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Original contribution

Low levels of glycoprotein 96 indicate a worse prognosis in early-stage hepatocellular carcinoma patients after hepatectomy☆,☆☆

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Summary Heat shock proteins are a highly conserved group of cellular proteins and are up-expressed in hepatocellular carcinoma (HCC). As a member of the heat shock protein-90 family, glycoprotein 96 (gp96) modulates immunity and tumorigenicity, is increased during the development of HCC from normal liver tissue, and is considered a pro-oncogenic chaperone. However, the prognostic value of gp96 has not been well clarified. The purpose of this study was to investigate the relationship between gp96 and survival of postoperative HCC patients. The expressions of gp96 protein and messenger RNA were measured by immunohistochemistry and real-time quantitative polymerase chain reaction, respectively. The relations between gp96 expression level and clinicopathological factors were analyzed. Kaplan-Meier survival and Cox regression analyses were used to identify factors associated with prognosis. All normal liver tissue exhibited low gp96 expression, whereas high gp96 expression was present in 54% of HCC tissues. The expression of gp96 protein was inversely correlated with TNM stage ($P = .037$) and tumor recurrence ($P = .004$). Low gp96 expression was an independent risk factor for poor postoperative disease-free survival (hazard ratio, 0.385; 95% confidence interval, 0.226-0.655; $P < .001$), and overall survival (hazard ratio, 0.345; 95% confidence interval, 0.187-0.637; $P = .001$). Stratification analysis indicated that high gp96 had better predictive value for tumor recurrence in HCC patients with normal serum α-fetoprotein levels or with TNM stage I and tumor differentiation I-II HCC. In conclusion, gp96 is a potential and reliable prognostic biomarker for tumor recurrence and overall survival in HCC patients after curative resection.

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1. Background

Hepatocellular carcinoma (HCC) is a very common and serious malignancy with high morbidity and high mortality; it is the sixth most common cancer and the third cause of cancer-related death globally [1]. Although most cases of HCC occur in Eastern Asia and sub-Saharan Africa, the incidence in some developed countries such as Japan, the United Kingdom, France, and the United States has been increasing [2]. The increasing incidence and the lack of effective treatment of HCC have made the disease a major worldwide health problem. Therefore, it is important to uncover the mechanisms of HCC and find effective biomarkers and therapeutic targets.

Heat shock proteins (HSPs) are a group of highly conserved proteins that are produced during stress and are involved in several aspects of cellular homeostasis. Glycoprotein 96 (gp96) is a major endoplasmic reticulum chaperone protein that assists in protein folding, processing, and secretion, and is the endoplasmic reticulum counterpart of HSP90 [3]. Because gp96 chaperons multiple essential proteins, such as TLRs (except TLR3) [4], Wnt co-receptor LRP6 [5], GARP [6], GPIb [7], and insulin-like growth factor [8], as well as most integrin subunits [9,10], which are involved in various stages of cancer development, gp96 is considered a pro-oncogenic chaperone.

Wu et al [11] reported that liver gp96 protein level increased with the development of HCC from normal liver tissue in a murine model. Rachidi et al [12] and Chen et al [13] found that gp96 was necessary for the development of HCC and considered it a pro-oncogenic chaperone. Yao et al [14] showed that gp96 was strongly expressed in HCC (73.3%; 22/30), and in particular in HCC in patients who were hepatitis B virus DNA positive (89.5%; 17/19). Lim et al [15] and Zhu et al [16] also reported that the expression of gp96 increased along with the stepwise progression of HCC development. Furthermore, Yao et al [14] and Lim et al [15] both reported that gp96 expression in HCC tissue was correlated with the degree of tumor differentiation and tumor size.

However, in the aforementioned studies, the sample sizes were small, and no information on the relation between gp96 protein level in HCC and survival rate was reported. Thus, the current study was to examine the prognostic value of gp96 in HCC patients who underwent curative resection.

2. Materials and methods

2.1. Study population

This study was approved by the ethics review committee of the First Affiliated Hospital of Sun Yat-sen University. Eighty-four patients with HCC who received hepatectomy at our center from June 2007 to October 2009 were included in this

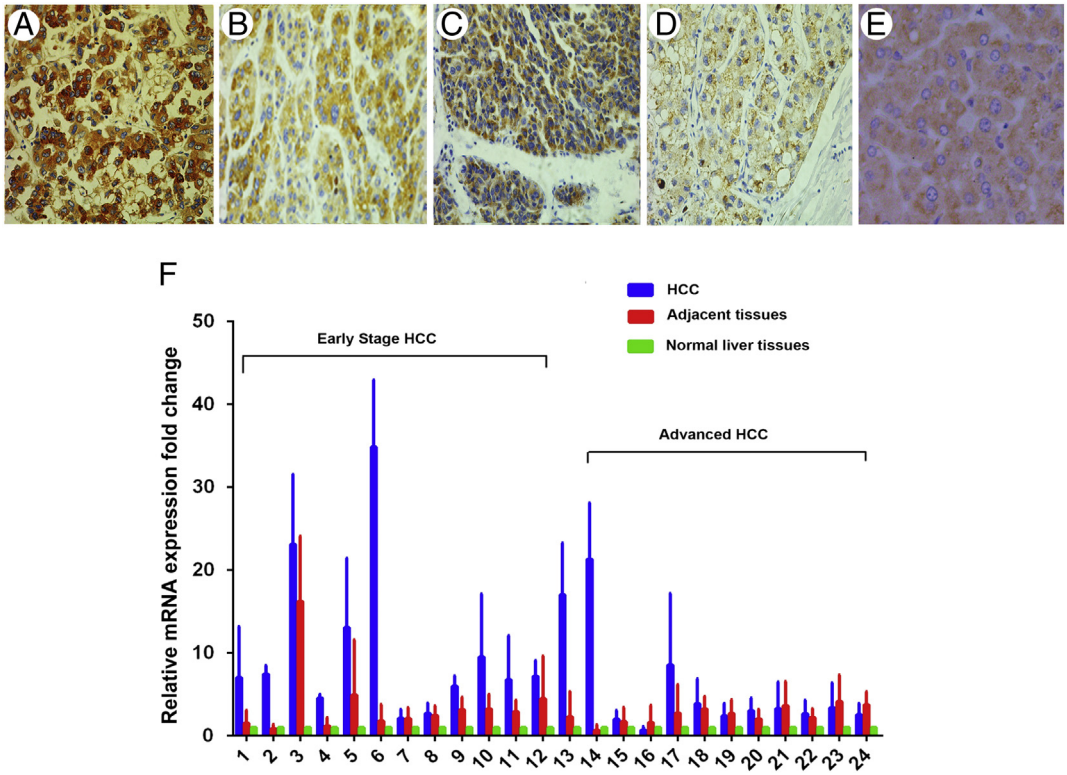

Fig. 1 The expression of gp96 protein and mRNA. High expression of gp96 protein in HCC tissues (A and B), low expression of gp96 protein in HCC tissues (C and D) and in the normal liver tissues (E; original magnification ×400), and the expression of gp96 mRNA (F).

study. The inclusion criteria were as follows: (1) TNM stage I, II, IIIA, or IIIB (International Union Against Cancer, seventh edition); (2) Child-Pugh class A and class B hepatic function; (3) 18 to 80 years old; and (4) provided written informed consent. The exclusion criteria were as follows: (1) TNM stage IIIC, IVA, or IVB; (2) Child-Pugh class C hepatic function; (3) second malignancy or history of second malignancy within the prior 5 years; (4) perioperative dysfunction of vital organs; (5) prior percutaneous ablation; (6) prior transcatheter arterial chemoembolization; and (7) received chemotherapy or radiotherapy within 1 month after surgery. All patients were followed up postoperatively as described in our previous study [17]. Normal liver tissue and HCC tissue were collected and prepared as described in our previous study [17].

Table 1 Correlation between gp96 and the clinicopathological characteristics in HCC tissues

Variables	Cases	gp96 expression in HCC tissues		*P*
		Low	High	
Age (y)				
≥60	16	5 (31.3%)	11 (68.7%)	.176
<60	68	34 (50.0%)	34 (50.0%)	
Sex				
Male	68	32 (47.1%)	36 (52.9%)	.811
Female	16	7 (43.8%)	9 (56.2%)	
HbsAg				
Positive	72	34 (47.2%)	38 (52.8%)	.721
Negative	12	5 (41.7%)	7 (58.3%)	
ALT (U/L)				
≥80	9	5 (55.6%)	4 (44.4%)	.855
<80	75	34 (45.3%)	41 (54.7%)	
PLT ($\times 10^9$)				
>100	74	34 (45.9%)	40 (54.1%)	.809
≤100	10	5 (50.0%)	5 (50.0%)	
Cirrhosis				
Yes	64	29 (45.3%)	35 (54.7%)	.714
No	20	10 (50.0%)	10 (50.0%)	
AFP (μg/L)				
≥20	36	16 (44.4%)	20 (55.6%)	.752
<20	48	23 (47.9%)	25 (52.1%)	
Tumor size (cm)				
≥5	50	27 (58.0%)	23 (42.0%)	.092
<5	34	12 (35.3%)	22 (64.7%)	
Tumor no.				
Single	62	25 (40.3%)	37 (59.7%)	.06
Multiple	22	14 (63.6%)	8 (36.4%)	
Differentiation				
I-II	62	30 (48.4%)	32 (51.6%)	.546
III	22	9 (40.9%)	13 (59.1%)	
TNM stage				
I	55	21 (38.2%)	34 (61.8%)	.037
II-III	29	18 (62.1%)	11 (37.9%)	
PVTT				
No	73	32 (43.8%)	41 (56.2%)	.220
Yes	11	7 (63.6%)	4 (36.4%)	
Tumor encapsulation				
Complete	64	28 (43.8%)	36 (56.2%)	.379
None	20	11 (55.0%)	9 (45.0%)	
Recurrence				
Yes	58	33 (56.9%)	25 (43.1%)	.004
No	26	6 (23.1%)	20 (76.9%)	
Complication				
No	73	33 (45.2%)	40 (54.8%)	.563
Yes	11	6 (54.5%)	5 (45.5%)	

Abbreviations: ALT, alanine aminotransferase; HbsAg, hepatitis B surface antigen; PLT, platelet.

2.2. Immunohistochemical analysis

Immunohistochemical analysis and slide evaluation were performed as previously described [17]. Rat monoclonal anti-gp96 antibody (ADI-SPA-850; Enzo Life Sciences, New York, USA) was used in the immunohistochemical analysis (1:100 dilution).

2.3. Evaluation of immunohistochemical staining

Immunohistochemical staining in the tissue was scored independently by 2 pathologists blinded to the clinical data using a semiquantitative immunoreactivity score reported previously [17]. The intensity of immunostaining was graded 0 to 3 (0, negative; 1, weak; 2, moderate; 3, strong; category A). The percentage of immunoreactive cells was classified as 0 (<5%), 1 (6%-25%), 2 (26%-50%), 3 (51%-75%), and 4 (76%-100%; category B). Multiplication of categories A and B resulted in an immunoreactivity score ranging from 0 to 12 for each tissue specimen. Sections with a total score of 0, 1, or 2 were defined as negative (−), a score of 3 or 4 was defined as weakly positive (+), a score of 6 or 8 was defined as moderately positive (++), and a score of 9 or 12 was defined as strongly positive (+++). For categorical analyses, the immunoreactivity was graded as low expression level (total score ≤8) or high expression level (total score >8).

2.4. Real-time quantitative polymerase chain reaction

From June 2016 to August 2016 at our center, fresh HCC tissue (n = 24), corresponding adjacent tissue (n = 24), and normal liver tissue (n = 4) were collected immediately after resection for detection of gp96 messenger RNA (mRNA) expression. Of the 24 HCC cases, 13 were TNM stage I (early stage HCC) and 11 were TNM stage II or III (advanced-stage HCC). RNA was extracted by TRIzol reagent (Thermo Fisher Scientific, Waltham, MA) according to a general protocol. The cDNA was synthesized using a cDNA synthesis kit (Takara Biotechnology, Shiga, Japan) in accordance with manufacturer's instructions. The cDNA was performed to real-time quantitative polymerase chain reaction (PCR) by using the SYBR Green PCR kit (Toyobo, Osaka, Japan), and the reaction was performed using a Bio-rad IQ5 PCR system (Bio-Rad, Hercules, CA). GAPDH was used as an internal control. The relative gene expressions were quantified and analyzed. The primers were obtained from the Genecopia Company (Guangzhou, China).

2.5. Statistical analysis

Normally distributed continuous variables were compared using the Student *t* test. Categorical variables were compared using the χ^2 test. Disease-free survival (DFS) was defined as the interval from surgery to recurrence, whereas overall survival (OS) was defined as the interval from surgery to HCC-associated death. The univariate and multivariate analyses and the Kaplan-Meier method were used to analyze survival rates, and survival curves were compared using a log-rank test. A *P* value <.05 was considered to indicate statistical significance. All analyses were performed using IBM SPSS software, version 20 (IBM, Armonk, NY).

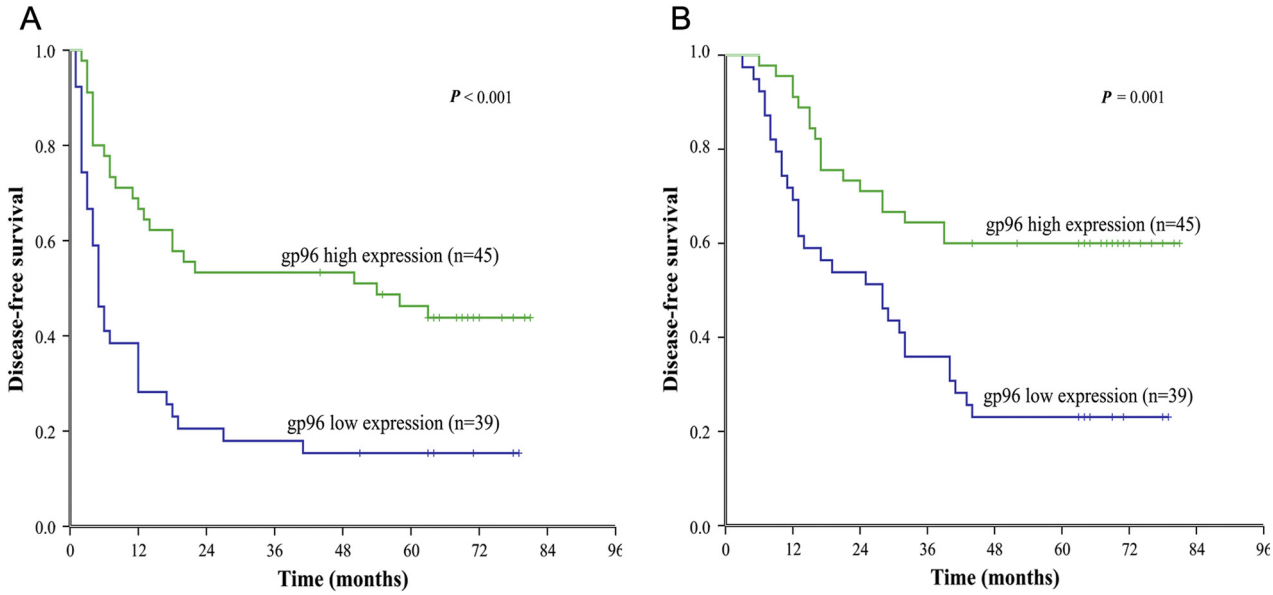

Fig. 2 Kaplan-Meier curves are shown for time to disease recurrence (A, $P < .001$, log-rank test) and OS (B, $P = .001$, log-rank test) among patients with high or low gp96 protein expression.

Table 2 Prognostic factors for DFS and OS by univariate analysis

Variables	n	DFS			*P*	OS			*P*
		1 y	3 y	5 y		1 y	3 y	5 y	
Age (y)									
<60	68	45.6%	32.4%	27.7%	.11	82.4%	48.5%	39.7%	.39
≥60	16	62.5%	56.3%	49.2%		75.0%	62.5%	56.3%	
Sex									
Male	68	47.1%	36.8%	30.5%	.43	79.4%	50.0%	41.2%	.48
Female	16	56.3%	37.5%	37.5%		87.5%	56.3%	50.0%	
HCC family history									
Yes	6	50.0%	33.3%	16.7%	.62	83.3%	50.0%	50.0%	.63
No	78	48.7%	37.2%	33.3%		79.5%	51.3%	42.3%	
HBsAg									
Positive	72	47.2%	38.9%	33.3%	.90	79.2%	50.0%	44.4%	.88
Negative	12	58.3%	25.0%	25.0%		91.7%	58.3%	33.3%	
PLT ($\times 10^9$)									
>100	74	44.6%	33.8%	28.2%	.07	78.4%	47.3%	39.2%	.08
≤100	10	80.0%	60.0%	60.0%		100.0%	80.0%	70.0%	
AFP (μg/L)									
<20	48	52.8%	38.9%	38.9%	.34	83.3%	50.0%	44.4%	.75
≥20	36	45.8%	35.4%	26.3%		79.2%	52.1%	41.7%	
Ascites									
No	68	52.9%	39.7%	34.1%	.14	83.8%	51.5%	44.1%	.55
Yes	16	31.3%	25.0%	25.0%		68.8%	50.0%	37.5%	
Cirrhosis									
Yes	64	50.0%	37.5%	32.9%	.78	76.6%	48.4%	42.2%	.49
No	20	45.0%	35.0%	30.0%		95.0%	60.0%	45.0%	
Tumor no.									
Single	62	59.7%	43.5%	36.9%	.003	85.5%	61.3%	51.6%	<.001
Multiple	22	18.2%	18.2%	18.2%		68.2%	22.7%	18.2%	
PVTT									
No	73	54.8%	41.1%	35.3%	<.001	87.7%	56.2%	47.9%	<.001
Yes	11	9.1%	9.1%	9.1%		36.4%	18.2%	9.1%	
Tumor size (cm)									
≥5	50	38.0%	30.0%	28.0%	.05	70.0%	40.0%	36.0%	.04
<5	34	64.7%	47.1%	39.2%		97.1%	67.6%	52.9%	
Tumor encapsulation									
None	20	30.0%	25.0%	15.0%	.01	60.0%	45.0%	30.0%	.08
Complete	64	54.7%	40.6%	37.7%		87.5%	53.1%	46.9%	
Resection margin (cm)									
<2	45	40.0%	26.7%	24.4%	.07	80.0%	40.0%	28.9%	.01
≥2	39	59.0%	48.7%	39.6%		82.1%	64.1%	59.0%	
Complication									
No	73	49.3%	38.4%	33.1%	.37	84.9%	53.4%	45.2%	.10
Yes	11	45.5%	27.3%	27.3%		54.5%	36.4%	27.3%	
Tumor differentiation									
I-II	62	54.8%	41.9%	35.1%	.21	85.5%	56.5%	48.4%	.04
III	22	31.8%	22.7%	22.7%		68.2%	36.4%	27.3%	
TNM stage									
I	55	60.0%	43.6%	38.0%	.01	90.9%	60%	52.7%	.001
II-III	29	27.6%	24.1%	20.7%		62.1%	34.5%	24.1%	
gp96 expression									
Low	39	28.2%	17.9%	15.4%	<.001	69.2%	35.9%	23.1%	<.001
High	45	66.7%	53.3%	46.3%		91.1%	64.4%	60.0%	

Abbreviations: HBsAg, hepatitis B surface antigen; PLT, platelet.

3. Results

3.1. Expression of gp96 protein and mRNA in HCC

To detect the level of gp96 protein expression in HCC, we performed immunohistochemical staining on 84 HCC tissues and 20 normal liver tissues. The gp96 protein expression was localized mainly in the cytoplasm, with some detected on the cell membranes. We found that the positive immunostaining of gp96 was present in all tissues; however, gp96 expression was low in all normal liver tissues, but a high level of gp96 expression was present in 45 (54%) of 84 HCC tissues (Fig. 1).

To investigate the expression level of gp96 mRNA in HCC tissues, real-time PCR was used. Twenty-four pairs of HCC and corresponding adjacent tissues and 4 normal liver tissue specimens were examined. The expression of gp96 mRNA was significantly increased in HCC tissue as compared with the corresponding adjacent tissues and normal liver tissue ($P = .027$ and $P = .026$). Moreover, gp96 mRNA expression in corresponding adjacent tissue was also greater than that in normal liver tissue ($P = .001$). In addition, gp96 mRNA expression in early-stage HCC was greater compared with advanced-stage HCC ($P = .012$; Fig. 1).

3.2. Correlation between gp96 protein expression and clinicopathological characteristics

To examine the prognostic value of gp96 protein expression, the relations between gp96 expression and 15 clinicopathological characteristics were analyzed (Table 1). The expression of gp96 was inversely correlated with TNM stage ($P = .037$) and tumor recurrence ($P = .004$). However, gp96 expression was not associated with the other clinicopathological characteristics including age, sex, hepatitis B surface antigen, alanine aminotransferase, platelet count, cirrhosis, α-fetoprotein (AFP), tumor number, tumor size, tumor differentiation, portal vein tumor thrombi (PVTT), tumor encapsulation, and postoperative complications (all, $P > .05$).

3.3. Low expression of gp96 in HCC tissue predicts poor outcomes

The patients were divided into 2 groups: high expression group (n = 45) and low expression group (n = 39) according to their gp96 expression profiles. The analysis showed that gp96 expression was positively correlated with DFS and OS. The 1-, 3-, and 5-year DFS rates in the high expression group were significantly higher than those in the low expression group (66.7%, 53.3%, 46.3% versus 28.2%, 17.9%, 15.4%, respectively; $P < .001$; Fig. 2A). In addition, the 1-, 3-, and 5-year OS rates of the high expression group were also markedly higher than those of the low expression group (91.1%, 64.4%, 60.0% versus 69.2%, 35.9%, 23.1%, respectively; $P = .001$; Fig. 2B).

3.4. Independent prognostic factors of HCC outcomes

To determine factors related to DFS and OS, gp96 and 15 clinicopathological factors were assessed by univariate analysis and the multivariate analysis. The univariate analysis showed that the significant prognostic factors for DFS were tumor number, PVTT, tumor encapsulation, TNM stage, and gp96 expression. The significant factors for OS were tumor number, PVTT, tumor size, resection margin, tumor differentiation, TNM stage, and gp96expression (all, $P < .05$; Table 2). Using Cox regression multivariate analysis, we found that PVTT ($P < .001$) and gp96 expression ($P < .001$) were significant independent prognostic factors for DFS, whereas tumor size ($P = .010$), resection margin ($P = .008$), tumor differentiation ($P = .001$), PVTT ($P = .001$), and gp96 expression ($P = .001$) were significant independent prognostic factors for OS (Table 3).

3.5. Low expression of gp96 predicts poor outcomes of HCC patients independent of AFP level

To further clarify the prognostic value of gp96 in patients with negative AFP expression, patients were subdivided into the AFP < 20 μg/L subgroup (n = 36) and the AFP ≥ 20 μg/L subgroup (n = 48). The results indicated that in the AFP < 20 μg/L subgroup, low gp96 expression predicted a worse 1-, 3-, and 5-year DFS (18.8%, 12.5%, 12.5% versus 80.0%, 60.0% 60.0%, respectively; $P = .001$) and a worse 1-, 3-, and 5-year OS (75.0%, 31.3%, 18.8% versus 90.0%, 65.0%, 65.0%, respectively; $P = .007$). Similarly, in the AFP ≥ 20 μg/L subgroup, low gp96 expression was associated with worse 1-, 3-, and 5-year DFS (34.8%, 21.7%, 17.4% versus 56.0%, 48.0% 34.9%, respectively; $P = .029$)

Table 3 Prognostic factors for DFS and OS by the multivariate Cox proportional hazards regression model

Variables	DFS			OS		
	HR	95% CI	*P*	HR	95% CI	*P*
Tumor differentiation				3.013	1.537-5.909	.001
Tumor size				2.346	1.222-4.502	.010
Resection margin				0.432	0.232-0.805	.008
PVTT	3.851	1.863-7.960	<.001	3.524	1.708-7.272	.001
gp96 expression	0.385	0.226-0.655	<.001	0.345	0.187-0.637	.001

Abbreviations: CI, confidence interval; HR, hazard ratio.

and a worse 1-, 3-, and 5-year OS (65.2%, 39.1%, 26.1% versus 92.0%, 64.0%, 56.0%, respectively; $P = .032$) compared with high gp96 expression (Fig. 3).

3.6. Low expression of gp96 predicts poor outcomes of HCC patients with early-stage disease

To determine the prognostic value of gp96 in patients with early-stage HCC, we analyzed whether the prognosis was different in TNM stage I patients with low or high gp96 expression. Patients with TNM stage I HCC were subdivided into 2 subgroups: high-expression gp96 subgroup (n = 34) and low-expression gp96 (n = 21) subgroup. Low gp96 expression was associated with poorer DFS (1-, 3-, and 5-year DFS: 42.9%, 23.8%, 19.0% versus 70.6%, 55.9%, 52.6%, respectively; $P = .013$) and poorer OS (1-, 3-, and 5-year OS: 85.7%, 47.6%, 33.3% versus 94.1%, 67.6%, 64.7%, respectively; $P = .035$). In addition, in TNM stage II-III patients, low gp96 expression also predicted a worse DFS (1-, 3-, and

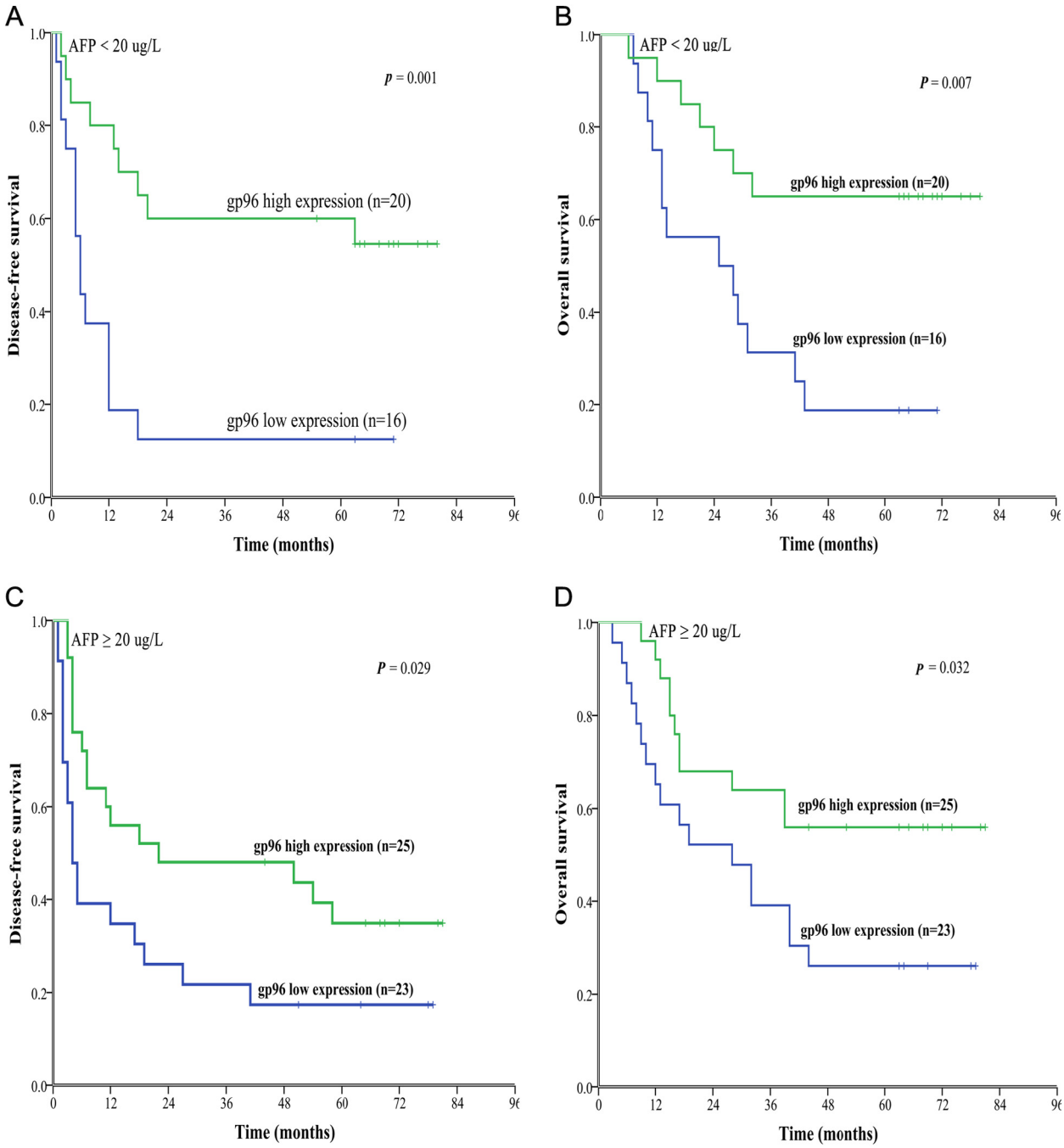

Fig. 3 After stratification analysis of AFP, AFP < 20 μg/L (A and B) and AFP ≥ 20 μg/L (C and D), Kaplan-Meier curves are shown for time to disease recurrence and OS among patients with high or low gp96 protein expression.

5-year DFS: 11.1%, 11.1%, 11.1% versus 54.5%, 45.5%, 18.2%; $P = .044$) and worse OS (1-, 3-, and 5-year OS: 50.0%, 22.2%, 11.1% versus 81.8%, 54.5%, 45.5%; $P = .044$).

We further analyzed the association of gp96 expression with tumor differentiation and found poorer DFS (1-, 3-, and 5-year DFS: 26.7%, 20.0%, 16.7% versus 81.3%, 62.5%, 52.4%; $P < .001$) and poorer OS (1-, 3-, and 5-year OS: 73.3%, 36.7%, 26.7% versus 96.9%, 75.0%, 68.8%; P .001) in the low-gp96 expression subgroup of tumor differentiation I-II HCC, but no significance in DFS ($P = .235$) and OS ($P = .202$) between the low- and high-gp96 expression subgroups of tumor differentiation III HCC (Fig. 4).

4. Discussion

The current study showed that gp96 was present in all liver tissues tested, suggesting that gp96 is indispensable for normal liver cells or HCC cells. Moreover, a tendency of increasing gp96 expression was found from normal liver tissue, to tissue adjacent to HCC, to HCC tissue. However, low expression of gp96 in HCC tissue correlated with late TNM stage and high recurrence rate and predicted a poor prognosis: shorter DFS and OS. Furthermore, a low level of gp96 expression was an independent prognostic factor for poor DFS or OS.

It is surprising and unclear why a high level of gp96 expression was associated with longer DFS and OS. It seems that the

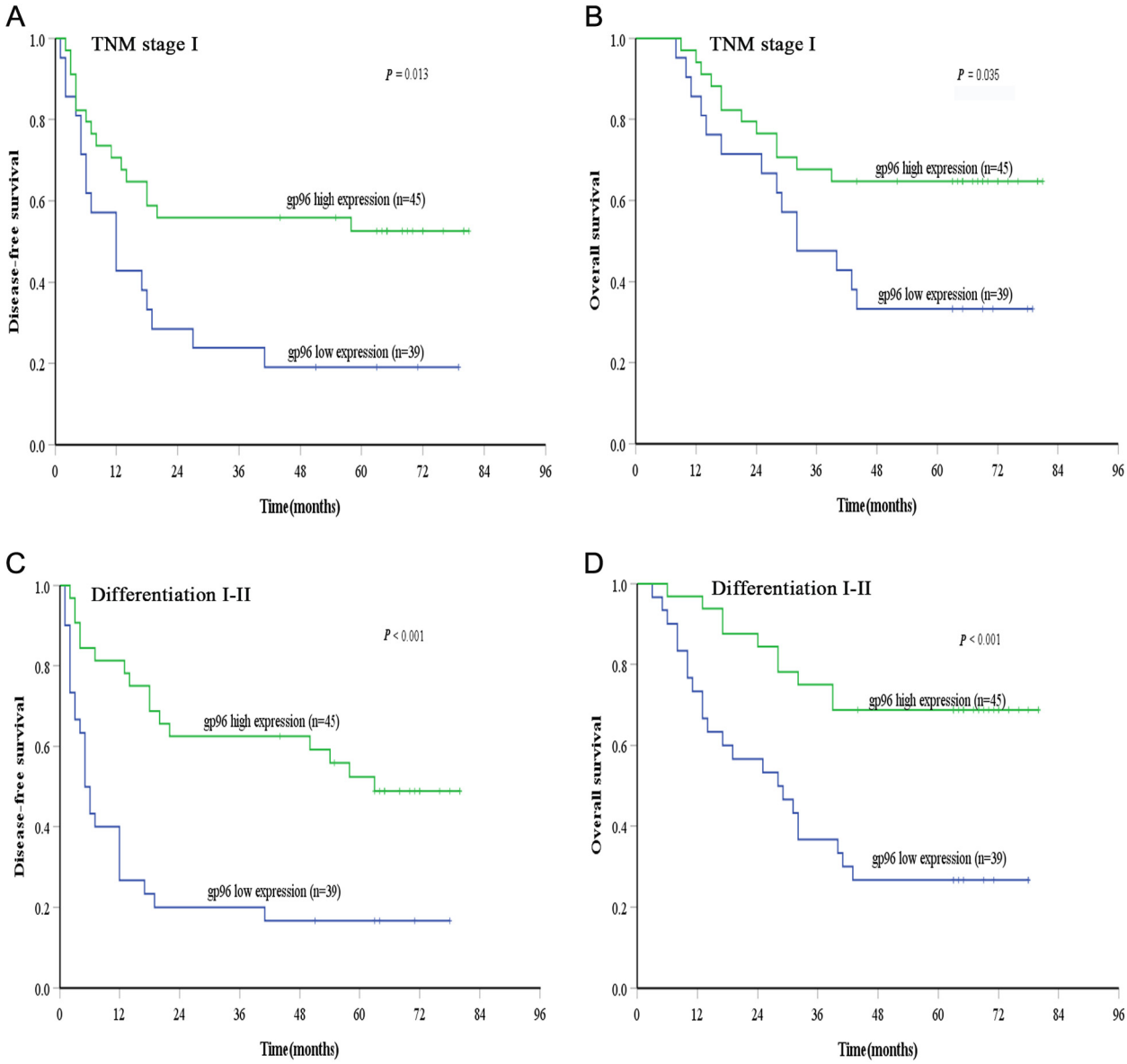

Fig. 4 After stratification analysis of early stage HCC, TNM stage I (A and B) and tumor differentiation I-II (C and D), Kaplan-Meier curves are shown for time to disease recurrence and OS among patients with high or low gp96 protein expression.

function of gp96 switches from tumor promotion to tumor suppression. Zhu et al [16,18,19] reported that gp96 was a special member of the HSP family and acts as a "Swiss army knife" with multiple functions. In both in immunity and tumorigenicity, gp96 can act as a chaperone to facilitate major histocompatibility complex (MHC) class I peptide loading and therefore increase the tumor peptides presented by MHC class I. Second, exogenous gp96-peptide complexes facilitate MHC class I peptide loading through the uptake of this complex by gp96 receptors on the cell surface. As such, a cytotoxic T lymphocyte (CTL) response could be induced and provoke cellular tumor immunity. In addition, gp96 is able to prime a unique and necessary helper role of natural killer (NK) cells for CTL functioning during the effector arm of the immune response. NK cells activated by gp96 were observed to upregulate the expression of many cytokines and chemokines associated with the enhancement of APCs and T cells, which enhance tumor rejection [20-22]. Zheng et al [22] reported that surface gp96 was involved in dendritic cell maturation leading to inflammation and an increase of antigen-presenting molecules and costimulators. The overexpression of surface gp96 in tumor cells resulted in tumor regression through T lymphocytes. Dai et al [23] further demonstrated in a subsequent study that surface expression of gp96 promotes antigen presentation, which leads to increased memory T-cell development. Together, these data suggest a role of tumor-specific surface gp96 in the activation of NK cells, dendritic cells, and CTL, and present a promising target for cancer immunotherapies.

An early study showed that immunotherapy with gp96 was highly effective in inducing an antitumor immune response in mice bearing methylcholanthrene-induced fibrosarcomas [24]. Another study showed that photodynamic therapy increased surface expression of HSPs, including gp96, which then contributed to therapeutic outcome via induction of inflammation and immune responses [25]. In addition, activated T cells and NK cells were shown to mediate antitumor immune responses against multiple myeloma [26] and lung cancer [27] when stimulated with dendritic cells pulsed with tumor-derived gp96.

It has been reported that gp96 is capable of maturating human monocyte–derived dendritic cells and expanding CTL-specific responses for hepatitis B virus infection or HCC [28,29]. Yang et al [30] observed that a hyposensitive to anticancer drugs for a longer period resulted in greater surface expression and release of gp96 in HCC cells, which might represent a crucial event in the antitumor immune response.

In addition to its role as a pro-oncogenic chaperon, gp96 also plays a crucial role of inducing the antitumor immune response and may become a promising target for cancer immunotherapies. We speculate that although gp96 is very important to liver cirrhosis and carcinogenesis, high expression of gp96 in HCC might be more easily induced than the activation of NK cells, dendritic cells, and CTL, and initiate an antitumor immune response and thus improve outcomes of patients with HCC.

AFP is a well-established biomarker for the diagnosis and prognosis of HCC. A high serum AFP level always correlates with recurrence and metastasis in patients with HCC. However, Shiota [31] reported that 30% of HCC patients did not exhibit a remarkable rise of serum AFP, and AFP may also initially increase in the early stages of HCC, but then decrease or even normalize before rising again through disease progression [32-34]. Hence, we lack a convenient and accurate marker to predict the prognosis of HCC patients with normal AFP levels. In the present study, stratification analysis of patients by AFP level revealed that the 1-, 3-, and 5-year DFS rates in patients with low gp96 expression were worse than those in patients with high gp96 expression. This result suggests that gp96 could be used as a convenient and precise prognostic indictor of postoperative recurrence or metastasis in HCC patients with the normal AFP levels. Because of the heterogeneity of genetic alterations in HCC, individuals with the same tumor stage can have different clinical outcomes [13]. It is very difficult to predict clinical outcomes of early-stage HCC using conventional indexes. In our study, for patients with TNM stage I HCC, the 1-, 3-, and 5-year DFS and OS rates in the low-expression gp96 subgroup were significantly lower than those in the high-expression gp96 subgroup. Moreover, for patients with advanced-stage HCC, the 1-, 3-, and 5-year DFS and OS rates in the low-expression gp96 subgroup were also significantly lower than those in the high-expression gp96 subgroup. This finding may help clinicians develop personal therapeutic strategies for patients with the same TNM stage and low gp96 expression.

There are some limitations to the current study. First, it was a retrospective, single-institution study with a relatively small number of patients. Hence, a well-designed, prospective study with a larger number of patients with HCC who underwent radical surgery is needed to confirm the results. Second, we were not able to split our data set into a training data set and a test data set for statistical validation because of the small number of patients. Third, it is not clear why a high gp96 expression activates antitumor immune responses and improves the prognosis of patients with HCC, rather than promote the development of HCC. The mechanism of this finding is worthy of future investigation.

In conclusion, although gp96 is expressed in all HCC tissue and the normal liver tissue, and gp96 expression in HCC tissues is significantly higher than that in normal liver tissue, high expression of gp96 protein predicts good outcomes of HCC patients who undergo hepatectomy. In addition, For patients with a normal AFP level and in those with early-stage HCC, gp96 also manifests a good predictive value. Thus, gp96 may be a potential prognostic marker for HCC and help the clinicians personalized therapies for HCC patients who are at high risk of recurrence after curative resection.

Authors' contributions

FF.J., Y.Z., Z.B.Z and Y.P.H were the main authors of the manuscript. They were involved in the conception, design and

coordination of the study as well as in data curation, formal analysis, interpretation of results, and drafting the manuscript. The funding was acquired by YPH. The investigation and methodology was completed by Y.G.. F.J., Y.Z., Z.B.Z and Y.P.H was in charge of project administration, resources, software and supervision. S.L.S, Q.H.C, J.W.H, S.Q.L, B.G.P, and L.J.L participated in the validation and visualization. F.J. and Y.P.H. completed the writing and original draft. All authors critically revised and edited the the content of the manuscript, and all authors approved the final manuscript.

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