

Significant correlation between expression level of HSP gp96 and progression of hepatitis B virus induced diseases

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Abstract

AIM: Gp96, also known as Grp94, is a member of heat shock protein (HSP) family and binds repertoires of peptides thereof eliciting peptide-specific T cell immune responses. It predominantly locates inside the endoplasmic reticulum (ER) with some cell surface expression in certain cancerous cells. Previous studies have shown that gp96 expression level was up-regulated in tumor cells, including hepatocellular carcinoma (HCC). However, relationship between the extent of gp96 expression and disease progression especially HBV-induced chronic infection, cirrhosis and hepatocellular carcinoma, has not been addressed before. As primary HCC can be induced and progressed from chronic hepatitis B virus (HBV) infection and HBV-induced cirrhosis, we designed an immunohistochemical experiment to test the correlation between gp96 expression level and HBV-induced disease progression, from chronic HBV infection, cirrhosis to HCC.

METHODS: We chose liver samples from different patients of hepatitis B virus induced diseases, including chronic hepatitis B (77 patients), cirrhosis (27 patients) and primary HCC (30 patients), to test the expression level of gp96 in different affected groups. Formalin-fixed, and paraffin-embedded liver tissues taken from these patients were immuno-stained by using an anti-gp96 monoclonal antibody for the expression level of gp96 protein in the sections. In addition, Western blotting of whole cell lysates derived from established human embryonic liver cell lines and several human HCC cell lines (Huh7, HepG2, SSMC-7721) was compared with the expression of gp96.

RESULTS: We found that the extent of elevated gp96 expression was significantly correlated with the disease progression, and was the highest in HCC patients, lowest in chronic HBV infection and was that of the cirrhosis in the middle.

CONCLUSION: Increased expression of gp96 might be used as a diagnostic or prognostic bio-marker for the HBV infection and HBV-induced diseases.

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INTRODUCTION

Heat shock protein (HSP) is a highly conserved group of cellular proteins and is up-expressed under hostile micro-environments, such as heat, hypoxia, acidosis, glucose deprivation, neoplasia and virus infection^[1-3]. It functions mainly as molecular chaperones to facilitate protein folding, allowing the exposed cells to adapt to gradual alterations in micro-environments and to survive. Recent studies have shown that expression of HSPs in cancerous cells is up-regulated in general but down-regulated in some cases^[4,5]. These have been used in some cases as disease-prognostic markers^[6-9]. However the molecular basis for over- or lower- expressions of HSPs in tumors is not completely understood. Based on their regulatory roles in cell apoptosis, HSPs have been divided into two groups: pro-apoptotic or anti-apoptotic HSPs^[10,11], e.g., HSP27 and HSP70 are anti-apoptotic, whereas HSP10 and HSP60 are pro-apoptotic^[10]. As such HSPs regulate cell apoptosis thereof, they modulate tumorigenicity and carcinogenesis.

Gp96 is a member of HSPs and shows anti-apoptotic effect in some tumor cells^[12]. It also activates both innate and adaptive immunity and currently is being tested extensively as an autologous therapeutic vaccine for tumors^[13-16]. Therefore it is a special member of the HSP family as a "Swiss-army knife" with multiple functions, both in immunity and tumorigenicity or carcinogenesis^[14,16]. We previously reported the identification of an HBV-specific HLA class I specific peptide bound to gp96 in HCC patients^[17,18], indicating its possible roles in immunity of HBV-induced HCC patients. At the same time, a Japanese group showed that gp96 expression level in HCC patients increased^[19] and implied its effect on tumorigenicity.

It is estimated that 350-400 million people worldwide are persistently infected with HBV and some of these persistent infected patients develop into cirrhosis or HCC in later stage^[20,21]. Therefore HBV is believed as one of the most successful human pathogens. Elucidation of the detailed pathogenesis mechanism of HBV infection, cirrhosis and HBV-induced HCC will shed light on virus-host interactions and ultimately lead to some prophylactic or therapeutic methods for the disease.

One previous report indicated that gp96 expression in HCC cells was highly up-regulated^[19]. However relationship between the extent of gp96 expression and disease progression, especially HBV-induced chronic infection, cirrhosis and HCC, has not been addressed before. Therefore we addressed this problem in the current study and the results showed that there existed a significant correlation between the expression level of gp96 and the disease progression in HBV-induced diseases. The results imply that gp96 might act as an anti-apoptotic factor in HBV-induced disease progression, thus facilitating the tumorigenicity.

MATERIALS AND METHODS

Human liver tissue samples

Seventy-two patients with chronic hepatitis B, 27 patients with liver cirrhosis and 30 patients with HCC were enrolled in this study. The patients were hospitalized in You-An Hospital, Beijing, China, between January 1998 and August 2002. All patients studied were infected with HBV examined with ELISA and were HBeAg positive in serum. The final diagnoses were confirmed by histological evaluation of the liver specimens. Sixteen control normal liver tissues were obtained from patients who underwent gastrectomy with all serological HBV-viral markers tested negative. None of the patients received irradiation or chemotherapy at the time of surgery. The liver specimens from patients with chronic hepatitis were obtained by needle biopsy and primary HCC and adjacent non-tumorous liver tissues were obtained by autopsy. All liver specimens were stained with hematoxylin and eosin and also with Masson's trichrome as well as Gordon Sweet's silver method for reticulin to confirm the histological diagnosis. In order to perform immunohistochemical study, liver specimens were fixed in 20% formalin and embedded in paraffin and cut into 5 μ m thick sections. All these manipulations were approved by the Institutional Bioethics Committee and informed consent was given by all patients and participants.

Cell lines and cell culture

Three established human HCC cell lines (Huh7, HepG2 and SSMC 7721) obtained from Cell Bank of Chinese Academy of Sciences and a human embryonic liver (HEL) cell line from Cell Bank of Peking Union Medical College were subjected to this study. HepG2, Huh7 and SSMC 7721 cells were grown in DMEM medium (Life Technologies, Inc) supplemented with 2 mmol/L L- glutamine and 100 mL/L fetal bovine serum (FBS). HEL cells were grown in DMEM medium containing 200 mL/L FBS. Cells were incubated at 37 °C under 50 mL/L CO₂ in a humidified atmosphere and media were changed on alternate days.

Immunohistochemistry

Formalin-fixed, paraffin-embedded liver specimens were deparaffinized in 3 times of xylene change (5 min each) followed by 2 changes in 100 mL/L ethanol. Immunohistochemistry for gp96 was performed using the avidin-biotin-peroxidase complex method (ABC immunostaining method). Endogenous peroxidase activity was blocked with a 15 min incubation in 3 mL/L methanol hydrogen peroxide. For gp96 antigen processing, sections were rehydrated and treated with 0.1 mmol/L citrate solution at 92 °C for 10 min. After 3 times of phosphate-buffered saline (PBS) change, sections were treated with normal goat serum for 20 min to block non-specific bindings. Then, a rat-derived monoclonal antibody (McAb) against gp96 (1:400 dilution; Neomarkers, USA) was added to the sections and incubated at 4 °C overnight. On the next day, both sets of tissue sections were rinsed with PBS 3 times (5 min each), then incubated with biotinylated goat anti-rat immunoglobulin (1:150 dilution; Goldenbridge Co.) at 37 °C for 20 min. After three times of PBS change, sections were soaked in streptavidin-biotinylated peroxidase complex at 37 °C for 20 min. Rinsed with PBS 3 times, the sections were finally incubated with diaminobenzidine-hydrogen peroxide to visualize the reaction products.

Immunoblotting

Whole cell lysate without trypsin treatment was prepared from culture cells using CCR5 lysis buffer (Promega, USA). After centrifugation, aliquots of each cell extracts containing an equal amount (15 μ g) was resolved by 100 g/L SDS-PAGE and electrophoretically transferred onto a nitrocellulose membrane.

Blots were probed with the anti-gp96 MAb (Neomarkers, USA). To verify that an equal amount of proteins was loaded onto the stocking gel, actin expression was simultaneously estimated in each example as the internal marker by Western blotting using anti-actin monoclonal antibody (Santa Cruz, USA). Protein concentration in the supernatants was measured using the Bradford assay (Bio-Rad laboratories Ltd, UK).

Gp96 expression and statistical analysis

For each tissue section, staining was assessed as negative, weakly positive or only focally positive (low-level expression), or strongly positive (high-level expression) and scored as 0, 1 and 2, respectively. When the stained cells accounted for $\geq 30\%$ of the total, the tissue was evaluated as strongly positive (grade 2). If the frequency of stained cells was $\leq 5\%$, the tissue was evaluated as negative (grade 0). The samples, with 5-30% of stained cells, were classified as weakly positive (grade 1).

Statistical significance of the data was analyzed using the chi-square test and set at $P < 0.05$.

RESULTS

Distribution of gp96 in normal, chronic hepatitis B, cirrhosis and HCC liver tissues

By using the ABC immunostaining method, we examined the presence of HSP gp96 in human liver parenchyma (Figure 1). Characteristically, HCC cells and normal hepatocytes were stained and the expressed styles could be diffusely cytoplasmic. In hepatitis liver tissues, the positive hepatocytes were often localized in periportal areas when the positive immunostaining was weak or few (Figure 1B). When more positive cells were stained, they often diffusely localized in the liver lobule. Moreover, the positive hepatocytes were markedly increased in regenerative liver cells. Cytoplasmic gp96 staining was significantly higher in HCC cells than in adjacent non-tumor liver cells (Figure 1G). In addition, nuclear staining for gp96 was also detected in a small number of HCC cells (Figure 1F), but not found in non-tumorous liver cells or cells derived from hepatitis, cirrhosis. This was consistent with the observation that gp96 localized in the nuclear envelope^[22]. Therefore the nuclear staining most likely reflected the outer nuclear membrane staining. It was observed that gp96 was detected not only in HCC cells and hepatocytes, but also expressed in lymphocytes (Figure 1H). Meanwhile, we observed specimens expressing gp96 in HCC with many leukocytes infiltrating tumour cells (Figure 1H). As a negative control, the second goat anti-rabbit antibody without prestaining of gp96 specific monoclonal antibody did not give any significant background staining, confirming the McAb specificity to gp96.

Table 1 Statistics of gp96 expression

Diagnosis	No	Negative	Weak	Strong	P-value ¹
CON	16	14	2	0	
CH	77	29	41	7	<0.01
LC	27	4	11	12	<0.01
HCC	30	0	9	21	<0.01

CON: control normal liver tissue; CH: chronic hepatitis; LC: liver cirrhosis; HCC: hepatocellular carcinoma. ¹Significance relative to control tissues.

Relations between histological findings and gp96 expression

To understand the association between intensity of gp96 staining and histological stages of chronic hepatitis, cirrhosis and primary HCC, semi-quantitative assessment of the gp96 expression level was performed according to the above criteria.

Typical staining grades (0, 1, 2) of the gp96 expression are shown in Figure 1 (1A, 1B and 1E respectively). As shown in Table 1, various staining patterns were observed for the gp96 expression. Of the specimens from 77 chronic hepatitis B and 27 liver cirrhosis, 62.3% and 85% were positive respectively. In 30 HCC tissues gp96 expression was positive (100%). These findings were statistically significant ($P < 0.01$) with respect to the control normal tissues. As shown in Figure 2, the expression level of gp96 was indeed correlated positively with their corresponding histological stages, and was the highest in HCC patients, lowest in chronic HBV infection and that of the cirrhosis was in the middle. However, there was no significant association between gp96 expression and patient ages, gender or other clinic-

pathologic characteristics (data not shown) in this study.

Expression of gp96 in several HCC cell lines

We examined the gp96 expression by Western blot in three HCC cell lines available (Huh7, HepG2 and SSMC-7721) with variable phenotypes and a human embryonic liver cell line (HEL) established by Cell Bank of Peking Union Medical College. As expected, gp96 expression was observed in all three HCC cell lines. However, in HEL cell line, there was no visible band in the immunoblot of crude materials (Figure 3). Furthermore, immunocytochemistry analysis also showed gp96 protein expression in SSMC-7721 cells but did not in HEL cells (Figure 4).

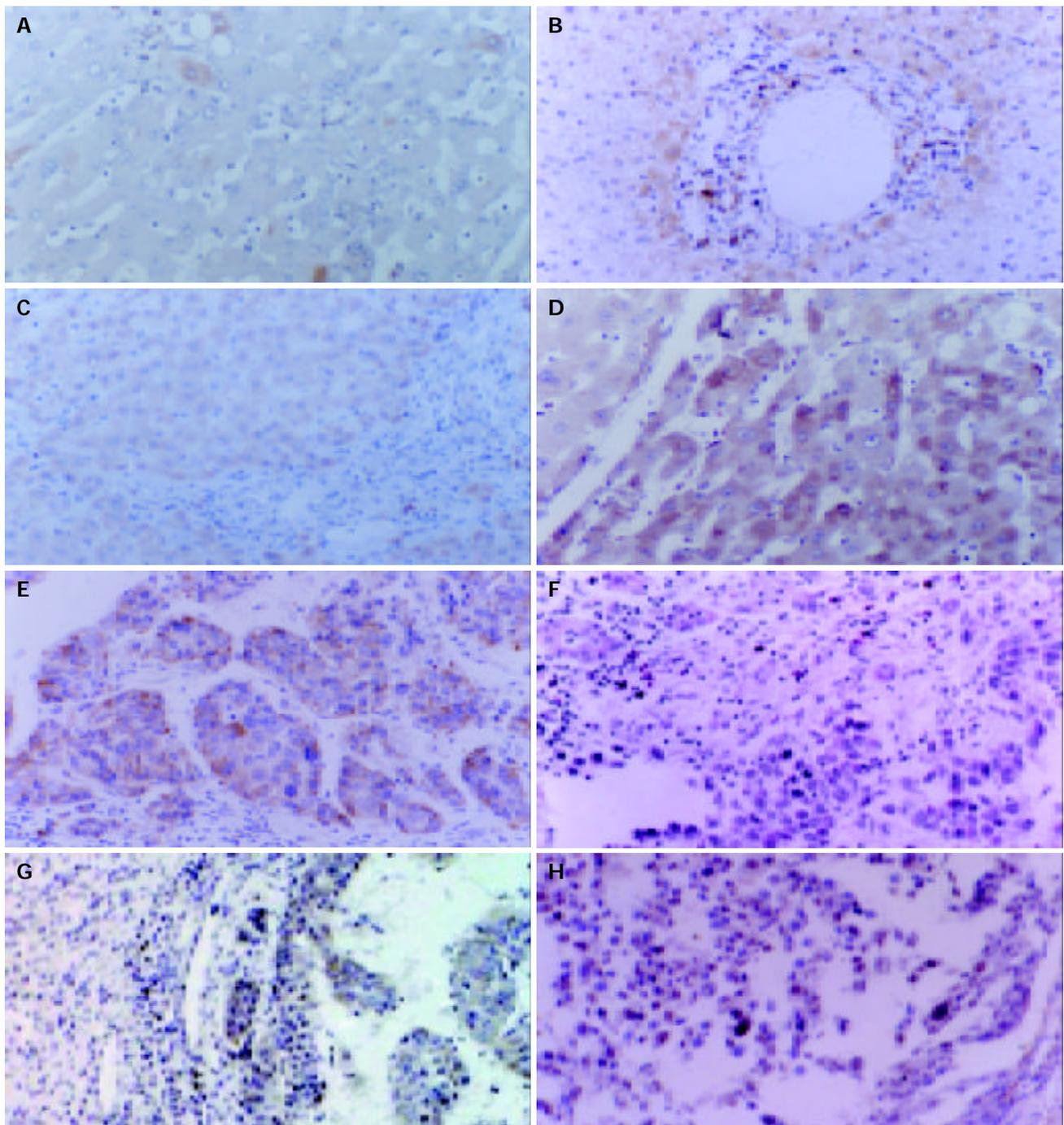


Figure 1 Sections of immunoperoxidase staining for gp96 in chronic hepatitis liver, liver cirrhosis and HCC. Several representative liver tissues with A: gp96 negative in chronic hepatitis, B: gp96 weakly positive in chronic hepatitis, C: gp96 strongly positive in chronic hepatitis, D: gp96 strongly detected in liver cirrhosis, E: gp96 strongly positive in HCC, F: gp96 positive in the nuclear and cytoplasmic staining, G: Cytoplasmic gp96 staining significantly higher in HCC cells than in adjacent non-tumorous liver cells, and H: gp96 expression in HCC accompanied by lymphocyte infiltration (indicated by arrow).

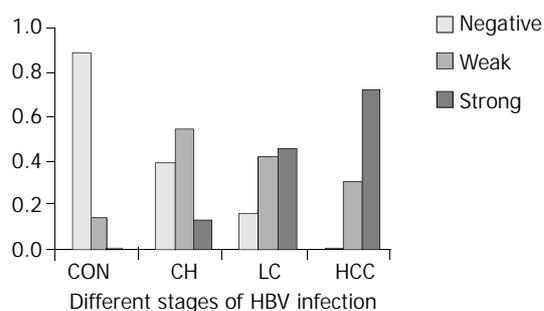


Figure 2 Correlation of gp96 expression and the progression of hepatitis B virus induced diseases. For the definition of gp96 staining density (negative, weak and strong), please see Materials and Methods. X-axis, different stages of HBV infection; Y axis, the percentage of classified samples numbers in total numbers of each group samples. CON: control normal liver tissue; CH: chronic hepatitis; LC: liver cirrhosis; HCC: hepatocellular carcinoma.

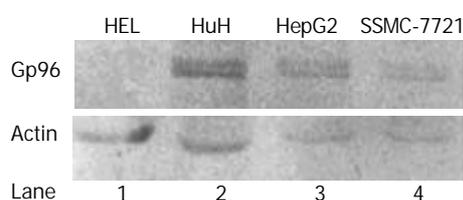


Figure 3 Western blotting analysis of gp96 expression in HCC cell lines and human embryonic liver cells (HEL). Clearly shown in the figure are high-level expression of gp96 in the HCC cell lines but not in HEL. Actin expression level was used as SDS-PAGE loading control.

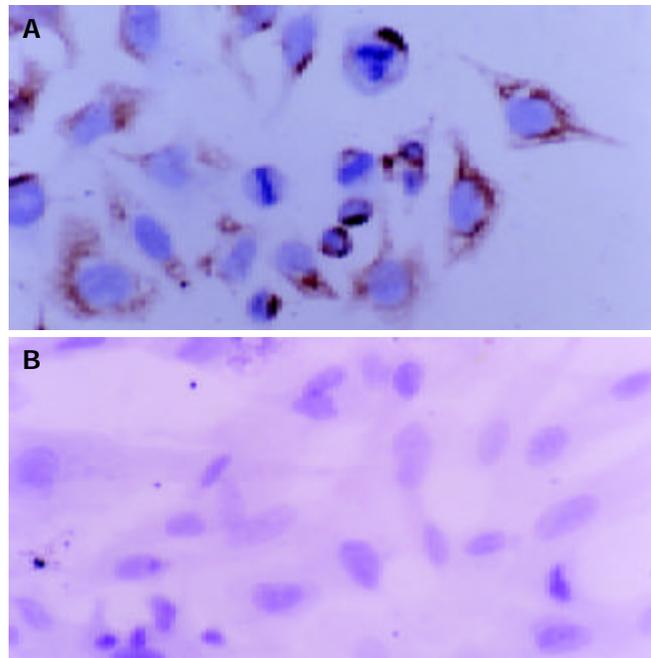


Figure 4 Immunocytochemical analysis of gp96 in HCC cell line SSMC-7721 (A) and HEL (B). High-level expression of gp96 was seen in SSMC-7721, but not in HEL.

DISCUSSION

In this study, the relationship between expression level of gp96 in liver tissues and the progression of HBV-induced diseases was quantitatively analyzed. It was clearly shown that there was a significant correlation. Gp96 expression increased as the HBV-induced disease progressed from chronic hepatitis to cirrhosis, then HCC. Elevated gp96 expression in tumour

cells, including HCC (not quantitatively) and human colorectal cancers, was reported earlier^[19,23], but its role in tumorigenicity is not clear. As the longevity of cirrhosis and HCC cells was observed compared to normal hepatocytes, it is plausible to propose that gp96 up-expression in these tumorous cells is closely related to the cell survival, thereof possibly preventing cell apoptosis. Some reports showed that gp96 might be a member of anti-apoptotic HSPs^[12]. Our current observation seems to support this notion. However, it is not clear yet if the gp96 up-expression in cirrhosis or HCC is the cause or the outcome of the disease progression. This needs to be addressed in future studies.

This obvious correlation has also raised another possibility, *i.e.*, whether evaluation of gp96 expression level can be used as a prognostic marker of HBV-induced diseases. HBV infection is a serious problem worldwide, especially in China. A substantial number of HBV carriers develop into cirrhosis and HCC, but the mechanism underlying the different outcomes among patients is not clear. There is an urgent need for identification of molecular biomarkers for the evaluation of HBV-carriers' prognostic factors. Although some studies suggested that HBxAg could be used as a pathological prognostic factor in the progression of HBV-induced diseases, the expression patterns were often contradictory^[24-26]. The expression level of other HSPs has been recognized as diagnostic or prognostic markers in certain tumours^[6-9,27-31]. Nonetheless, use of gp96 expression in this purpose has not been addressed before. Gp96 was constitutively expressed at very low levels in ER of a variety of normal cell types^[23] but the expression was dramatically enhanced by stressful conditions. Our data (Figure 4) are consistent with the previous observations as we did not detect any gp96 expression in HEL cell line but easily seen in HCC cell lines. Our results, combined with the earlier observation^[19], suggested that immunohistochemical detection of increased gp96 expression in liver tissues was associated with an increased risk of HBV chronic infection development into cirrhosis or HCC. In other words, increased expression of gp96 in liver tissues might be a valuable diagnostic or prognostic marker for chronic HBV-infection patients though further work needs to be done for a conclusive implication.

Gp96 is not an oncofetal protein as HEL cells showed negative staining in this study. Gp96 expression was not associated with cell differentiation stages either but with cell malignancy. This finding was correlated with the data from tissue specimens in which enhanced expression occurred in tumor tissues rather than in (even neighbouring) normal tissues. The role of gp96 in HCC tumorigenicity is not clear, but some studies have suggested that high-level expression of gp96 could contribute to tumorigenicity of other tumours^[23,32,33]. Gp96 expression was also reported to increase the immunity to tumours^[34-38]. At least the complex preparation of gp96 and bound-peptides has been being successfully used as autologous tumour vaccines^[13-16]. Our data however did not indicate a positive correlation of gp96 up-expression and host immunity against HBV-induced diseases as the high-level expression was associated with a worse clinical outcome. Why does not high expression level of gp96 in HCC or chronic HBV infection confer protection immunity on HCC or HBV-induced diseases? It is a complicated scenario. As to the other edge of the "Swiss-army-knife", the protection of gp96 against tumours was believed that gp96 could act as a chaperone to facilitate MHC class I peptide loading, therefore increasing the tumour peptides presented by MHC class I^[16,39]. Because of the down-regulation of MHC class I on tumorous cell surfaces, cytotoxic T cells (CTL) were in general in a low density in chronic HBV infection or HCC^[40], but exogenously introduced gp96-peptide complexes would help class I peptide loading through the up-taking of this complex by gp96 receptors on the cell surfaces, *e.g.* CD91^[16]. Through this way CTL response could be induced and the

cellular tumour immunity was provoked. The high expression level of gp96 in cirrhosis and HCC was complicated by other factors because their effects could confer immunity on HCC.

In conclusion, there is a positive correlation between HSP gp96 expression in liver tissues and HBV-induced disease progression. Its mechanism and possible application in HBV-infection patients should be further studied.

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