patients with persistently normal alanine aminotransferse levels. *Gut* 2002; **51**: 585–90.
17 Okanoue T, Yasui K, Sakamoto S *et al.* Circulating HCV RNA, HCV genotype, and liver histology in asymptomatic individuals reactive for anti-HCV antibody and their follow-up study. *Liver* 1996; **16**: 241–7.
18 Okanoue T, Makiyama A, Nakayama M *et al.* A follow-up study to determine the value of liver biopsy and need for antiviral therapy for hepatitis C virus carriers with persistently normal serum aminotransferase. *J Hepatol* 2005; **43**: 599–605.
19 Bacon BR. Treatment of patients with hepatitis C and normal serum aminotransferase levels. *Hepatology* 2002; **36**: S179–84.
20 Zeuzem S, Diago M, Gane E *et al.* Peginterferon alfa-2a (40 kilodaltons) and ribavirin in patients with chronic hepatitis C and normal aminotransferase levels. *Gastroenterology* 2004; **127**: 1724–32.
21 Manns MP, McHutchison JG, Gordon SC *et al.* Peginterferon alfa-2b plus ribavirin compared to interferon alfa-2b plus ribavirin for the treatment of chronic hepatitis C: a randomized controlled trial. *Lancet* 2001; **359**: 958–65.
22 Fried MW, Shiffman ML, Reddy KR *et al.* Peginterferon alfa-2a plus ribavirin for chronic hepatitis C virus infection. *N Engl J Med* 2002; **347**: 975–82.
23 Puoti C, Magrini A, Stati T *et al.* Clinical, histological, and virological features of hepatitis C virus carriers with persistently normal or abnormal alanine aminotransferase levels. *Hepatology* 1997; **26**: 1393–8.
24 Prati D, Taidoli E, Zanela A *et al.* Updated definition of healthy ranges for serum alanine aminotransferase levels. *Ann Intern Med* 2002; **137**: 1–9.
25 Desmet VJ, Gerber M, Hoofnagle JH *et al.* Classification of chronic hepatitis: diagnosis, grading and staging. *Hepatology* 1994; **19**: 1513–20.
26 Ishak K, Baptista L, Bianchi L *et al.* Histological grading and staging of chronic hepatitis. *J Hepatol* 1995; **22**: 696–9.
27 MacSween RNM, Anthony PP, Sheuer PJ. *Pathology of the Liver*. Edinburgh: Churchill Livingstone, 1987.
28 Tsukiyama-Kohara K, Yamaguchi K, Maki N *et al.* Antigenicities of group 1 and 2 hepatitis C virus polypeptides: molecular basis of diagnosis. *Virology* 1993; **192**: 430–7.
29 Simmonds P, Alberti A, Alter HJ *et al.* A proposed system for the nomenclature of hepatitis C virus genotypes. *Hepatology* 1994; **19**: 1321–4.
30 Makiyama A, Itoh Y, Kasahara A *et al.* Characteristics of patients with chronic hepatitis C who develop hepatocellular carcinoma after sustained response to interferon. *Cancer* 2004; **101**: 1616–22.
31 Healey CJ, Chapman RWG, Fleming KA. Liver histology in hepatitis C virus infection: a comparison between patients with persistently normal or abnormal transaminase. *Gut* 1993; **37**: 274–8.
32 Ohkoshi S, Tawaraya H, Kuwana K *et al.* A retrospective study of hepatitis C virus carriers in a local endemic town in Japan. *Dig Dis Sci* 1995; **40**: 465–71.
33 Puoti C, Castellacci R, Montagness F *et al.* Histological and virological features and follow-up of HCV carriers with normal aminotransferase levels: the Italian Study of the Asymptomatic C Carriers (ISACC). *J Hepatol* 2002; **37**: 117–23.
34 Yano M, Kumada H, Kage M *et al.* The long-term pathological evolution of chronic hepatitis C. *Hepatology* 1996; **332**: 1463–6.
35 Poynard T, Bedossa P, Opolon P. Natural history of liver fibrosis progression in patients with chronic hepatitis C. The OBSVIRC, METAVIR, CLINVIR, and DOSVIRC. *Lancet* 1997; **346**: 825–32.
36 Takahashi M, Yamada G, Miyamoto R, Doi H, Endo H, Tsuji T. Natural course of chronic hepatitis C. *Am J Gastroenterol* 1993; **88**: 240–3.
37 Ghany MG, Kleiner DE, Alter H *et al.* Progression of fibrosis in chronic hepatitis C. *Gastroenterology* 2003; **124**: 97–104.
38 Mathurin P, Moussalli J, Cardanei J-F *et al.* Slow progression rate of fibrosis in hepatitis C virus patients with persistently normal alanine transaminase activity. *Hepatology* 1998; **27**: 868–72.
39 Hui C-K, Belaye T, Montegrade K, Wright TL. A comparison in the progression of liver fibrosis in chronic hepatitis C between persistently normal and elevated transaminase. *J Hepatol* 2003; **38**: 511–17.
40 Prati D, Shiffman ML, Diago M *et al.* Viral and metabolic factors influencing alanine aminotransferase activity in patients with chronic hepatitis C. *J Hepatol* 2006; **44**: 679–85.
41 Tarao K, Ohkawa S, Tamai S, Miyakawa K. Sustained low serum GPT level below 80 INU in HCV- associated cirrhotic patients by multiagents prevent development of hepatocellular carcinoma. *Cancer* 1994; **73**: 1149–54.
42 Boccato S, Pistis R, Noventa F, Guido M, Benvegnu L, Alberti A. Fibrosis progression in initially mild chronic hepatitis C. *J Viral Hepat* 2006; **13**: 297–302.
43 Cadranel JF, Rufat P, Degos F. Practices of liver biopsy in France: results of a prospective nationwide survey. For the Group of Epidemiology of the French Association for the Study of the Liver (AFEF). *Hepatology* 2000; **32**: 477–81.
44 Castera L, Negre I, Samii K, Buffet C. Pain experienced during percutaneous liver biopsy. *Hepatology* 1999; **30**: 1529–30.
45 Afdal NH. Diagnosing fibrosis in hepatitis C: is the pendulum swinging from biopsy to blood test? *Hepatology* 2003; **37**: 972–4.
46 Imbert-Bismut F, Ratziu V, Pieroni L, Charlotte F, Benhamou Y, Poynard T. Biochemical markers of liver fibrosis in patients with hepatitis C virus infection: a prospective study. *Lancet* 2001; **357**: 1069–75.

47 Sandrin L, Fourquet B, Hasquenoph JM *et al.* Transient elastography: a new invasive method for assessment of hepatic fibrosis. *Ultrasound Med Biol* 2003; 29: 1705–13.
48 Castera L, Vergniol J, Foucher J *et al.* Prospective comparison of transient elastography, fibrotest, APRI, and liver biopsy for the assessment of fibrosis in chronic hepatitis C. *Gastroenterology* 2005; 128: 343–50.
49 Saito H, Tada S, Nakamoto N *et al.* Efficacy of non-invasive elastometry on staging of hepatic fibrosis. *Hepatol Res* 2004; 29: 97–103.
50 Colletta C, Smirne C, Fabris C *et al.* Value of two noninvasive methods to detect progression of fibrosis among HCV carriers with normal aminotransferase. *Hepatology* 2005; 42: 838–645.
51 Castera L, Foucher J, Bertet J, Couzigou P. FibroScan and FibroTest to assess liver fibrosis in HCV with normal aminotransferse. *Hepatology* 2006; 43: 373–4.
52 Alberti A. Towards more individualized management of hepatitis V virus patients with initially or persistently normal alanineaminotransferase levels. *J Hepatol* 2005; 42: 266–74.

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Association of hepatitis B virus subgenotypes and basal core promoter/precore region variants with the clinical features of patients with acute hepatitis

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Background. In endemic areas, including Japan, basal core promoter (BCP) and precore (PC) variants of hepatitis B virus (HBV) have been reported to be associated with the clinical outcome of acute hepatitis B patients. However, the associations of BCP/PC variants with clinical outcomes have not been observed in non-endemic areas. HBV subgenotypes, which show geographic variations in prevalence, may underlie this discrepancy in clinical outcomes. Little is known about the differences in the clinical and virological features of HBV subgenotypes and BCP/PC variants. The aim of this study was to investigate the distributions of subgenotypes and BCP/PC variants to identify clinical differences in acute hepatitis B patients. ***Methods.*** One hundred thirty-nine patients with acute hepatitis were enrolled. Nested polymerase chain reaction was used to amplify the pre-S region of HBV for genotyping and the BCP/PC regions for variant screening. ***Results.*** HBV subgenotypes A1 (n = 3), A2 (n = 28), B1 (n = 3), B2 (n = 9), C1 (n = 5), C2 (n = 84), C variant (n = 1), D2 (n = 3), and H (n = 3) were detected. BCP/PC variants were not associated with progression to chronic hepatitis. Patients infected with subgenotype C2 who progressed to fulminant hepatic failure frequently carried variants at nucleotides non-T1753 and non-T1754 and T1762, A1764, and A1896. ***Conclusions.*** BCP/PC variants would be associated with progression to fulminant hepatitis in subgenotype C2. Knowledge of HBV subgenotypes and BCP/PC variants is useful for developing strategies to treat acute hepatitis B patients.

Key words: hepatitis B virus, fulminant hepatic failure, subgenotypes, basal core promoter/precore region variants

Introduction

Approximately 350 million people worldwide are infected with hepatitis B virus (HBV).[1] HBV infection has a variety of clinical courses, including self-limited acute hepatitis, fulminant hepatic failure, chronic hepatitis, and progression to cirrhosis and hepatocellular carcinoma.[2] Therefore, HBV infection is a significant global health problem. HBV has been classified into eight major genotypes on the basis of divergence of 8% of the full-length nucleotide sequence, and the prevalence of each genotype differs by region.[3,4] Each genotype shows different responses to antiviral treatments and different virological characteristics;[5–7] therefore, HBV genotype information may be useful for developing strategies to treat HBV-related liver disease. Moreover, HBV genotypes have been subdivided into subgenotypes that differ in their geographic distribution.[4] Therefore, HBV subgenotypes can be used to study geographic distributions in greater detail than can simple genotypes. Recently, the prevalence and geographic distribution of HBV subgenotypes in Japanese HBV carriers, including patients with acute hepatitis, were reported.[8,9] However, the effects of HBV subgenotypes on the clinical course of acute hepatitis have not been well documented. Several studies have reported that variants of the basal core promoter (BCP) and precore (PC) regions may be associated with progression to fulminant hepatic failure.[10–12] However, the roles of BCP and PC variants in acute hepatitis are controversial.[13,14] The prevalences of BCP and PC region variants depend on genotype, and some researchers have proposed that genotype differences influence clinical outcome.[15–17] Therefore, one reason that may explain the discrepant results for the roles of BCP and PC variants in acute hepatitis may be HBV genotype and subgenotype differences. Therefore, the relationships of BCP and PC region variants with clinical features need to be considered with respect to the HBV subgeno-

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types. The aim of this study was to investigate the distribution of BCP and PC variants in each HBV subgenotype to identify the clinical and virological differences in Japanese patients with acute hepatitis.

Materials and methods

Patients

One hundred thirty-nine patients with acute hepatitis B who were treated from 1998 to 2006 at Nagoya University Hospital and Ogaki Municipal Hospital and whose samples were stored were enrolled in the present study. The patients consisted of 102 men and 37 women with a mean age of 36.8 (range, 16–75) years. Acute hepatitis B was diagnosed based on positive hepatitis B surface antigen (HBsAg) and high titers of IgM class antibody to hepatitis B core antigen (anti-HBc); elevated serum alanine aminotransferase levels; and negative for antibodies against hepatitis A virus, hepatitis C virus, Epstein–Barr virus, and cytomegalovirus. To discriminate between the development of chronic hepatitis after initial infection and acute onset during chronic infection, the presence of HBsAg in serum before admission was confirmed from previous medical records that included information regarding screening for HBsAg related to blood donation, delivery, or employment. A negative HBsAg result in a previous record and a persistently positive HBsAg after an observation period of 6 months was considered to indicate the development of chronic hepatitis. All patients with acute self-limited hepatitis were confirmed to be negative for HBsAg and/or positive for HBs antibody during the observation period. Fulminant hepatic failure was defined as the development of hepatic encephalopathy and a less than 40% prolongation of the prothrombin time during the course of acute hepatitis.[18] Informed consent was obtained from each patient, and the study was carried out in accordance with the 1975 Helsinki Declaration.

Genetic analysis of HBV

HBsAg levels were measured with a commercially available kit (AxSYM HBsAg(V2); Abbott Japan, Tokyo, Japan). Antibody titers against hepatitis A virus and hepatitis C virus were measured with a commercial microparticle enzyme immunoassay (AxSYM HAVAB-M 2.0, AxSYM Anti-HCV; Abbott Japan).

HBV DNA was isolated from peripheral blood with a QIAamp DNA Mini Kit (Qiagen, Hilden, Germany). Nested polymerase chain reaction (PCR) analysis and direct sequencing of the pre-S and precore/core regions were performed as previously reported.[9,19] A mutation mixture was defined as viral mutants that constituted 50% or more of the total viral population. The neighbor-joining method[20] was used for phylogenetic analysis of the preS region to classify HBV into subgenotypes. Bootstrap resampling and reconstruction (100 replicates) were performed to confirm the reliability of the phylogenetic tree analysis.

Statistical analyses

The data are expressed as mean ± standard deviation (SD) or as odds ratio (OR) and 95% confidence interval (95% CI). Contingency table analysis with Fisher's exact probability test was used for comparisons between groups. Chi-square distributions of HBV subgenotypes, BCP/PC variants, and clinical course were analyzed. $P < 0.05$ was considered statistically significant. SPSS software (SPSS, Chicago, IL, USA) was used for statistical analysis.

Results

Patient clinical outcome included acute self-limited hepatitis ($n = 121$), fulminant hepatic failure ($n = 10$), and progression to chronic hepatitis ($n = 8$). Clinical characteristics by outcome are shown in Table 1. The distribution of HBV subgenotypes by phylogenetic

Table 1. Clinical characteristics

	Acute self-limited hepatitis ($n = 121$)	Fulminant hepatic failure ($n = 10$)	Progression to chronic hepatitis ($n = 8$)	P value
Age (years)	36.6 ± 13.9	43.8 ± 14.9	32.8 ± 7.1	NS
Sex (male/female)	89/32	6/4	7/1	NS
AST (IU/l)	1410.3 ± 1134.1	5532.0 ± 6692.7	302.4 ± 221.1	<0.05
ALT (IU/l)	2198.3 ± 1219.5	5310.8 ± 4805.5	552.8 ± 412.3	<0.05
PT (%)	78.3 ± 19.8	29.1 ± 26.1	86.3 ± 19.6	<0.05
Treatment (yes/no)	10/111	10/0	1/7	<0.05

Data are expressed as mean ± SD
Treatment means lamivudine or steroid
AST, aspartate aminotransferase; ALT, alanine aminotransferase; PT, prothrombin time; NS, not significant

Table 2. Relationship between hepatitis B virus (HBV) subgenotypes and clinical course

HBV subgenotypes / Clinical course	A1	A2	B1	B2	C1	C2	Cv	D2	H
Acute self-limited hepatitis	3	23	3	7	5	75	0	3	2
Fulminant hepatic failure	0	0	0	2	0	7	1	0	0
Progression to chronic hepatitis	0	5	0	0	0	2	0	0	1

Data are number of patients
$P < 0.05$

Table 3. Association between HBV subgenotypes and major variants in the basal core promoter (BCP) and precore (PC) regions

HBV subgenotypes	A1	A2	B1	B2	C1	C2	Cv	D2	H	*P* value
Sequence at nt 1753 and 1754										
TT	3	26	1	9	3	69	1	2	0	
others	0	1	2	0	1	9	0	0	2	NS
Sequence at nt 1762 to 1764										
AGG	2	27	3	6	3	58	1	2	2	
TGA	1	0	0	3	1	16	0	0	0	
others	0	0	0	0	0	4	0	0	0	NS
Sequence at nt 1856 to 1858										
CCT	0	0	3	9	3	78	1	2	0	
CCC	3	27	0	0	0	0	0	0	2	
TCC	0	0	0	0	1	0	0	0	0	NS
Sequence at nt 1895 to 1897										
TGG	3	27	2	8	4	67	1	2	2	
TAG	0	0	1	1	0	11	0	0	0	NS

Data are number of patients
nt, nucleotide; NS, not significant

analysis is shown in Fig. 1. The distribution of HBV subgenotypes was A1 ($n = 3$), A2 ($n = 28$), B1 ($n = 3$), B2 ($n = 9$), C1 ($n = 5$), C2 ($n = 84$), C variant ($n = 1$), D2 ($n = 3$), and H ($n = 3$). The relationship between HBV subgenotype and clinical course is shown in Table 2. Among the 10 patients who developed fulminant hepatic failure, 7 were infected with subgenotype C2, 2 were infected with subgenotype B2, and 1 was infected with subgenotype C variant. No other HBV subgenotypes were found in the patients with fulminant hepatic failure. The 2 fulminant hepatic failure patients with subgenotype B2 had an immunocompromised status secondary to liver or kidney transplantation. Among patients who progressed to chronic hepatitis, subgenotype A2 (17.9%) was found more frequently than subgenotype C2 (2.6%) (OR, 0.123; 95% CI, 0.022–0.675; $P = 0.006$). BCP and PC sequences could be obtained in 129 of the 139 patients; variants in the BCP and PC regions were frequently found at nucleotides 1753, 1754, 1762, 1764, 1858, 1896, and 1899. Other regions were well conserved. The associations between the HBV subgenotypes and these major variants in the BCP and PC regions are summarized in Table 3. C1858 and T1896 were closely associated. C1858 prevents mutation of nucleotides T1896 to A1896. As a result, subgenotypes A1, A2, and H, which carry C1858, did not have the A1896 mutation. Subgenotype C1 has both the C1858 and T1858 variants. BCP and PC variants are presented by clinical course in Table 4. BCP and PC variants were not detected in patients who developed chronic hepatitis, indicating that BCP and PC variants were not related to progression to chronic hepatitis. Patients infected with subgenotype C2 who progressed to fulminant hepatic failure were frequently found to carry mutations at nucleotides 1753 and 1754 (OR, 0.025; 95% CI, 0.004–0.169; $P < 0.0001$). For subgenotype C2, T1762 and A1764 were detected more frequently in patients with fulminant hepatic failure (100%) than in patients with acute self-limited hepatitis (13.9%) ($P < 0.0001$). In subgenotype C2, development of fulminant hepatic

Fig. 1. Results of phylogenetic analysis of 139 sequences from the preS region of hepatitis B virus (HBV) of acute hepatitis patients and 58 reference strains from a database shown by accession number. Phylogenetic analysis was performed using the neighbor-joining method with Woolly monkey HBV (AF046996) as the outgroup. Percentages of bootstrap values greater than 90% are shown on the nodes. *Bar* indicates genetic distance

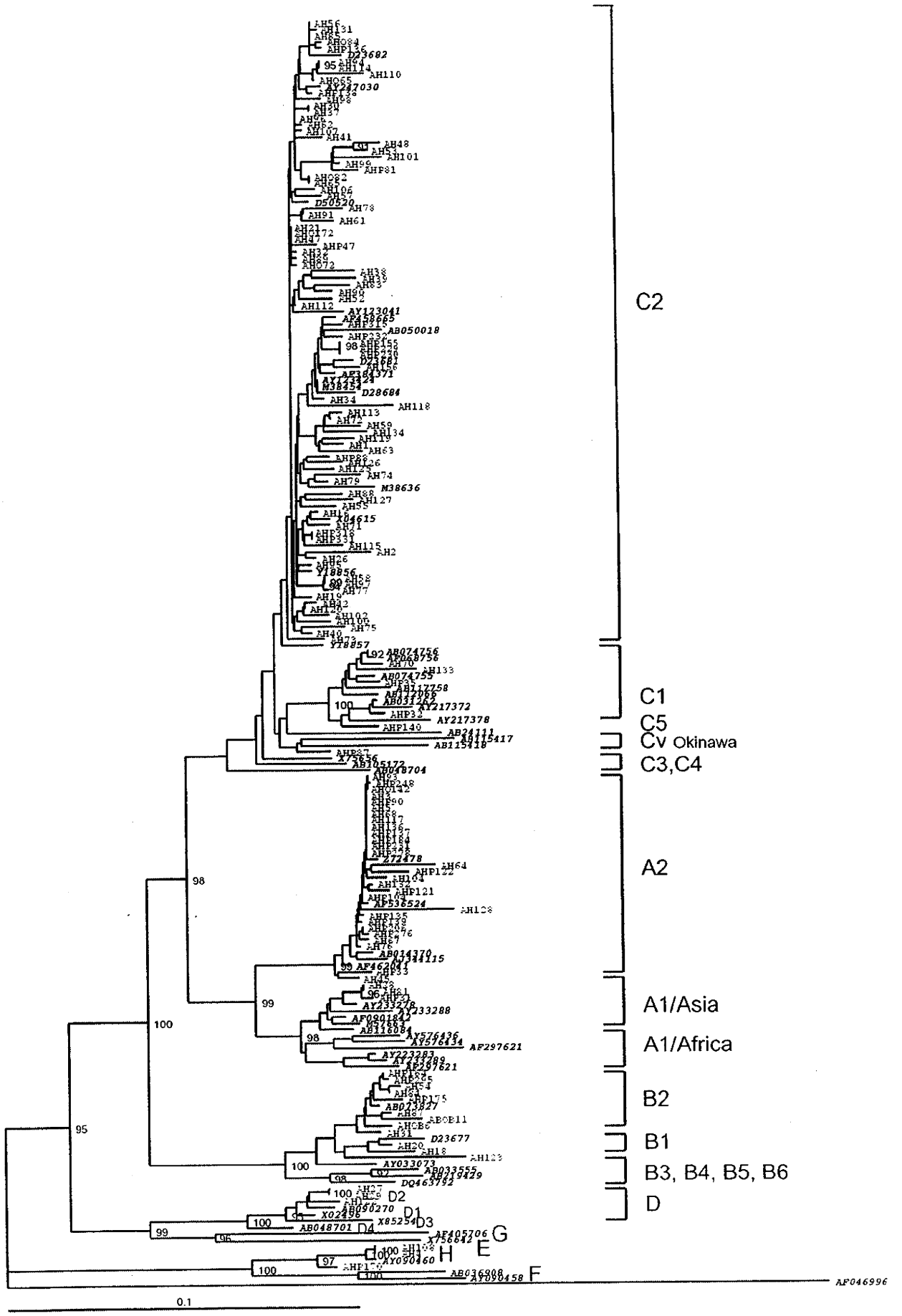
C2
C1
C5
Cv Okinawa
C3,C4
A2
A1/Asia
A1/Africa
B2
B1
B3, B4, B5, B6
D
D2
D1
D3
D4
G
E
H
F
AF046996
0.1

Table 4. HBV subgenotypes and major variants in the BCP and PC regions and clinical course

	Progression to chronic hepatitis				Fulminant hepatic failure				Acute self-limited hepatitis								
	All	A2	C2	H	All	B2	C2	Cv	All	A1	A2	B1	B2	C1	C2	D2	H
nt 1753 and 1754																	
TT	7	5	2	0	5	2	2	1	102	3	21	1	7	3	65	2	0
Others	1	0	0	1	5	0	5	0	9	0	1	2	0	1	4	0	1
P value				<0.05				NS									<0.05
nt 1762 to 1764																	
AGG	8	5	2	1	2	1	0	1	94	2	22	3	5	3	56	2	1
TGA	0	0	0	0	8	1	7	0	13	1	0	0	2	1	9	0	0
Others	0	0	0	0	0	0	0	0	4	0	0	0	0	0	4	0	0
P value				ND				<0.05									NS
nt 1856 to 1858																	
CCT	2	0	2	0	10	2	7	1	84	0	0	3	7	3	69	2	0
CCC	6	5	0	1	0	0	0	0	26	3	22	0	0	0	0	0	1
TCC	0	0	0	0	0	0	0	0	1	0	0	0	0	1	0	0	0
P value				<0.05				ND									<0.05
nt 1895 to 1987																	
TGG	8	5	2	1	4	1	2	1	103	3	22	2	6	4	63	2	1
TAG	0	0	0	0	6	1	5	0	8	0	0	1	1	0	6	0	0
P value				ND				NS									NS

Data are number of patients
NS, not significant; ND, not determined; nt, nucleotide

failure occurred more frequently in patients with A1896 (40.0%) than in those with G1896 (3.2%) (OR, 0.038; 95% CI, 0.006–0.240; $P < 0.0001$). Five of 7 patients with fulminant hepatic failure and 1 of 69 patients with acute self-limited hepatitis carried the T1762, A1764, and A1896 mutations simultaneously (OR, 0.006; 95% CI, 0.001–0.077; $P < 0.0001$). One patient with fulminant hepatic failure but without T1762, A1764, or A1896 was classified as subgenotype C Okinawa variant from the pre-S region sequence.

Discussion

BCP and PC variants have been reported to have a variety of effects on the clinical course of patients with HBV-related liver diseases. In most of these reports, genotypes and/or subgenotypes were not distinguished, and therefore there may have been bias because of genotypic differences. We hypothesized that differences in clinical features result from differences in HBV subgenotypes and HBV genotypes. In the present study, the relationships between HBV subgenotypes, BCP and PC region variants, and the clinical course of patients with acute hepatitis B were investigated. HBV subgenotypes A1 ($n = 3$), A2 ($n = 28$), B1 ($n = 3$), B2 ($n = 9$), C1 ($n = 5$), C2 ($n = 84$), C variant ($n = 1$), D2 ($n = 3$), and H ($n = 3$) were detected in the present study, as well as in previous reports.[8,9] However, the sample numbers of the present study were too small to allow comparison of development of fulminant hepatic failure and progression to chronic hepatitis among HBV subgenotypes. Therefore, we focused on genotype C, which was the predominant genotype in the present study, and further classified genotype C into subgenotypes to address subgenotype bias. Genotype C is the most common HBV genotype in Asia, where it is endemic, and is classified into five subgenotypes: C1 (also known as Cs), C2 (also known as Ce), C3, C4, and C5. Each subgenotype has a different geographic distribution. Subgenotype C1 is found in Southeast Asia, including Vietnam, Thailand, and southern areas of China. Subgenotype C2 is found in Far East Asia, including Japan, Korea, and Northern China. Subgenotype C3 is found in Oceania, and subgenotype C4 is found in Australia. Subgenotype C5 was recently identified in the Philippines.[4,21] In the present study, the predominant subgenotype in Japanese patients with acute hepatitis B was C2 ($n = 84$, 60.4%), which is consistent with epidemiological studies of chronic HBV infection.[22] Five patients with subgenotype C1 were also identified. Because of increased human migration, the distributions of the HBV subgenotypes are gradually changing in Japanese patients with acute hepatitis B.[8,9,23–26] The association of fulminant hepatic failure with BCP and PC variants in HBV subgenotype C2 was examined first. The T1762, A1764, and A1896 variants were significantly associated with fulminant hepatic failure in patients with subgenotype C2. Therefore, these mutations may be useful for predicting the clinical course of patients with acute hepatitis B from subgenotype C2. The results of the present study are consistent with those of previous reports from

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.

References

1. Kao JH, Chen DS. Global control of hepatitis B virus infection. Lancet Infect Dis 2002;2:395–403.
2. Ganem D, Prince AM. Hepatitis B virus infection: natural history and clinical consequences. N Engl J Med 2004;350: 1118–29.
3. Okamoto H, Tsuda F, Sakugawa H, Sastrosoewignjo RI, Imai M, Miyakawa Y, et al. Typing hepatitis B virus by homology in nucleotide sequence: comparison of surface antigen subtypes. J Gen Virol 1988;69:2575–83.
4. Norder H, Courouce AM, Coursaget P, Echevarria JM, Lee SD, Mushahwar IK, et al. Genetic diversity of hepatitis B virus strains

derived worldwide: genotypes, subgenotypes, and HBsAg subtypes. Intervirology 2004;47:289–309.
5. Wai CT, Chu CJ, Hussain M, Lok AS. HBV genotype B is associated with better response to interferon therapy in HBeAg(+) chronic hepatitis than genotype C. Hepatology 2002;36:1425–30.
6. Erhardt A, Blondin D, Hauck K, Sagir A, Kohnle T, Heintges T, et al. Response to interferon alfa is hepatitis B virus genotype dependent: genotype A is more sensitive to interferon than genotype D. Gut 2005;54:1009–13.
7. Janssen HL, van Zonneveld M, Senturk H, Zeuzem S, Akarca US, Cakaloglu Y, et al. Pegylated interferon alfa-2b alone or in combination with lamivudine for HBeAg-positive chronic hepatitis B: a randomised trial. Lancet 2005;365:123–9.
8. Ozasa A, Tanaka Y, Orito E, Sugiyama M, Kang JH, Hige S, et al. Influence of genotypes and precore mutations on fulminant or chronic outcome of acute hepatitis B virus infection. Hepatology 2006;44:326–34.
9. Hayashi K, Katano Y, Takeda Y, Honda T, Ishigami M, Itoh A, et al. Comparison of hepatitis B virus subgenotypes in patients with acute and chronic hepatitis B and absence of lamivudine-resistant strains in acute hepatitis B in Japan. J Med Virol 2007;79: 366–73.
10. Omata M, Ehata T, Yokosuka O, Hosoda K, Ohto M. Mutations in the precore region of hepatitis B virus DNA in patients with fulminant and severe hepatitis. N Engl J Med 1991;324: 1699–704.
11. Liang TJ, Hasegawa K, Rimon N, Wands JR, Ben-Porath E. A hepatitis B virus mutant associated with an epidemic of fulminant hepatitis. N Engl J Med 1991;324:1705–9.
12. Sato S, Suzuki K, Akahane Y, Akamatsu K, Akiyama K, Yunomura K, et al. Hepatitis B virus strains with mutations in the core promoter in patients with fulminant hepatitis. Ann Intern Med 1995;122:241–8.
13. Feray C, Gigou M, Samuel D, Bernuau J, Bismuth H, Brechot C. Low prevalence of precore mutations in hepatitis B virus DNA in fulminant hepatitis type B in France. J Hepatol 1993;18: 119–22.
14. Laskus T, Rakela J, Nowicki MJ, Persing DH. Hepatitis B virus core promoter sequence analysis in fulminant and chronic hepatitis B. Gastroenterology 1995;109:1618–23.
15. Kao JH, Chen PJ, Lai MY, Chen DS. Basal core promoter mutations of hepatitis B virus increase the risk of hepatocellular carcinoma in hepatitis B carriers. Gastroenterology 2003;124:327–34.
16. Imamura T, Yokosuka O, Kurihara T, Kanda T, Fukai K, Imazeki F, et al. Distribution of hepatitis B viral genotypes and mutations in the core promoter and precore regions in acute forms of liver disease in patients from Chiba, Japan. Gut 2003;52:1630–7.
17. Liu CJ, Chen BF, Chen PJ, Lai MY, Huang WL, Kao JH, et al. Role of hepatitis B viral load and basal core promoter mutation in hepatocellular carcinoma in hepatitis B carriers. J Infect Dis 2006;193:1258–65.
18. Perrillo RP, Aach RD. The clinical course and chronic sequelae of hepatitis B virus infection. Semin Liver Dis 1981;1:15–25.
19. Lindh M, Hannoun C, Dhillon AP, Norkrans G, Horal P. Core promoter mutations and genotypes in relation to viral replication and liver damage in East Asian hepatitis B virus carriers. J Infect Dis 1999;179:775–82.
20. Saitou N, Nei M. The neighbor-joining method: a new method for reconstructing phylogenetic trees. Mol Biol Evol 1987;4:406–25.
21. Sakamoto T, Tanaka Y, Orito E, Co J, Clavio J, Sugauchi F, et al. Novel subtypes (subgenotypes) of hepatitis B virus genotypes B and C among chronic liver disease patients in the Philippines. J Gen Virol 2006;87:1873–82.
22. Orito E, Ichida T, Sakugawa H, Sata M, Horiike N, Hino K, et al. Geographic distribution of hepatitis B virus (HBV) genotype in patients with chronic HBV infection in Japan. Hepatology 2001;34:590–4.
23. Joh R, Hasegawa K, Ogawa M, Ishikawa K, Iizuka A, Naritomi T, et al. Genotypic analysis of hepatitis B virus from patients with fulminant hepatitis: comparison with acute self-limited hepatitis. Hepatol Res 2003;26:119–24.
24. Kobayashi M, Suzuki F, Arase Y, Akuta N, Suzuki Y, Hosaka T, et al. Infection with hepatitis B virus genotype A in Tokyo, Japan during 1976 through 2001. J Gastroenterol 2004;39:844–50.
25. Yotsuyanagi H, Okuse C, Yasuda K, Orito E, Nishiguchi S, Toyoda J, et al. Distinct geographic distributions of hepatitis B virus genotypes in patients with acute infection in Japan. J Med Virol 2005;77:39–46.
26. Takeda Y, Katano Y, Hayashi K, Honda T, Yokozaki S, Nakano I, et al. Difference of HBV genotype distribution between acute hepatitis and chronic hepatitis in Japan. Infection 2006;34: 201–7.
27. Michitaka K, Horiike N, Chen Y, Yatsuhashi H, Yano M, Kojima N, et al. Infectious source factors affecting the severity of sexually transmitted acute hepatitis due to hepatitis B virus genotype C. Intervirology 2005;48:112–9.
28. Chisari FV, Ferrari C. Hepatitis B virus immunopathogenesis. Annu Rev Immunol 1995;13:29–60.
29. Huy TT, Ushijima H, Quang VX, Win KM, Luengrojanakul P, Kikuchi K, et al. Genotype C of hepatitis B virus can be classified into at least two subgroups. J Gen Virol 2004;85:283–92.
30. Chan HL, Tsui SK, Tse CH, Ng EY, Au TC, Yuen L, et al. Epidemiological and virological characteristics of 2 subgroups of hepatitis B virus genotype C. J Infect Dis 2005;191:2022–32.
31. Tanaka Y, Orito E, Yuen MF, Mukaide M, Sugauchi F, Ito K, et al. Two subtypes (subgenotypes) of hepatitis B virus genotype C: a novel subtyping assay based on restriction fragment length polymorphism. Hepatol Res 2005;33:216–24.
32. Lindh M, Andersson AS, Gusdal A. Genotypes, nt 1858 variants, and geographic origin of hepatitis B virus: large-scale analysis using a new genotyping method. J Infect Dis 1997;175:1285–93.
33. Huy TT, Ushijima H, Sata T, Abe K. Genomic characterization of HBV genotype F in Bolivia: genotype F subgenotypes correlate with geographic distribution and T(1858) variant. Arch Virol 2006;151:589–97.
34. Chan HL, Tse CH, Ng EY, Leung KS, Lee KH, Tsui SK, et al. Phylogenetic, virological, and clinical characteristics of genotype C hepatitis B virus with TCC at codon 15 of the precore region. J Clin Microbiol 2006;44:681–7.
35. Huy TT, Ushijima H, Quang VX, Ngoc TT, Hayashi S, Sata T, et al. Characteristics of core promoter and precore stop codon mutants of hepatitis B virus in Vietnam. J Med Virol 2004;74: 228–36.
36. Sugauchi F, Orito E, Ichida T, Kato H, Sakugawa H, Kakumu S, et al. Epidemiologic and virologic characteristics of hepatitis B virus genotype B having the recombination with genotype C. Gastroenterology 2003;124:925–32.

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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, 4×10^5 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 (1×10^3 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).

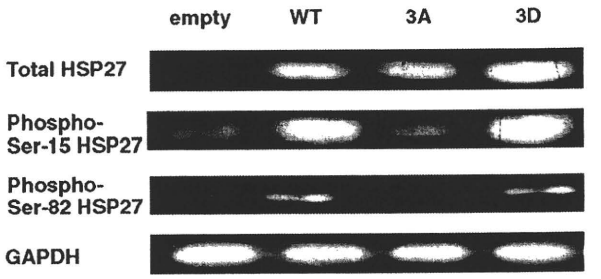

FIGURE 1. **Phosphorylated HSP27 overexpressed both the wild-type and the aspartate mutant HSP27-transfected HCC cells.** The protein expression levels of endogenous as well as the overexpressed wild-type and mutant HSP27s in the HuH7 cells were determined by Western blotting using anti-HSP27 antibodies, 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

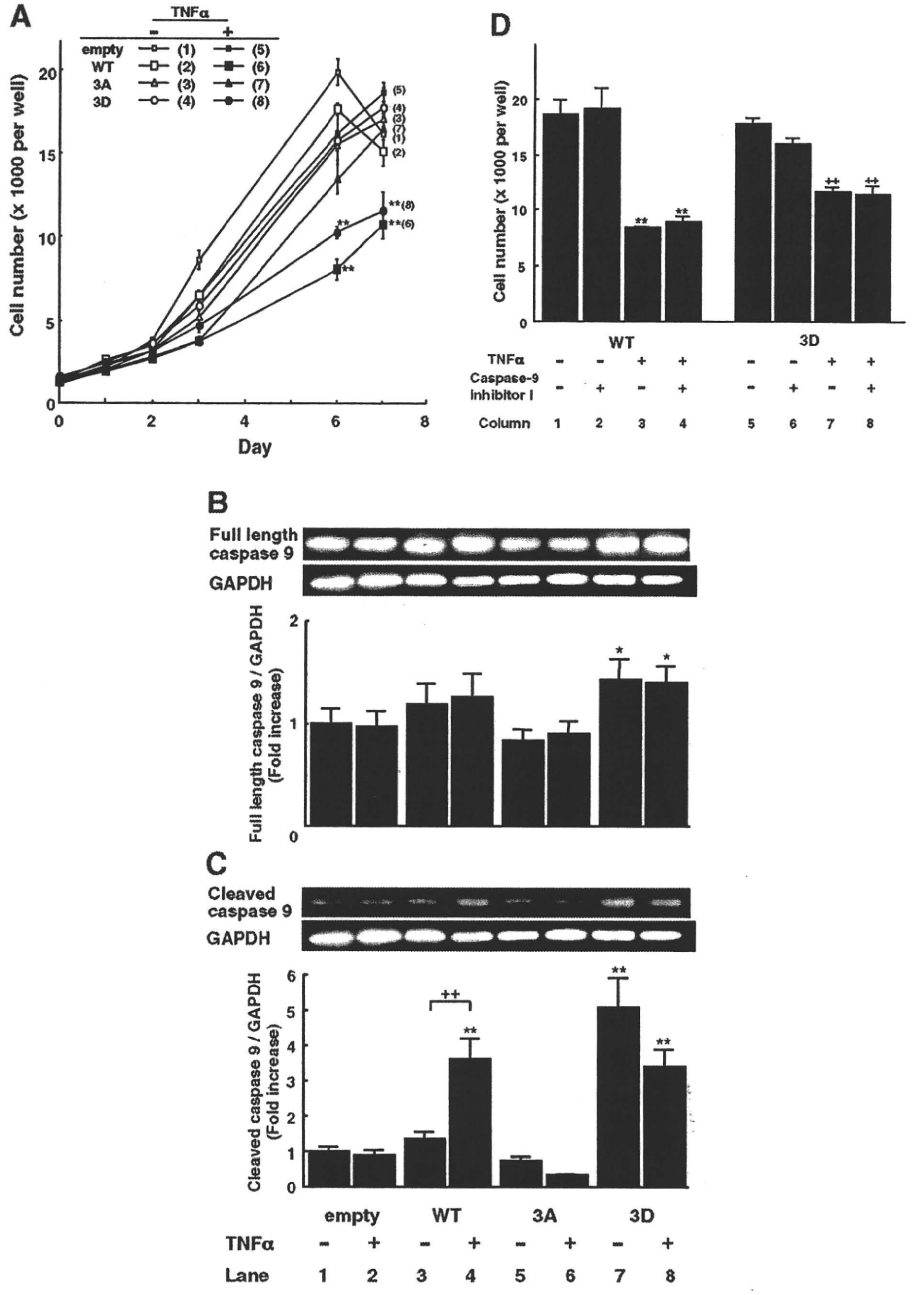

FIGURE 2. **Cell growth of the phosphorylated HSP27-overexpressed HCC cells was suppressed in the presence of TNFα and was not associated with caspase 9 activation.** *A*, cell growth curve. HuH7 cells were stably transfected either with empty (*curves 1* and *5*), WT (*curves 2* and *6*), 3A (*curves 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

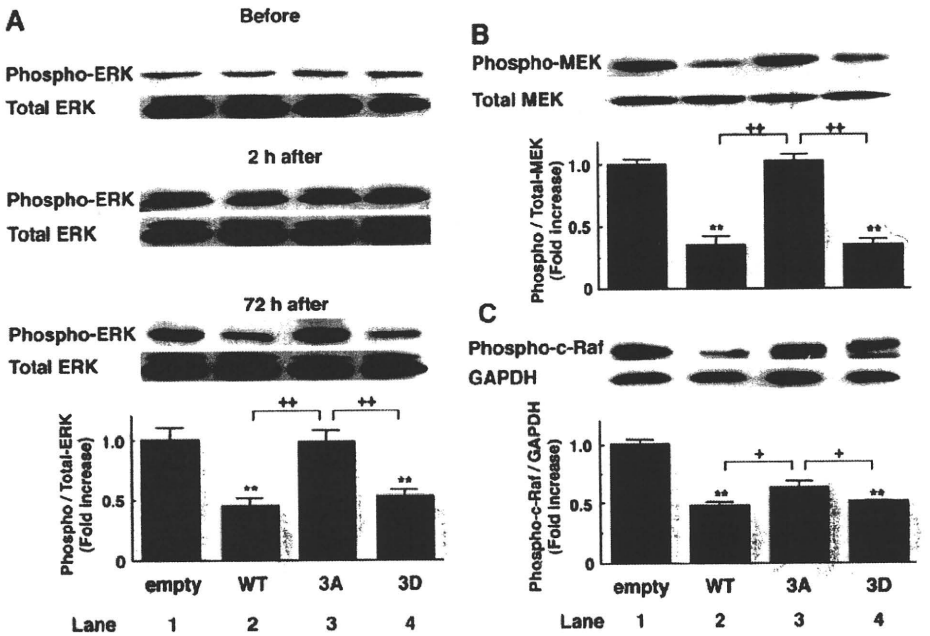

FIGURE 3. **Repression of the sustained activation of c-Raf-MEK-ERK signal transduction in the presence of TNFα by phosphorylated HSP27 in HCC cells.** The effects of phosphorylated HSP27 on the levels of phospho-ERK (*A*), phospho-MEK (*B*), and phospho-c-Raf (*C*) in HuH7 cells in the presence of 1 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

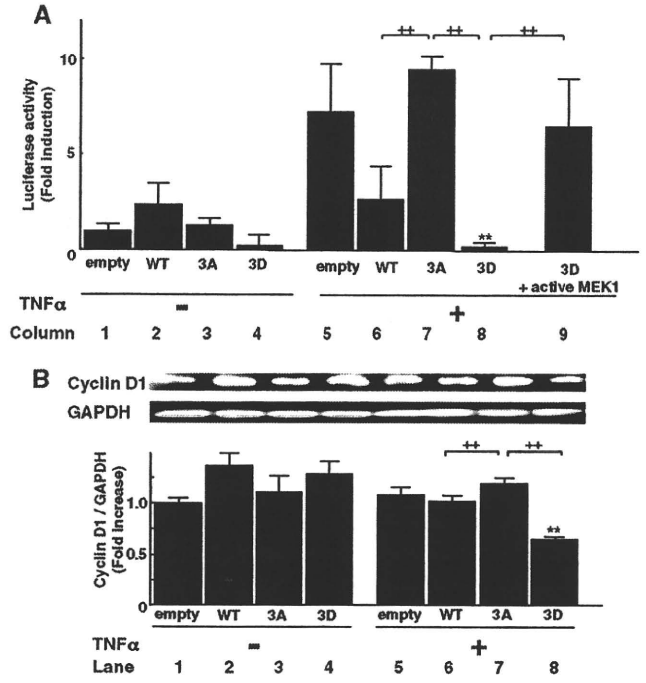

FIGURE 4. **Inhibition of the AP-1 transactivation activity and cyclin D1 protein expression by phosphorylated HSP27 in HCC cells.** *A*, AP-1 transactivation activities in HuH7 cells were determined 72 h after stimulation with (*columns 5–9*) or without (*columns 1–4*) TNFα. HuH7 cells were stably transfected 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*; ++, $p < 0.01$.

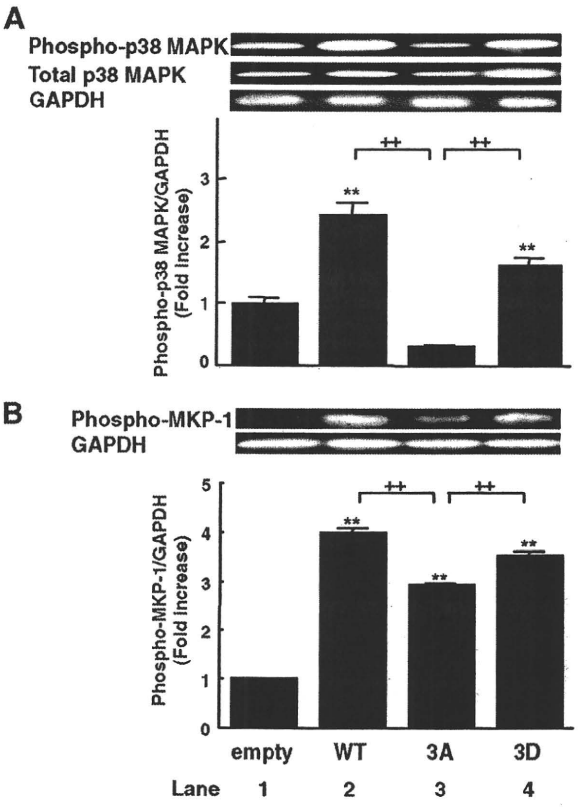

FIGURE 5. **The enhanced activation and the increased amount of p38 MAPK, and the induction of the phospho-MKP-1 protein expression in the presence of TNFα by phosphorylated HSP27 in HCC cells.** HuH7 cells were stably transfected either with empty (*lanes* and *column 1*), WT (*lanes* and *column 2*), 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-

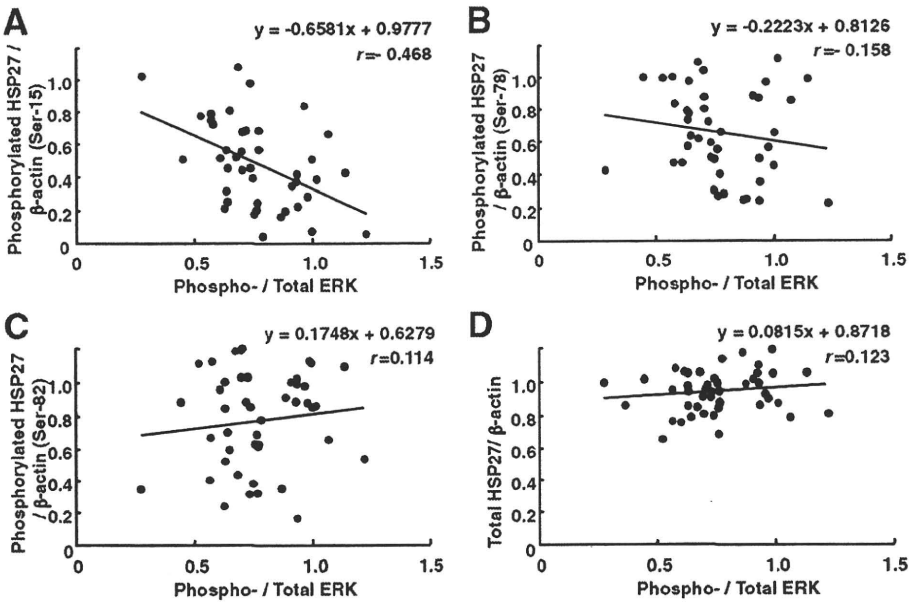

FIGURE 6. **Significant inverse correlation between the levels of phosphorylated HSP27 (Ser-15) and activation of ERK in human HCC tissues.** Correlation between the expression levels of phospho-ERK and the levels of phosphorylated HSP27 (Ser-15) (*A*), phosphorylated HSP27 (Ser-78) (*B*), phosphorylated 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.

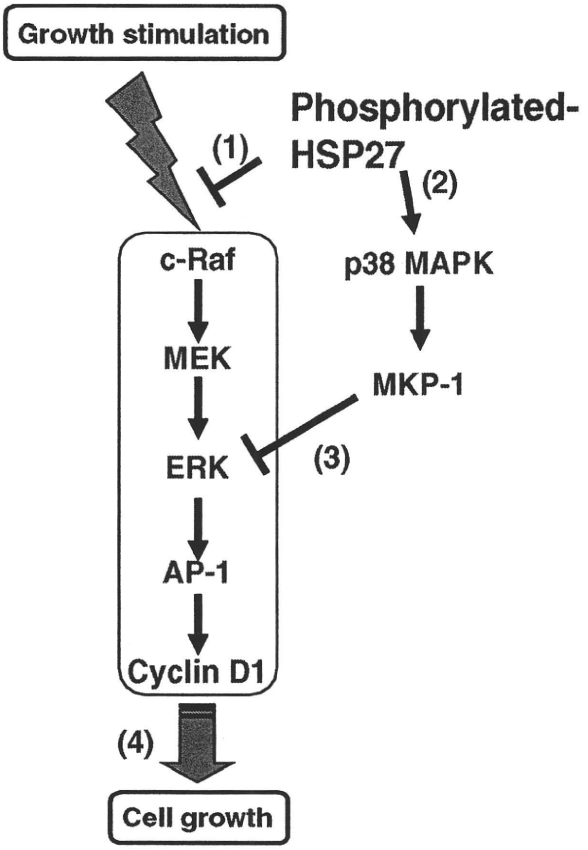

FIGURE 7. **Schematic representation of the effect of phosphorylated HSP27 on HCC cell growth under inflammatory conditions.** Phosphorylated HSP27 in an irritated liver with HCC inhibits sustained activation of c-Raf-MEK-ERK system (*1*) while also up-regulating the MKP-1 induction and activation via 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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REFERENCES

1. Garrido, C., Brunet, M., Didelot, C., Zermati, Y., Schmitt, E., and Kroemer, G. (2006) *Cell Cycle* **5,** 2592–2601
2. Lambert, H., Charette, S. J., Bernier, A. F., Guimond, A., and Landry, J. (1999) *J. Biol. Chem.* **274,** 9378–9385
3. Benjamin, I. J., and McMillan, D. R. (1998) *Circ. Res.* **83,** 117–132
4. Welch, W. J. (1985) *J. Biol. Chem.* **260,** 3058–3062
5. Beere, H. M. (2005) *J. Clin. Invest.* **115,** 2633–2639
6. Wullaert, A., van Loo, G., Heyninck, K., and Beyaert, R. (2007) *Endocr. Rev.* **28,** 365–386
7. Parcellier, A., Schmitt, E., Gurbuxani, S., Seigneurin-Berny, D., Pance, A., Chantôme, A., Plenchette, S., Khochbin, S., Solary, E., and Garrido, C. (2003) *Mol. Cell. Biol.* **23,** 5790–5802
8. Haddad, J. J., and Land, S. C. (2002) *Br. J. Pharmacol.* **135,** 520–536
9. Park, K. J., Gaynor, R. B., and Kwak, Y. T. (2003) *J. Biol. Chem.* **278,** 35272–35278
10. Kojima, S., Okuno, M., Matsushima-Nishiwaki, R., Friedman, S. L., and Moriwaki, H. (2004) *Int. J. Oncol.* **24,** 797–805
11. Cano, E., and Mahadevan, L. C. (1995) *Trends Biochem. Sci.* **20,** 117–122
12. Ito, Y., Sasaki, Y., Horimoto, M., Wada, S., Tanaka, Y., Kasahara, A., Ueki, T., Hirano, T., Yamamoto, H., Fujimoto, J., Okamoto, E., Hayashi, N., and Hori, M. (1998) *Hepatology* **27,** 951–958
13. Lavoie, J. N., L'Allemain, G., Brunet, A., Müller, R., and Pouysségur, J. (1996) *J. Biol. Chem.* **271,** 20608–20616
14. Hui, L., Bakiri, L., Mairhorfer, A., Schweifer, N., Haslinger, C., Kenner, L., Komnenovic, V., Scheuch, H., Beug, H., and Wagner, E. F. (2007) *Nat. Genet.* **39,** 741–749
15. Yasuda, E., Kumada, T., Takai, S., Ishisaki, A., Noda, T., Matsushima-Nishiwaki, R., Yoshimi, N., Kato, K., Toyoda, H., Kaneoka, Y., Yamaguchi, A., and Kozawa, O. (2005) *Biochem. Biophys. Res. Commun.* **337,** 337–342
16. Takai, S., Matsushima-Nishiwaki, R., Tokuda, H., Yasuda, E., Toyoda, H., Kaneoka, Y., Yamaguchi, A., Kumada, T., and Kozawa O. (2007) *Life Sci.* **81,** 585–591
17. Kubisch, C., Dimagno, M. J., Tietz, A. B., Welsh, M. J., Ernst, S. A., Brandt-Nedelev, B., Diebold, J., Wagner, A. C. C., Göke, B., Williams, J. A., and Schäfer, C. (2004) *Gastroenterology* **127,** 275–286
18. Mansour, S. J., Matten, W. T., Hermann, A. S., Candia, J. M., Rong, S., Fukasawa, K., Vande Woude, G. F., and Ahn, M. G. (1994) *Science* **265,** 966–970
19. Noda, T., Kumada, T., Takai, S., Matsushima-Nishiwaki, R., Yoshimi, N., Yasuda, E., Kato, K., Toyoda, H., Kaneoka, Y., Yamaguchi, A., and Kozawa, O. (2007) *Oncol. Rep.* **17,** 1309–1314
20. Matsushima-Nishiwaki, R., Okuno, M., Adachi, S., Sano, T., Akita, K., Moriwaki, H., Friedman, S. L., and Kojima, S. (2001) *Cancer Res.* **61,** 7675–7682
21. Maeda, S., Kamata, H., Luo, J. L., Leffert, H., and Karin, M. (2005) *Cell* **121,** 977–990
22. Ataseven, H., Bahcecioglu, I. H., Kuzu, N., Yalniz, M., Celebi, S., Erensoy, A., and Ustundag, B. (2006) *Mediators Inflamm.* **2006,** 1–6
23. Adachi, S., Okuno, M., Matsushima-Nishiwaki, R., Takano, Y., Kojima, S., Friedman, S. L., Moriwaki, H., and Okano, Y. (2002) *Hepatology* **35,** 332–340
24. Su, B., and Karin, M. (1996) *Curr. Opin. Immunol.* **8,** 402–411
25. Davis, R. J. (1994) *Trends Biochem. Sci.* **19,** 470–473
26. Wang, X., and Liu, Y. (2007) *Cell Signal.* **19,** 1372–1382
27. Lee, K. H., Lee, C. T., Kim, Y. W., Han, S. K., Shim, Y. S., and Yoo, C. G. (2005) *J. Biol. Chem.* **280,** 13179–13186
28. Gaur, U., and Aggarwal, B. B. (2003) *Biochem. Pharmacol.* **66,** 1403–1408
29. Cho, H. N., Lee, Y. J., Cho, C. K., Lee, S. J., and Lee, Y.-S. (2002) *Cell Death Differ.* **9,** 448–456
30. Lee, Y. J., Cho, H. N., Jeoung, D. I., Soh, J. W., Cho, C. K., Bae, S., Chung, H. Y., Lee, S. J., and Lee, Y. S. (2004) *Free Radic. Biol. Med.* **36,** 429–444
31. Taylor, R. P., and Benjamin, I. J. (2005) *J. Mol. Cell. Cardiol.* **38,** 433–444
32. Sun, X., Welsh, M. J., and Benndorf, R. (2006) *Cell Stress Chaperones* **11,** 61–70
33. Schmitz, K. J., Wohlschlaeger, J., Lang, H., Sotiropoulos, G. C., Malago, M., Steveling, K., Reis, H., Cicinnati, V. R., Schmid, K. W., and Baba, H. A. (2008) *J. Hepatol.* **48,** 83–90
34. Hsieh, Y. H., Wu, T. T., Huang, C. Y., Hsieh, Y. S., Hwang, J. M., and Liu, J. Y. (2007) *Cancer Res.* **67,** 4320–4327
35. Xu, L., Chen, S., and Bergan, R. C. (2006) *Oncogene* **25,** 2987–2998
36. Iyoda, K., Sasaki, Y., Horimoto, M., Toyama, T., Yakushijin, T., Sakakibara, M., Takehara, T., Fujimoto, J., Hori, M., Wands, J. R., and Hayashi, N. (2003) *Cancer* **97,** 3017–3026
37. Cuenda, A., and Rousseau, S. (2007) *Biochim. Biophys. Acta* **1773,** 1358–1375
38. Torres, M., and Forman, H. J. (2003) *Biofactors* **17,** 287–296
39. Garg, A. K., and Aggarwal, B. B. (2002) *Mol. Immunol.* **39,** 509–517
40. Mehlen, P., Kretz-Remy, C., Préville, X., and Arrigo, A. P. (1996) *EMBO J.* **15,** 2695–2706
41. Kim, S. K., Woodcroft, K. J., Oh, S. J., Abdelmegeed, M. A., and Novak, R. F. (2005) *Biochem. Pharmacol.* **70,** 1785–1795
42. Alford, K. A., Glennie, S., Turrell, B. R., Rawlinson, L., Saklatvala, J., and Dean, J. L. E. (2007) *J. Biol. Chem.* **282,** 6232–6241
43. Liu, P., Kimmoun, E., Legrand, A., Sauvanet, A., Degott, C., Lardeux, B., and Bernuau, D. (2002) *J. Hepatol.* **37,** 63–71
44. Gollob, J. A., Whilhelm, S., Carter, C., and Kelley, S. L. (2006) *Semin. Oncol.* **33,** 392–406
45. Wilhelm, S. M., Carter, C., Tang, L., Wilkie, D., McNabola, A., Rong, H., Chen, C., Zhang, X., Vincent, P., McHugh, M., Cao, Y., Shujath, J., Gawlak, S., Eveleigh, D., Rowley, B., Liu, L., Adnane, L., Lynch, M., Auclair, D., Taylor, I., Gedrich, R., Voznesensky, A., Riedl, B., Post, L. E., Bollag, G., and Trail, P. A. (2004) *Cancer Res.* **64,** 7099–7109

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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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1. Introduction

Hepatocellular carcinoma (HCC) is a common malignancy, especially in southern and eastern Asia. The incidence of HCC is also increasing in the United States [1,2]. The development of various scanning techniques and the identification of sensitive and specific tumor markers for HCC have contributed not only to the detection of HCC, but also to the evaluation of its progression and the determination of patient prognosis.

Three tumor markers specific for HCC are currently used in Japanese clinics: alpha-fetoprotein (AFP), *Lens culinaris* agglutinin A-reactive fraction of AFP (AFP-L3), and des-gamma carboxy prothrombin (DCP), which is also referred to as protein induced by vitamin K absence-II (PIVKA-II). Previous reports have detailed the usefulness of each of these tumor markers in the detection and diagnosis of HCC, the evaluation of tumor progression, and the determination of patient prognosis [3–7]. The elevation of each tumor marker has been reported to indicate poor prognosis and decreased survival rates [8]. However, the prognostic value of these tumor markers in distinct patient subpopulations who underwent potential curative treatments for HCC has not been well studied.

In the present study, we attempted to evaluate the effect of the pretreatment elevation of these tumor markers for HCC (AFP, AFP-L3, and DCP) on the outcome of patients who underwent curative treatments: hepatectomy and locoregional thermal ablation that includes percutaneous microwave thermocoagulation (PMCT) and radiofrequency ablation (RFA).

2. Patients and methods

2.1. Patients

A total of 3725 patients were initially diagnosed with HCC at one of the five institutions that participated in the study between July 1994 and December 2004. Three tumor markers for HCC (AFP, AFP-L3, and DCP) were measured at the time of diagnosis, and drugs that would influence the serum DCP levels, such as warfarin or vitamin K, were not being taken by 2600 of the 3725 patients [8]. Of these 2600 patients, 736 received hepatectomy and 945 received locoregional ablative therapies as an initial treatment for HCC. Of the patients who underwent hepatectomy, 345 were enrolled in this study, while 391 were excluded as their maximum tumor size was greater than 3 cm or their number of tumors was greater than 3 (Fig. 1A). With regard to the patients who underwent locoregional ablative therapies, we first excluded 344 patients who received percutaneous ethanol injections because percutaneous ethanol injection is reported to be less effective as a therapy for HCC compared to other locoregional ablative therapies [9]. As a result, the patients in the study were primarily those who underwent PMCT ($n = 123$) or RFA ($n = 478$). We defined these patients as patients who underwent locoregional thermal ablation (LTA). We further excluded 139 patients in whom the maximum tumor size was greater than 3 cm or the number of tumors was greater than 3. Finally, we excluded 6 patients in whom the pretreatment remnant liver function was estimated as class C according to the Child-Pugh classification [10]. A total of 456 patients were therefore enrolled in this study (Fig. 1B). These patients were treated by hepatectomy and LAT solely, and no patients received an addition of other kinds of therapy for HCC as multimodality treatment until the recurrent HCC developed. The study protocol was approved by the Institutional Ethics Review Board at each of the participating institutions and was in compliance with the Declaration of Helsinki. Written informed consent for the use of information on the pretreatment tumor marker values for future study on patient outcome was obtained from each patient prior to treatment.

2.2. Diagnosis of HCC, treatment, and follow-up

Patients were diagnosed with HCC based on histologic examination of tumor tissue taken from resected specimens in 345 patients who underwent hepatectomy. In patients treated by LTA, the diagnosis of HCC had been made based on fine-needle biopsy of specimens from 198 of the 456 patients (43.4%). In the remaining 258 patients, the diagnosis was made based on clinical criteria [11,12]: a pertinent clinical background (association with liver cirrhosis or viral hepatitis) and typical imaging findings. Typical imaging features of HCC include a mosaic pattern with a halo by B-mode ultrasonography; hypervascularity on angiographic images; and a high-density mass on arterial phase dynamic computed tomography (CT) images and 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 T1- and T2-weighted imaging associated with superparamagnetic iron oxide-enhanced magnetic resonance imaging were performed.

All patients underwent the treatment for HCC within 2 weeks after the diagnosis of HCC. In patients who underwent LTA, dynamic CT was performed 1 to 3 days after the last session of ablation to evaluate the efficacy of treatment. Complete ablation was defined by CT findings as non-enhancement in the entire lesion with a safety margin in the surrounding liver parenchyma. Patients received additional sessions of ablation until complete ablation was confirmed in each nodule.

Patients were prospectively followed up from 0.8 months to 175.1 months (median follow-up period, 26.6 months). All 801 patients were followed up at one of the five participating institutions.

2.3. Measurement of tumor markers and cut-off levels

AFP, AFP-L3, and DCP were measured in serum samples obtained from each patient at the time of HCC diagnosis. The serum AFP levels were determined by enzyme-linked immunosorbent assay with a commercially available kit (ELISA-AFP, International Reagents, Kobe, Japan). Serum AFP-L3 levels were measured by lectin-affinity electrophoresis coupled with antibody-affinity blotting (AFP Differentiation Kit L, Wako Pure Chemical Industries, Ltd., Osaka, Japan) and are expressed as a percentage (AFP-L3 level/total AFP level X 100) [13,14]. The serum DCP levels were determined by sensitive enzyme immunoassay (Eitest PIVKA-II kit, Eisai Laboratory, Tokyo, Japan) according to the manufacturer's instructions [15–17]. We designated