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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.