

Increased expression of heat shock protein-binding protein 1 and heat shock protein 70 in human hepatocellular carcinoma tissues

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Abstract. Heat shock protein-binding protein 1 (HspBP1) is a co-chaperone that inhibits heat shock 70-kDa protein (Hsp70) activity. In mouse neuroblastomas and lung tumors, the protein levels of HspBP1 and Hsp70 are elevated by a similar amount compared to non-tumor tissues. However, no studies have been reported regarding the levels of HspBP1 in human cancer tissues. Our previous proteomic study demonstrated that the expression of Hsp70 was increased in human hepatitis C virus-related hepatocellular carcinoma (HCV-HCC) tissues. Here, we investigated the expression of HspBP1 in human HCV-HCC. Immunoblotting analysis of HspBP1 and Hsp70 was performed in human HCV-HCC tissues from 20 patients. In 80% of the patients, Hsp70 increased an average of 3.55-fold, and in 50% of the patients, HspBP1 increased an average of 2.02-fold. Comparison and analysis of expression and clinical data revealed a significant difference between moderately-differentiated HCC and non-tumor tissues. In addition, there was a significant difference between the ratio of HspBP1 to Hsp70 levels and tumor size (<3 cm vs. ≥ 3cm) with larger tumors having a lower ratio. This ratio was significantly lower in moderately-differentiated HCC tissues than in non-tumor HCC tissues. In conclusion, HspBP1 was up-regulated in human HCV-HCC, an increase which correlated with the increase of Hsp70 levels. The ratio of HspBP1 to Hsp70 in HCC may provide novel information

concerning the characterization of tumors, tumor progression and resistance to treatment.

Introduction

Hepatocellular carcinoma (HCC) is one of the most malignant tumors worldwide. Most notably, chronic hepatitis and liver cirrhosis caused by infection with hepatitis C virus (HCV) are correlated with HCC in Japan. An urgent goal in this area is the identification of reliable biomarkers for the early diagnosis of HCC. Proteomic studies have been carried out to find new markers for HCC and to understand the pathogenesis of this disease (1-10). We previously reported proteomic profiling focused on HCC with HCV infection (6-8). These studies revealed that members of the heat shock protein 70 family, including heat shock 70-kDa protein (Hsp70), heat shock cognate 71-kDa protein (Hsc71), 75-kDa glucose-regulated protein (GRP75) and 78-kDa glucose-regulated protein (GRP78), were all up-regulated in HCV-related HCC (6). Heat shock proteins are induced by various stresses and serve as molecular chaperones that function to maintain protein structure, transport proteins and aid in the formation of protein complexes, as well as playing a role in proteolysis (11).

Heat shock protein-binding protein 1 (HspBP1) was isolated and characterized as a protein which regulates Hsp70 activity. The expression of HspBP1 was shown to increase in mouse neuroblastoma and lung tumors (12-14). Here, we demonstrate that the changes in the protein expression of HspBP1 are correlated with changes in Hsp70 expression in human HCV-related HCC tissues, and discuss the biological significance of HspBP1 in HCC.

Materials and methods

Tissue specimens. Tumor and paired non-tumor liver specimens from 20 patients who had undergone partial hepatectomy at the Yamaguchi University Hospital for HCC between 1998 and 2000 were included in this study. The clinical tumor data of these patients is summarized in Table I. TNM staging was

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Table I. Clinical tumor data of HCC.

No.	Main tumor size (cm)	TNM stage ^a	Differentiation	Vascular invasion	Mono/multi
1	2.8	II	Moderate	Yes	Multi
2	2.5	II	Moderate	No	Multi
3	2.5	I	Well	Yes	Mono
4	12.0	IV ^a	Moderate	No	Multi
5	5.0	II	Moderate	Yes	Multi
6	7.0	III	Moderate	No	Multi
7	2.9	I	Moderate	No	Mono
8	3.4	II	Poor	Yes	Multi
9	1.6	I	Well	No	Mono
10	1.2	I	Well	No	Mono
11	3.5	II	Moderate	No	Multi
12	3.8	I	Moderate	No	Mono
13	2.0	II	Moderate	Yes	Multi
14	9.0	II	Moderate	Yes	Mono
15	3.2	II	Well	No	Mono
16	1.5	III	Well	No	Multi
17	10.0	IV ^a	Poor	Yes	Multi
18	8.5	III	Poor	No	Multi
19	12.0	IV ^a	Poor	Yes	Mono
20	2.5	II	Poor	Yes	Mono

^aTNM staging according to the Liver Cancer Study Group of Japan criteria (ref. 15).

based on the criteria of the Liver Cancer Study Group of Japan (15). HCC tumors from 5, 10 and 5 patients were well, moderately and poorly-differentiated, respectively. All patients were serologically positive for anti-HCV antibody and negative for hepatitis B surface antigen. Normal control liver tissues were derived from patients without hepatitis who had undergone partial hepatectomy for metastatic liver tumors of colon cancer.

Sample preparation. Resected liver tissues were homogenized in lysis buffer (1% NP-40, 1 mM sodium vanadate, 1 mM PMSF, 50 mM Tris, 10 mM NaF, 10 mM EDTA, 165 mM NaCl, 10 μ g/ml leupeptin and 10 μ g/ml aprotinin) using a Potter type homogenizer with a Teflon tip and incubated at 4°C for 1 h. The homogenate was separated by centrifugation at 15,000 x g for 30 min to yield a supernatant and stored at -80°C until used.

Immunoblotting. Protein samples (20 μ g) of the supernatant were submitted to SDS-PAGE. Proteins were separated on 10-20% gradient polyacrylamide gels at 15 mA/gel. A control sample was included on each gel. After separation by SDS-PAGE, proteins were transferred electrophoretically onto a polyvinylidene fluoride membrane (Immobilon; Millipore Corp., Bedford, MA), and the membranes were blocked overnight at 4°C with TBS containing 5% skim milk. The primary antibodies were goat anti-human Hsp70 antibody (1:200) (Santa Cruz Biotechnology, Santa Cruz, CA), 1 μ g/ml sheep anti-HspBP1 antibody (Novus Biologicals, Littleton, CO) and rabbit anti-actin antibody (1:200) (Santa Cruz Bio-

technology). The membranes were incubated for 1 h, rinsed four times with TBS containing 0.05% Tween-20 and visualized using a chemiluminescence reagent (ECL; Amersham Pharmacia Biotechnology, Uppsala, Sweden).

Image analysis. The positions of the protein bands on the gels were defined using an Agfa Arcus 1200™ image scanner (Agfa-Gevaert N.V., Mortsel, Belgium) and analyzed with Image Gauge ver. 3.45 software (Fuji Film Science Lab, Minami Ashigara, Japan).

Results

The protein expression of Hsp70 and HspBP1 in tumor and non-tumor tissues from 20 patients bearing HCV-related HCC was investigated. The major HspBP1 band was ~40 kDa in size, but a slightly smaller band was also present in the samples analyzed (Fig. 1A). All analysis was conducted using the 40-kDa upper band. The expression of Hsp70 and HspBP1 in tumor tissues was increased by >1.5-fold in 16 (80%) of the patients and in 10 (50%) of the patients, respectively. The levels of Hsp70 and HspBP1 were significantly elevated 3.55-fold (0.87-8.73) and 2.02-fold (0.66-6.53), respectively, in the tumor tissues compared to non-tumor tissues. Statistical analysis showed a significant increase in both Hsp70 and HspBP1 in HCC by the t-test (Fig. 1D and E). The increases in the levels of Hsp70 and HspBP1 in tumor and non-tumor tissues revealed a significant correlation in the 40 samples as shown in Fig. 2 ($R^2=0.286$, $p<0.001$).

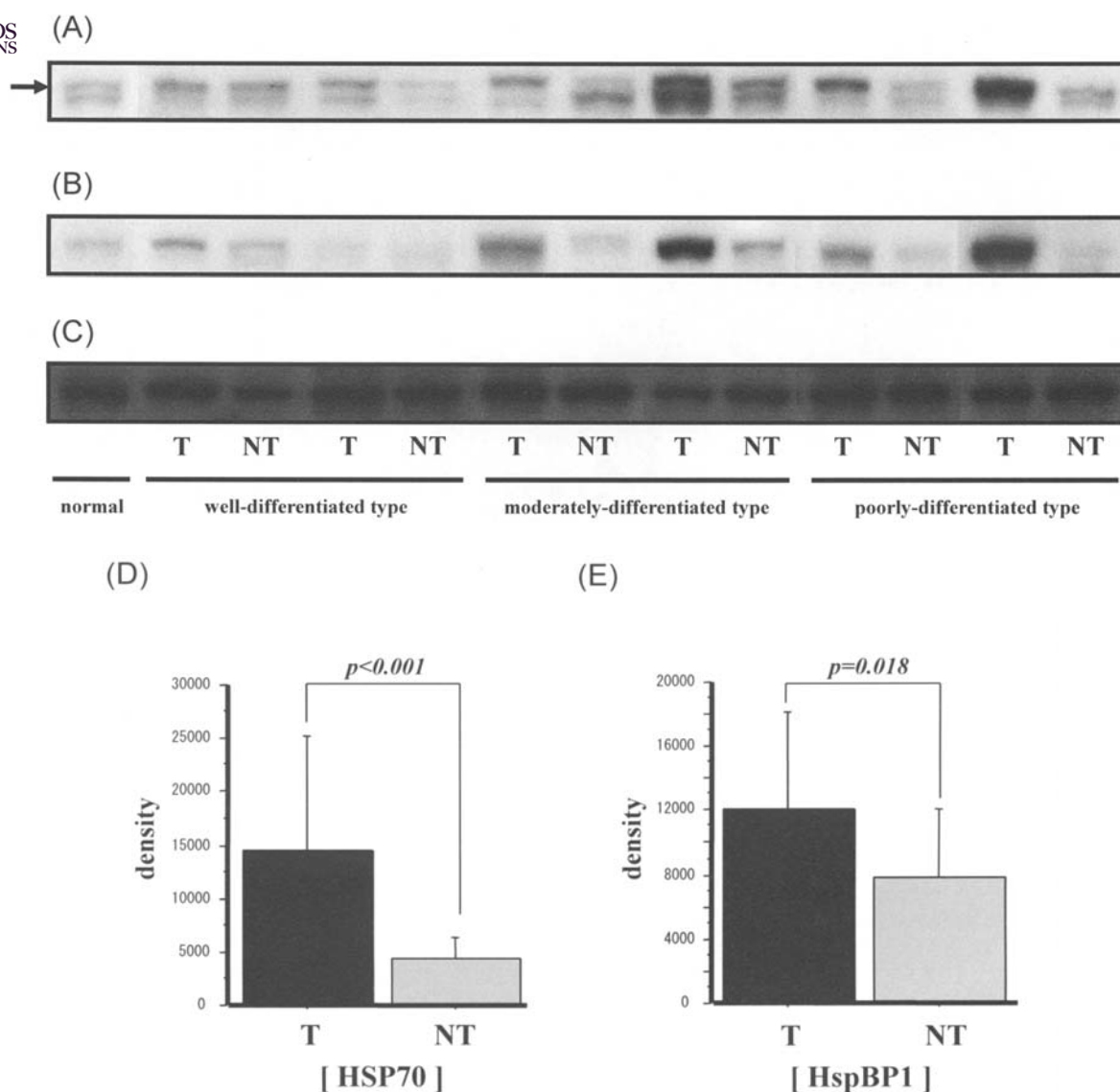


Figure 1. HspBP1 and Hsp70 levels in human HCC tissues. Western blot analysis was used to measure HspBP1 (A), Hsp70 (B), and actin (C) in tumor (T) and non-tumor (NT) tissues. Two representative samples of each type of differentiated HCC are shown. The arrow indicates the position of HspBP1. Normal tissues were obtained from patients who had undergone partial hepatectomy for metastatic liver tumors. Comparison of the amounts of Hsp70 (D) and HspBP1 (E) in tumor tissues vs. non-tumor tissues.

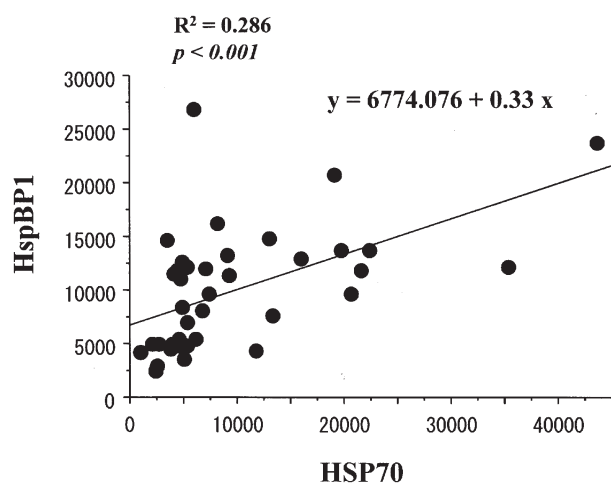


Figure 2. Correlation between the expression of HspBP1 and Hsp70. HspBP1 expression correlated with the expression of Hsp70 in tumor and non-tumor tissues ($p < 0.001$, $R^2 = 0.286$).

The correlation between the protein expression of Hsp70 and HspBP1 in tumor and non-tumor tissues and the clinical data of HCC, such as tumor size, staging, differentiation, vascular invasion and the number of tumors, was examined. Differentiation was the only feature that demonstrated a significant difference. Hsp70 was significantly up-regulated from the comparison of moderately-differentiated HCC with non-tumor tissues (Fig. 3). The expression of HspBP1 in HCC was higher in comparison to non-tumor tissues, but there was no significant difference among the groups. The relation between the ratio of expression of HspBP1 to Hsp70 in tumor tissues (HspBP1 T/HSP T) and clinical HCC data was investigated. There was a significant difference in this ratio for tumor size (< 3 cm vs. ≥ 3 cm) (Fig. 4). Large HCC had a lower ratio of HspBP1 T/HSP T. This ratio was lowest in moderately-differentiated HCC among the three types of differentiated HCC and significantly lower than in non-tumor tissues (Fig. 5).

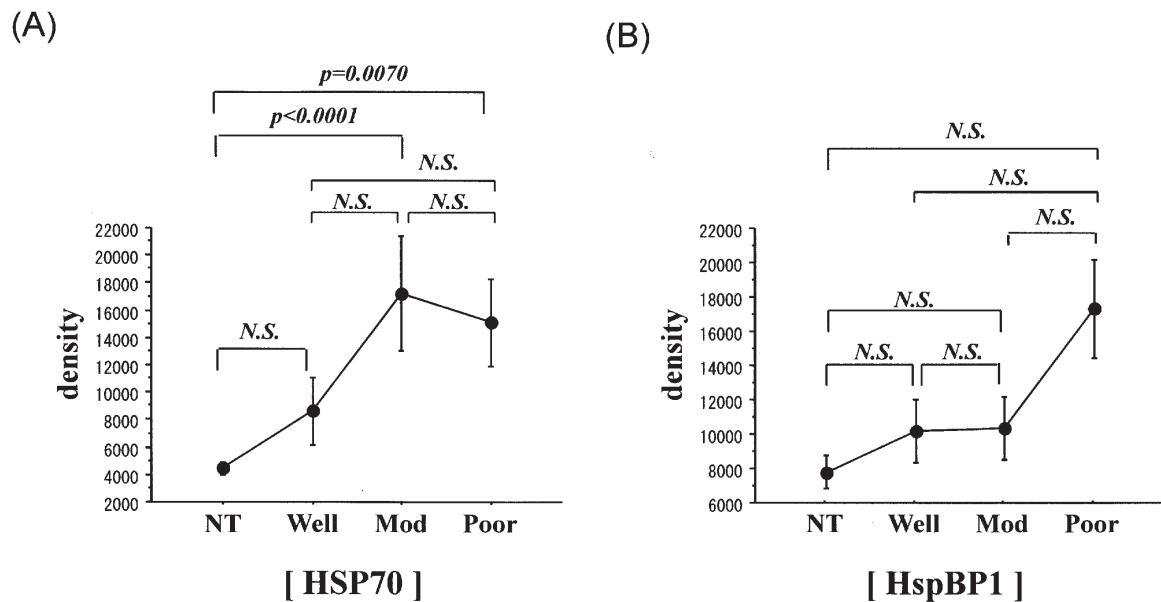


Figure 3. Quantitative analysis of the expression of Hsp70 (A) and HspBP1 (B) by type of differentiated HCC. This quantitative analysis was performed by ANOVA with the Bonferroni-Dunn test; $p < 0.0083$ was considered significant. The expression of HspBP1 demonstrated no significant difference among all groups although there was a significant difference between tumor (T) and non-tumor (NT) tissues as determined by the t-test (Fig. 1). The expression of Hsp70 was significantly up-regulated in moderately- (Mod) and poorly- (Poor) differentiated HCC compared to non-tumor tissues. N.S., no significant difference.

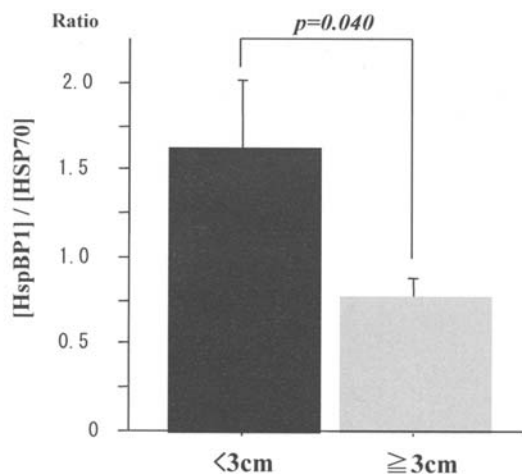


Figure 4. Correlation between the ratio of Hsp70 to HspBP1 and tumor size. The ratio of HspBP1 to Hsp70 in tumors <3 cm and ≥3 cm.

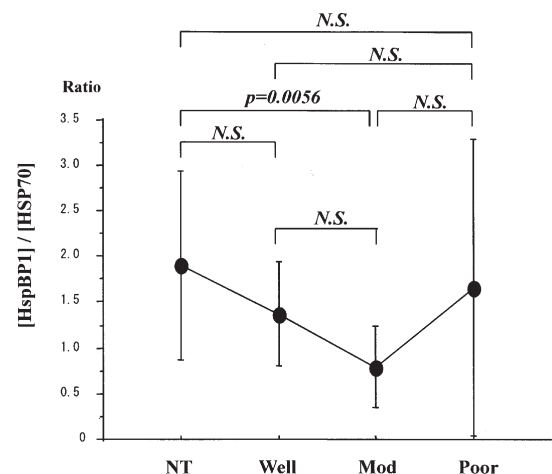


Figure 5. Quantitative analysis of the ratio of HspBP1 to Hsp70 in the types of differentiated HCC. This quantitative study was performed by ANOVA with the Bonferroni-Dunn test; $p < 0.0083$ was considered significant. The ratio was significantly lower in moderately-differentiated (Mod) compared to non-tumor (NT) tissues. N.S., no significant difference.

Discussion

We investigated the expression of Hsp70 and its inhibitor, HspBP1 in human HCV-related HCC tissues. Although the fluctuation of HspBP1 in tumors has been studied, until now, no study has been performed on human cancer tissues. For the first time, we analyzed the expression of HspBP1 in human HCV-related HCC tissues, and demonstrated that it was increased in tumor compared to non-tumor tissues.

It is known that Hsp70 expression is increased in several cancer cell lines and tissues, such as HCV-related HCC (6,16-21), and has been reported that the elevation of Hsp70 in certain cancer tissues leads to high malignant potential, poor prognosis or poor therapeutic outcome (16,21,22). HspBP1 binds the ATPase domain of Hsp70 and inhibits its

activity by nucleotide exchange (14). Hsp70 regulates cell growth or transformation in cancer cells (23,24). Moreover, previous reports have revealed that Hsp70 in cancer tissues has an anti-apoptotic effect, and several studies have shown that increasing the level of Hsp70 prevents apoptosis whereas decreasing the level promotes it (23-28). One possibility is that the amount of HspBP1 in tumor tissues may decrease the anti-apoptotic effect of Hsp70.

The present study showed that the expression of Hsp70 and HspBP1 were correlated in human HCV-related HCC tissues. The expression of Hsp70 in cancer tissues from well-differentiated to poorly-differentiated types was significantly increased compared with non-tumor tissues. In addition, the



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 Expression of HspBP1 in tumor tissues was significantly lower than in non-tumor tissues. However, it was evident that HspBP1 was not significantly up-regulated in poorly-differentiated HCC. Previous data have demonstrated the importance of the ratio of the expression of HspBP1 to Hsp70 (12). The ratio in moderately-differentiated HCC had the lowest values among the three differentiated types. There was no significant difference in the ratio between non-tumor tissues and well-differentiated HCC. This ratio in poorly-differentiated HCC was greater than in moderately-differentiated HCC (Fig. 5). Although the reason for this discrepancy was unclear, these two proteins might have reacted differently to stress.

We investigated the relation between the ratio of HspBP1 to Hsp70 and HCC clinical data. The ratio showed a relationship to both tumor differentiation and tumor size. The ratio was significantly lower in larger tumors. These data indicate that the ratio of the expression of HspBP1 to Hsp70 may influence the progression of HCV-related HCC.

HspBP1 was up-regulated in human HCV-related HCC in this study, and therefore may have use as a tumor marker. Moreover, the ratio of the expression of HspBP1 to Hsp70 is a potentially important marker for tumor progression. The Hsp70 inhibitory activity of HspBP1 may provide a therapeutic means for controlling HCC by increasing HspBP1 or decreasing Hsp70, thereby regulating tumor growth or the apoptosis of tumor cells.

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