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Full Length Research Paper

Significance of clinicopathology and expression of heat shock protein 72 and glycoprotein 96 in human hepatocellular carcinomas

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Heat shock protein 72 (HSP72) and glycoprotein 96 (gp96) are highly expressed in cancer tissues. Recent studies indicate the possible roles of HSP72 and gp96 in the development and progression of gastrointestinal carcinomas, but detailed information is still ambiguous. The aim of the study is to investigate the correlation between clinicopathology and immunolocalization of HSP72 and gp96 in human hepatocellular carcinoma. The expression of HSP72 and gp96 was studied in human hepatocellular carcinomas with or without metastasis as well as in tissues adjacent to cancer by way of immunohistochemistry. Messenger ribonucleic acid (RNA)-gene expression levels of HSP72 and gp96 were determined by quantitative real-time real-time reverse transcriptase polymerase chain reaction (RT-PCR) after mRNA extraction. The expression of HSP72 and gp96 has a correlation with the differentiation of hepatocellular carcinoma. HSP72 and gp96 expression in hepatocellular carcinomas with lymph node and organ metastasis was significantly higher than those with non-metastasis. The results indicate that there exists a significant correlation between the expression of HSP72 and gp96 and the progression of hepatocellular carcinomas. HSP72 and gp96 expression were significantly associated with the presence of tumor infiltration, lymph node and remote metastasis. The expression characters of HSP72 and gp96 in tumors may contribute to study the pathogenesis and progression of hepatocellular carcinoma.

Key words: Heat shock protein 72(HSP72), glycoprotein 96(gp96), hepatocellular carcinoma, clinicopathology, prognosis.

INTRODUCTION

The heat shock protein (HSP) family is a highly conserved group of cellular proteins and is up-regulated under stress conditions, such as heat, hypoxia, serum deprivation, neoplasia and virus infection (Argon and Simen, 1990; Morimoto, 1993; Schlesinger, 1990). It functions as molecular chaperone and biochemical regulator to mediate cell growth, apoptosis, protein homeostasis and cellular targets of peptides (Morimoto, 1993). Aside from their response to heat shock and chemical or physical stress stimuli, HSPs have been reported to be overexpressed in a wide range of human tumors including breast, endometrial, ovarian, colon, lung and prostate (Ciocca and Calderwood, 2005). Studies have also shown that HSP expression have a close

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Abbreviations: HSP, Heat shock protein; **PBS,** phosphate buffered saline; **DAB,** 3,3'-diaminobenzedine solution; **cDNA,** complementary deoxyribonucleic acid; **RNA,** messenger ribonucleic acid; **RT-PCR,** polymerase chain reaction; **CT,** concentration x time; **CTL,** cytotoxic T lymphocyte.

relationship with carcinoma prognosis (Ciocca and Calderwood, 2005; Lebret et al., 2003). They may combine with oncogene products to form complexes and transport them into intracellular special sites and promote cancer cell proliferation and heterogeneous differentiation (Dorsey and Tchounwou, 2003; Villaseca et al., 1997). Recent studies have also shown that, HSP72 and gp96 are highly expressed in cancer tissues and have been used as prognostic markers in some tumors (Bausero et al., 2004; Gabai et al., 2005; Wang et al., 2002; Wang et al., 2007b; Wang et al., 2008).

Study indicates the possible roles of HSP72 and gp96 in the development and progression of gastrointestinal carcinomas but detailed information is still ambiguous (Maehara et al., 2000). Hepatocellular carcinoma is one of the most malignant cancers, and there may be a correlation between the progression of hepatocellular carcinoma and over-expression of HSP72 and gp96, but now, there are few reports about expression of HSP72 and gp96 in hepatocellular carcinoma. The present study aims at estimating the extent of the expression of HSP72 and gp96 proteins in tumoral specimens obtained from liver cancer patients. The study also aims at evaluating the association between the extent of expression of HSP and various clinicopathological parameters, tumor proliferative capacity. The results showed that there exists a significant correlation between the expression of HSP72 and gp96 and the progression in hepatocellular carcinoma.

MATERIALS AND METHODS

Immunochemistry reagents

Mouse anti-human HSP72 monoclonal antibody was obtained from StressGen Biotechnologies (Victoria, British Columbia, Canada) and mouse anti-human gp96 monoclonal antibody was purchased from Santa Cruz Biotechnology, Inc (Santa Cruz, CA, USA). EnVisionTM kits were purchased from Dako Corp (Carpinteria, CA, USA).

Tissue samples

This investigation was approved by the Ethics Committee on Human Study at Shaanxi University of Chinese Medicine (2004-4B). Paraffin specimens of primary hepatocellular carcinoma from 140 patients undergoing liver resection were collected from the affiliated Hospital, Shaanxi University of Chinese Medicine, Xianyang, China between 2000 to 2008. None of the patients received any kind of anti-cancer treatment or other therapies prior to surgery. The patients consisted of 96 males and 44 females, with a mean age of 54.5 ± 5.6 years, ranging from 28 to 80 years. Routine pathological diagnosis showed that all cases were primary hepatocellular carcinoma. Tumors were categorized as: nodular type in 78 (55.7%), massive type in 37 (26.4%), and diffuse type in 25 (17.9%) out of 140 cases. 57 cases were well-differentiated type and 83 cases were poorly differentiated. Among the cases, 92 cases had regional lymph node metastases, and 54 cases had remote metastases. The specimens were fixed in 10% buffered formalin and embedded in paraffin. Serial sections, 5-µm-thick, were cut and placed on poly-lysine coated glass slides.

Immunohistochemical staining methods

All sections were deparaffinized and rehydrated with graded alcohols. Endogenous peroxidase was then blocked with 3 mL/L H_2O_2 diluted in methanol for 30 min at room temperature. Antigen retrieval was performed by treating the slides in citrate buffer in a microwave for 10 min. The slides were incubated in a moist chamber with HSP72 mouse monoclonal antibody (1:100) and gp96 mouse monoclonal antibody (1:100) at 4°C overnight. After a complete wash in phosphate buffered saline (PBS), the slides were incubated with horseradish peroxidase labeled goat anti-mouse antibody (1:100) for 45 min at 37°C. After a complete wash in PBS, the slides were developed in 0.5 g/L freshly prepared 3,3'-diaminobenzedine solution (DAB) (Sigma Co, St.Louis, Mo, USA) for 8 min, and then counterstained with hematoxylin, dehydrated, air dried, and mounted. Normal mouse IgG was used to substitute for the primary antibody as a negative control. No specific immuno-reactivity was detected in these tissue sections. Two of the authors initially determined the fields simultaneously using a double-headed light microscope. The evaluation of HSP72 and gp96 positive cells was performed on high-power fields (× 400) using a standard light microscope. Only distinctive intranuclear or intra-cytoplasm immunoreactivity was considered positive. In each case, more than 1000 cells were counted and the percentage of immunoreactivity was independently determined. When interobserver differences were greater than 5%, the immunostained slides were re-examined simultaneously using a using a double-headed light microscope and the percentage of positive cells were determined. When interobserver differences were less than 5%, the mean value was obtained as the positive rate.

Quantitative real-time reverse transcriptase polymerase chain reaction (RT-PCR)

Microdissection, RNA extraction, complementary deoxyribonucleic acid (cDNA) synthesis and RT-PCR were performed as described previously (Specht et al., 2001). Following short hematoxylin staining, a minimum of 5000 cells of defined carcinoma areas were scraped off the glass slides with a sterile blade under light microscopic control. Hemorrhagic or necrotic areas were excluded. The microdissected tumor tissues and tissues adjacent to tumor were transferred into a sterile 1.5-ml tube containing RNA lysis buffer. Lysis was carried out at 60°C for 24 h until the tissue was completely solubilized. Real-time PCR was performed with the Applied Biosystems Prism 7900 SDS instrument using a qPCR SYBR Green Core Kit (Eurogentec) according to the manufacturer's instructions. RNA was extracted from the tissues using TRIzol (Invitrogen/Life Technologies) and made into cDNA using the

SuperScript III Reverse Transcriptase (Invitrogen/Life Technologies). Primer pairs were designed using the Primer Premier 5.0 program. The primer pairs spanning one intron were as follows: beta-2-microglobulin (F: 5'-TCCGACATGTCTC-GCTCCGTGCA-3', R: 5'-GATGCTCTAGCTCTGTACATTATC-3'), HSP72 (F: 5′-TCCTCAATGGCCAAAGCCGCGAC-3′, R: 5′-CCAC-CTCTCCTCCATCTAATCAG-3′), gp96 (F: 5′-CAAA-CTATGAGGGCCCTGAC-3′, R: 5′-GCAGCTTTTCTACTTAACTC-3′). Reactions were performed in a total volume of 10 µl, including 5 µl of SYBR Green Supermix (BioRad Laboratories, Ltd), 0.2 µl of each primer of a 10 µM stock, 0.5 µl of the reverse transcribed cDNA template and 4.3 µl ultrapure grade water. The cycling conditions were as follows: predenaturation (95°C for 10 min) and PCR amplification (45 cycles of 95°C for 15 s, 60°C for 1 min). All reactions were carried out in triplicate for every sample. The standard template cDNA and two reactions of negative control were repeated on every plate. Beta-2-microglobulin was used for normalization, and relative quantification was analyzed using BioRad iQ5 Optical System Software (BioRad Laboratories, Ltd).

Table 1. Relationship between immunoreactivity of HSP72 and gp96 and clinical features of patients with hepatocellular carcinomas.

Variables	n	HSP72 (%)	gp96 (%)
Age (yrs)[a]			
< 60	85	64(45.7)	62(44.2)
> 60	55	68(48.6)	60(42.9)
Gender[b]			
Male	96	92(65.7)	89(63.6)
Female	44	40(28.6)	33(23.6)
Tumor type[c]			
Nodular	78	75(53.6)	71(50.7)
Massive	37	34(24.3)	31(22.1)
Diffuse	25	23(16.4)	20(14.3)
Lymph node metastasis[d]			
Presence	92	92(65.7)	92(65.7)
Absence	48	40(28.6)	30(21.4)
Remote metastasis[e]			
Presence	54	54(38.6)	54(38.6)
Absence	86	78(55.7)	68(48.6)

[a]P >0.05, <60 years groups versus >60 years groups; [b]P <0.05, male groups versus female groups; [c]P <0.05, nodular groups versus massive and diffuse groups; [d]P <0.05, [e]P <0.05, metastasis groups versus non-metastasis groups.

All results are presented as means ± SD. Relative expression levels of target genes were determined by the relative standard curve method. Based on the concentration x time (CT) value and the corresponding standard curve, the mRNA quantity of each sample was calculated by determining the ratio between the amounts of the gene of interest and beta-2-microglobulin.

Statistical analysis

HSP72 and gp96 expression differences between hepatocellular carcinomas and tissues adjacent to cancer were analyzed statistically using u test. The relationship between expression of HSP72 and gp96 in hepatocellular carcinoma tissue with or without metastasis was analyzed statistically using χ^2 test. P <0.05 was considered statistically significant.

RESULTS

Immunolocalization of HSP72 and gp96 in hepatocellular carcinomas and adjacent tissues to cancer

The results of immunohistochemistry of HSP72 and gp96 were summarized in Table 1. HSP72 immunoreactivities were detected in 132 of 140 primary tumors (94.3%) and in 26 of 140 mucous membranes adjacent to cancers (18.6%). Gp96 detected in hepatocellular carcinoma and in mucous membrane adjacent to cancer was 87.1 and 16.4% respectively. Owing to the heterogeneous in hepatocellular carcinoma, HSP72 and gp96 expressed differently in cell plasma and nuclei. HSP72 was mainly stained in the nuclei of cancer cells and gp96 proteins were mainly presenting a cytoplasmic and nuclei pattern of staining. Representative immunostainings for HSP72 and gp96 are illustrated in Figure 1. HSP72 and gp96 positive rates in hepatocellular carcinoma groups were significantly higher than that in adjacent tissues to cancer (P <0.05).

Relationship between clinicopathology and expression of HSP72, gp96 in hepatocellular carcinomas

Results showed that HSP72 and gp96 expressed higher in low differentiation of hepatocellular carcinomas than that in tissues adjacent to cancers (P <0.05). HSP72 and gp96 positive rates in lymph node metastasis and remote metastasis groups were 100%. There were significant differences of HSP72 and gp96 expression between metastasis groups and non-metastasis groups (P <0.05).

To assess if HSPs bear a pronounced prognostic effect in patient subgroups, we conducted an extensive analysis of HSP72 and gp96 protein expressions. We stratified by tumor grading (poorly versus well differentiated), nodal status (absence versus presence of lymph node

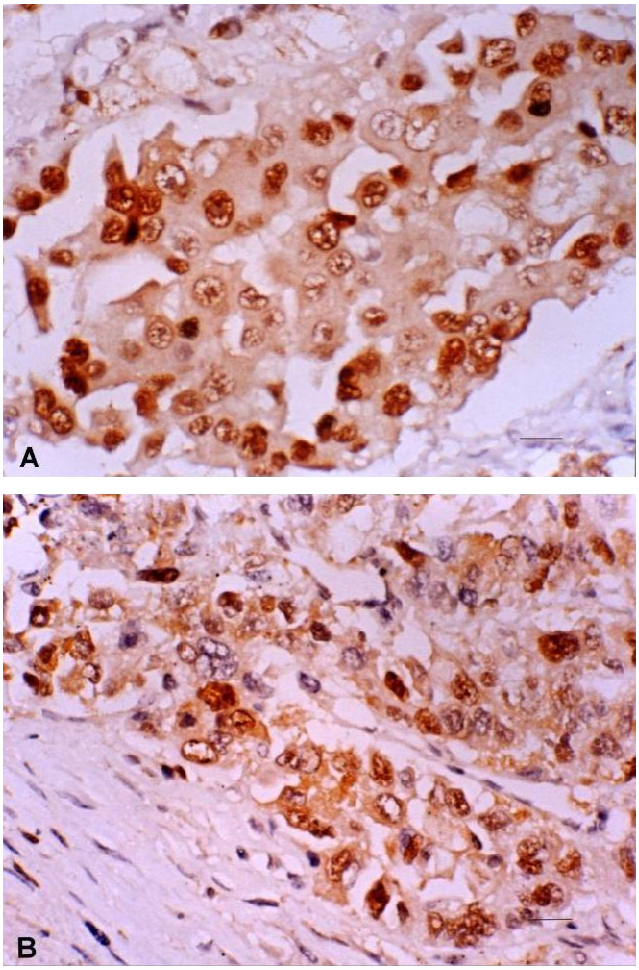

Figure 1. Immunohistochemistry for HSP72 and gp96 in hepatocellular carcinoma cells (counterstained with hematoxylin), bars 40 µm. (A) Distinctive nuclei immunoreactivity was detected for HSP72 in hepatocellular carcinoma cells; (B) gp96 immunostained in cancer cell cytoplasm, and partially stained in nuclei of cancer cells.

metastases), presence of organ metastases and histo-pathological type (nodular and massive versus diffuse). In cross-tables, HSP72 and gp96 expression were signifi-cantly associated with the presence of organ metastases and lymph node positivity (Table 2). The expression of HSP72 and gp96 has a correlation with the differentiation of hepatocellular carcinoma. These results suggest that, there exists a significant correlation between expression of both HSP72 and gp96 and progression of hepatocellular carcinomas.

Gene Expression of HSP72 and gp96 mRNA in hepatocellular carcinomas

According to the selection criteria mentioned above, mRNA analyis was performed on tissue of 140 tumors and tissues adjacent to cancer. Quantitative real time-RT-PCR analysis showed that, HSP72 and gp96 mRNA in both cancer and normal tissues were detectable. There was a significant, positive correlation between the relative gene expression levels of HSP72 and gp96 ($p = 0.001$; $r = 0.421$). Table 3 showed that, expression levels of HSP72 and gp96 mRNA in tumors with advanced stages were higher than in early tumor stages and adjacent tissues to cancer ($p<0.01$). Furthermore, significant higher HSP72 and gp96 mRNA levels were found in the poorly differentiated tumors as compared to well differentiated tumors ($p <0.01$).

Correlation of mRNA-gene expression levels and immunohistochemistry

HSP72 and gp96 mRNA expression showed a significant, positive correlation with HSP72 and gp96 protein-

Table 2. Relationship between clinicopathology and immunoreactivity of HSP72 and gp96 in hepatocellular carcinomas.

Pathologic types	n	HSP72		gp96	
		- (%)	+ (%)	- (%)	+ (%)
Tissues adjacent to cancers	140	114 (81.4)	26 (18.6)	117 (83.6)	23 (16.4)
Hepatocellular carcinomas[a]	140	8 (5.7)	132 (94.3)	18 (12.9)	122 (87.1)
Tumor Differentiation[b]					
Well differentiated	57	8 (14.0)	49 (86.0)	18 (31.6)	39 (68.4)
Poorly differentiated	83	0 (0)	83 (100)	0 (0)	83 (100)
Lymph node metastasis[c]					
yes	92	0 (0)	92 (100)	0 (0)	92 (100)
no	48	14(29.2)	26 (54.2)	7 (14.6)	23 (47.9)
Remote metastasis[d]					
yes	54	0 (0)	54 (100)	0 (0)	54 (100)
no	86	13(15.1)	65 (75.6)	8(9.3)	60 (69.8)

[a]P <0.05, [b]P <0.05, versus tissues adjacent to cancers; [c]P <0.05, [d]P <0.05, versus non-metastasis groups.

Table 3. Relative expression levels of HSP72 and gp96 gene expression and clinicopathologic features (means ± SD).

Group	Cases	HSP72/β-2-microglobulin	gp96/β-2-microglobulin
Tissue			
Adjacent tissues to cancer	140	0.54 ± 0.32	0.46 ± 0.51
Carcinoma[a]	140	1.42 ± 0.53	1.26 ± 0.48
Differentiation			
Well differentiation	57	0.73 ± 0.59	0.68 ± 0.46
Poorly differentiation[b]	83	1.64 ± 0.45	1.38 ± 0.37
Metastesis			
Lymph node metastasis	92	0.92 ± 0.35	0.83 ± 0.26
Remote metastasis[c]	54	1.87 ± 0.52	1.65 ± 0.39

[a]P < 0.01, versus tissues adjacent to cancer; [b]P <0.01, versus well differentiation; [c]P <0.01, versus lymph node metastasis.

expression evaluated by immunohistochemistry (p =0.01). Particularly, patients with a high HSP72 and gp96 protein expression rate showed significant higher mRNA-levels than patients with low or moderate gene expression levels (p <0.01) (Table 4).

DISCUSSION

In this study, we examined the expressions of HSP72 and gp96 in 140 hepatocellular carcinoma samples by immunohistochemistry. The results showed that almost all of the detected hepatocellular carcinomas expressed HSP72, and majority of tumors expressed gp96, which had significant differences compared with that in tissues adjacent to cancers. By way of immunohistochemistry and quantitative real-time RT-PCR, we found that there was a definite correlation between expression of both HSP72 and gp96 and development of hepatocellular carcinomas.

The HSP family is group of highly conserved proteins synthesized after heat induction or other stressors (Argon and Simen, 1990; Morimoto, 1993; Schlesinger, 1990). In mammalian cells, this system is divided into two predominant categories, which appear to be structurally and functionally related: the HSPs and the glycoproteins (gps) (Schlesinger, 1990). During the growth and development of normal cells, HSP70 is constitutively expressed at low levels but the expression was dramatically enhanced by stressful conditions (Morimoto, 1993). Heat shock protein 72, belonging to the family of HSP70, is a highly conserved protein synthesized under

Table 4. Correlation between HSP72 and gp96 gene expression and immunohistochemistry cases staining gene-expression level (mean ± SD).

Group	Cases	HSP72/β-2-microglobulin	Cases	gp96/β-2-microglobulin
Tissue				
Adjacent tissues to cancer	26	0.63 ± 0.52	23	0.52 ± 0.37
Carcinoma[a]	132	1.37 ± 0.43	122	1.18 ± 0.42
Differentiation				
Well differentiation	49	0.75 ± 0.54	39	0.59 ± 0.41
Poorly differentiation[b]	83	1.64 ± 0.45	83	1.38 ± 0.37
Metastesis				
Lymph node metastasis	92	0.92 ± 0.35	92	0.83 ± 0.26
Remote metastasis[c]	54	1.87 ± 0.52	54	1.65 ± 0.39

[a]P <0.01, versus tissues adjacent to cancer; [b]P <0.01, versus well differentiation; [c]P <0.01, versus lymph node metastasis.

various stresses. In non-transformed cells at normal conditions, Hsp72 is expressed at very low levels. It is, however, present at elevated levels in the major fraction of tumors and in many transformed cell lines (López-Cotarelo et al., 2000; Kato et al., 2000; Volloch and Sherman, 1999). It is commonly assumed that, in tumor cells the expression of HSP72 at elevated levels is the consequence of oncogenic transformation and enhanced expression of HSP72 has a close relationship with epithelial carcinoma cells growth (Hwang et al., 2003; López-Cotarelo et al., 2000; Volloch and Sherman, 1999). Up-regulated expression of the HSP70 family in tumor cells may be a requirement to serve as molecular chaperones in regulating and stabilizing oncofetal protein and mutant oncogene products during tumor growth process (Hwang et al., 2003; Lee, 2001; Volloch and Sherman, 1999). In normal cells, gp96 expression could also be induced by various stresses to function as molecular chaperones (Lee, 2001; Linderoth et al., 2001). Some researchers have implied that enhanced expression of gp96 has a close relationship with cancer cells growth (Wang et al., 2007b; Wang et al., 2008). High level expression of gp96 could contribute to tumorigenicity of certain tumors (Fu and Lee, 2006; Wang et al., 2008). Recent studies have shown that HSP72 and gp96 are highly expressed in cancer tissues and have been used as prognostic markers in some tumors, such as gastric cancers, colonic tumors, breast cancers, lung cancers and so on, which have also been verified to be associated with the development and progression of the above-mentioned carcinomas (Bausero et al., 2004; Gabai et al., 2005; Wang et al., 2002; Wang et al., 2007b; Wang et al., 2008). However, few reports have studied the expression of gp96 in hepatocellular carcinomas, especially during the course of tumor growth and differentiation, in simultaneous comparison with HSP72. In this experiment, we found that HSP72 and gp96 were highly expressed when hepatocellular carcinomas progressed, but their roles in hepatocellular carcinoma are not clear. It is reasonable to propose that HSP72 and gp96 up-regulation in these tumor cells are closely related with tumor cell survival and proliferation. Recent studies have suggested that, HSPs take part in cell growth and proliferation in several ways such as signal transduction and cell cycle regulation through combining certain proto-oncogene products. This indicates that these proliferating cells need higher level of HSPs to maintain the stability of tumor proteome (Fisher et al., 2000; Liu et al., 1999). It is believed that tumor cells are a group of highly proliferative heterogeneous cells which progress gradually through mutant oncogene products (King et al., 2001; Renan, 1990). Continuous expression of HSP in tumor cells may be required to serve as molecular chaperones in regulating and stabilizing these products during tumor growth process. At the same time, the existence of mutant or oncogene products may stimulate HSP synthesis (Dorsey and Tchounwou, 2003; Villaseca et al., 1997).

The present study further supports the clinical significance of HSP72 and gp96 expression in the progression of hepatocellular carcinoma. In the study, HSP72 and gp96 expression were found to be associated with important clinicopathological characteristics for patients' management. Consistently, HSP72 and gp96 expression were significantly associated with the presence of tumor size, lymph node and organ metastasis. Our results showed that, not only the expression of both HSP72 and gp96 in hepatocellular carcinoma was higher than that in tissues adjacent to cancer, but also the expression of both HSP72 and gp96 in hepatocellular carcinomas with metastasis was definitely higher than that of hepatocellular carcinomas without metastasis. The expression of both HSP72 and gp96 in hepatocellular carcinoma was related to the differentiated tissue type of hepatocellular cancer. The results indicate that up-regulation of HSP72 and gp96 is likely to have some

relationship with progression, invasion and metastasis of hepatocellular carcinomas.

Studies revealed that, considerable expression of HSPs was found in tumor cells, showing that HSPs may be induced by other stresses and participate in broader array of defenses during cell growth and cell differen-tiation of tumors (Dorsey and Tchounwou, 2003; Lebret et al., 2003; Villaseca et al., 1997). Thus, it may be presumed that under various stimuli and stressful conditions, in order to avoid the damage caused by deleterious factors such as nitrosamines, hepatitis B or C virus-oncogenesis evocator, liver cells have to transcribe and translate high levels of HSPs in order to sustain normal metabolism and functions of cells. Under these conditions, liver cells should synthesize HSPs rapidly to exert a protective role for themselves. The progression of hepatocellular carcinoma is a gradual process under the long-term influence of various stimuli. During the process, inducible HSP synthesis increases gradually (Wang et al., 2007a). This viewpoint was confirmed by our results, in that HSP72 and gp96 were expressed at a higher level in hepatocellular carcinoma than that in tissues adjacent to cancer. The expression levels of HSP72 and gp96 may be useful prognostic markers for hepatocellular carcinoma.

Numerous investigations have been verified that HSP70 and gp96 are potent stimulators of immune responses (Castelli et al., 2004; Faure et al., 2004; Graner et al., 2003). The classical mechanisms of HSP70 and gp96 against tumors are believed that they may act as chaperones to facilitate major histocompatibility complex-1 (MHC-I) peptide loading, therefore increasing the tumor peptides presented by MHC-I (Berwin and Nicchitta, 2001; Sastry and Linderoth, 1999; Wells et al., 1998). Studies have shown that HSP72-associated peptides can also anchor antigen on the cell membrane and directly present it to natural killer cells or γδ T cells as superantigen without being dependent on the stimulation of MHC-I molecules (Multhoff et al., 1997; Zhang et al., 2005). Through this way, cytotoxic T lymphocyte (CTL) responses could be induced and the anti-tumor immunity was activated. Our data shows high-level expression of HSP72 and gp96 in hepatocellular carcinomas, and there was a significant correlation between their expression and progression, metastasis of tumors. These results raise the possibility that expression of HSP72 and gp96 in hepatocellular carcinomas may provide a useful link between immunity and tumor therapy against these cancers.

Conclusions

In this study, we examined the expressions of HSP72 and gp96 in hepatocellular carcinoma samples by way of immunohistochemistry and quantitative real-time RT-PCR. Human hepatocellular carcinomas existed with high level of expression of HSP72 and gp96. HSP72 and gp96 expression were significantly associated with the presence of tumor differentiation, lymph node and remote metastasis. There is a close correlation between the overexpression of HSP72 and gp96 and progression of hepatocellular carcinomas. The expression characters of HSP72 and gp96 in hepatocellular carcinoma may be useful to study the pathogenesis and progression of hepatocellular carcinoma.

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