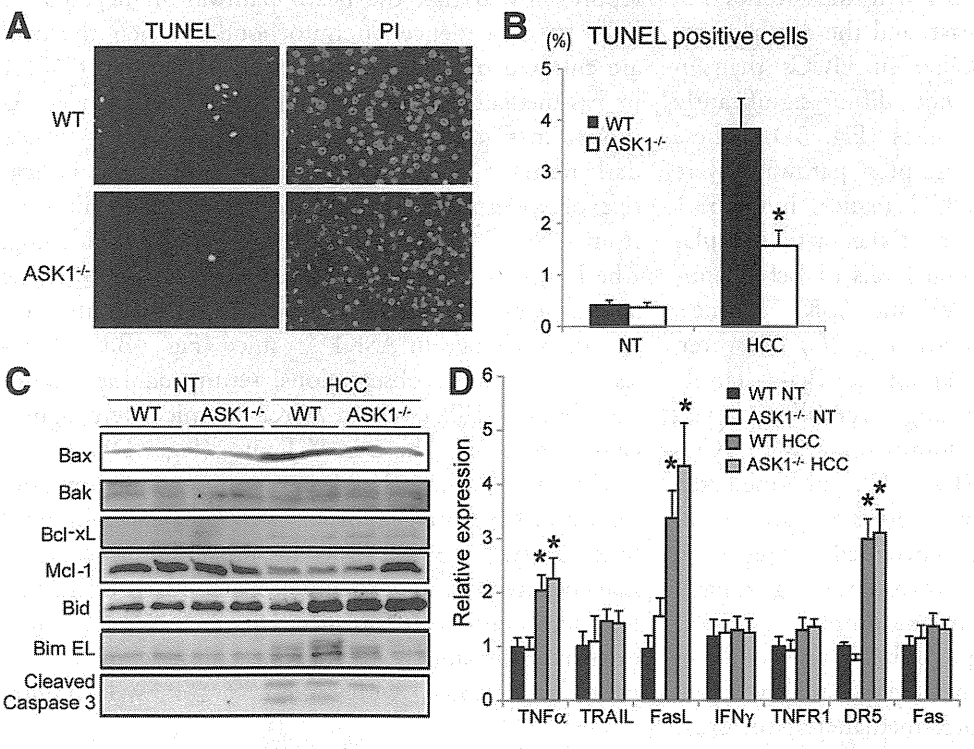

Fig. 3. Apoptosis is suppressed in ASK1$^{-/-}$ HCC tissues. (A) Representative TUNEL-stained sections of HCC tissues from WT and ASK1$^{-/-}$ mice. (B) Frequencies of TUNEL-positive apoptotic cells in nontumor and HCC liver tissues. Data are expressed as means ± SEM (n = 9 per group). *P < 0.05, compared with HCC tissue from WT mice. (C) Western blot analysis of the indicated proteins in nontumor and HCC liver tissue from WT and ASK1$^{-/-}$ mice. (D) Relative mRNA levels for death ligands and receptors in nontumor and HCC liver tissues from WT and ASK1$^{-/-}$ mice determined by real-time PCR. Data are expressed as means ± SEM (n = 9 per group). *P < 0.05, compared with nontumor tissue from WT mice.

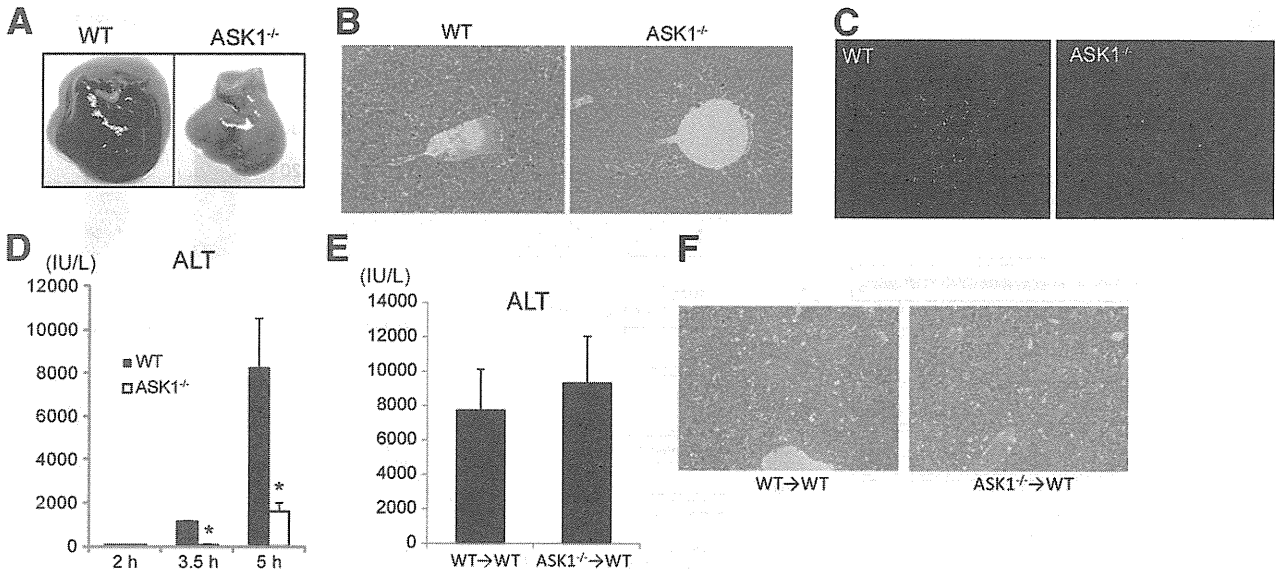

Fig. 4. Involvement of ASK1 in Fas-induced hepatocyte apoptosis. Mice were injected intraperitoneally with the anti-Fas antibody Jo2 (0.4 μg/g). (A) Representative images of WT and ASK1$^{-/-}$ mouse livers 5 hours posttreatment. (B) Histological images of H&E-stained liver obtained 5 hours posttreatment. (C) TUNEL-stained liver sections from WT and ASK1$^{-/-}$ mice taken 5 hours posttreatment. (D) Serum ALT levels 2, 3.5, and 5 hours posttreatment. Data are expressed as means ± SEM (n = 3 per group at 2 hours; n = 5 per group at 3 and 5 hours). *$P <$ 0.05, compared with WT mice. (E,F) Analysis of the role of ASK1 in hematopoietic cells in Fas-induced liver injury. (E) Serum ALT levels 5 hours after Jo2 administration to bone marrow-chimeric mice. WT mice were transplanted with WT or ASK1$^{-/-}$ mouse-derived bone marrow cells. Data are expressed as means ± SEM (n = 5 per group). (F) Histological image of H&E-stained liver obtained 5 hours after Jo2 administration.

terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) assay. As shown in Fig. 3A,B, significantly fewer apoptotic tumor cells were found in ASK1$^{-/-}$ HCCs than in WT HCCs. Consistent with this, caspase-3 activation was significantly attenuated in ASK1$^{-/-}$ HCCs (Fig. 3C). Messenger RNA (mRNA) levels for the death ligands tumor necrosis factor-α (TNF-α) and FasL and the death receptor TRAIL-R2/DR5 were higher in HCCs than in nontumor tissues, but did not differ significantly between WT and ASK1$^{-/-}$ HCCs (Fig. 3D). These findings indicate that death receptor pathways were activated in DEN-induced HCC tissues, but ASK1 does not regulate the expression of the main modulators. Furthermore, the expression levels of Bcl-2 families were almost identical in WT and ASK1$^{-/-}$ mice, as shown by western blot analysis (Fig. 3C). However, slower migration of the proapoptotic Bcl-2 family member BimEL band, indicating hyperphosphorylation of BimEL, was more predominant in WT HCCs than with ASK1$^{-/-}$ HCCs (Fig. 3C). JNK-mediated Bim phosphorylation has been reported to play an important role in death receptor-mediated apoptosis in the liver,[19,20] and defective death receptor signaling is considered to be a cause of tumor immune escape.[21] Based on these results, we hypothesized that the loss of ASK1 might accelerate hepatocarcinogenesis by allowing cells to escape death receptor-mediated apoptosis.

ASK1 Is Involved in Fas-Induced Hepatocyte Apoptosis. To evaluate whether ASK1 plays a role in Fas-mediated hepatocyte apoptosis, WT and ASK1$^{-/-}$ mice were injected intraperitoneally with agonistic anti-Fas antibody (Jo2), which causes severe liver damage through apoptotic Fas signaling. Because a recent report showed that the death pathway in hepatocytes loses its dependence on mitochondria when the cells are cultured on plates,[22] we assessed the role of ASK1 in Fas-mediated apoptosis using an *in vivo* model. As shown in Fig. 4A, the liver from WT mice turned dark red at 5 hours after injection, which was indicative of widespread hemorrhage. In contrast, the liver from ASK1$^{-/-}$ mice showed only slight reddening. The histological examination revealed extensive hepatic apoptosis and hemorrhage in WT mice, but only focal apoptotic change in ASK1$^{-/-}$ mice (Fig. 4B,C). Consistent with these observations, serum alanine aminotransferase (ALT) levels in ASK1$^{-/-}$ mice were significantly lower than those in WT mice (Fig. 4D).

On the other hand, secondary inflammatory responses have been reported to modulate Jo2-induced liver injury.[23] To rule out the possibility that ASK1 may be involved in Jo2-induced secondary inflammatory responses, we performed Jo2-induced liver injury experiments using bone marrow chimeric mice. WT mice transplanted with ASK1$^{-/-}$ or WT mouse-