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Attenuated phosphorylation of heat shock protein 27 correlates with tumor progression in patients with hepatocellular carcinoma

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Abstract

Heat shock protein 27 (HSP27) is expressed at high levels in human hepatocellular carcinoma (HCC). We examined correlations of total HSP27 and serine phosphorylated (Ser-15, Ser-78, and Ser-82) HSP27 levels in HCC tissues with clinical and pathologic characteristics in 48 resected HCC specimens. The levels of total and Ser-phosphorylated HSP27 were evaluated by Western blot analysis. Immunohistochemical analysis of HSP27 expression was also performed on some samples. Phosphorylation of HSP27 was detected in all 48 HCC tissues. Levels of phosphorylated HSP27 were correlated inversely with tumor size, microvascular invasion of HCC, and tumor stage by TNM classification. In contrast, only microvascular invasion showed an inverse correlation with total HSP27 levels. The decrease in phosphorylated HSP27 in progressed HCC was also observed by immunohistochemistry. Levels of phosphorylated HSP27 gradually decreased in parallel with HCC progression. Our findings suggest that phosphorylated HSP27 may have a suppressive role in progression of human HCC.

Keywords: Liver; Hepatocellular carcinoma; Heat shock protein 27; Phosphorylation; Western blot; Immunohistochemistry; Tumor stage; Tumor size; Microvascular invasion; Tumor progression

Hepatocellular carcinoma (HCC) is a common malignancy, ranking fifth in frequency worldwide, and it causes more than one million deaths annually [1,2]. The overall survival of patients with HCC is still unsatisfactory even after hepatectomy. Factors believed to be associated with patient prognosis and progression of HCC reportedly include histologic grade, tumor stage according to TNM classification, tumor size, intrahepatic metastasis, vascular invasion, α-fetoprotein (AFP) levels, and *des*-γ-carboxy prothrombin (DCP) levels [3–5]. The most suitable prognostic factor of patients with HCC, however, has not yet been identified. It is, therefore, required to search for other markers indicating high risk HCC.

Heat shock proteins (HSPs) are produced by cells exposed to biological stressors such as heat and chemicals [6]. HSPs are classified as high-molecular-weight (HMW) HSPs, such as HSP70, HSP90, and HSP110, or low-molecular-weight (LMW) HSPs, which have molecular masses from 10 to 30 kDa. HMW HSPs act as molecular chaperones in protein folding, oligomerization, and translocation [6]. Though the functions of LMW HSPs, such as HSP27 and α-B-crystallin, are not

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as well characterized as those of the HMW HSPs, it is thought that they may also have chaperone functions [6]. LMW HSPs show significant amino-acid sequence similarity across the alpha-crystallin domain. In a previous study [7], we showed that HSP27 is constitutively expressed in various tissues and cells, especially in skeletal muscle and smooth muscle cells where it may be essential. In addition, we showed that several physiological factors induce HSP27 activity in vascular smooth muscle cells and osteoblasts [8,9].

HSP27 activity is regulated by post-translational modifications such as phosphorylation [6,10]. It has been reported that mouse HSP27 is phosphorylated at two serine residues (Ser-15 and Ser-82), whereas human HSP27 is phosphorylated at three serine residues (Ser-15, Ser-78, and Ser-82) [6]. Phosphorylation of HSP27 is catalyzed by means of the mitogen-activated protein kinase superfamily [6]. Recently, up-regulation of HSPs, including HSP27, was reported in HCC and correlation between HSP27 expression levels and histological grade and survival of patients with HCC [11]. It was also reported that HSP27 functions in tissue protection by interacting intracellular signaling to suppress apoptosis [12]. In addition, phosphorylated HSP27 is translocated from the cytosol to the nucleus in hippocampal progenitor cells, and prevents apoptosis [13]. However, the exact role and mechanism of regulation of HSP27 in HCC tissue remain to be clarified. To our knowledge, analysis of HSP27 phosphorylation in HCC has not been reported.

In the present study, we examined total HSP27 levels and levels of phosphorylated HSP27 at three serine residues (Ser-15, Ser-78, and Ser-82) in HCC samples from 48 patients with respect to HCC characteristics associated with tumor progression.

Materials and methods

Patients. Forty-eight patients (42 men, 6 women, mean age, 67.4 ± 8.2 years), who had been diagnosed with HCC at the Department of Gastroenterology, Ogaki Municipal Hospital, Ogaki, Japan, underwent hepatic resection between September 2002 and March 2005. Chronic hepatitis was present in 23 patients, and cirrhosis was present in 25 patients. Twelve patients were infected with hepatitis B virus, and 33 patients were infected with hepatitis C virus. The remaining three patients had evidence of alcoholic cirrhosis. No patient underwent preoperative chemotherapy.

The resected HCC specimens were obtained according to a protocol approved by the Committee for the Conduct of Human Research at Ogaki Municipal Hospital. Informed consent was obtained from all patients.

Surgical specimens. Primary HCC tissues were obtained from all patients by surgical resection at the Department of Surgery, Ogaki Municipal Hospital. The excised tissue was divided into two parts, and one part was fixed with 20% neutral formalin overnight. The fixed tissue was then dehydrated with 100% methyl alcohol and xylene, and embedded in paraffin wax. Three-micron-thickness of this tissue was used for immunohistochemical staining. The other part of the resected tissue was snap-frozen in liquid nitrogen and stored at −80 °C until used for Western blot analysis.

Measurement of serum tumor markers. Serum AFP levels were determined by liquid-phase binding assay (LBA AFP-L3, Wako Pure Chemical Industries, Osaka, Japan). The cut-off value considered positive for AFP was 20 ng/mL as described previously [14,15]. DCP was measured by means of a high-sensitivity method with a DCP reagent (Picolumi PIVKA-II, Eisai, Tokyo, Japan) and an apparatus that measures electrochemiluminescence automatically (Picolumi 8200, Sanko Pure Chemicals, Tokyo, Japan) [16]. A cut-off of 40 mAU/mL was used [16,17].

Pathologic evaluations. The pathologic features of HCC were evaluated by two of the authors (N.Y. and Y.K.) without knowledge of the HSP27 status of the tumor. The specimen was stained with hematoxylin and eosin, and the entire specimen was examined. Differentiation of HCC was classified as well-, moderately, or poorly differentiated HCC on the basis of the classification by International Working Party [18]. Vascular invasion and infiltration to the tumor capsule were evaluated macroscopically.

Western blot analysis. Snap-frozen samples were homogenized and sonicated in lysis buffer containing 62.5 mM Tris–HCl (pH 6.8), 2% sodium dodecyl sulfate (SDS), 50 mM dithiothreitol, and 10% glycerol. The linear range of loading volume in Western blot analysis was tested with serially diluted protein samples. SDS–polyacrylamide gel electrophoresis (PAGE) was performed by the method of Laemmli on 10% polyacrylamide gels [19]. Protein samples (10–15 μg) were subjected to SDS–PAGE and transferred to polyvinylidene difluoride (PVDF) membranes. Western blot analysis was performed as described previously [20] with polyclonal antibodies against HSP27 and Ser-78 phosphorylated HSP27 (Stressgen Biotechnologies, Victoria, BC, Canada) and phospho-Ser-15 HSP27 (Affinity BioReagents, Golden, CO). Peroxidase-conjugated antibodies against rabbit IgG were used as secondary antibodies against the above-mentioned primary antibodies. Primary antibodies against phospho-Ser-82 HSP27 (Biomol Research Laboratories, Plymouth Meeting, PA) and β-actin (Sigma, St. Louis, MO) were detected with peroxidase-conjugated antibodies against mouse IgG as secondary antibodies. Peroxidase activity on PVDF membranes was visualized on X-ray film with the ECL Western blotting detection system. Band intensities were determined by integrating the optical density over the band area (band volume) with NIH image software. Total HSP27 and phosphorylated HSP27 levels were normalized to those of β-actin.

Immunohistochemical analyses. Immunohistochemical staining of some specimens was done with the streptavidin–biotin complex method to investigate expression and localization of total HSP27 and phospho-Ser-15 HSP27. Primary antibodies were anti-HSP27 rabbit polyclonal antibody (1:2000, Stressgen) and anti-phospho-Ser-15 HSP27 (1: 2000, Affinity BioReagents). Briefly, deparaffinized sections were treated with 3% H_2O_2 in methanol for 10 min to inhibit endogenous peroxidase activity. Sections were immersed in 0.05 M citrate buffer (pH 6.0), heated in a microwave oven for 15 min, and then incubated with primary antibody for 2 h at room temperature. Each section was treated sequentially with biotinylated secondary antibodies (anti rabbit-IgG) and streptavidin–peroxidase complex (DakoChemMate, Kyoto, Japan). Finally, immune complexes were visualized with 3,3′-diaminobenzidine tetrahydrochloride as a chromogen. Mayer's hematoxylin was used as a counterstain. Negative controls (isotype-matched irrelevant antibodies or preimmune goat serum as primary antibodies) were run simultaneously. The results of staining were evaluated by two independent examiners (H.T. and M.I.), and differences in interpretation were resolved by consensus.

Statistical analysis. Patient clinical data are expressed as means ± SD. Levels of phosphorylated HSP27 were analyzed as a continuous variable. The data were analyzed with the SPSS software program (Release 11.5.1J standard version; SPSS Japan, Tokyo, Japan). One-way analysis of variance (ANOVA) was used to determine the significance of differences between protein expression and grade of tumor differentiation or tumor stage. The correlation between HSP27 phosphorylation levels and features of HCC was analyzed by Student's t test. Nonparametric data were analyzed with the Mann–Whitney U test, Kruskal–Wallis test, or Spearman's correlation coefficient (r). The relation between HSP27 phosphorylation levels and AFP or DCP was analyzed with Spearman's correlation coefficient (r). All p values were derived from two-tailed tests and $p < 0.05$ was accepted as statistically significant.

Results

Comparisons between total and phosphorylated HSP27 levels according to characteristics of HCC

Levels of total HSP27 or HSP27 phosphorylated at three Ser residues (Ser-15, Ser-78, and Ser-82) were compared with the clinical and pathologic characteristics of patients with HCC, including age, sex, underlying liver disease, etiology, number of tumors, tumor size, vascular invasion, infiltration to the tumor capsule, tumor stage (evaluated according to the TNM classification of the International Union Against Cancer) [21], differentiation, and AFP and DCP levels. We found no significant difference in total HSP27 levels with respect to the clinical and pathologic characteristics, except that levels were significantly lower in HCC with vascular invasion than in HCC without vascular invasion (0.948 ± 0.104 vs. 0.879 ± 0.092, $p = 0.029$).

Comparisons of the levels of serine phosphorylated HSP27 revealed significant differences in levels of all three types of serine phosphorylated HSP27 with respect to tumor size (Ser-15, $p = 0.003$; Ser-78, $p = 0.002$; Ser-82, $p = 0.004$), vascular invasion (Ser-15, $p = 0.002$; Ser-78, $p = 0.009$; Ser-82, $p = 0.029$), and tumor stage (Ser-15, $p = 0.001$; Ser-78, $p = 0.003$; Ser-82, $p = 0.008$; Table 1). We found no differences in levels of phosphorylated HSP27 with respect to patient age, sex, underlying liver disease, etiology, number of tumors, infiltration to the tumor capsule, differentiation, or AFP and DCP levels.

Total and serine phosphorylated HSP27 levels according to differentiation and tumor stage are shown in Figs. 1 and 2. A trend toward decreased phosphorylation of HSP27 was observed at all three serines examined as tumor stage increased.

Immunohistochemical analysis of total and phosphorylated HSP27 in HCC specimens

Immunohistochemical staining was performed for total HSP27 and Ser-15-phosphorylated HSP27 in a subset of the resected HCC specimens classified as stages I and IV (Fig. 3). Total HSP27 immunoreactivity was high in cytoplasm of both stage I (Fig. 3A) and IV (Fig. 3B) HCC tissues. In contrast, immunoreactivity for Ser-15-phosphorylated HSP27 was markedly attenuated in cytoplasm in stage IV HCC in comparison with that in stage I HCC (Figs. 3C and D).

Discussion

In the present study, we showed that attenuation of serine phosphorylation of HSP27 at three sites (Ser-15, Ser-78, and Ser-85) is associated with tumor stage in patients with HCC. In addition, we found that phosphorylation of HSP27 was attenuated in the larger HCCs and in HCC with vascular invasion, both of which are indicative of an advanced tumor. To the best of our knowledge, this is the first report of a significant relation between phosphorylated HSP27 levels and progression of HCC, including tumor stage.

HSPs function as chaperone proteins [6], and HSPs are essential for survival of cancer cells in various types of cancers [22–24]. HSP27 can interfere with transduction of apoptotic signal at several points [25]. HSP27 is thought to preserve mitochondrial integrity and reduce cytochrome c release; HSP27 can bind directly to cytochrome c, thus preventing activation of procaspase-9. HSP27 can also bind to procaspase-3 and inhibit its activation. Up-regulation of HSPs has been reported in several cancers, including breast, kidney, and bladder cancers and leukemia [22,26–28]. In addition, overexpression of HSPs in tumor

Table 1
Levels of phosphorylated HSP27 and characteristics of HCC

Phosphorylation site	Ser-15	Ser-78	Ser-82
Tumor size			
$\leqslant$20 mm ($n = 15$)	0.484 ± 0.157	0.787 ± 0.200	0.939 ± 0.158
>20 mm and $\leqslant$50 mm ($n = 26$)	0.329 ± 0.187	0.501 ± 0.217	0.689 ± 0.270
>50 mm ($n = 7$)	0.226 ± 0.120	0.450 ± 0.361	0.519 ± 0.306
	($p = 0.003$)	($p = 0.002$)	($p = 0.004$)
Vascular invasion			
Negative ($n = 33$)	0.419 ± 0.195	0.648 ± 0.255	0.809 ± 0.256
Positive ($n = 15$)	0.240 ± 0.105	0.441 ± 0.066	0.596 ± 0.288
	($p = 0.002$)	($p = 0.009$)	($p = 0.029$)
Tumor stage			
Stage I ($n = 9$)	0.527 ± 0.126	0.768 ± 0.209	0.970 ± 0.187
Stage II ($n = 22$)	0.405 ± 0.194	0.618 ± 0.262	0.773 ± 0.264
Stage III ($n = 10$)	0.234 ± 0.114	0.546 ± 0.255	0.596 ± 0.256
Stage IV ($n = 7$)	0.206 ± 0.081	0.289 ± 0.137	0.563 ± 0.280
	($p = 0.001$)	($p = 0.003$)	($p = 0.008$)

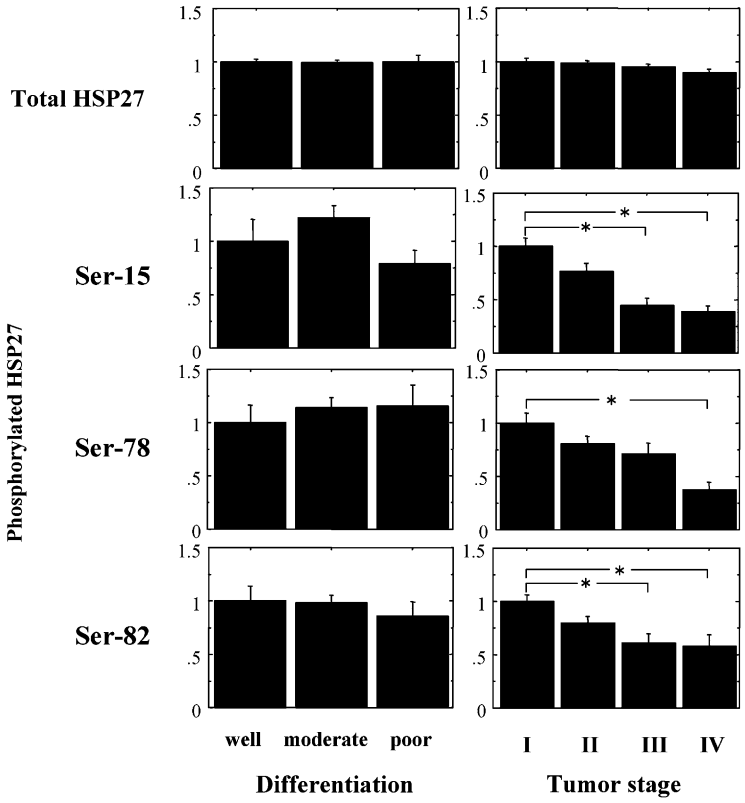

Fig. 1. Total and phosphorylated HSP27 levels in patients with hepatocellular carcinoma (HCC) Protein extracts from 48 HCC specimens were analyzed with antibodies against HSP27, Ser-15-phosphorylated HSP27, Ser-78-phosphorylated HSP27, Ser-82-phosphorylated HSP27, and β-actin. Signal intensities on X-ray film were quantified with NIH image software. The histogram shows quantitative representations of the levels of total and phosphorylated HSP27 after normalization to levels of β-actin. Values on the vertical axis represent means ± standard error (SE) of four independent experiments. The values were calculated as the average values of well-differentiated HCC (left column) and those of stage I HCC (right column) to be 1.0. *$p < 0.05$.

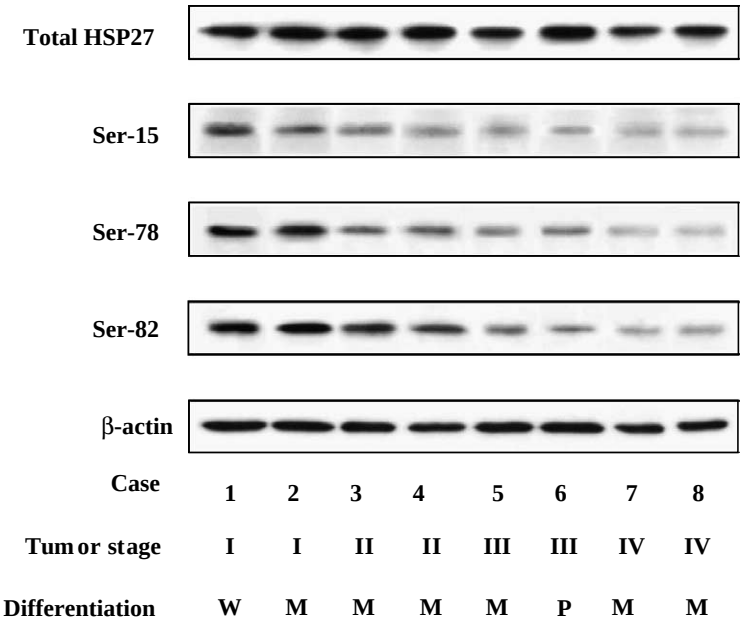

Fig. 2. Total and phosphorylated HSP27 levels in eight representative patients with HCC according to tumor stage and differentiation Protein extracts were analyzed with antibodies against total HSP27, Ser-15-, Ser-78-, or Ser-82-phosphorylated HSP27, and β-actin. W, well-differentiated HCC; M, moderately differentiated HCC; P, poorly differentiated HCC.

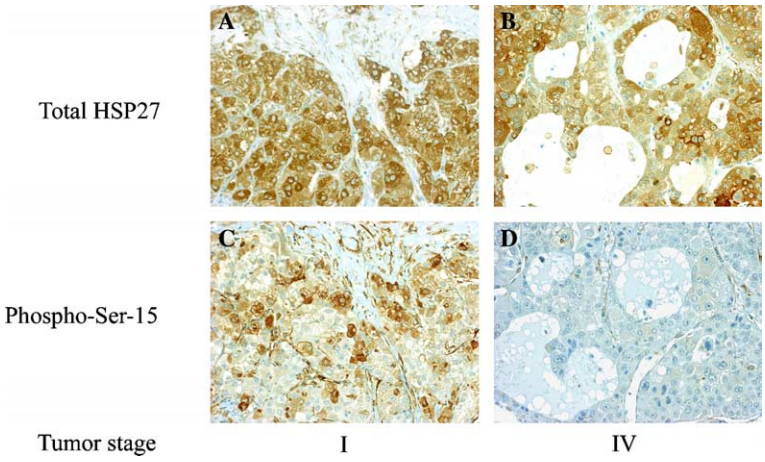

Fig. 3. Immunohistochemical analysis of levels of total and Ser-15-phosphorylated HSP27 in patients with HCC Protein extracts were analyzed with antibodies against HSP27 (A,B) and Ser-15-phosphorylated HSP27 (C,D). Phosphorylation of HSP27 at Ser-15 was reduced in stage IV HCC tissue in comparison with that in stage I HCC, whereas total HSP27 was similar in both stages.

tissues has been suggested to have prognostic value in patients with breast, kidney, or bladder cancer [26,29]. With respect to HCC, King and colleagues [11] reported that HSP27 levels are correlated with histological grade and patient survival. In contrast, Lim and colleagues [30] found no relation between HSP27 expression and several prognostic factors of HCC, including histological grade. In the present study, we found no association between total HSP27 levels and progression of HCC, with the exception of vascular invasion.

It is generally recognized that HSP27 occurs on two forms, an aggregated form and a small dissociated form, in unstressed cells [6,31]. The functions of HSP27 are regulated not only by these forms but also by post-translational modifications such as phosphorylation [6]. Human HSP27 is phosphorylated at three serine residues, Ser-15, Ser-78, and Ser-82 [6]. We previously reported that HSP27 dissociates into dimers concomitant with phosphorylation of the aggregated form (large 700-kDa oligomers) and that dephosphorylation of dissociated HSP27 causes aggregation [32]. It has been reported that dissociation of HSP27 from the aggregate form decreases the chaperone function of HSP27 [6]. In the present study, we used Western blot analysis to quantitate the levels of HSP27 phosphorylated at each of the three serines and to compare these levels with characteristics of HCC, including tumor size, vascular invasion, and tumor stage. We showed that phosphorylation of HSP27 at all three serine residues decreases with advances in tumor stage. Furthermore, immunohistochemical analysis revealed that phosphorylation of HSP27 at Ser-15 decreases with increases in tumor stage, which is consistent with our Western blot data. It has been reported that increased phosphorylation of HSP27 is correlated with growth inhibition in osteoblasts [33]. Spector and colleagues [34] showed that HSP27 phosphorylation increases during retinoic acid-induced differentiation of human leukemic HL-60 cells. It is possible that phosphorylated HSP27 in hepatic cancer cells has protective effects and prevents these cells from advancing. Further studies are needed to clarify the exact role of serine phosphorylated HSP27.

In conclusion, our present results suggest that serine phosphorylation of HSP27 decreases with increases in tumor stage in patients with HCC and that phosphorylated HSP27 may have a suppressive effect on the advancement of human HCC.

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