areas where subgenotype C2 predominates.[10,12] However, 1753 and 1754 mutations have not been previously associated with fulminant hepatic failure,[8] possibly because of the small number of patients in the present study and the fact that these may be weak associations. Thus, discussion of the association of fulminant hepatic failure with the T1762, A1764, and A1896 BCP/PC mutations should also consider HBV subgenotypes.[27] In the present study, of the 9 patients who progressed to fulminant hepatic failure, 8 were infected with subgenotype C2 carrying a T1762, A1764, or A1896 mutation, while 1 lacked the T1762, A1764, and A1896 mutations and was infected with the Okinawa variant of genotype C. It is possible that this minor variant of subgenotype C could affect the clinical course independent of the BCP and PC variants. The contributions of the subgenotype variants to the clinical features of acute hepatitis patients need to be clarified. However, host immune responses against HBV are also important in the pathogenesis of HBV infection.[28] Although host factors may influence progression to fulminant hepatic failure, these factors are complicated and difficult to evaluate clinically.

Acute hepatitis B patients with subgenotype C1 were also identified. Several reports have assessed differences in clinical and virological features between C1 and C2 subgenotypes.[29-31] The C1858 variant is detected frequently in subgenotype C1 but not in C2. C1858 is found mainly in genotype A and also occurs in genotypes C and F, but not in genotypes B, D, and E.[32,33] A study of 174 patients in Hong Kong with subgenotype C1 found the C1858 variant in 151 patients and the T1858 variant in 23 patients. Of 40 patients with subgenotype C2, 1 had C1858 and the remaining 39 patients had T1858.[34] In the present study, T1858 was found in HBV subgenotype C2, and both T1858 and C1858 were found in subgenotype C1. Nucleotides 1858 and 1896 form a pair in the hairpin loop of the encapsidation sequence of HBV. C1858 and T1896 pair to form a stable structure, whereas C1858 and A1896 mutations do not occur in the same strain. In contrast, T1858 tolerates the T to A transition at nucleotide 1896, which creates a TAG stop codon in the precore region. Among patients with HBV subgenotypes C1 and C2 with T1858, those with A1896 are at high risk for progression to fulminant hepatic failure. However, typing of nucleotide 1896 was not useful for predicting progression to fulminant hepatic failure in patients infected with subgenotype C1 with C1858. A patient with fulminant hepatic failure infected with subgenotype C1 with the C1858 but not the A1896 variant has been reported; this case supports our hypothesis.[35] Similarly, fulminant hepatic failure caused by genotype A was not related to nucleotide A1896 mutation, because genotype A has mainly C1858, which prevents mutation of T1896 to A1896. BCP and PC variants, especially nucleotide 1858 and 1896 variants and HBV subgenotype C, may serve as predictive markers for fulminant hepatic failure. However, to predict fulminant hepatic failure, other variants should be evaluated for patients with HBV subgenotypes A1, A2, and C1 that have the C1858 mutation.

With respect to HBV genotype B, Ozasa et al.[8] reported that subgenotype Bj (also known as B1) is strongly associated with the development of fulminant hepatic failure. Imamura et al.[16] reported that genotype B is more prevalent in patients with fulminant hepatic failure than in those with acute self-limited hepatitis. However, they did not compare differences between subgenotypes Ba (also known as B2) and Bj (B1). On the basis of the nucleotide sequence at nucleotide 1838, 15 of 22 of their acute hepatitis patients with genotype B could be classified as subgenotype Ba (B2) and 7 as subgenotype Bj (B1) [A1838 is Ba (B2) and G1838 is Bj (B1)].[36] Of their 15 acute hepatitis patients with Ba, 6 (40%) developed fulminant hepatic failure, whereas 4 of their 7 (57.1%) acute hepatitis patients with Bj (B1) developed fulminant hepatic failure (OR, 2.00; 95% CI, 0.324–12.329; $P = 0.384$). No statistically significant differences in subgenotype B were noted in their report. In the present study, 2 patients with subgenotype Ba (B2) who developed fulminant hepatic failure were immunocompromised, and no patients with subgenotype Bj (B1) developed fulminant hepatic failure, although the number of patients was very small. Further studies are needed to clarify the influence of subgenotypes of HBV genotype B. The roles of BCP and PC variants in acute hepatitis may differ between subgenotypes. The relationships of other HBV subgenotypes and BCP and PC variants to progression to fulminant hepatic failure should be studied further.

In conclusion, the T1762, A1764, and A1896 variants of HBV subgenotype C2 may play important roles in progression to fulminant hepatic failure, and the combination of HBV subgenotype with BCP and PC variant analyses would be useful for predicting clinical outcome and developing treatment algorithms for patients with acute hepatitis B.

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HEPATOLOGY

# Characteristics and prognosis of patients in Japan with viral marker-negative hepatocellular carcinoma

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## Abstract

**Background and Aim:** The characteristics and prognosis of patients with hepatitis virus marker-negative hepatocellular carcinoma (HCC) is not fully elucidated in Japan. We investigated the characteristics and prognosis of HCC patients in whom no markers for hepatitis virus infection were detected, in comparison with those of HCC patients with hepatitis virus infection.

**Methods:** Viral markers for hepatitis B and C virus (HBV and HCV) infection were measured in 1152 patients in whom initial HCC was diagnosed between 1991 and 2004. Patient characteristics, characteristics of HCC and survival were compared between patients in whom no marker was positive (viral marker-negative HCC) and those in whom chronic HBV or HCV infection was confirmed by viral markers (viral HCC).

**Results:** Overall, 119 patients (10.3%) were shown to have viral marker-negative HCC. Hepatocellular carcinoma was detected under surveillance in a significantly smaller percentage of patients with viral marker-negative HCC than of patients with viral HCC ($P < 0.0001$). The tumor was significantly larger ($P < 0.0001$) and vascular invasion was significantly more prevalent ($P = 0.0003$) in patients with viral marker-negative HCC than in those with viral HCC. The survival rate of patients with viral marker-negative HCC was significantly lower than that of patients with viral HCC ($P = 0.0378$).

**Conclusion:** The patients with HCC in whom hepatitis viral infection had not been confirmed tended not to be under surveillance, resulting in the detection of HCC at more advanced stage and with a poorer prognosis. Efforts to identify patients without hepatitis virus infection who should be under surveillance for HCC will be necessary in the future.

## Introduction

Hepatocellular carcinoma (HCC) is one of the most common malignancies, especially in southern and eastern Asia. Currently in Japan, HCC is the third-leading cause of death from cancer. The most important risk factor for development of HCC worldwide is chronic hepatitis caused by hepatitis B virus (HBV) or hepatitis C virus (HCV).[1–4] Most HCC develop in the presence of chronic hepatitis or cirrhosis, each of which occurs in Japan most often as a complication of chronic hepatitis B or C.[1,2] Currently, up to 80% of patients with HCC are infected with HCV.[5]

In Japan, absence of hepatitis virus infection has been confirmed in small number of patients with HCC.[5] In these patients, the characteristics and prognosis of HCC might differ from those in patients with chronic hepatitis virus infection. In the present study, we attempted to clarify the prevalence, characteristics and prognosis of HCC in patients without the detection of hepatitis virus infection in comparison with those of patients with hepatitis virus (HBV or HCV) infection.

## Methods

A total of 1152 patients were diagnosed as having initial HCC (not recurrence) and treated at Ogaki Municipal Hospital between 1991 and 2004, these were 847 men and 305 women, with a mean age of 65.9 ± 9.1 years (range 29–93 years). Hepatocellular carcinoma was diagnosed on the basis of histologic examination of resected tumor tissue or biopsy specimens in 429 cases (37.2%). Diagnosis in the remaining 723 cases was based on clinical criteria:[6,7] pertinent clinical background (liver cirrhosis or chronic hepatitis) and typical imaging findings. Typical imaging features of HCC include a mosaic pattern with a halo on B-mode ultrasonographic images, hypervascularity on angiographic images and a high-density mass on arterial phase dynamic computed tomography (CT) images with a low-density mass on portal phase dynamic CT images obtained with a helical or multidetector row CT scanner. When findings typical of HCC were not obtained by means of dynamic CT or angiography, CT during hepatic arteriography and CT during arterial portography or superparamagnetic

iron oxide-enhanced T1- and T2-weighted magnetic resonance imaging were performed. In cases without typical imaging features, biopsy was performed to confirm the diagnosis of HCC.

Chronic HBV or HCV infection was tested at the time of HCC diagnosis. Hepatitis B virus infection was identified by positivity for serum HBV surface antigen. Hepatitis C virus infection was identified by positivity for serum HCV antibody and confirmed by positivity for serum HCV RNA. Patients were assigned to one of two groups according to the detection of hepatitis virus infection: patients with viral HCC in whom HBV or HCV infection was detected and patients with viral marker-negative HCC in whom HBV or HCV infection was not detected.

Surveillance status of each patient before diagnosis, remnant liver function at the time of HCC diagnosis, characteristics of HCC including maximum tumor size, number of tumors and vascular invasion, stage of HCC and patient survival were determined on the basis of clinical records. The Child–Pugh classification[8] was used as an indicator of remnant liver function. Vascular invasion was assessed by means of dynamic CT and angiography in cases in which pathologic evaluation had not been performed. Stage of HCC were evaluated according to the recently proposed CLIP[9] and JIS[10] scoring systems, and BCLC classification,[11] which incorporate both tumor extension and liver function factors. Patients were also classified into one of two groups according to whether they were under surveillance for liver tumor before the initial diagnosis of HCC: those in whom HCC was detected under surveillance (including surveillance at our center [$n = 464$] or under surveillance by a primary-care physician who referred them to us because liver tumor was suspected [$n = 401$]) and those not under surveillance until admission for HCC ($n = 287$).

### Statistical analyses

Values are expressed as mean ± SD. Differences in distributions between groups were analyzed by $\chi^2$ test. Differences in mean quantitative values were analyzed by Mann–Whitney *U*-test. The date of HCC diagnosis was defined as time zero for calculation of survival rates. Surviving patients and patients who died from a cause other than liver disease were censored. Patients who died from an HCC-related cause or liver failure were not censored. The Kaplan–Meier method[12] was used to calculate survival rates, and the log-rank test[13] was used to analyze differences in survival. The Cox proportional hazards model[14] was used for multivariate analysis of factors related to survival. The variables analyzed were patient age and sex, the presence or absence of surveillance before the diagnosis of HCC, Child–Pugh class (A *vs* B, C), tumor stage (stage I *vs* II, III, IV) and the presence or absence of hepatitis virus infection. Data analyses were performed with the JMP statistical software package, version 4.0 (Macintosh version; SAS Institute, Cary, NC, USA). All *P*-values were derived from two-tailed tests, and $P < 0.05$ was accepted as statistically significant.

The entire study was approved by the hospital ethics committee and carried out in compliance with the Helsinki Declaration.

## Results

Hepatitis B virus or HCV infection was not detected in 119 of the 1152 patients (10.3%) with HCC. The numbers of cases of viral HCC and viral marker-negative HCC are shown per year in Fig. 1. The percentage of patients with viral marker-negative HCC was consistently around 10%, with some fluctuation. We found no particular increase or decrease in this percentage during the observation period.

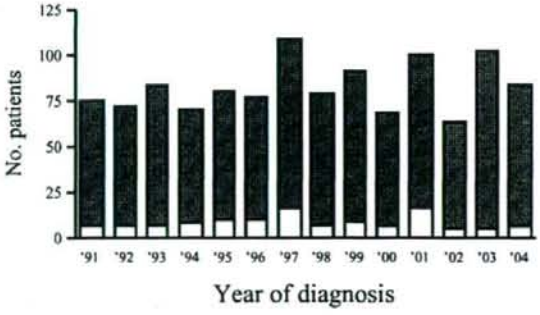


**Figure 1** Number of patients in whom initial hepatocellular carcinoma (HCC; not recurrence) was diagnosed at our hospital per year (1991–2004). The annual incidence of viral marker-negative HCC was around 10% during the entire observation period. ■, viral HCC patients; □, viral marker-negative HCC patients.

### Patient characteristics and characteristics of hepatocellular carcinoma with and without hepatitis virus infection

Characteristics of patients and HCC at the time of diagnosis are shown in Table 1 according to the detection or non-detection of HBV or HCV. Patients with viral marker-negative HCC were significantly older ($P = 0.0123$) and had a significantly more prevalent history of regular alcohol intake ($P = 0.0021$). In patients with viral marker-negative HCC, 21 patients had alcoholic cirrhosis and two had autoimmune hepatitis. The other 96 patients with viral marker-negative HCC did not have alcoholic cirrhosis, primary biliary cirrhosis, autoimmune hepatitis, or iron overload, and the definitive etiology was unknown.

No difference was found between the two groups in remnant liver function (Child–Pugh class). Maximum tumor size was significantly greater ($P < 0.0001$) and there was a significantly higher prevalence of suspected vascular invasion (by imaging diagnosis in some patients; $P = 0.0003$) in patients with viral marker-negative HCC than in patients with viral HCC, but there was no difference in the number of tumors. As for stage of patients, both CLIP scores ($P = 0.0348$) and JIS scores ($P = 0.0198$) were lower in patients with viral HCC. Also, the patients of earlier stage by BCLC classification were more prevalent in the group with viral HCC than in the group with viral marker-negative HCC ($P = 0.0023$). When we compared these factors between patients with HBV infection and those with HCV infection, we found no difference in all remnant liver function, characteristics of HCC or stage of HCC (data not shown).

A significantly smaller number of viral marker-negative HCC (*vs* viral HCC) patients had been under surveillance for HCC before the detection and diagnosis of HCC ($P < 0.0001$). The


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**Table 1** Characteristics of hepatocellular carcinoma (HCC) in patients with and without hepatitis virus infection

| | Viral HCC ($n = 1033$) | Viral marker-negative HCC ($n = 119$) | *P*-value |
|---|---|---|---|
| Age (years ± SD) | 65.7 ± 9.1 | 67.7 ± 9.7 | 0.0123 |
| Sex (M/F) | 756 (73.2)/277 (26.8) | 91 (76.5)/28 (23.5) | 0.5095 |
| Regular alcohol intake (yes/no) | 274 (26.5)/759 (73.5) | 48 (40.3)/71 (59.7) | 0.0021 |
| Surveillance before HCC (yes/no) | 796 (77.1)/237 (22.9)† | 69 (58.0)/50 (42.0)† | <0.0001 |
| Child–Pugh class (A/B/C) | 532 (51.5)/385 (37.3)/116 (11.2)‡ | 64 (53.8)/38 (31.9)/17 (14.3)¶ | 0.4096 |
| Maximum tumor size (cm ± SD) | 3.76 ± 3.01 | 5.96 ± 4.55 | <0.0001 |
| (≤2 cm/>2cm and ≤5cm/>5 cm) | 369 (35.7)/386 (37.4)/278 (26.9) | 29 (24.4)/29 (24.4)/61 (51.2) | <0.0001 |
| Number of tumors ($n$ ± SD) | 2.26 ± 2.31 | 2.76 ± 3.92 | 0.9030 |
| (single/multiple) | 514 (49.8)/519 (50.2) | 59 (49.6)/60 (50.4) | 0.9706 |
| Vascular invasion (absent/present) | 836 (80.9)/197 (19.1) | 79 (66.4)/40 (33.6) | 0.0003 |
| CLIP score | 1.72 ± 1.59 | 2.04 ± 1.66 | 0.0348 |
| BCLC classification (A/B/C/D) | 565 (54.7)/209 (20.2)/165 (16.0)/94 (9.1) | 49 (41.2)/21 (17.6)/35 (29.4)/14 (11.8) | 0.0023 |
| JIS score | 2.00 ± 1.41 | 2.32 ± 1.41 | 0.0198 |
| Treatment | 226 (21.9)/231 (22.3)/343 (33.2)/ | 29 (24.4)/21 (17.7)/28 (23.5)/16 (13.4)/ | 0.0192 |
| (surgery/LAT/TACE/others/none) | 70 (6.8)/163 (15.8) | 25 (21.0) | |

†427 of 796 patients (53.6%) were outpatients under surveillance at our liver center; the other 369 (46.4%) were under surveillance of a primary-care physician.

‡37 of 69 patients (53.6%) were outpatients under surveillance at our liver center; the other 32 (46.4%) were under surveillance of a primary-care physician.

‡Child–Pugh class A includes 256 patients without cirrhosis.

¶Child–Pugh class A includes 34 patients without cirrhosis.

Number of patients is shown unless otherwise indicated (percentage in parentheses).

BCLC, Barcelona Clinic Liver Cancer; CLIP, Cancer of the Liver Italian Program; JIS, Japan Integrated Staging; LAT, locoregional ablative therapies; TACE, transcatheter arterial chemoembolization.

percentage of patients in whom HCC was detected under surveillance is shown per observation period (i.e. 1991–1995, 1996–2000 and 2001–2004) in Fig. 2. The percentage of patients with viral HCC in whom HCC was diagnosed under surveillance increased significantly over time ($P = 0.0333$). In contrast, we found no increase over time in the percentage of patients with viral marker-negative HCC in whom HCC was diagnosed under surveillance ($P = 0.8603$). In patients under surveillance before the diagnosis of HCC, the percentage of patients under surveillance at our center is exactly the same (53.6%) between patients with viral HCC (427 of 796 patients) and those with viral marker-negative HCC (37 of 69 patients). For patients under surveillance at our center, all cirrhotic patients were followed up with ultrasonography every 3 months and, in addition, CT or dynamic magnetic resonance imaging was performed every 6 months in order to prevent the failure of HCC detection by ultrasonography. Regular monitoring of tumor markers (alpha-fetoprotein and des-gamma-carboxy prothrombin) was also performed. Patients without cirrhosis followed up with 6–12 months interval by ultrasonography and a measurement of tumor markers.[15] For patients under surveillance at a primary-care physician, the manner of surveillance simply depended on the physician. We did not supervise the care of these patients until they were referred to us.[15]

Characteristics of viral HCC and viral marker-negative HCC in patients with cirrhosis ($n = 862$) and in those without cirrhosis ($n = 290$) are shown in Table 2. Cirrhosis was evaluated clinically on the basis of laboratory data (serum albumin, serum bilirubin, prothrombin and platelet) and imaging findings (splenomegaly), except for 257 patients who were treated by surgery. Eighteen of 34 patients with viral marker-negative HCC without cirrhosis were

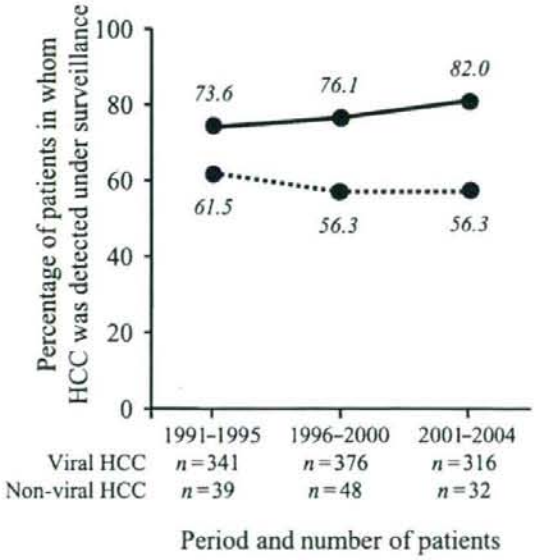


**Figure 2** Changes in the percentage of patients in whom hepatocellular carcinoma (HCC) was detected under surveillance. There was a significant increase in the percentage among patients (—) with viral HCC ($n = 1033$, $P = 0.0333$). In contrast, there was no increase in the percentage among patients (·····) with viral marker-negative HCC ($n = 119$, $P = 0.8603$).

**Table 2** Characteristics of hepatocellular carcinoma (HCC) in patients with and without hepatitis virus infection according to the presence or absence of cirrhosis.

| Patients with cirrhosis | Viral HCC ($n = 777$) | Viral marker-negative HCC ($n = 85$) | *P*-value |
|---|---|---|---|
| Surveillance before HCC (yes/no) | 591 (76.1)/186 (23.9)[†] | 51 (60.0)/34 (40.0)[‡] | 0.0020 |
| Maximum tumor size (cm ± SD) | 3.66 ± 2.87 | 4.90 ± 4.21 | 0.1012 |
| (≤2 cm/>2cm and ≤5cm/>5 cm) | 276 (35.5)/290 (37.3)/211 (27.2) | 25 (29.4)/23 (27.1)/37 (43.5) | 0.0062 |
| Vascular invasion (absent/present) | 620 (79.8)/157 (20.2) | 58 (68.2)/27 (31.8) | 0.0198 |
| Treatment (surgery/LAT/TAE/others/none) | 119 (15.3)/187 (24.1)/279 (35.9)/ 48 (6.2)/144 (18.5) | 17 (20.0)/17 (20.0)/24 (28.2)/8 (9.4)/19 (22.4) | 0.3185 |
| **Patients without cirrhosis** | **Viral HCC ($n = 256$)** | **Viral marker-negative HCC ($n = 34$)** | ***P*-value** |
| Surveillance before HCC (yes/no) | 205 (80.1)/51 (19.9)[§] | 18 (52.9)/16 (47.1)[¶] | 0.0009 |
| Maximal tumor size (cm ± SD) | 4.06 ± 3.39 | 8.29 ± 4.44 | <0.0001 |
| (≤2 cm/>2cm and ≤5cm/>5 cm) | 93 (36.3)/96 (37.5)/67 (26.2) | 4 (11.8)/6 (17.6)/24 (70.6) | <0.0001 |
| Vascular invasion (absent/present) | 216 (84.4)/40 (15.6) | 21 (61.8)/13 (38.2) | 0.0030 |
| Treatment (surgery/LAT/TAE/others/none) | 107 (41.8)/44 (17.2)/64 (25.0)/ 22 (8.6)/19 (7.4) | 12 (35.3)/4 (11.8)/4 (11.8)/8 (23.5)/6 (17.6) | 0.0216 |

[†]314 of 591 (53.1%) patients were outpatients under surveillance at our liver center; the other 277 (46.9%) were under surveillance of a primary-care physician.

[‡]33 of 51 patients (64.7%) were outpatients under surveillance at our liver center; the other 18 (35.3%) were under surveillance of a primary-care physician.

[§]113 of 205 patients (55.1%) were outpatients under surveillance at our liver center; the other 92 (44.9%) were under surveillance of a primary-care physician.

[¶]2 of 18 patients (11.1%) were outpatients under surveillance at our liver center; the other 16 (88.9%) were under surveillance of a primary-care physician.

Number of patients is shown unless otherwise indicated (percentage in parenthesis).

LAT, locoregional ablative therapies; TACE, transcatheter arterial chemoembolization.

under surveillance. All these patients were receiving routine periodic ultrasonography examination of the liver with 1-year interval because of the liver damage (the elevation of serum alanine aminotransferase activity) of unknown etiology. Differences in maximum tumor size and the prevalence of suspected vascular invasion between viral HCC and viral marker-negative HCC were more marked in patients without cirrhosis than in those with cirrhosis.

### Patient survival

The survival rate of patients with viral marker-negative HCC was significantly lower than that of patients with viral HCC ($P = 0.0378$; Fig. 3). By multivariate analysis, the surveillance before the diagnosis of HCC is a factor that affects patient survival independent of remnant liver function (Child–Pugh class) and of tumor progression (tumor stage of Liver Cancer Study Group of Japan[16]). In contrast, the presence or absence of hepatitis virus infection did not have an effect on patient survival in multivariate analysis (Table 3).

When survival was compared between patients with viral marker-negative HCC and those with viral HCC according to the presence or absence of cirrhosis, the difference in survival was significant among patients without cirrhosis ($P = 0.0016$), but not among patients with cirrhosis ($P = 0.2031$; Fig. 4). Among patients with viral HCC, the survival rate of those without cirrhosis was significantly higher than that of those with cirrhosis ($P < 0.0001$). In contrast, among patients with viral marker-negative HCC, there was no difference in survival between patients with and without cirrhosis ($P = 0.6205$; Fig. 5).

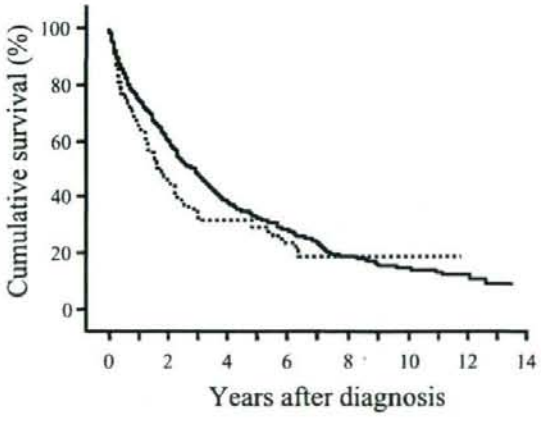


**Figure 3** Survival of patients (—) with viral hepatocellular carcinoma (HCC; $n = 1033$) and patients (·····) with viral marker-negative HCC ($n = 119$). The survival rate of patients with viral HCC was higher than that of patients with viral marker-negative HCC ($P = 0.0378$).

## Discussion

Nearly 90% of patients in Japan with HCC are chronically infected with HBV or HCV. Although the percentage is small, there is a subpopulation of Japanese patients with HCC in whom no



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**Table 3** Multivariate analyses of factors associated with patient survival

| Factor | Parameter estimate | Standard error | X | Risk ratio (95% confidence interval) | *P*-value |
|---|---|---|---|---|---|
| Age | 0.0085 | 0.0048 | 3.17 | 1.0085 (0.9991–1.0179) | 0.0749 |
| Sex | | | | | |
| Male | | | | 1 | |
| Female | −0.1390 | 0.0464 | 9.34 | 0.8702 (0.7936–0.9519) | 0.0022 |
| Surveillance before HCC | | | | | |
| No | | | | 1 | |
| Yes | −0.0844 | 0.0424 | 3.98 | 0.9191 (0.8458–0.9988) | 0.0459 |
| Child–Pugh class | | | | | |
| A | | | | 1 | |
| B | 0.3500 | 0.0437 | 63.88 | 1.4190 (1.3025–1.5460) | <0.0001 |
| C | 0.6690 | 0.0567 | 115.79 | 1.9523 (1.7444–2.1790) | <0.0001 |
| Tumor stage† | | | | | |
| Stage I | | | | 1 | |
| Stage II | 0.2722 | 0.0637 | 19.19 | 1.3128 (1.1605–1.4903) | <0.0001 |
| Stage III | 0.5559 | 0.0665 | 74.65 | 1.7435 (1.5326–1.9894) | <0.0001 |
| Stage IV | 1.1395 | 0.0707 | 282.81 | 3.1251 (2.7245–3.5956) | <0.0001 |
| Hepatitis virus infection | | | | | |
| No | | | | 1 | |
| Yes | 0.0589 | 0.0611 | 0.90 | 1.0607 (0.9374–1.1916) | 0.3424 |

†by Liver Cancer Study Group of Japan.
HCC, hepatocellular carcinoma.

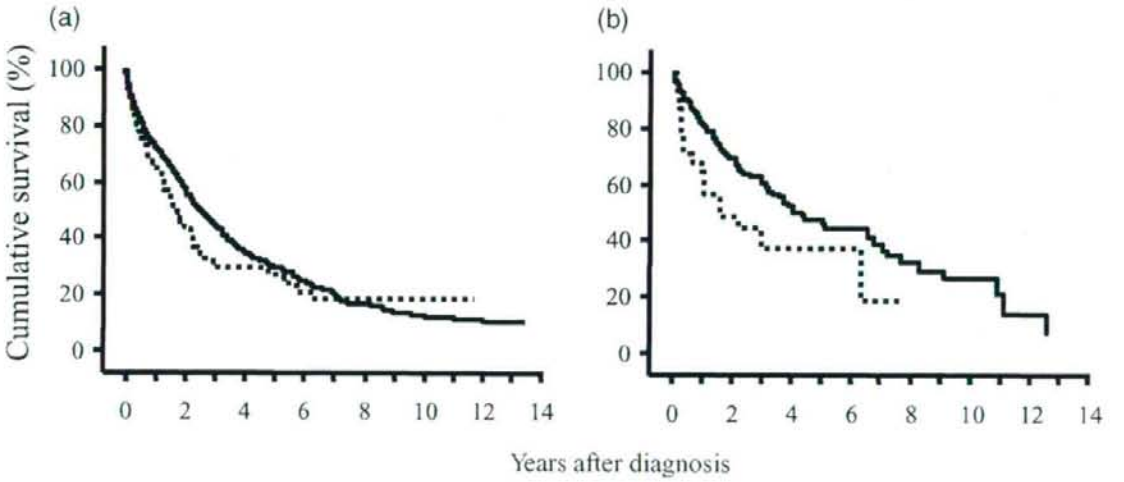


**Figure 4** Survival rates of patients (—) with viral hepatocellular carcinoma (HCC) and patients (- - -) with viral marker-negative HCC in relation to cirrhosis. (a) Survival of patients with viral HCC ($n = 777$) and patients with viral marker-negative HCC ($n = 85$), all with cirrhosis. No significant difference was observed between these two groups ($P = 0.2031$). (b) Survival of patients with viral HCC ($n = 256$) and patients with viral marker-negative HCC ($n = 34$), all without cirrhosis. Among these patients, the survival rate of patients with viral HCC was significantly higher than that of patients with viral marker-negative HCC ($P = 0.0166$).

hepatitis virus infection is found. The percentage is reportedly much higher in Western countries.[17–19]

There have been studies of the mechanism underlying development of HCC in patients with no apparent hepatitis virus infection. Few studies, however, have investigated in detail the characteristics and prognosis of patients with HCC in whom no hepatitis virus infection is detected. Some studies reported favorable survival of patients with viral marker-negative HCC, including preserved liver function and absence of multicentric carcinogenesis, in comparison with that of patients with viral

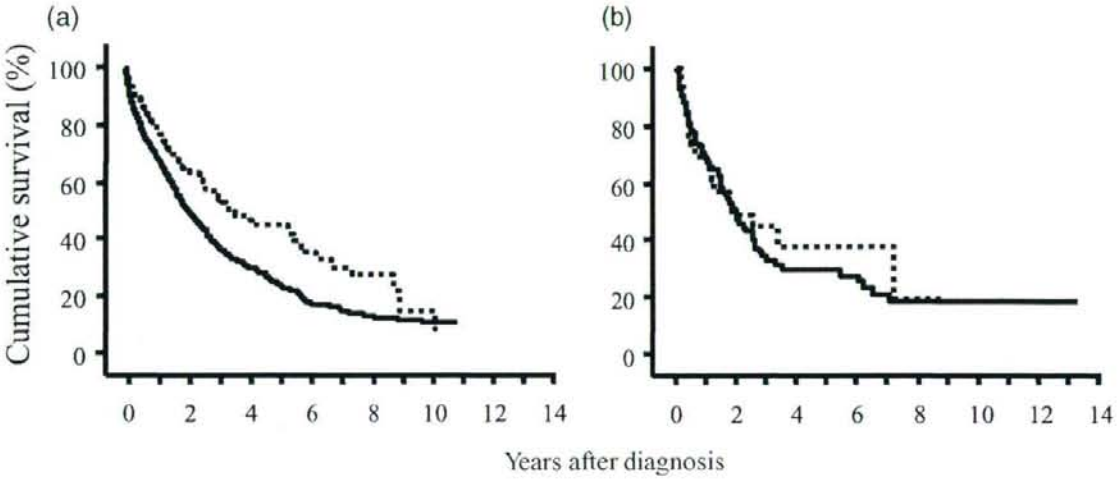


**Figure 5** Survival rates of patients (—) with cirrhosis and patients (·····) without cirrhosis in relation to hepatitis virus infection. (a) Survival of patients with ($n = 777$) and without ($n = 256$) cirrhosis, all having viral hepatocellular carcinoma (HCC). The survival rate of patients without cirrhosis was significantly higher than that of patients with cirrhosis ($P < 0.0001$). (b) Survival of patients with ($n = 85$) and without ($n = 34$) cirrhosis, all having viral marker-negative HCC. No significant difference was observed between these two groups ($P = 0.6205$).

HCC.[20–23] These studies, however, focused on patients who were treated by hepatic resection and do not reflect the entire population of patients with viral marker-negative HCC.

In the present study, we analyzed the characteristics and prognosis of patients with HCC in whom no hepatitis virus was detected. The infection of hepatitis virus was evaluated by means of examinations that are used routine clinical settings: serum HBV surface antigen for HBV infection, and serum HCV antibody and HCV RNA for HCV infection. These examinations do not necessarily reflect the infection with HBV or HCV accurately due to their limitation of sensitivity, especially in case of HBV infection. Some studies have reported occult HBV infection in patients with viral marker-negative HCC.[24–27] In the present study, however, we did not investigate this occult infection. Hepatitis virus infection was usually examined by routine serologic and virologic analyses (positivity for serum HBV surface antigen, HCV antibody, or HCV RNA) and further analyses for occult viral infection was not performed in daily clinical settings. The patients in whom HBV or HCV was not detected with routine examination therefore were defined as those without hepatitis virus infection. The purpose of the present study was to investigate the characteristics and prognosis of the patients with HCC who were defined as not having hepatitis viral infection.

The results of our present study clearly show viral marker-negative HCC to be more advanced than viral HCC at the time of diagnosis: maximum tumor size was greater, the prevalence of vascular invasion was higher and survival rate was lower. In our previous studies showing improved survival of patients with HCC over the past few decades, the contribution of early detection of HCC to the improved patient prognosis and the importance of surveillance of patients at high risk for development of HCC were also shown.[15,28] Our present study shows a lower percentage of patients under surveillance before the detection and diagnosis of viral marker-negative HCC. In addition, among patients with viral marker-negative HCC, we observed no increase in the rate of surveillance over time, whereas, among patients with viral HCC, we observed a significant increase in the rate of surveillance. Increased awareness of the risk of HCC in patients with cirrhosis or chronic viral hepatitis could have contributed to the increase in the number of patients under surveillance. Awareness of the risk of developing HCC spread especially after the 1990s, when HCV was identified and many patients were admitted to the hospital for examination and treatment of hepatitis virus infection. In contrast, it is difficult to identify patients at high risk for development of HCC if no hepatitis virus infection is detected, especially when they do not have symptomatic liver disease such as cirrhosis. Early-stage HCC is usually asymptomatic, and early detection of HCC is difficult without periodic surveillance. Therefore, patients who are not under surveillance for HCC and who are admitted to the hospital after HCC becomes symptomatic usually have advanced-stage disease. This accounts for the significantly larger tumors and greater prevalence of vascular invasion in patients with viral marker-negative HCC than in those with viral HCC. The surveilled patients in groups with viral HCC and viral marker-negative HCC are the mixture of patients under surveillance at out liver center and those at a primary-care physician. The surveillance at a primary-care physician is likely to be less intensive than that at out liver center, and subsequently the survival rate of patients under surveillance at a primary-care physician is lower than that of patients under surveillance at our center.[15] However, the percentage of patients under surveillance at our center is exactly the same between in surveilled patients with viral HCC and in those with viral marker-negative HCC, and therefore the mixture of the two kinds of surveilled patients would not have


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affected the comparison of patient survival between viral HCC and viral marker-negative HCC.

The advanced disease stage and decreased survival in association with viral marker-negative HCC versus viral HCC was marked among patients without clinically evaluated cirrhosis. We did not find a difference in survival between viral marker-negative patients with and without cirrhosis, but a significant difference was observed between these two subgroups among patients with viral HCC. The benefit of well-preserved remnant liver function did not contribute to survival of patients with viral marker-negative HCC without cirrhosis, because the HCC was advanced when it was found. Viral marker-negative HCC without cirrhosis is usually asymptomatic until the HCC progresses to an advanced stage. Although some of the patients were being followed up for abnormal liver function before the detection of HCC, most were not under surveillance for HCC. Indeed, only two of 18 patients with viral marker-negative HCC without cirrhosis were under surveillance at our liver center, whereas the other 16 patients were under surveillance of a primary-care physician. Such surveillance for HCC would typically be less intensive than at our liver center.[15]

There are several reported risk factors for the development of HCC, in addition to HBV or HCV infection. Heavy drinkers are reported to be at high risk for the development of HCC and should be under surveillance even in the absence of hepatitis virus infection.[29] Non-alcoholic steatohepatitis may be another risk factor for the development of HCC.[17,30-33] Detailed analysis of risk factors for development of HCC other than hepatitis viral infection is important to identify patients at high risk for development of HCC in the absence of the detection of hepatitis virus infection.

In conclusion, when HCC is diagnosed in patients in Japan without the detection of hepatitis virus infection, it is generally more advanced and has a poorer prognosis than that of patients with hepatitis virus infection. This could be due to the lower percentage of patients under surveillance for HCC before its detection and diagnosis. The increase in the number of patients under close surveillance has contributed to improved survival in cases of viral HCC. In contrast, it is difficult to identify patients without hepatitis virus infection that are at high risk for developing HCC. Further studies are needed to find a strategy for identifying patients that should be placed under close surveillance so that viral marker-negative HCC will be detected in the early stage. Such a strategy will be of even greater importance in other parts of the world where viral hepatitis is not a predominant cause of HCC.

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# Phosphorylated Heat Shock Protein 27 Represses Growth of Hepatocellular Carcinoma via Inhibition of Extracellular Signal-regulated Kinase*

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**Heat shock protein 27, one of the low molecular weight stress proteins, is recognized as a molecular chaperone; however, other functions have not yet been well established. Phosphorylated heat shock protein 27 levels inversely correlate with the progression of human hepatocellular carcinoma. This study shows that phosphorylated heat shock protein 27 interferes with cell growth of the hepatocellular carcinoma-derived HuH7 cells in the presence of the proinflammatory cytokine, tumor necrosis factor-α, via inhibition of the sustained activation of the extracellular signal-regulated kinase signal pathway. The activities of Raf/extracellular signal-regulated kinase and subsequent activator protein-1 transactivation and the induction levels of cyclin D1 were lower in HuH7 cells transfected with phosphorylated heat shock protein 27 than those with unphosphorylated heat shock protein 27. Moreover, phosphorylated heat shock protein 27 up-regulated the levels of p38 mitogen-activated protein kinase and mitogen-activated protein kinase phosphatase-1, an inhibitory protein of extracellular signal-regulated kinase. These results indicate that phosphorylated heat shock protein 27 might suppress the extracellular signal-regulated kinase activity in the hepatocellular carcinoma cells via two separate pathways in an inflammatory state. The extracellular signal-regulated kinase activity is inversely correlated with phosphorylated heat shock protein 27 at serine 15 and also in human hepatocellular carcinoma tissues *in vivo*. Because the extracellular signal-regulated kinase signal pathway is a major proliferation signal of hepatocellular carcinoma, activator protein-1 activation is an early event in hepatocarcinogenesis. These findings strongly suggest that the control of the phosphorylated heat shock protein 27 levels could be a new therapeutic strategy especially to counter the recurrence of hepatocellular carcinoma.**

The mammalian small stress protein, heat shock protein (HSP)[2] 27, is a widely expressed 27-kDa protein, and it is one of 10 members of the human low molecular weight HSP family. HSPs are classified into high molecular weight HSPs such as HSP70 and HSP90, and low molecular weight HSPs with molecular masses from 10 to 30 kDa based on their apparent molecular sizes. Low molecular weight HSPs have significant similarities in terms of amino acid sequences, known as the α-crystallin domain and WDPF motif (1, 2). The high molecular weight HSPs act as molecular chaperones in protein folding, oligomerization, and translocation (1). Although the functions of low molecular weight HSPs are not as well characterized as those of the high molecular weight HSPs, it is recognized that they may have chaperone activities (1). The functions of HSP27 are regulated by post-translational modifications such as phosphorylation (3, 4). Mouse HSP27 is phosphorylated at two sites (Ser-15 and Ser-82), whereas human HSP27 is phosphorylated at three sites (Ser-15, Ser-78, and Ser-82) (3). Ser-78 and Ser-82 of HSP27 are adjacent to the amino-terminal sequence of the α-crystallin domain, whereas Ser-15 is on the amino terminus of the WDPF motif. HSP27 can form oligomers up to 1000 kDa and interfere with cell death induced by several stimuli (1, 5). The oligomerization is regulated by phosphorylation of Ser-78 and/or Ser-82 and the WDPF motif, although phosphorylation of Ser-15 is unrelated to oligomerization (2). HSP27 is reportedly phosphorylated through the following activation of the p38 mitogen-activated protein kinase (MAPK) pathway by the MAPK-activated protein kinase (MAPKAP) 2 and 3 (1). Phosphorylated HSP27 forms a dimer, and the chaperone function is diminished (1). However, the role of phosphorylated HSP27 has not yet been precisely elucidated.

Proinflammatory stimuli, such as tumor necrosis factor-α (TNFα), are involved in the pathophysiology of viral hepatitis, alcoholic liver disease, and nonalcoholic fatty liver disease (6). TNFα plays a dichotomous role in the liver, where it not only acts as a mediator of cell death but also induces hepatocyte proliferation and liver regeneration. HSP27 was reported to be able to suppress TNFα-induced apoptosis and enhance NF-κB activity via promotion of the proteasome-dependent degradation of IκB in a human leukemic cell line (7). Otherwise, TNFα

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[2] The abbreviations used are: HSP, heat shock protein; AP-1, activator protein-1; ERK, extracellular signal-regulated kinase; HCC, hepatocellular carcinoma; IKK, IκB kinase; JNK, c-Jun $NH_2$-terminal kinase; MAPK, mitogen-activated protein kinase; MAPKAP, mitogen-activated protein kinase-activated protein kinase; MEK, MAPK/ERK kinase; MKK, mitogen-activated protein kinase kinase; MKP-1, mitogen-activated protein kinase phosphatase-1; TNFα, tumor necrosis factor-α; WT, wild type; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

activates MAPK that enhances phosphorylation of HSP27 (8). Phosphorylated HSP27 inhibits IκB kinase (IKK) and reduces IκB degradation, thus resulting in the suppression of the NF-κB activation in HeLa cells (9). Accumulating evidence indicates that the phosphorylation of HSP27 holds the key to the TNFα related liver diseases.

Hepatocellular carcinoma (HCC) commonly arises in the liver with chronic inflammation and ranks fifth in frequency on a worldwide basis, thus causing more than 1 million deaths annually (10). The overall survival of patients with HCC even after resection is still unsatisfactory because of frequent recurrence. The recurrence rate at 5 years after the curative treatment may exceed 70% (10). This high recurrence rate is not because of local recurrence or metastasis from the original lesion but rather from second primary lesions (10). However, the most suitable prognostic factor that suggests patients with HCC are at high risk for early recurrence has not yet been identified. MAPKs are essential components of intracellular signal transduction and are activated by phosphorylation in response to various extracellular stimuli, including growth factors, cytokines, and environmental stress. Among the MAPK family, extracellular signal-regulated kinase (ERK) is a key molecule that transfers signals into the nuclei to induce proliferation and differentiation (11). In HCC, the ERK are activated, and they up-regulate cyclin D1 expression, which thus stimulates progression (12). Conversely, p38 MAPK negatively regulates cyclin D1 and antagonizes the c-Jun $NH_2$-terminal kinase (JNK)-c-Jun pathway to suppress HCC development (13, 14). Previous studies showed that the level of phosphorylated HSP27 in human HCC tissues inversely correlates with the tumor size and the TNM stage of HCC (15). In addition, a pro-apoptotic, tumor-suppressive molecule protein kinase Cδ regulates HSP27 phosphorylation at a point upstream of p38 MAPK in the human HCC-derived cell line, HuH7 cells (16). However, the exact role and regulatory mechanism of HSP27 in human HCC remain to be clarified.

This study aimed to clarify the role of phosphorylated HSP27 in HCC and to analyze the proliferation of the HCC cells transfected with unphosphorylatable or phospho-mimic mutants of human HSP27. The results showed that phosphorylated HSP27 repressed the HCC cell proliferation in the presence of proinflammatory cytokine, TNFα, via inhibition of the Raf-ERK kinase (MEK)-ERK signaling pathway and the activation of p38 MAPK-MAPK phosphatase-1 (MKP-1) pathway.

## EXPERIMENTAL PROCEDURES

*Plasmids*—Wild-type (WT) and mutant human HSP27s subcloned into pcDNA3.1 mammalian expression vector were kindly provided by Dr. C. Schäfer (Klinikum Grosshadern, Ludwig-Maximilians University Munich, Munich, Germany). For mutant HSP27 vectors, the cDNA of HSP27 had been mutated at serine residues 15, 78, and 82 to aspartate (3D) to imitate the phosphorylated HSP27 form or mutated at the same residues to alanine (3A) to prevent phosphorylation of HSP27 (17). A constitutively active MEK1 cDNA was the generous gift from Dr. N. G. Ahn (Howard Hughes Medical Institute, University of Colorado, Boulder) (18).

*Antibodies and Chemicals*—HSP27 antibodies, phosphorylated HSP27 (Ser-15) antibodies, and phosphorylated HSP27 (Ser-78) antibodies were purchased from StressGen Biotechnologies Corp. (Atlanta, GA). Phosphorylated HSP27 (Ser-82) antibodies were obtained form Biomol (Plymouth Meeting, PA). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) antibodies and β-actin antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA) and Sigma, respectively. Caspase 9 antibodies, ERK (p44/p42 MAPK) antibodies, phospho-ERK antibodies, MEK antibodies, phospho-MEK antibodies, phospho-c-Raf antibodies, cyclin D1 antibodies, p38 MAPK antibodies, phospho-p38 MAPK antibodies, and phospho-MKP-1 (Ser-359) antibodies were purchased from Cell Signaling Technology, Inc. (Danvers, MA). Recombinant human TNFα was a kind gift from Dainippon Pharmaceutical Co., Ltd. (Osaka, Japan). Caspase-9 inhibitor I (benzyloxycarbonyl-LEHD-fluoromethyl ketone), a cell-permeable and irreversible inhibitor of caspase 9, was purchased from Merck.

*Cell Culture and Stable Transfections*—Human HCC-derived HuH7 cells, which originated from well differentiated HCC tissues, were obtained from the Japanese Cancer Research Resources Bank. HuH7 cells were maintained in RPMI 1640 medium (Sigma) supplemented with 1% fetal calf serum. For stable transfections, $4 \times 10^5$ HuH7 cells were cultured in 6-well dishes and then transfected with 2 μg of the WT or mutant HSP27 plasmids that expresses geneticin (G418) resistance using 12 μl of UniFECTOR transfection reagent (B-Bridge International, Mountain View, CA) in 1 ml of RPMI 1640 medium without fetal calf serum per well. One ml/well medium with 10% fetal calf serum was added 5 h after transfection. The cells were subcultured and grown in the presence of 1 mg/ml of G418 (EMD Chemicals, Inc., San Diego) 2 days later. After about 2 weeks, single G418-resistant colonies were obtained by serial dilution in 96-well dishes. The colonies then were maintained and analyzed individually for the expression of HSP27s.

*Cell Growth Assay*—Empty vector-transfected, WT, or mutant HSP27s stably expressing HuH7 cells were plated on 96-well dishes ($1 \times 10^3$ cells/well). Twenty four h after seeding, the cells were treated with or without 1 nM TNFα for the indicated time, and cell numbers were counted using the trypan blue dye exclusion method or using WST-1 reagent (Roche Diagnostics) according to the manufacturer's instructions. To investigate the influence of caspase 9 on the cell growth, WT, or the 3D HSP27, stably expressed HuH7 cells were treated with caspase-9 inhibitor I simultaneously with or without 1 nM TNFα for 6 days.

*Western Blotting*—The cultured cells, which overexpressed WT or mutant HSP27s, were stimulated with or without TNFα for the indicated time. The cells or the snap-frozen human HCC samples were lysed, homogenized, and sonicated in lysis buffer containing 62.5 mM Tris/HCl (pH 6.8), 2% SDS, 50 mM dithiothreitol, and 10% glycerol. A Western blot analysis was performed as described previously (16, 19). Band intensities were visualized on x-ray film with the ECL Western blotting detection system (GE Healthcare). The protein band intensities were determined by integrating the optical density over the band area (band volume) using NIH image software. The samples from the cell cultures to be quantitatively compared by

Western blots were run in the same gel. Values represent the amount of phospho-ERK or phospho-MEK divided by those of total ERK or total MEK, respectively. The values represent the amount of full-length and cleaved caspase 9, phospho-c-Raf, cyclin D1, phospho-p38 MAPK, and phospho-MKP-1 divided by those of GAPDH. To quantify the protein from the HCC tissue extracts, 0.25 μl of MagicMark XP Western protein standard (Invitrogen), the marker protein, was run in every gel. Based on the intensity of the marker protein band on x-ray film, the proteins of the tissue samples were quantitatively compared. After being normalized by the intensity of the marker protein, values represent the amount of phospho- and total HSP27s or phospho-ERK divided by those of β-actin or total-ERK, respectively. The data of the normalized values of the protein bands were statistically analyzed as described under "Statistics."

*Luciferase Reporter Assay*—A reporter plasmid, activator protein-1 (*AP-1*)-*Luc* was kindly provided by Dr. S. Kojima (RIKEN, Wako, Japan). The cells were stimulated with or without 1 nM TNFα for 48 h before transfection. At 5 h after transfection, another 24 h of stimulation of TNFα was performed. Transient transfection with the *AP-1-Luc* reporter (1 μg/35-mm dish) and measurement of luciferase activity of cell lysates were performed using UniFECTOR transfection reagent and a dual luciferase reporter assay system (Promega Corp., Madison, WI) as described previously (20). The cells were cotransfected with *pRL-CMV* (*Renilla* luciferase; 100 ng/35-mm dish) as an internal standard to normalize transfection efficiency. To examine the involvement of MEK-ERK system in AP-1-mediated transactivation activity within the 3D HSP27 mutant overexpressed cells, active MEK1 was cotransfected with the reporter plasmid.

*Tissue Specimens*—HCC tissues were obtained by surgical resection from 44 patients infected with hepatitis viruses B (10 cases) or C (31 cases) and 3 patients with alcoholic cirrhosis at the Department of Surgery, Ogaki Municipal Hospital. No patient had previously undergone chemotherapy. The resected tissues were snap-frozen in liquid nitrogen and then stored at −80 °C until used for the Western blot analysis. The resected HCC specimens were obtained according to protocol approved by the Committee for the Conduct of Human Research at Ogaki Municipal Hospital. Informed consent was obtained from all patients.

*Statistics*—Data are expressed as the means ± S.D. Statistical significance of the data from the cell cultures was analyzed using one-way analysis of variance, followed by Dunnett's test, and the patient clinical data were analyzed using the Pearson correlation coefficient ($r$). All $p$ values were derived from two-tailed tests, and $p < 0.05$ was accepted as statistically significant. A Pearson correlation coefficient of $|r| > 0.400$ was accepted as a positive correlation.

## RESULTS

*Expression of HSP27 in Wild-type, Unphosphorylated Type, or Phospho-mimic Type HSP27-transfected HuH7 Cells*—To investigate the effect of phosphorylated HSP27 on HCC cell growth, human HCC-derived HuH7 cells were stably transfected with cDNAs of mutant HSP27s with alanine 15, alanine 78, and alanine 82 (3A) that mimicked the unphosphorylated type or with aspartate 15, aspartate 78, and aspartate 82 (3D) that mimicked the phosphorylated type. For comparison purposes, HuH7 cells were also transfected with wild-type (WT) HSP27 cDNA or an empty pcDNA3.1 vector (empty). A Western blot analysis demonstrated that HSP27 was overexpressing in WT, 3A, or 3D HSP27 cDNA-transfected HuH7 cells (Fig. 1). The empty vector-transfected cells only expressed intact HSP27 proteins. Anti-phospho-Ser-15 HSP27 antibodies and anti-phospho-Ser-82 HSP27 antibodies reacted with the HSP27 protein that was overexpressed in both WT and 3D HSP27 vector-transfected cells (Fig. 1). The phosphorylated HSP27 protein level in WT HSP27 cDNA-transfected HuH7 cells was almost the same as that in 3D HSP27 cDNA-transfected cells. The antibodies for human-specific phospho-Ser-78 HSP27 also reacted with the HSP27 in WT or 3D HSP27 cDNA-transfected cells as the antibodies for other phosphorylated forms (data not shown). On the other hand, the overexpressed HSP27 protein in the 3A HSP27 cDNA-transfected cells did not react with the phospho-HSP27 antibodies (Fig. 1).

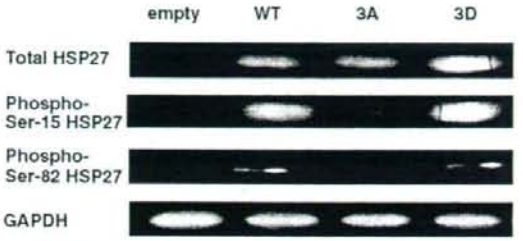


FIGURE 1. **Phosphorylated HSP27 overexpressed both the wild-type and the aspartate mutant HSP27-transfected HCC cells.** The protein expression levels of endogenous as well as the overexpressed wild-type and mutant HSP27s in the HuH7 cells were determined by Western blotting using anti-HSP27 antibodies, anti-phospho-HSP27 (Ser-15) antibodies, anti-phospho-HSP27 (Ser-82) antibodies, and anti-GAPDH antibodies. HuH7 cells were stably transfected either with empty, wild-type HSP27-expressing (*WT*), its alanine mutant-expressing (*3A*), or its aspartate mutant-expressing (*3D*) vectors.

*Phosphorylated HSP27 Retarded the HCC Cell Growth in the Presence of TNFα*—To clarify the relationship between phosphorylation of HSP27 and HCC cell growth, we first studied whether the cell growth of phosphorylated HSP27-overexpressed HuH7 cells was suppressed compared with that of unphosphorylated HSP27-overexpressed cells. HCC commonly arises in the liver with chronic inflammation (10, 21). In the liver, the levels of TNFα, a proinflammatory stimuli, in patients with cirrhosis and HCC have been reported to be significantly higher than those in normal individuals (22). Therefore, the cell growth of phosphorylated HSP27-overexpressed HuH7 cells was examined both in the presence and in the absence of TNFα. In the absence of TNFα, all WT, 3A, or 3D HSP27-overexpressed cell lines and the empty vector-transfected cell line showed almost the same growth curve (Fig. 2*A*, *curves 1–4*). Even in the presence of 1 nM TNFα, the empty vector or 3A HSP27 vector-transfected HuH7 cells also exhibited almost similar growth rate as in the absence of TNFα (Fig. 2*A*, *curves 5* and *7*). However, the cell growth of WT and 3D HSP27-overexpressed HuH7 cells was remarkably delayed in

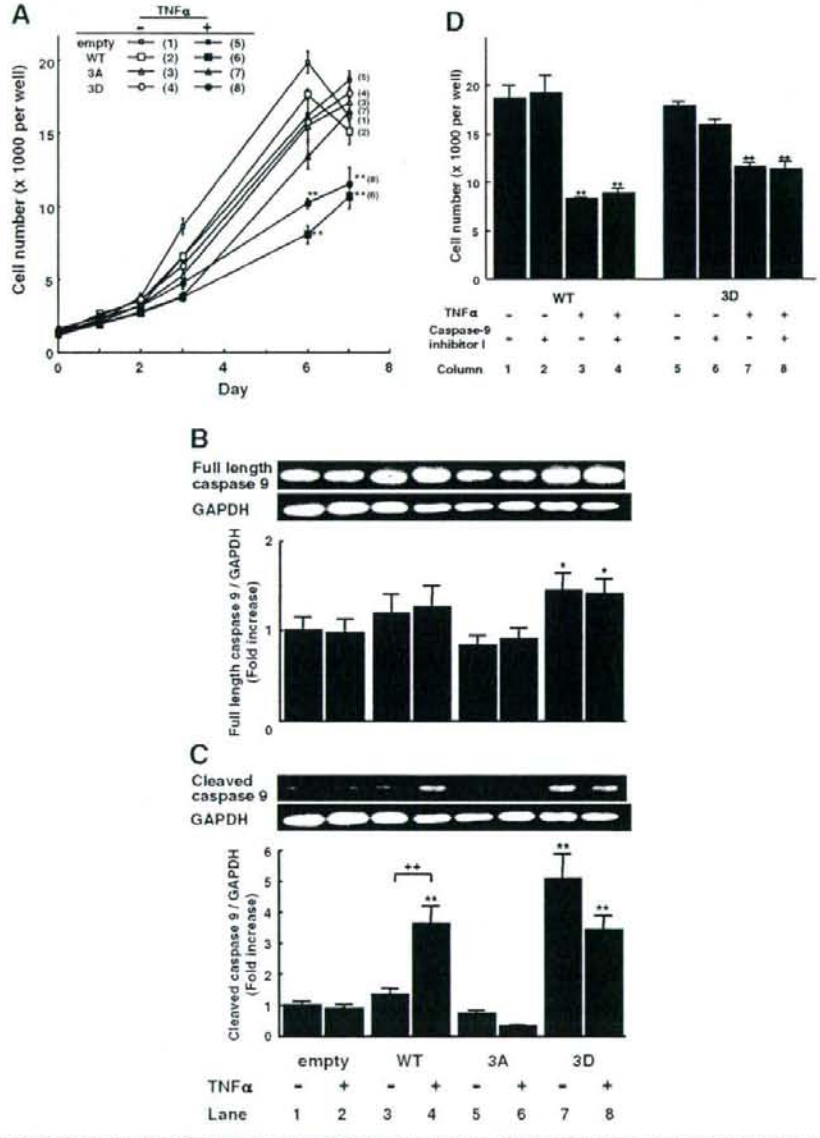


FIGURE 2. **Cell growth of the phosphorylated HSP27-overexpressed HCC cells was suppressed in the presence of TNFα and was not associated with caspase 9 activation.** *A*, cell growth curve. HuH7 cells were stably transfected either with empty (*curves 1* and *5*), WT (*curves 2* and *6*), 3A (*curves 3* and *7*), or 3D (*curves 4* and *8*) HSP27 vectors. These cells were cultured either in the absence (*curves 1–4*) or in the presence (*curves 5–8*) of 1 nM TNFα. Data are the mean ± S.D. ($n = 6$). The levels of full-length (*B*) and cleaved (*C*) caspase 9 of the HuH7 cells that were stably transfected either with empty (*lanes* and *columns 1* and *2*), WT (*lanes* and *columns 3* and *4*), 3A (*lanes* and *columns 5* and *6*), or 3D (*lanes* and *columns 7* and *8*) HSP27 vectors were determined by a Western blot analysis. The cells were stimulated with vehicle (*lanes* and *columns 1, 3, 5,* and *7*) or 1 nM TNFα (*lanes* and *columns 2, 4, 6,* and *8*) for 2 h. Values represent the amount of full-length (*B*) or cleaved (*C*) caspase 9 divided by those of GAPDH and were plotted as fold induction in comparison with those in the empty vector-transfected cells without TNFα stimulation (mean ± S.D., $n = 3$). *D*, effects of caspase-9 inhibitor I on cell growth. WT or 3D HSP27 vectors-transfected HuH7 cells were cultured in the absence (*columns 1, 2, 5,* and *6*) or in the presence (*columns 3, 4, 7,* and *8*) of 1 nM TNFα with (*columns 2, 4, 6,* and *8*) or without (*columns 1, 3, 5,* and *7*) 20 μM caspase-9 inhibitor I for 6 days. Data are the mean ± S.D. ($n = 6$). **, $p < 0.01$ *versus curves 1–5* and *7* at the indicated day (*A*). *, $p < 0.05$; **, $p < 0.01$ *versus column 1*; ++, $p < 0.01$ (*B* and *C*). **, $p < 0.01$ *versus column 1*; ++, $p < 0.01$ *versus column 5* (*D*).

cell growth of HCC under inflammatory conditions. Nonphosphorylated HSP27 is an inhibitor for caspase-dependent apoptosis (1, 5). It inhibits the interaction of cytochrome *c*, which is released from the permeabilized mitochondria, and pro-caspase 9. To study the relationship of apoptosis and the growth retardation of phosphorylated HSP27-overexpressed HuH7 cells, the activities of caspase 9 were examined in WT, 3A, or 3D HSP27-transfected HuH7 cells in the presence of TNFα. Regardless of the presence or the absence of TNFα, the amount of the full-length caspase 9 increased in 3D HSP27 cDNA-transfected HuH7 cells (Fig. 2*B*, *lanes 7* and *8*). The cleaved and activated caspase 9 significantly increased in WT HSP27 cDNA-transfected HuH7 cells after 2 h of stimulation of TNFα (Fig. 2*C*, *lane 4*). However, the increased activation of caspase 9 was shown also in the 3D HSP27-overexpressed cells in the absence of TNFα (Fig. 2*C*, *lane 7*). The cleavage of caspase 9 in the 3D HSP27-overexpressed cells in the presence of TNFα was even weaker than in the absence of TNFα (Fig. 2*C*, *lanes 7* and *8*). The similar tendency of the caspase 9 activities in the 2-h TNFα-stimulated cells was also shown in the cells after 72 h of stimulation of TNFα (data not shown). To confirm the cell growth retardation of WT and 3D HSP27-overexpressed HuH7 cells in the presence of TNFα is not related to caspase 9, we investigated whether caspase-9 inhibitor I, an irreversible inhibitor of caspase 9, restored the cell growth of WT and 3D HSP27 in the presence of TNFα. As shown in Fig. 2*D*, caspase-9 inhibitor I, which alone had little effect on the cell number, did not affect the cell growth of both WT and 3D HSP27-overexpressed HuH7 cells treated with 1 nM TNFα for 6 days (*columns 4* and *8*, in comparison with *columns 3* and *7*, respectively). Caspase-9 inhibitor I at a dose of 50 μM was toxic (data not shown). Therefore, it seems unlikely that caspase-dependent apoptosis caused the growth retardation of the phosphorylated HSP27-overexpressed cells in the presence of TNFα.

comparison with that of 3A HSP27-overexpressed HuH7 cells in the presence of TNFα 6 days after incubation (Fig. 2*A*, *curves 6* and *8*). Phosphorylated HSP27 therefore seems to inhibit the

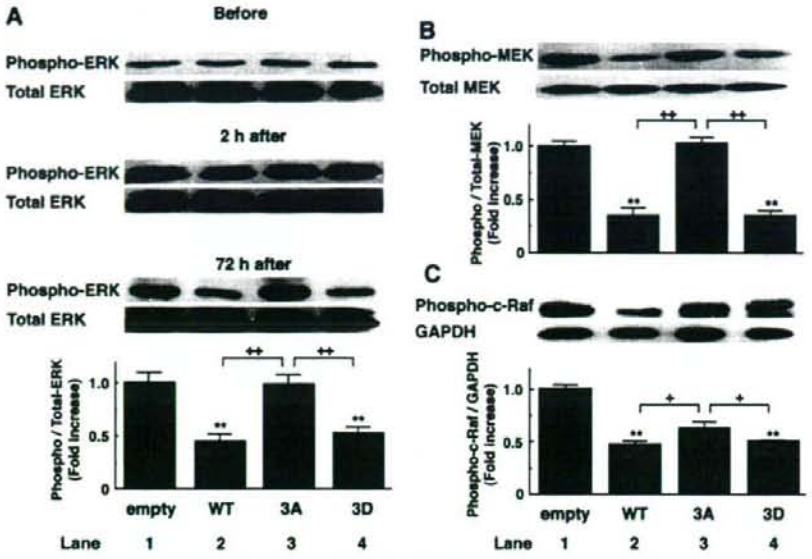


FIGURE 3. **Repression of the sustained activation of c-Raf-MEK-ERK signal transduction in the presence of TNFα by phosphorylated HSP27 in HCC cells.** The effects of phosphorylated HSP27 on the levels of phospho-ERK (*A*), phospho-MEK (*B*), and phospho-c-Raf (*C*) in HuH7 cells in the presence of 1 nM TNFα were determined by Western blot analysis. HuH7 cells were stably transfected either with empty (*lanes* and *columns 1*), WT (*lanes* and *columns 2*), 3A (*lanes* and *columns 3*), or 3D (*lanes* and *columns 4*) HSP27 vectors. *A*, levels of phospho- and total ERK in the HuH7 cells were determined before and 2 and 72 h after stimulation of TNFα. *B*, levels of phospho- and total MEK were determined using the same extract 72 h after stimulation with TNFα as in *A*. *C*, levels of phospho-c-Raf were determined 48 h after stimulation with TNFα. The relative intensity of the bands of the 72 h of stimulation in *A* and the bands in *B* and *C* were analyzed by densitometry (*columns 1–4*). Values represent the amount of phospho-ERK, phospho-MEK, or phospho-c-Raf divided by those of total ERK, total MEK, or GAPDH, respectively, and were plotted as fold induction in comparison with those in the empty vector-transfected cells (mean ± S.D., $n = 3$). **, $p < 0.01$ *versus* column 1; +, $p < 0.05$; ++, $p < 0.01$.

*Phosphorylated HSP27 Inhibited Prolonged Activation of ERK Signal Transduction in the HCC Cells*—ERK has been reported to act as a potent proliferative factor of HCC and be constitutively activated in the human HCC cells and tissues (12, 23). Does the cell growth retardation of the phosphorylated HSP27-overexpressed cells correlate with the ERK activity? The basal levels of phosphorylated ERK were similar among all HSP27 cDNA-transfected cells (Fig. 3*A*). Although the ERK phosphorylation levels in all HSP27 cDNA-transfected cells were similarly increased after 2 h of stimulation with 1 nM TNFα, phospho-ERK levels in both WT and the 3D HSP27-overexpressed HuH7 cells significantly decreased in comparison with those in 3A HSP27 cDNA or empty vector-transfected HuH7 cells after 72 h of stimulation with TNFα (Fig. 3*A*). Total ERK proteins were expressed at almost the same levels among all HSP27 cDNA-transfected cells regardless of whether or not they were stimulated with TNFα. The ERK activity is regulated by upstream kinases MEK and c-Raf. As shown in Fig. 3*B*, a significant decline of MEK activity in WT and the 3D HSP27-overexpressed HCC cells was observed in comparison with that in 3A HSP27 cDNA or empty vector-transfected cells after 72 h of stimulation of TNFα. Furthermore, significant attenuation of c-Raf activity was also shown in WT and the 3D HSP27-overexpressed HCC cells following 48 h of TNFα stimulation (Fig. 3*C*). Therefore, phosphorylated HSP27 might act as a repressor for prolonged activation of ERK signaling pathway at a point upstream of c-Raf in the HCC cells.

*Transactivation Activities of AP-1 and Cyclin D1 Expression Were Suppressed in the 3D HSP27-overexpressed HCC Cells*—ERK contributes to the induction of AP-1 transcriptional activity, and AP-1 activates the cyclin D1 promoter to induce cell proliferation (13, 24). Therefore, the effect of the phosphorylated HSP27 on AP-1 transactivation activity was assessed (Fig. 4*A*). After 72 h of stimulation with TNFα, WT and the 3D HSP27-overexpressed HuH7 cells expressed significantly less transactivation activity of AP-1 than 3A HSP27-introduced cells (Fig. 4*A*, *columns 6* and *8*, in comparison with *column 7*). A remarkable decrease of AP-1 transactivation activity was observed especially in 3D HSP27 cDNA-transfected cells (Fig. 4*A*, *column 8*). To confirm that the attenuation of this AP-1 transactivation activity occurred because of the ERK signaling pathway, constitutive active MEK1 cDNA was transfected into the 3D HSP27-overexpressed HuH7 cells. The active MEK1 restored AP-1 transactivation activity of the 3D HSP27-overexpressed HuH7 cells to the similar level as the 3A HSP27-overexpressed or empty vector-transfected HuH7 cells (Fig. 4*A*, *column 9* in comparison with *columns 7* or *5*). In the absence of TNFα, no significant difference of the AP-1 transactivation activity among empty vector and all HSP27 cDNAs-transfected cells was shown (Fig. 4*A*, *columns 1–4*). Therefore, phosphorylated HSP27 presumably reduced AP-1-mediated cell proliferation via ERK signaling pathway in the HCC tissues under inflammatory conditions. Next, cyclin D1 protein expression levels in empty vector and all HSP27 cDNA-transfected cells in the presence and absence of TNFα were examined. In WT and the 3D HSP27-transfected cells treated with TNFα for 72 h, cyclin D1 levels significantly decreased in comparison with those in the 3A HSP27-transfected cells (Fig. 4*B*, *columns* and *lanes 6* and *8* in comparison with *column* and *lane 7*). In the absence of TNFα, no significant difference of cyclin D1 protein level among empty vector and all HSP27 cDNA-transfected cells was shown (Fig. 4*B*, *columns 1–4*). Therefore, phosphorylated HSP27 presumably reduced AP-1-mediated cell proliferation via ERK signaling pathway in the HCC tissues.

*Increased Expression Level and Activation of p38 MAPK That Were Followed by the Induction of Active MKP1 Were Observed in the Phosphorylated HSP27-overexpressed HCC Cells*—In eukaryotic cells, there are another two MAPKs, p38 MAPK and

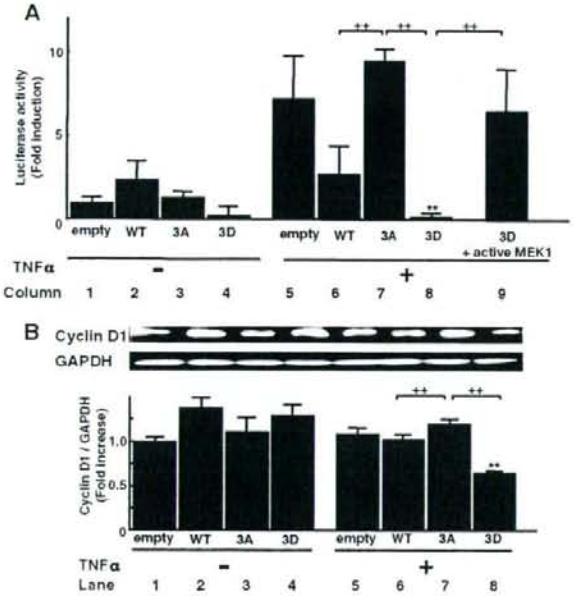


FIGURE 4. **Inhibition of the AP-1 transactivation activity and cyclin D1 protein expression by phosphorylated HSP27 in HCC cells.** *A*, AP-1 transactivation activities in HuH7 cells were determined 72 h after stimulation with (*columns 5–9*) or without (*columns 1–4*) TNFα. HuH7 cells were stably transfected either with empty (*columns 1* and *5*), WT (*columns 2* and *6*), 3A (*columns 3* and *7*), or 3D (*columns 4*, *8*, and *9*) HSP27 vectors. The cells were transiently transfected with AP-1-luciferase reporter gene alone (*columns 1–8*) or a combination with active MEK1 vector (*column 9*) along with *pRL-CMV* (*Renilla* luciferase) as an internal standard using lipofection. The cells were stimulated with or without 1 nM TNFα for 48 h before transfection. Five h after transfection, the cells were stimulated for another 24 h with or without 1 nM TNFα. The luciferase activity in cell lysates was measured and plotted as fold induction in comparison with the activity in empty vector-transfected cells in the absence of TNFα (*column 1*) after they were normalized to *Renilla* luciferase activity. Values are the mean ± S.D. ($n = 6$). *B*, protein levels of cyclin D1 were determined 72 h after in the absence (*lanes* and *columns 1–4*) or presence (*lanes* and *columns 5–8*) of TNFα. HuH7 cells were stably transfected either with empty (*lanes* and *columns 1* and *5*), WT (*lanes* and *columns 2* and *6*), 3A (*lanes* and *columns 3* and *7*), or 3D (*lanes* and *columns 4* and *8*) HSP27 vectors. The relative intensity of the bands was analyzed by densitometry (*columns 1–8*). Values represent the amount of cyclin D1 divided by those of GAPDH and were plotted as fold induction in comparison with those in the empty vector-transfected cells in the absence of TNFα (mean ± S.D., $n = 3$). **, $p < 0.01$ *versus column 1*; ++, $p < 0.01$.

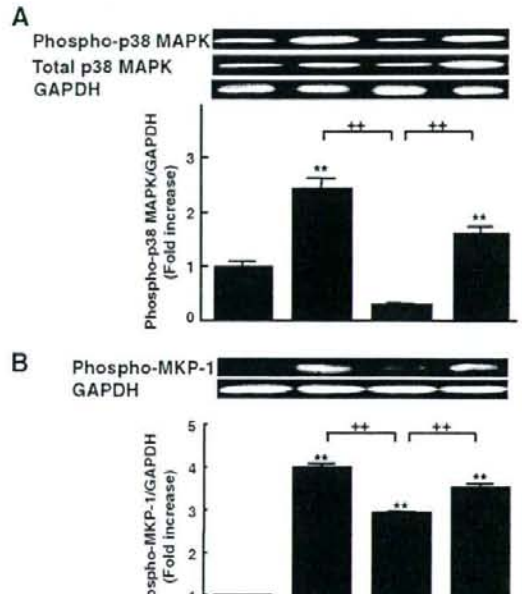


FIGURE 5. **The enhanced activation and the increased amount of p38 MAPK, and the induction of the phospho-MKP-1 protein expression in the presence of TNFα by phosphorylated HSP27 in HCC cells.** HuH7 cells were stably transfected either with empty (*lanes* and *column 1*), WT (*lanes* and *column 2*), 3A (*lanes* and *column* 3), or 3D (*lanes* and *column 4*) HSP27 vectors. *A*, levels of phospho- and total p38 MAPK of HuH7 cells were determined by a Western blot analysis. The cells were treated with 1 nM TNFα for 2 h. *B*, levels of phospho-MKP-1 of HuH7 cells were determined by a Western blot analysis. The cells were stimulated with 1 nM TNFα for 72 h. Values represent the amount of phospho-p38 MAPK and phospho-MKP-1 divided by those of GAPDH and were plotted as fold induction in comparison with those in the empty vector-transfected cells (mean ± S.D., $n = 3$). **, $p < 0.01$ *versus column 1*; ++, $p < 0.01$.

JNK, in addition to ERK (11). Although p38 MAPK and JNK are less sensitive to growth signals than ERK, their activation is preferentially triggered by pro-inflammatory cytokines and environmental stresses (25). Therefore, p38 MAPK activities were next examined in the unphosphorylated and in the phosphorylated HSP27-overexpressed HCC cells. Because the maximum activity of ERK was observed after 2 h stimulation with TNFα, the amount of p38 MAPK and phosphorylated p38 MAPK in the cells was also examined after 2 h of stimulation with TNFα. Fig. 5*A* shows that the p38 MAPK level was increased in WT and especially in the 3D HSP27-overexpressed HuH7 cells in the presence of TNFα in comparison with that in the 3A HSP27 cDNA or empty vector-transfected cells. The increased levels of p38 MAPK were also observed even in the absence of TNFα in both WT and the 3D HSP27-overexpressed HuH7 cells (data not shown). Furthermore, TNFα stimulation significantly induced the p38 MAPK phosphorylation in both WT and the 3D HSP27 cDNA-transfected HuH7 cells in comparison with the empty vector and 3A HSP27 cDNA-transfected cells (Fig. 5*A*, *lanes 2* and *4* in comparison with *lanes 1* or *3*). The similar tendency of enhanced activation of p38 MAPK in WT and 3D HSP27-introduced HuH7 cells was also observed in 72-h TNFα-stimulated cells, although the activity was less than after 2 h of stimulation (data not shown). HSP27 is phosphorylated by the p38 MAPK pathway (1). On the other hand, phosphorylated HSP27 enhanced p38 MAPK expression and activation in this experiment. This is probably the first report showing such a positive feedback from phosphorylated HSP27 to p38 MAPK. The activation of p38 MAPK is reported to induce MKP-1, a phosphatase that inactivates ERK (26). The effect of phosphorylated HSP27 was analyzed on an active form of MKP-1, phosphorylated MKP-1 (27). Phosphorylated MKP-1 was significantly induced in WT and the 3D HSP27-overexpressed cells after 72 h stimulation of TNFα in comparison with that in the 3A HSP27 cDNA-transfected cells (Fig. 5*B*, *lanes 2* and *4* in comparison with *lane 3*). Phosphoryl-

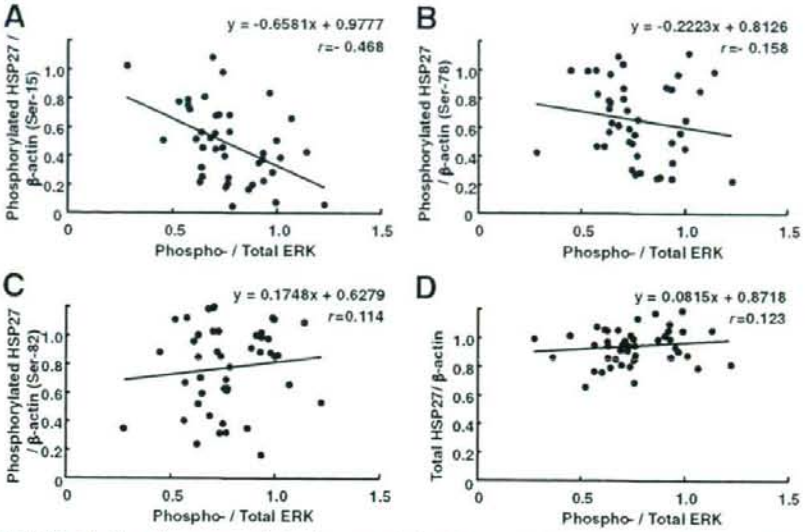


FIGURE 6. **Significant inverse correlation between the levels of phosphorylated HSP27 (Ser-15) and activation of ERK in human HCC tissues.** Correlation between the expression levels of phospho-ERK and the levels of phosphorylated HSP27 (Ser-15) (*A*), phosphorylated HSP27 (Ser-78) (*B*), phosphorylated HSP27 (Ser-82) (*C*), and total HSP27 in human HCC tissues (*D*). The expression levels of phospho-ERK and total ERK, phosphorylated and total HSP27, and β-actin were determined by the band intensities obtained from a Western blot analysis. Based on the intensity of the same concentration of the marker protein that runs in every gel, the values of the tissue samples protein on separate gels were normalized. The values represent the amount of phospho-ERK, and phosphorylated and total HSP27s were divided by those of total ERK and β-actin, respectively. Data were analyzed with Pearson's correlation coefficient ($r$). $|r| \geq 0.400$ was accepted as a positive correlation. *A*, $p < 0.01$.

ated MKP-1 was also induced in 3*A* HSP27 as well as WT and 3D HSP27 in HCC cells, although the induction was weaker (Fig. 5*B*, *lane 3* in comparison with *lane 1*). The phosphorylated MKP-1 expression slightly increased in the WT and the 3D HSP27-transfected cells also in the absence of TNFα (data not shown). Not only the presence of the phosphorylated HSP27 but also the total amount of HSP27 might play some part in the induction of active MKP-1. The activity of JNK, the other MAPK, was not significantly changed in these experiments (data not shown).

*The Levels of Phosphorylated HSP27 (Ser-15) Inversely Correlated with the Levels of ERK Activation in Human HCC Tissues*—The ERK activities were down-regulated by phosphorylated HSP27 in the human HCC cells as described above. Therefore, the *in vivo* correlation between the levels of ERK activity and the levels of phosphorylated HSP27 was investigated in HCC tissues. The levels of phosphorylated HSP27 (Ser-15) were significantly inversely correlated with the levels of activated ERK ($r = -0.468, p < 0.001$; Fig. 6*A*). On the contrary, the levels of phosphorylated HSP27 (Ser-78), phosphorylated HSP27 (Ser-82), and total HSP27 did not correlate with those of the activated ERK (Fig. 6, *B–D*, respectively).

## DISCUSSION

The influence of post-translational modification, such as phosphorylation on the function of HSP27, is precisely unknown. In this study, phosphorylated HSP27 reduced the cell growth rate of the HuH7 cells in the presence of TNFα (Fig. 2*A*). HSP27 reportedly inhibits the caspases to protect the cells from apoptosis, and the phosphorylation status of HSP27 influences that function (1, 5). A transcription factor, NF-κB, has been implicated in suppression of apoptosis, cell survival, proliferation, viral replication, inflammation, tumorigenesis, and metastasis, and all members of the TNF superfamily are known to activate it (28). The ability of HSP27 to interact with IKKβ has been reported to be enhanced via the TNFα-induced activation of MAPK-dependent phosphorylation of HSP27, thus leading to the enhanced inhibition of IKK activity, reduced IκB degradation, and consequent suppression of NF-κB activation in HeLa cells (9). The overexpression of phosphorylated HSP27 increased caspase 9 protein levels and activity (Fig. 2, *B* and *C*). Phosphorylated HSP27 may therefore increase apoptosis in the HCC cells. However, the caspase activities in the phosphorylated HSP27-overexpressed cells were not enhanced by TNFα (Fig. 2*C*), and the caspase inhibitor did not restore the cell growth retardation of WT and phosphorylated HSP27-overexpressed cells in the presence of TNFα (Fig. 2*D*). These results suggest that the influence of some mechanisms other than apoptosis might play important roles in the control of the HCC cell proliferation by phosphorylated HSP27 in the presence of an inflammatory cytokine like TNFα. However, the HCC cell growth retardation by phosphorylated HSP27 may be caused, in part, by the suppression of NF-κB.

In human HCC, it is generally accepted that the activation of the ERK signal pathway leads to a mitogenic effect (12, 20). This study presented novel evidence that phosphorylated HSP27 inhibits the sustained activation of the c-Raf-MEK-ERK pathway in an inflammatory environment (Fig. 3). The phosphorylation of HSP27 significantly correlated with the activity of ERK in not only HCC cells *in vitro* but also the specimens in patients with HCC *in vivo* (Fig. 6). There have so far been few reports addressing the influence of HSP27 on the ERK activation, except for the study by Lee and co-workers (29, 30) where the overexpression of HSP25, the same species as human HSP27 in the mouse, was shown to down-regulate ERK expression, while also inhibiting their activation in mouse fibroblast L929 cells by a reduction in reactive oxygen species. Contrary to our results, HSP25 overexpression attenuated the $H_2O_2$-ERK pathway-mediated apoptosis in their experiments. The role of the HSP27-ERK pathway might be different in the mouse fibroblasts as compared with the human HCC cells. The attenuation of phosphorylated HSP27, especially phosphorylated at Ser-15, is correlated with HCC progression (15). In addition, the expression levels of HSP20, one of the low molecular weight HSP family

(31), decreases in parallel with HCC progression and a significant correlation we observed between the levels of HSP20 and phosphorylated HSP27 at Ser-15 but not at Ser-78 and Ser-82 in human HCC tissues (19). This study revealed a significant inverse correlation between the levels of phosphorylated HSP27 (Ser-15) and ERK activity (Fig. 6). Based on these findings, it is probable that the phosphorylation at Ser-15 of HSP27 is important for repressing HCC cell growth activity. It has been shown that phosphorylation at Ser-15, but not Ser-78 and Ser-82, of HSP27 results in the conformational changes of HSP27 and the alteration of the direct interaction of HSP27 with other HSPs (32). Therefore, the alteration of direct interaction of HSP27 with HSP20 and/or other factors by the phosphorylation at Ser-15 may affect the ERK signal transduction pathway in HCC, thus resulting in the suppression of proliferation.

Activated ERK is generally known to translocate into the nuclei and induce/activate transcription factors such as AP-1, which in turn increase the transcriptional activity of genes relevant for cell cycle progression, such as cyclin D1. In this study, the suppression of ERK activity by phosphorylated HSP27 attenuated the AP-1 transactivation activity and the expression of cyclin D1 (Fig. 4). ERK activation in human HCC is known to play an important role in multistep hepatocarcinogenesis, especially in the progression of HCC, at least in part through cyclin D1 up-regulation primarily induced by MAPK/ERK via c-Fos (12, 33). In addition, active MEK1 restored the AP-1 activity levels suppressed by phosphorylated HSP27 to the control levels. Therefore, the suppression of AP-1-cyclin D1 signal transduction via the inhibition of ERK activity might be the significant mechanism for the proliferative control of the HCC cell by phosphorylated HSP27.

Although HSP27 phosphorylation is generally known to be a reversible process catalyzed by the MAPKAP2, a downstream substrate of p38 MAPK (1), phosphorylated HSP27 surprisingly induced p38 MAPK (Fig. 5*A*). The activation of the p38 MAPK cascade is preferentially triggered by pro-inflammatory cytokines, such as TNFα and environmental stress. The α-isoform of protein kinase C, an essential molecule of malignant cancer cells, has been reported to activate p38 MAPK while also stimulating cell migration and invasion in poorly differentiated human HCC cell lines (34). Transforming growth factor β mediated the activation of p38 MAPK, and its downstream HSP27 may increase the invasive potential and matrix metalloprotease (MMP)-2 expression in human prostate cancer cells (35). On the contrary, p38 MAPK and p38 MAPK kinase (MKK3) have been shown to significantly inhibit mitogen-induced cyclin D1 expression in the constitutively active Raf-1 and estrogen receptor fusion protein stably expressed CCL39 cells (13). The association of human HCC with nearby normal tissues has been shown to reduce p38 MAPK and MKK6 activities especially in larger tumors (36). Moreover, it has been reported recently that p38 MAPKα suppresses liver cancer development by antagonizing the JNK-c-Jun pathway (14). There are at least four isoforms of p38 MAPK that have been identified and characterized, and the activation of p38 MAPK is mediated by MKK3, -4, and -6 (37). The role of p38 MAPK and its isoforms in HCC cell growth have not yet been established. It has been shown that p38 MAPK induces MKP-1, a major negative regulator for ERK (26). This study also showed that phosphorylated HSP27 activated p38 MAPK and subsequently induced phosphorylated MKP-1 (Fig. 5*B*). These findings suggest that the cross-talk among phosphorylated HSP27, p38 MAPK, and MKP-1 might also regulate ERK activity in addition to the down-regulation of c-Raf-MEK-ERK signal transduction by phosphorylated HSP27, thus resulting in the suppression of HCC cell proliferation. The potential mechanism of phosphorylated HSP27 in HCC shown here is summarized in Fig. 7.

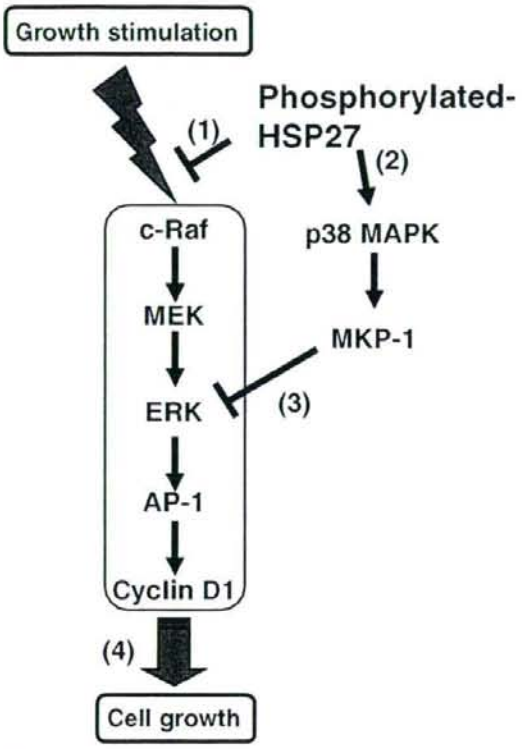


FIGURE 7. **Schematic representation of the effect of phosphorylated HSP27 on HCC cell growth under inflammatory conditions.** Phosphorylated HSP27 in an irritated liver with HCC inhibits sustained activation of c-Raf-MEK-ERK system (*1*) while also up-regulating the MKP-1 induction and activation via induction and activation of p38 MAPK (*2*). The induced MKP-1 also inhibits ERK activity (*3*). The repression of ERK activity and subsequent AP-1/cyclin D1 down-regulation led to the suppression of HCC cell growth (*4*).

A number of studies have shown that the redox state plays a role in the regulation of TNFα intracellular signaling and ERK activation (38, 39). HSP27 has been reported to regulate the intracellular reactive oxygen species and/or glutathione level (30, 40). However, reactive oxygen species inhibitors do not suppress TNF-induced AP-1 activation (40). No change in the total glutathione levels is observed with the decrease in ERK phosphorylation in response to oxidative stress in primary cultured rat hepatocytes (41). It was recently reported that HSP27 is required for interleukin-1-activated transforming growth factor-β-activated kinase-1 (TAK1) and downstream signaling by p38 MAPK, JNK, and their activator kinases and IKKβ (42). TAK1 is the most upstream common signaling protein of both interleukin-1 and TNFα. However, the activation of ERK

induced by interleukin-1 appears to be independent of HSP27 (42). Further studies should therefore investigate where and how phosphorylated HSP27 acts at a point upstream of c-Raf.

The activation of AP-1 is known as an early event in HCC (43). In this experiment, phosphorylated HSP27 overexpression repressed prolonged activation of ERK signal transduction and down-regulated AP-1 activity, thus resulting in the suppression of HCC cell growth. These results suggest that phosphorylated HSP27 has a stronger influence in an earlier stage of the liver carcinogenesis. The remarkably high incidence of secondary liver cancer is actually responsible for the poor prognosis of liver cancer (10). At least one-third of post-therapeutic recurrence is because of the *de novo* cancer through multicentric carcinogenesis. The prevention of the recurrence of HCC at early stage is therefore urgently needed to enhance long term survival. Recently, an oral multikinase inhibitor, sorafenib, was developed, and the clinical trials against human HCC are in progress (44). Sorafenib blocks tumor cell proliferation by targeting Raf/MEK/ERK signaling at the level of Raf kinase (45). The HCC cell growth retardation via inhibition of ERK pathway by phosphorylated HSP27 shown here could therefore be a novel promising therapeutic strategy to prevent the recurrence of HCC.

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# Prognostic value of pretreatment levels of tumor markers for hepatocellular carcinoma on survival after curative treatment of patients with HCC☆

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***Background/Aims*: We evaluated the prognostic value of the pretreatment elevation of tumor markers for hepatocellular carcinoma (HCC) in patients who underwent curative treatment.**

***Methods*: We studied 801 patients who had been diagnosed as initial HCC and fulfilled the following criteria: maximum tumor size, ⩽3 cm; number of tumors, ⩽3; remnant liver function, Child-Pugh class A or B; treated by hepatectomy or locoregional thermal ablation (LTA); and alpha-fetoprotein (AFP), *Lens culinaris* agglutinin A-reactive fraction of AFP (AFP-L3), and des-gamma carboxy prothrombin (DCP) were measured at diagnosis. We analyzed the effects of elevated tumor markers on patient survival in these 2 distinct groups with different types of treatment, i.e. hepatectomy and LTA.**

***Results*: By multivariate analysis in 345 patients who underwent hepatectomy, no tumor marker significantly affected decreased survival rate. In the 456 patients who underwent LTA, the elevation of AFP-L3 ($p = 0.0171$) and DCP ($p = 0.0004$) significantly affected decreased survival rate; DCP elevation had the strongest effect on patient survival.**

***Conclusions*: The prognostic value of pretreatment tumor marker elevation was different in patients who underwent the curative treatment according to the type of treatment. Pretreatment elevation of AFP-L3 and DCP had prognostic values only in patients treated with LTA.**

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*Abbreviations:* AFP, alpha-fetoprotein; AFP-L3, Lens-culinaris agglutinin A-reactive fraction of AFP; CT, computed tomography; DCP, des-gamma-carboxy prothrombin; HBV, hepatitis B virus; HCC, hepatocellular carcinoma; HCV, hepatitis C virus; LTA, locoregional thermal ablation; PIVKA-II, protein induced by vitamin K absence-II; PMCT, percutaneous microwave thermocoagulation; RFA, radiofrequency ablation.


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