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• *LIVER CANCER* •

Expression of heat shock proteins (HSP27, HSP60, HSP70, HSP90, GRP78, GRP94) in hepatitis B virus-related hepatocellular carcinomas and dysplastic nodules

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Abstract

AIM: Expression of heat shock proteins (HSPs) is frequently up-regulated in hepatocellular carcinoma (HCC), which evolves from dysplastic nodule (DN) and early HCC to advanced HCC. However, little is known about the differential expression of HSPs in multistep hepatocarcinogenesis. It was the purpose of this study to monitor the expression of HSPs in multistep hepatocarcinogenesis and to evaluate their prognostic significance in hepatitis B virus (HBV)-related HCC.

METHODS: Thirty-eight HCC and 19 DN samples were obtained from 52 hepatitis B surface antigen-positive Korean patients. Immunohistochemical and dot immunoblot analyses of HSP27, HSP60, HSP70, HSP90, glucose regulated protein (GRP)78, and GRP94 were performed and their expression at different stages of HCC development was statistically analyzed.

RESULTS: Expression of HSP27, HSP70, HSP90, GRP78, and GRP94 increased along with the stepwise progression of hepatocarcinogenesis. Strong correlation was found only in GRP78 (Spearman's $r = 0.802$). There was a positive correlation between the expressions of GRP78, GRP94, HSP90, or HSP70 and prognostic factors of HCC. Specifically, the expression of GRP78, GRP94, or HSP90 was associated significantly with vascular invasion and intrahepatic metastasis.

CONCLUSION: The expressions of HSPs are commonly up-regulated in HBV-related HCCs and GRP78 might play an important role in the stepwise progression of HBV-related hepatocarcinogenesis. GRP78, GRP94, and HSP90 may be important prognostic markers of HBV-related HCC, strongly suggesting vascular invasion and intrahepatic metastasis.

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Key words: Heat shock protein; Hepatocellular carcinoma; Dysplastic nodule; Hepatocarcinogenesis; Immunohistochemistry; Dot immunoblot analysis

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INTRODUCTION

Worldwide hepatocellular carcinoma (HCC) is a common malignant tumor that takes the lives of about one million people annually. Even after resection, the overall survival rate of patients with HCC is still unsatisfactory due to frequent recurrences[1]. Hepatitis B virus (HBV) is one of the known risk factors for HCC, but it is not yet clear how this factor leads to HCC[1]. HCC is characterized by multistage process of tumor progression. Recently, dysplastic nodule (DN) has been described as a precancerous lesion in the multistep hepatocarcinogenesis and is divided into low grade and high grade DN depending on the degree of cytologic atypia on histological examination[2]. Early HCC does not destroy the underlying liver structure and is uniformly composed of well differentiated cancer cells with little cellular atypia. At the tumor-nontumor boundary, well-differentiated cancer cells proliferate as though they are replacing normal hepatocytes ('replacing growth')[3,4]. The appearance of a regenerative nodule in the liver might be the first step of

hepatocarcinogenesis, subsequently developing into advanced HCC through low-grade dysplastic nodule (LGDN), high-grade dysplastic nodule (HGDN), and early HCC (in a multistep fashion)[5]. However, the molecular mechanisms implicated in the progression to HBV-related HCC remain largely unknown.

Mammalian cells express a family of highly conserved proteins in response to heat as well as many other stressful stimuli[6]. This family of stress proteins include heat shock proteins (HSPs) and glucose regulated proteins (GRPs)[7,8]. These proteins are multifunctional molecular chaperones. In neoplasms, expression of HSP has been implicated in the regulation of apoptosis, in immune response against tumors, and in multidrug resistance[9,10]. Increased HSP levels make cells to be more resistant to apoptosis. HSP, being one of the most immunogenic molecules known, can also act by increasing cellular immunity[11]. Understanding the roles of HSPs in carcinogenesis has important implications regarding tumor behavior and potential prognostics[12].

Up-regulated expression of HSPs has been reported in several cancers (e.g., breast cancer, renal cancer, various leukemias, bladder cancer, *etc.*)[13-16]. The overexpression of HSPs in tumorous tissues has been implicated to have prognostic value in patients with breast, renal, and bladder cancer[13,17]. Recently, we have observed up-regulation of HSP60, HSP70, HSP90, GRP78, and GRP94 in HCCs by proteome analysis[18,19]. Correlation between HSP27 expression and histological grade and survival of patients with HCC has been reported[20]. Tanaka *et al*[21], reported that the expression of GRP94 mRNA increased along with the histological grade of the HCC. However, the prognostic significance of the expression of HSPs in HCC is largely unknown at this time and so further studies are needed. Also, it is not known whether the expression of HSPs play a role in the initiation or progression of HBV-related multistep hepatocarcinogenesis. In the present study, in order to determine whether the expression of the HSPs is involved in HBV-related hepatocarcinogenesis, and if so, to which stage it is linked, we performed immunohistochemical (IHC) and dot immunoblot analyses of HSP27, HSP60, HSP70, HSP90, GRP78, and GRP94 on a series of hepatocellular tumors which includes premalignant lesions.

MATERIALS AND METHODS

Tissue specimen and histopathology

Immediately after hepatectomy for primary HCC, freshly removed liver tissues were serially sliced from top to bottom edge at 4-5 mm intervals and then examined by a pathologist (C.K.P.) for the presence of nodular lesions. Any nodules greater than 10 mm in diameter and any bulging nodules different in color macroscopically, from the surrounding liver, regardless of size, were snap-frozen in liquid nitrogen and stored at -80 ℃. Subsequent sections from the same nodule were fixed in 10% neutral formalin for confirming morphological diagnosis. Early HCC was matched to the diagnostic criteria of Liver Cancer Study Group of Japan[22]. Advanced HCCs were graded histologically according to the Edmondson and Steiner's criteria[23], and DNs were subdivided into LGDN and HGDN by two pathologists (C.K.P. and K.J.), according to the guidelines of the International Working Party[2]. From this, 38 HCCs (early HCC, 5; Edmondson grade I HCC, 12; grade II, 11; grade III, 10 cases) and 19 DNs (LGDN, 11; HGDN, 8) were obtained from 52 Korean patients at Samsung Medical Center between 2000 and 2003. Informed consent was obtained from each patient included in the study. Non-tumorous tissues were taken far from the tumor as possible and were snap-frozen in liquid nitrogen and stored at -80 ℃. Non-tumorous liver revealed cirrhosis in 48 patients and chronic hepatitis in four. None of the patients had any preoperative chemotherapy. All patients were seropositive for hepatitis B surface antigen but had no serum antibody against hepatitis C virus. Among the 38 patients with HCC, 28 were men and 10 were women with a mean age of 50 years (range 25-65 years). Thirteen of the 14 patients with DNs were men and 1 was a woman with a mean age of 52 years (range 33-62 years). The histopathologic features of HCCs examined were histological differentiation, tumor size, tumor capsule formation, microvascular invasion, major portal vein invasion, intrahepatic metastasis, and tumor stage. Microvascular invasion was considered as present when at least one or more endothelial cells or the tunica media of the vessel were recognized to surround a neoplastic cell group. Intrahepatic metastasis and tumor capsule formation were matched to the criteria of the Liver Cancer Study Group of Japan[22]. The tumor stage was determined according to the AJCC cancer staging criteria[24]. Blocks of normal liver were prepared as controls from five patients with metastatic colonic carcinoma of the liver.

Immunohistochemistry

Formalin-fixed, paraffin-embedded tissue including both HCC or DN and adjacent non-tumorous liver were sectioned with 4 μm thickness. IHC study was performed using the streptavidin-biotin complex method and TechMate™ 1 000 automated staining system (DakoChemmate, Glostrup, Denmark). Primary antibodies used and working dilutions employed are as follows: HSP27 mouse monoclonal antibody (mAb) (SPA-800, StressGen, Victoria, Canada) (1∶200), HSP60 mouse mAb (SPA-806, StressGen) (1∶1 500), HSP70 mouse mAb (sc-24, Santa Cruz, San Francisco, USA) (1∶200), HSP90 mouse mAb (sc-13 119, Santa Cruz) (1∶800), GRP78 goat polyclonal antibody (pAb) (sc-1050, Santa Cruz) (1∶30), and GRP94 rat mAb (SPA-850, StressGen) (1∶1 600). Deparaffinized sections were treated with 3% hydrogen peroxide in methanol for 10 min to inhibit endogenous peroxidase. Sections were immersed in 0.05 mol/L citrate buffer (pH 6.0) and heated in a microwave oven for 10 min. Sections were then incubated with the primary antibody for 60 min at room temperature. Each section was treated sequentially with biotinylated secondary antibody (anti-mouse or anti-goat immunoglobulin) and streptavidin–peroxidase complex (DakoChemmate). 3,3'-diaminobenzidine tetrahydrochloride was used as a chromogen, and then Mayer's hematoxylin counterstain was applied. Negative controls (isotype-matched irrelevant antibody or preimmune goat serum as primary antibody) were made to run simultaneously. The results of staining were evaluated by two independent pathologists (C.K.P. and K.J.) and the difference in interpretation was resolved by consensual agreement. At least equally intensive nuclear or cytoplasmic staining

compared with bile ducts was considered positive[25]. Positive cells were counted by monitoring at least 1 000 cells in HCC or DN and non-tumorous liver from more than five high power fields where positive cells were present at a relatively uniform density. For each tissue section, staining was assessed as negative (0-5% positive cells), + (6-25% positive cells), ++ (26-50% positive cells), +++ (51-75% positive cells), or ++++ (>75% positive cells).

Immunoblot analysis

Sample preparation and immunoblot analysis were performed as previously described[19]. All samples were diluted at 2 μg/μL. Protein samples (10-20 μg) were subjected to SDS-PAGE and transferred to polyvinylidene difluoride membrane. Primary antibodies, β-actin mAb (Sigma, St. Louis, USA) (1∶5 000), HSP27 mAb (StressGen) (1∶1 000), HSP60 mAb (StressGen) (1∶10 000), HSP70 mAb (Santa Cruz) (1∶1 000), HSP90 mAb (Santa Cruz) (1∶1 000), GRP78 mAb (Santa Cruz) (1∶500), and GRP94 mAb (StressGen) (1∶1 000) were diluted in PBS/5% skim milk/0.1% Tween 20.

Dot immunoblot analysis

Before performing dot immunoblot analysis, each antibody was validated for specificity by performing immunoblot analysis as described above, using the same tissue protein sample. The linear range of loading volume in the dot immunoblot analysis was tested using serially diluted protein samples. Using Bio Dot™ (Bio-Rad, Hercules, USA), protein samples (0.8-1.6 μg) were loaded onto each well of dot blot apparatus and transferred to polyvinylidene difluoride membrane overnight at 4 ℃. Every protein sample was loaded as a triplicate. Same primary antibodies were used in immunoblot analysis above. The rest of the experimental procedure was the same except that the membrane was washed for 2 h with PBS/0.13% Tween 20. Using the ImageMaster 2D Elite software 4.01 (Amersham, Upsala, UK), the intensity of the dot was determined by integrating the optical density over the spot area (dot volume). The changes in the expression of HSPs were evaluated by dividing the dot volume of tumor by that of non-tumorous tissue.

Statistical analysis

The relationship between the expression of HSPs and hepatocellular tumors including DNs was analyzed by calculating Spearman's correlation coefficient (r). The relationship between the enhancement of expression of HSPs and hepatocellular tumors in dot immunoblot analysis was analyzed by Jonckheere-Terpstra test. Correlation between the expression of HSPs and prognostic factors of HCCs was analyzed by Jonckheere-Terpstra test, Mann-Whitney test, or Kruskal-Wallis test. P values of less than 0.05 were considered statistically significant.

RESULTS

Immunohistochemical analysis

In our earlier proteome analysis, we observed that the expression of many HSPs increased in HCC[18,19]. However, the determination of how the level of expression changes during the stepwise progression of hepatocarcinogenesis was not identified. So, we performed IHC analysis on a series of hepatocellular tumors including DNs. Immunoreactivity for HSP27, HSP60, or HSP90 was observed only in the cytoplasm of hepatocytes of tumor tissues. Immunoreactivity for HSP70 was observed in both the nucleus and cytoplasm, and, in most cases, the intensity of staining of the cytoplasm corresponded to that of the nucleus. Immunoreactivity for GRP78 or GRP94 was observed mostly in the cytoplasm, but was also observed in the nucleus although rarely. The bile duct epithelium always showed cytoplasmic immunoreactivity and thus served as an internal standard of positive staining (Figure 1D). There was a higher expression of HSP27, HSP90, GRP78, or GRP94 in HCC than in the adjacent non-tumorous liver. The expression of HSP60 in HCC was similar to that in the non-tumorous liver. There was either no immunoreactivity or focal staining for HSP70 in the non-tumorous liver (Table 1). Immunoreactivity for HSPs in normal livers was similar to that in the non-tumorous liver. Positive immunoreactivity (>5% positive hepatocytes) for HSP27 was demonstrated in 10.5% of DNs and 76.3% of HCCs. The proportion of positive immunoreactivity was 100% in DNs and 97.4% in HCCs for HSP60, 0% in DNs and 68.4% in HCCs for HSP70, 5.3% in DNs and 55.3% in HCCs for HSP90, 63.2% in DNs and 94.7% in HCCs for GRP78, and 68.4% in DNs and 86.8% in HCCs for GRP94 (Table 1 and Figure 1).

Expression of HSP27, HSP70, HSP90, GRP78, and GRP94 increased along with the stepwise progression of hepatocarcinogenesis (from LGDN to HCC grade III) (Spearman's r = 0.6-0.802). Strong correlation was found only in GRP78 (Spearman's r = 0.802) (Figures 1F-H). Expression of HSP60 decreased along with the stepwise progression of hepatocarcinogenesis (P = 0.012), but with weak correlation (Spearman's r = -0.329) (Table 1).

Dot immunoblot analysis

It was deemed necessary to confirm the IHC results by immunoblot analysis. However, conventional immunoblot analysis involves separation of the tissue proteins by SDS-PAGE and takes much time. Therefore, faster dot immunoblot analysis was used. In dot immunoblot analysis, the expression level of a certain protein can be determined simultaneously from 96 tissue samples using 96 wells on a plate. Smaller amount of protein is needed than by immunoblot analysis (dot immunoblot, 0.8-1.6 μg; immunoblot, 20-30 μg).

Before investigating the expression of six HSPs (HSP27, HSP60, HSP70, HSP90, GRP78, GRP94) in hepatocellular tumors by dot immunoblot analysis, validity of the dot immunoblot method was established by analyzing the expression levels of HSP27, HSP60, and HSP90 in the same tissue protein samples by both the dot immunoblot analysis and the immunoblot analysis. Figure 2 shows that consistent results are obtained by the two methods for all three proteins (P = 0.000, P = 0.034, P = 0.003 for HSP27, HSP60, HSP90, respectively). Figure 2 also shows the reproducibility by the three replicate dot immunoblot analyses of the same sample.

Dot immunoblot analysis showed up-regulation (≥1.5-fold increase) in 15.8% of DNs and 68.4% of HCCs for HSP27, 15.8% of DNs and 52.6% of HCCs for HSP60,

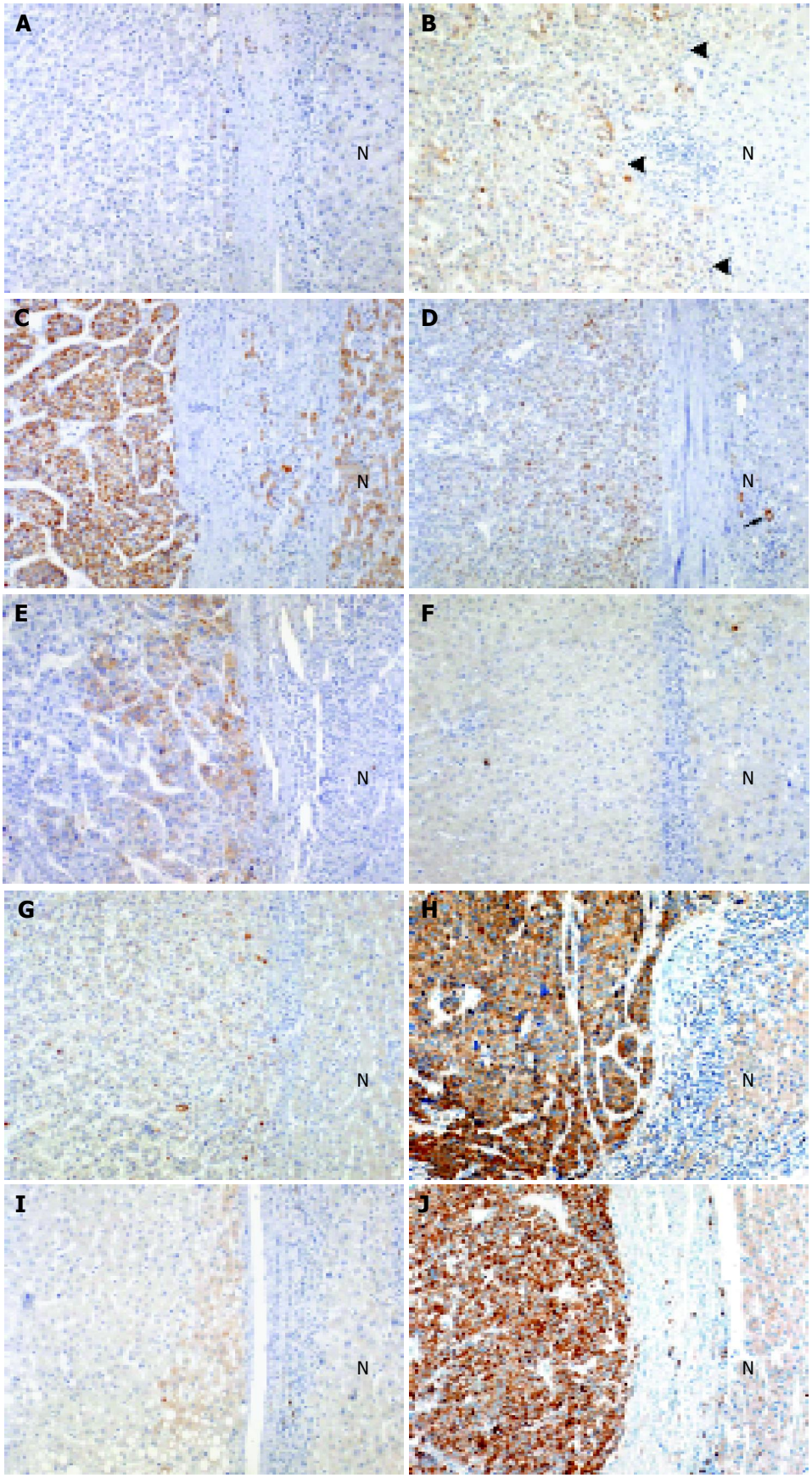

Figure 1 IHC staining of HSP in DN and HCC (original magnification, ×100). A: Rare immunoreactivity for HSP27 in high-grade DN; B: 25% immunoreactivity for HSP27 in early HCC. The borders between early HCC and non-tumorous liver are indicated by arrowheads; C: 96% immunoreactivity for HSP60 in HCC Grade II; D: 30% immunoreactivity for HSP70 in HCC Grade I. Bile duct epithelium shows cytoplasmic immunoreactivity (arrow); E: 35% immunoreactivity for HSP90 in HCC Grade II; F: Rare immunoreactivity for GRP78 in low-grade DN; G: 10% immunoreactivity for GRP78 in HCC Grade I; H: 93% immunoreactivity for GRP78 in HCC Grade III; I: 15% immunoreactivity for GRP94 in high-grade DN; J: 95% immunoreactivity for GRP94 in HCC Grade III. N, non-tumorous liver.

Table 1 Expression of HSPs in hepatocarcinogenesis obtained by IHC analysis

HSP	Percentage of positive cells (%)	NL	DN		HCC				*P* (*r*)
		(*n* = 57)	LGDN (*n* = 11)	HGDN (*n* = 8)	E-HCC (*n* = 5)	GI (*n* = 12)	GII (*n* = 11)	GIII (*n* = 10)	
HSP27	0-5	42	11	6		5	2	2	
	6-25	12		2	4	5	3	3	0.000[1]
	26-50	3					2	1	(0.600)
	51-75				1	1	2		
	>75					1	2	4	
HSP60	0-5							1	
	6-25								0.012[1]
	26-50	3					3		(–0.329)
	51-75	2						1	
	>75	52	11	8	5	12	8	8	
HSP70	0-5	57	11	8	3	4	3	2	
	6-25				1	3	2		0.000[1]
	26-50				1	3	2	1	(0.669)
	51-75					1	2	4	
	>75					1	2	3	
HSP90	0-5	56	10	8	5	8	4		
	6-25	1	1			2	4	3	0.000[1]
	26-50					2	2	3	(0.707)
	51-75						1	2	
	>75							2	
GRP78	0-5	5	6	1	1	1			
	6-25	22	5	7	4	5	2		0.000[1]
	26-50	17				1	2	1	(0.802)
	51-75	9				3	2	1	
	>75	4				2	5	8	
GRP94	0-5	16	3	3	2	1	2		
	6-25	26	7	4	2	2			0.000[1]
	26-50	9	1	1	1	7	3	2	(0.708)
	51-75	3				1	3		
	>75	3				1	3	8	

NL: non-tumorous liver; DN: dysplastic nodule; LGDN: low-grade DN; HGDN: high-grade DN; HCC: hepatocellular carcinoma; E: early; G: Edmondson-Steiner's grade; *n*: number of cases; *P*: Spearman correlation (from LGDN to HCC GIII); (*r*): Correlation coefficient; [1]Value was statistically significant.

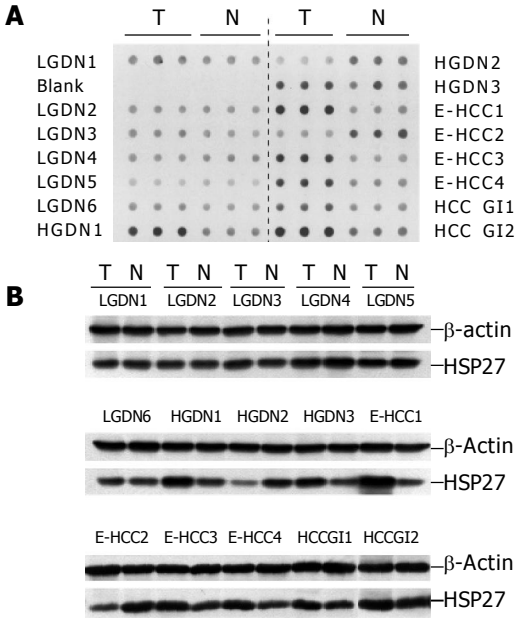

Figure 2 Examples of dot immunoblot and immunoblot analysis of HSP27 in the same tissue samples. A: Dot immunoblot analysis of HSP27 in tumor (T) and non-tumorous tissue (N) of 15 patients (LGDN, six; HGDN, three; E-HCC, four; HCC GI, two cases); B: Immunoblot analysis of HSP27 in tumor and non-tumorous tissue of 15 patients. Dot immunoblotting and immunoblotting with HSP27 monoclonal antibody were performed as described in Materials and Methods. β-actin was used as a reference. The results of dot immunoblot analysis revealed the same expression patterns as the immunoblot analysis. LGDN: low-grade dysplastic nodule, HGDN: high-grade dysplastic nodule, E: early, HCC: hepatocellular carcinoma, G: Edmondson-Steiner's grade.

31.6% of DNs and 65.8% of HCCs for HSP70, 42.1% of DNs and 73.7% of HCCs for HSP90, 52.6% of DNs and 71.1% of HCCs for GRP78, and 57.9% of DNs and 65.8% of HCCs for GRP94 (Table 2).

Expression of HSP27 increased along with the stepwise progression of hepatocarcinogenesis (Spearman's $r = 0.407$). Expression of HSP60, HSP90, and GRP78 increased likewise ($P<0.05$), but the correlation was weak (Spearman's $r<0.4$) (Table 2). There was a significant correlation between the expression levels of HSP27, HSP60, HSP90, and GRP78 and hepatocellular tumors including DNs ($P = 0.018$, $P = 0.004$, $P = 0.021$, $P = 0.005$, respectively) (Table 3).

Correlation between the expression of HSPs and prognostic factors of HCC

IHC analysis showed a positive correlation between the expression of GRP78 or GRP94 and poorer histological grade of differentiation, tumor size of >2 cm, lack of tumor capsule, microvascular invasion, major portal vein invasion, intrahepatic metastasis, and higher tumor stage. Expression of HSP90 was significantly associated with six prognostic factors except for tumor size. Expression of HSP70 was significantly correlated with poorer histological grade of differentiation, lack of tumor capsule, and microvascular invasion. Specifically, there was a significant correlation between the expression of GRP78, GRP94, or HSP90 and microvascular invasion, major portal vein invasion, and intrahepatic metastasis (Table 4).

Table 2 Expression of HSPs in hepatocarcinogenesis obtained by dot immunoblot analysis

HSP	Tumor/ non-tumor	DN		HCC				*P* (*r*)
		LGDN (*n* = 11)	HGDN (*n* = 8)	E-HCC (*n* = 5)	GI (*n* = 12)	GII (*n* = 11)	GIII (*n* = 10)	
HSP27	< 0.5		1	1				
	0.5–1.5	9	6		3	4	4	0.002[1]
	1.5–2.5	2	1	2	6	2	2	(0.407)
	> 2.5			2	3	5	4	
HSP60	< 0.5				1			
	0.5–1.5	10	6	4	4	5	4	0.004[1]
	1.5–2.5	1	2	1	4	6	4	(0.372)
	> 2.5				3		2	
HSP70	< 0.5							
	0.5–1.5	6	7	3	3	2	5	0.100
	1.5–2.5	5		2	4	8	4	(0.220)
	> 2.5		1		5	1	1	
HSP90	< 0.5			1				
	0.5–1.5	5	6	3	2	2	2	0.007[1]
	1.5–2.5	6	2	1	4	7	6	(0.353)
	> 2.5				6	2	2	
GRP78	< 0.5		1				1	
	0.5–1.5	5	3	3	2	3	2	0.007[1]
	1.5–2.5	5	4	1	4	3		(0.355)
	> 2.5	1		1	6	5	7	
GRP94	< 0.5						1	
	0.5–1.5	7	1	2	1	5	4	0.571
	1.5–2.5	2	6	3	5	4	1	(0.077)
	> 2.5	2	1		6	2	4	

DN: dysplastic nodule; LGDN: low-grade DN; HGDN: high-grade DN; HCC: hepatocellular carcinoma; E: early; G: Edmondson-Steiner's grade; *n*: number of cases; *P*: Spearman Correlation; (*r*): Correlation coefficient; [1]Value was statistically significant.

DISCUSSION

The present study confirms the results from earlier proteomic analysis and shows that HSPs are frequently up-regulated in HCCs. Up-regulation of HSPs in various cancers suggest that they might be involved in tumorigenesis[8]. Enhancement of tumorigenesis by overexpression of HSP27 and HSP70 has been implicated in a rodent model[26-29]. HSPs are known to be essential for the survival of cancer cells in different cancers[16,28,29]. HSPs as molecular chaperones might sustain cancer cells by modulating the activity of different proteins involved in cell cycle and apoptosis. In fact many HSPs are known to regulate apoptosis and even prevent apoptosis induced by anticancer drugs[8]. For example, there has been an implication that HSP27 prevents depolymerization of F-actin by cytochalasin D and subsequent cytochrome C release[30]. HSP70 helps to maintain the genomic stability of telomerase[31]. HSP90 plays a role in breast and prostate cancer by maintaining the functional quality of proteins involved in the progression of cancer[31,32]. Schamhart *et al*[33], reported that HCC cell lines respond to heat stress with a transient increase in the synthesis of HSPs (molecular weights of 107, 89, 70, 68, and 27 ku). Up-regulation of HSP27 and HSP70 in HCCs in microarray studies has been reported[34,35]. Takashima *et al*[36], reported that HSP70,

Table 3 Enhancement of HSP expression relative to non-tumorous tissue in hepatocarcinogenesis obtained by dot immunoblot analysis

Group	*n*	Enhancement of expression (mean±SD)					
		HSP27	HSP60	HSP70	HSP90	GRP78	GRP94
LGDN	11	1.25±0.31	1.07±0.43	1.37±0.45	1.55±0.45	1.65±0.85	1.61±1.02
HGDN	8	1.22±0.72	1.15±0.44	1.32±0.58	1.20±0.32	1.42±0.71	2.45±1.87
E-HCC	5	2.52±1.57	0.93±0.41	1.18±0.49	1.04±0.64	1.72±1.16	1.63±0.54
HCCGI	12	2.17±1.06	1.96±1.20	2.69±1.72	2.76±1.70	7.41±8.65	13.70±25.68
HCCGII	11	3.26±3.19	1.58±0.41	1.80±0.55	1.95±0.56	2.65±1.30	1.87±0.90
HCCGIII	10	2.68±2.34	2.14±1.58	1.66±0.84	2.22±1.33	4.54±4.79	3.02±2.70
P		0.018[1]	0.004[1]	0.123	0.021[1]	0.005[1]	0.373

LGDN: low-grade dysplastic nodule; HGDN: high-grade dysplastic nodule; E: early; HCC: hepatocellular carcinoma; G: Edmondson-Steiner's grade; *n*: number of cases; SD: standard deviation; *P*: Jonckheere-Terpstra test; [1]Value was statistically significant.

Table 4 Correlation between HSP immunostaining and prognostic factors in 38 HCCs

	P					
	HSP27	HSP60	HSP70	HSP90	GRP78	GRP94
Histological grade						
E–HCC (5)						
HCC GI (12)	0.150	0.085	0.009[1]	0.000[1]	0.000[1]	0.000[1]
HCC GII (11)						
HCC GIII (10)						
Tumor size						
≤2 cm (10)	0.097	0.158	0.077	0.079	0.000[1]	0.019[1]
>2 cm (28)						
Encapsulation						
E-HCC (5)						
Positive (27)	0.434	0.634	0.049[1]	0.001[1]	0.011[1]	0.005[1]
Negative (6)						
Microvascular invasion						
Positive (16)	0.413	0.339	0.042[1]	0.000[1]	0.002[1]	0.000[1]
Negative (22)						
Major portal vein invasion						
Positive (3)	0.271	0.489	0.739	0.012[1]	0.050[1]	0.028[1]
Negative (35)						
Intrahepatic metastasis						
Positive (7)	0.798	0.189	0.158	0.000[1]	0.010[1]	0.050[1]
Negative (31)						
Tumor stage						
I (25)						
II (10)	0.800	0.816	0.128	0.000[1]	0.002[1]	0.000[1]
III (3)						

E: early; G: Edmondson-Steiner's grade; ( ): number of cases; *P*: Jonckheere-Terpstra test, Mann–Whitney test, or Kruskal–Wallis test; [1]Value was statistically significant.

HSP70.1, GRP75, and GRP78 were simultaneously up-regulated in hepatitis C virus-related HCCs in proteomic and immunoblot analysis. However, it has not yet been known whether the expression of HSPs is involved in HBV-related multistep hepatocarcinogenesis. We performed IHC and dot immunoblot analyses of HSP27, HSP60, HSP70, HSP90, GRP78, and GRP94 on a series of hepatocellular tumors including premalignant lesions. IHC analysis is used often in clinical studies to investigate the expression of specific proteins. However, immunohistochemistry is inadequate for a quantitative comparison of protein expression in both non-tumorous and tumorous tissues. Thus we performed dot immunoblot analysis which is suitable for quantification of protein expression in multiple samples, in order to supplement the IHC results. Both immunohistochemistry and dot immunoblot analysis showed an overall increase in HSP27, HSP90, and GRP78 correlating with the stepwise progression of hepatocarcinogenesis ($P<0.05$). Dot immunoblot analysis enabled us to quantitatively compare the level of protein expression at various stages of hepatocellular tumor (Table 3).

In IHC analysis, we showed that HSP27, HSP70, HSP90, GRP78, and GRP94 were expressed increasingly correlating with the stepwise progression of HBV-related hepatocarcinogenesis. Strong correlation was found only in GRP78 (Spearman's $r = 0.802$). Moderate correlation (Spearman's $r = 0.4$-0.75) was found in HSP27, HSP70, HSP90, and GRP94. It is possible that HSP expression increases as a result of tumorigenesis, due to stimulation of HSPs by a stressful environment. However, the role of HSPs involved in hepatocarcinogenesis is unknown, at this time. Correlation between HSP27 expression and histological grade in HCC has been reported[20]. Chuma *et al*[25], reported that HSP70 expression gradually increased according to the stepwise progression of hepatocarcinogenesis, which is consistent with the result of this study. Shuda *et al*[37], reported that mRNA and protein expression of GRP78 was up-regulated as the histological grade of HCC increased and that its expression could be regulated by endoplasmic reticulum stress response elements. Tanaka *et al*[21], reported that the expression of GRP94 mRNA increased as the histological grade of HCC increased. To our knowledge, this is the first report of the correlations between the expression of HSP27, HSP90, GRP78, and GRP94 and hepatocellular tumors including premalignant lesions. In this study, GRP78 and GRP94 were commonly up-regulated in DN, although their expression levels were not that high. We think that GRP78 and GRP94 might play a role in the early stage of HBV-related hepatocarcinogenesis.

Progression of HCCs often leads to vascular invasion and intrahepatic metastasis. A tumor capsule may act as a barricade preventing the spread of cancer cells and may have a positive role in the prognosis of HCC[38]. There was a positive correlation between the expression of GRP78, GRP94, HSP90, or HSP70 and prognostic factors of HBV-related HCC, which implies that the expression of these HSPs is closely correlated with tumor progression and aggressive behavior of HCC. In this study, there was no relation between the HSP27 expression and prognostic factors of HBV-related HCC. This is at variance with a previous report by King *et al*[20], who found correlation between HSP27 expression and histological grade of HCC in cases with high rate of HBV infection. The relation between histological grade and HSP27 expression in HBV-related HCC needs further analysis. To our knowledge, this is the first report of the correlation between the expression of GRP78, GRP94, HSP90, and HSP70 and prognostic factors of HCC. Although the implication of the overexpression of GRP78, GRP94, and HSP90 in HCCs is not clear at present, results of this study suggest that the above HSPs could be important prognostic markers of HBV-related HCC, which strongly suggests the presence of vascular invasion and intrahepatic metastasis.

In conclusion, the expressions of HSPs are commonly up-regulated in HCCs and their expression patterns tend to be closely associated with stepwise progression of HBV-related hepatocarcinogenesis. GRP78 might play an important role in the stepwise progression of HBV-related hepatocarcinogenesis. Expression of GRP78, GRP94, HSP90, or HSP70 is closely correlated with tumor progression and aggressive behavior of HBV-related HCC. Specifically, GRP78, GRP94, and HSP90 may be important prognostic markers which strongly suggest the presence of vascular invasion and intrahepatic metastasis.

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