

Expression levels of heat shock protein 20 decrease in parallel with tumor progression in patients with hepatocellular carcinoma

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Abstract. Heat shock protein (HSP) 20, a low-molecular-weight HSP, is constitutively expressed in various tissues, such as smooth muscle, skeletal muscle, and liver. However, the characteristics and function of HSP20 have not been precisely understood. In the present study, we investigated correlations of expression levels of HSP20 in hepatocellular carcinoma (HCC) tissues and the surrounding tissues with clinical and pathologic characteristics in 53 resected HCC specimens. Although HSP20 was detected in all 53 HCC tissues, the expression levels were reduced compared with those in the adjacent non-tumor tissues. The expression levels of HSP20 were inversely correlated with tumor stage by TNM classification ($p < 0.01$), presence of microvascular invasion ($p < 0.05$), and tumor size ($p < 0.05$). Our findings strongly suggest that HSP20 may play a role against the progression of human HCC.

Introduction

Cells produce heat shock proteins (HSPs), when exposed to various kinds of biological stress such as heat and chemicals (1). HSPs are classified into high-molecular-weight HSPs

such as HSP70, HSP90 and HSP110, and low-molecular-weight HSPs with molecular masses from 10-30 kDa such as HSP20, HSP27 and α B-crystallin according to apparent molecular sizes. It is well recognized that high-molecular-weight HSPs act as molecular chaperones in protein folding, oligomerization and translocation (1). Though 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 also have chaperone functions (1). The human genome codes for 10 low-molecular-weight HSPs (2). In their C-terminal half, these proteins share a sequence element of ~100 amino acid residues called the α -crystallin domain, and toward their N-terminal end, they share a less conserved but nevertheless similar domain (3). HSP20 was co-purified with HSP27 and α B-crystallin from skeletal muscle, and it was identified as a member of the crystallin family (4). Although HSP20 is not induced by heat or chemical stress, it is highly expressed in normal skeletal and smooth muscle, heart and liver tissues where it may be essential, but the exact role of HSP20 remains to be clarified (4).

Hepatocellular carcinoma (HCC) is a common malignancy worldwide, and it causes more than one million deaths annually (5,6). Factors that indicate tumor progression in association with patient outcome reportedly include tumor size, number of tumors, vascular invasion that can be evaluated pathologically and imaging diagnosis (7-10). Tumor markers for HCC such as α -fetoprotein levels and *des- γ -carboxy* prothrombin levels are reported to be additional indicators of tumor progression associated with patient survival (10-13). However, these factors are not sufficient to accurately discriminate the tumor progression of HCC patients towards the accurate prediction of patient survival. It is, therefore, necessary to further investigate other indicators for the evaluation of tumor progression and for the prediction of patient outcome.

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To date, it has been reported that expression of certain HSPs can be correlated with the carcinogenic process as well as with the degree of differentiation and cell proliferation, and moreover, they have been implicated in the regulation of apoptosis (14,15). In addition, evidence is accumulating about the usefulness of the prognostic implications of HSPs in certain cancer types, especially high-molecular-weight HSPs (14,15). We have recently shown that attenuated phosphorylation of HSP27 correlates with tumor progression in patients with HCC (16). Among low-molecular-weight HSPs, HSP27 has been the most extensively studied, but to the best of our knowledge there has been no report about the relationship of HSP20 and tumor progression. Therefore, in the present study, we tried to investigate the relationship between HSP20 and HCC in 53 resected HCC specimens.

Materials and methods

Patients. Fifty-three patients (46 men, 7 women, mean age: 66.9 ± 8.4 years), having been diagnosed with HCC at the Department of Gastroenterology, Ogaki Municipal Hospital, Ogaki, Japan underwent hepatic resection between September 2002 and August 2005. Liver cirrhosis was present in 24 patients, and chronic hepatitis was present in 29. Fourteen patients were infected with hepatitis B virus, and 34 were infected with hepatitis C virus. The remaining 5 patients had evidence of alcoholic cirrhosis. No patient had previously undergone preoperative chemotherapy.

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.

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% methanol and xylene and embedded in paraffin wax. A 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.

Pathological evaluations. The pathological features of HCC were evaluated by two of the authors (N.Y. and Y.K.) without knowledge of the HSP20 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 the International Working Party (17). 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

Table I. Comparison of the protein levels of HSP20 with the clinical and pathological characteristics of 53 patients with HCC.

	p value	
	Tumor tissue	Non-tumor tissue
Gender		
male (n=46), female (n=7)	0.896	0.627
Underlying disease		
liver cirrhosis (n=25), chronic hepatitis (n=28)	0.957	0.010 ^a
Etiology of liver disease		
HBV (n=13), HCV (n=35), alcoholic (n=5)	0.662	0.482
Number of tumors		
solitary (n=40), multiple (n=13)	0.374	0.718
Tumor size (mm)		
<20 (n=12), 20-50 (n=32), >50 (n=9)	0.048 ^a	0.697
Vascular invasion		
negative (n=35), positive (n=18)	0.040 ^a	0.669
Infiltration to capsule		
negative (n=28), positive (n=25)	0.203	0.673
Tumor stage		
I (n=9), II (n=25), III (n=11), IV (n=8)	0.003 ^a	0.449
Histological classification (differentiation)		
well- (n=11), moderately (n=35), poorly (n=7)	0.858	0.636

HBV, hepatitis B virus; HCV, hepatitis C virus; ^ap<0.05.

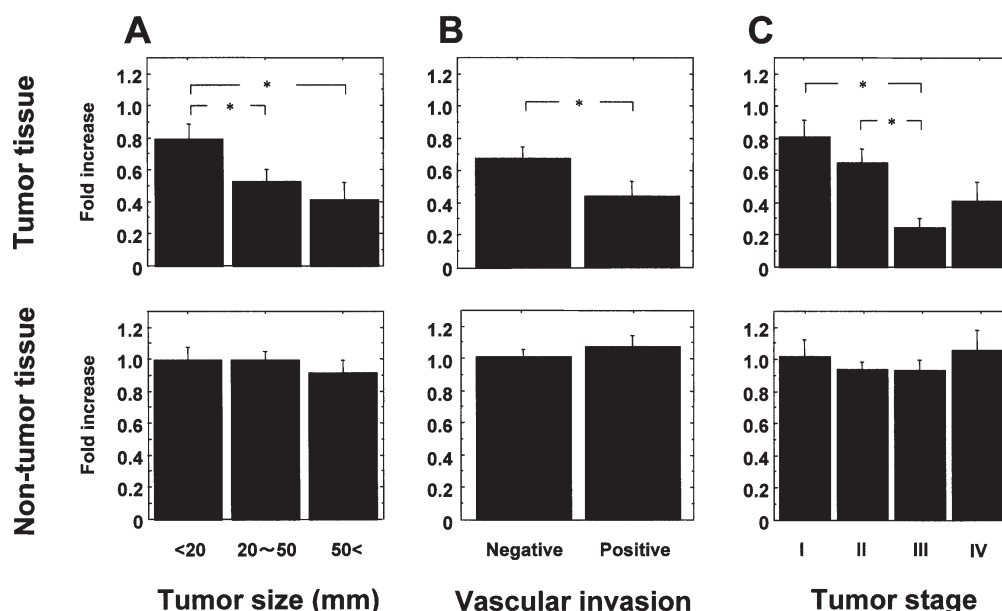


Figure 1. HSP20 levels in patients with HCC. Protein extracts from 53 HCC specimens (tumor and adjacent non-tumor tissue, respectively) were analyzed with antibodies against HSP20 and β -actin. Signal intensities on X-ray film were quantified with NIH image software. The histograms show quantitative representations of the levels of HSP20 after normalization to levels of β -actin. Values on the vertical axis represent the mean \pm SE of independent experiments. The values were calculated as the average values with those of small (tumor size <20 mm) HCC (left column), negative vascular invasion (center column) and tumor stage I HCC (right column) equal to 1.0. * $p < 0.05$.

(pH 6.8), 2% sodium dodecyl sulfate (SDS), 50 mM dithiothreitol, and 10% glycerol. Western blot analysis was performed as described previously (18) with polyclonal antibodies against HSP20 and HSP27 (Stressgen Biotechnologies, Victoria, British Columbia, Canada). Peroxidase-conjugated antibodies against rabbit IgG were used as secondary antibodies against the above-mentioned primary antibodies. Primary antibodies against β -actin (Sigma-Aldrich Co, 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 (GE Healthcare UK Ltd, Buckinghamshire, UK). Protein band intensities were determined by integrating the optical density over the band area (band volume) with NIH image software. HSP20 levels were normalized to those of β -actin.

Immunohistochemical analysis. Immunohistochemical staining of some specimens was performed with the streptavidin-biotin complex method to investigate expression and localization of HSP20. Primary antibodies were anti-HSP20 rabbit polyclonal antibodies (Stressgen Biotechnologies, Golden, CO). 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 antibodies for 2 h at room temperature. Each section was treated sequentially with biotinylated secondary antibodies (anti-rabbit-IgG) and streptavidin-peroxidase complex (Dako Chem Mate, Kyoto, Japan). Finally, immune complexes were visualized with 3,3'-diaminobenzidine tetrahydrochloride as a chromogen. Mayer's hematoxylin was used as a counterstain.

Statistical analysis. Patient clinical data were expressed as mean \pm SD. 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. Nonparametric data were analyzed with the Mann-Whitney U test, Kruskal-Wallis test, or Spearman's correlation coefficient (r). All p values were derived from two-tailed tests and $p < 0.05$ was accepted as statistically significant. A Spearman's correlation coefficient of $r \geq 0.400$ was accepted as a positive correlation.

Results

Correlations of HSP20 levels according to characteristics of HCC. The levels of HSP20 were compared with the clinical and pathological characteristics of 53 patients with HCC, including gender, underlying liver disease, etiology, number of tumors, tumor size, vascular invasion, infiltration to the tumor capsule, and tumor stage (evaluated according to the TNM classification of the International Union Against Cancer) (19), and histological classification (Table I). Comparisons of the levels of HSP20 revealed significant differences with respect to tumor size ($p = 0.048$), vascular invasion ($p = 0.040$) and tumor stage ($p = 0.003$) in tumor tissues, while there were no significant differences in HSP20 levels in adjacent non-tumor tissues, except in those tissues with underlying liver disease (Table I). In the non-tumor tissues, the levels of HSP20 in liver cirrhotic tissue were significantly higher than those in chronic hepatitis patient tissue (Table I).

HSP20 levels according to tumor size, vascular invasion and tumor stage are shown in Fig. 1. A trend toward decreased expression levels of HSP20 in tumor tissues was

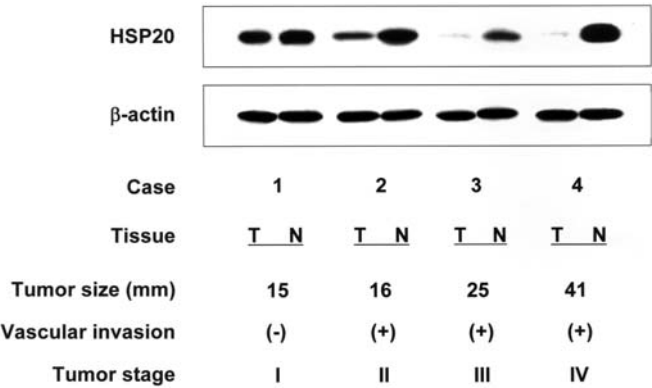


Figure 2. HSP20 levels in four representative patients with HCC according to tumor size, vascular invasion and tumor stage. Comparison with those in non-tumor tissue. Protein extracts were analyzed with antibodies against HSP20 and β -actin. T, tumor tissue; N, adjacent non-tumor tissue.

observed as tumor size, vascular invasion and tumor stage increased, suggesting that the levels of HSP20 in the adjacent non-tumor tissues were higher than those in the tumor tissues (Fig. 1A, B and C; upper panel). On the other hand, HSP20 levels in the adjacent non-tumor tissues were not correlated with these factors, suggesting that the levels of HSP20 in the tumor tissues were attenuated in parallel with HCC progression (Fig. 1A, B and C; lower panel). Western blot images of HSP20 expression in 4 representative patients with HCC according to tumor size, vascular invasion and tumor stage are shown in Fig. 2.

Immunohistochemical analysis of HSP20 in HCC specimens. To confirm our results from Western blot analysis, we performed immunohistochemical analysis of HSP20 in HCC tumor and non-tumor tissues. Immunohistochemical staining of HSP20 in stage-IV-HCC specimens containing tumor and non-tumor tissue is shown in Fig. 3. Immunoreactivity for HSP20 in tumor tissue was markedly lower than that in non-tumor tissue.

Comparisons between the levels of HSP20 and the levels of phosphorylated HSP27 in HCC tumor tissues. HSP27, a low-

molecular-weight HSP, is phosphorylated at three serine residues (Ser-15, Ser-78 and Ser-82) (1). We previously reported that attenuation of phosphorylated HSP27 (Ser-15, Ser-78 and Ser-82) in tumor tissue correlates with HCC progression (16). Therefore, we investigated the correlation between the levels of HSP20 and the levels of phosphorylated HSP27 that had been determined in the previous study. The levels of phosphorylated HSP27 (Ser-15) were significantly correlated with the levels of HSP20 ($r=0.505$, $p<0.001$; Fig. 4A). On the contrary, the levels of phosphorylated HSP27 (Ser-78), phosphorylated HSP27 (Ser-82) or total HSP27 were not correlated with those of HSP20 (Fig. 4B, C and D, respectively).

Discussion

In the present study, we showed that attenuation of HSP20 levels correlated with tumor progression in tumor tissues of patients with HCC. In addition, the HSP20 levels correlated inversely with tumor size and vascular invasion of HCC, both of which are indications of an advanced tumor. To the best of our knowledge, this is the first report of a significant relation between HSP20 levels and progression of HCC.

Recently, we reported that attenuation of phosphorylated HSP27 is correlated with HCC progression (16). It is recognized that HSP27, HSP20 and α B-crystallin form one type of complex (3,20). It has been shown that phosphorylation of HSP27 is associated with the disassembly of HSP27 complexes (21,22). In the present study, we found significant correlation between the levels of HSP20 and that of phosphorylated HSP27 (Ser-15), but not Ser-78 and Ser-82. Although the differential role of the three phosphorylation sites are not known, our findings suggest that HSP20 and phosphorylated HSP27 (Ser-15) may have suppressive effects on HCC progression. In addition, these results suggest that phosphorylated HSP27 (Ser-78) and phosphorylated HSP27 (Ser-82) may have different roles in HCC progression. Further investigations are required not only to clarify the exact role of HSP20, but also to determine whether these HSPs can be prognostic factors in HCC. Moreover, HSPs not only have prognostic implications but also have therapeutic implications for cancer (14). Among HSPs, the use of the HSP90 inhibitor,

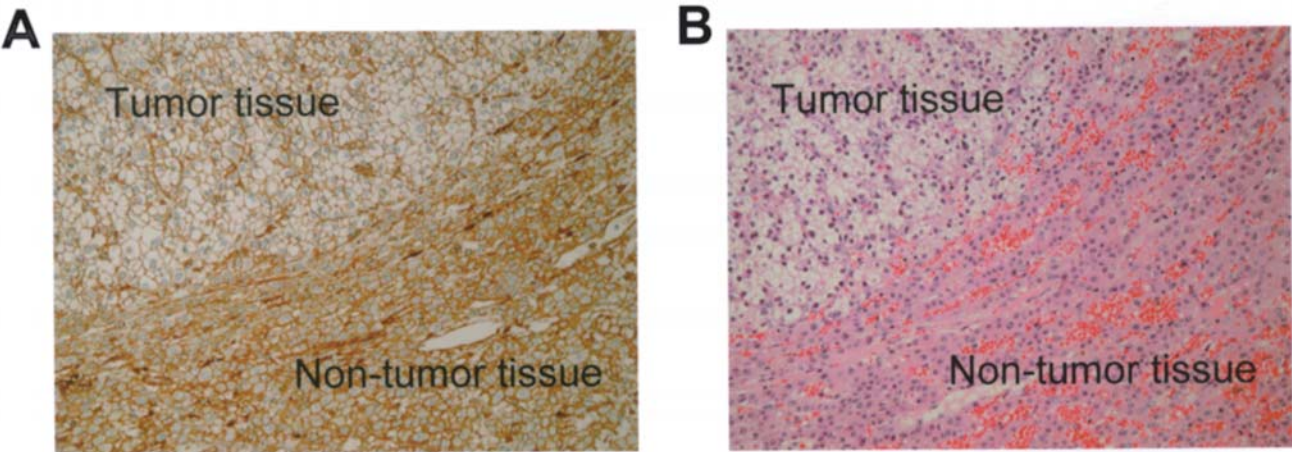


Figure 3. Immunohistochemical analysis of HSP20 in a patient with HCC (tumor stage IV) and adjacent non-tumor tissue (chronic hepatitis) (A). The same patient specimen stained with hematoxylin and eosin. (B).

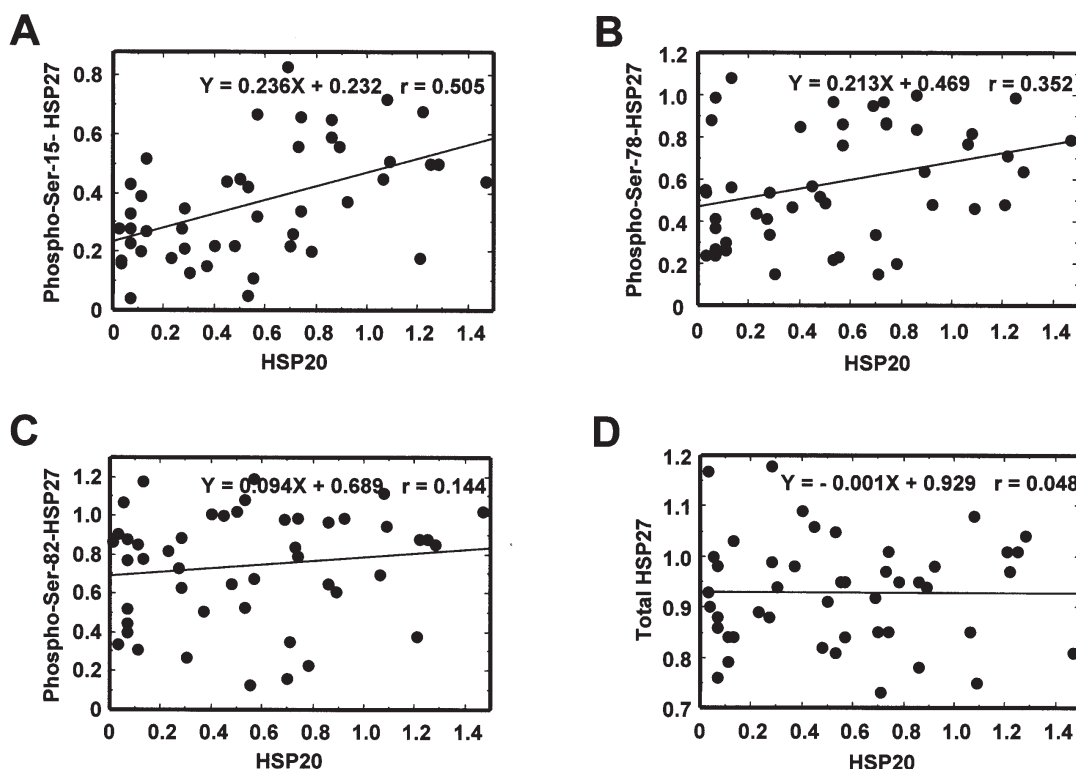


Figure 4. Correlations between the levels of HSP20 and the levels of (A) Ser-15-phosphorylated HSP27, (B) Ser-78-phosphorylated HSP27, (C) Ser-82-phosphorylated HSP27, and (D) total HSP27. The expression levels of HSP20, phosphorylated HSP27, and total HSP27 were determined by the band intensities obtained from Western blot analysis, and then normalized to those of β -actin.

which is under phase I trial has been extensively studied (14,15). Although the role of HSP20 in HCC is not precisely known, further investigations would help us to use HSP20 as a target for cancer therapy.

In conclusion, our present results strongly suggest that expression levels of HSP20 decrease with progression in tumor stages in patients with HCC and that HSP20 may have a suppressive effect on the advancement of human HCC.

Acknowledgements

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