Aberrant expression of Golgi protein 73 is indicative of a poor outcome in hepatocellular carcinoma

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Abstract. Golgi protein 73 (GP73), a resident Golgi type-II membrane protein, is often upregulated in hepatocytes. In the present study, shRNA-mediated suppression of GP73 expression in hepatocellular carcinoma (HCC) cell lines (MHCC97H, HCCLM3) resulted in a significant inhibition of cell motility and invasion and also led to the regression of epithelial-mesenchymal transition phenotypes. In contrast, overexpression of GP73 in the SMMC7721 cell line retrieved the expression of EMT markers, and promoted cell motility and invasion. High expression of GP73 was also found in HCC tissues with metastasis, as detected by western blot and immunohistochemistry analyses. Kaplan-Meier survival analysis showed that the survival of patients with high GP73 expression was significantly poorer than that of patients with low GP73 expression (p=0.027). Our findings demonstrated an important role of GP73 in HCC metastasis, and indicated that GP73 is a candidate target for HCC therapy.

Introduction

HCC is the fifth most common cancer and the third leading cause of cancer-related mortality globally (1,2). The 5-year survival rate of HCC patients is poor due to tumor recurrence and metastasis (3). The high incidence of metastasis continues to be the main obstacle of the treatment efficacy of HCC. However, the molecular mechanisms of hepatocarcinogenesis and metastasis remain unclear. Elucidation of the detailed mechanisms of HCC cell growth and metastasis is crucial to improve HCC therapeutic intervention. Epithelial-mesenchymal transition (EMT), usually occurring in the critical phases of embryonic development, is the differentiation switch from adherent epithelial cells into contractile and motile mesenchymal cells (4,5). Currently, EMT of cancer cells is thought to attribute much to cancer invasion and metastasis, making it a hallmark of tumor progression (6). Although the molecular mechanism underlying tumor metastasis is still not well elucidated, investigations into this process have led to the hypothesis that many molecules are involved in EMT and play pivotal roles in tumor invasion and metastasis (7).

GP73 is a resident Golgi-specific membrane protein which is highly expressed in HCC patients. Actually, in viral and non-viral liver diseases, expression of GP73 was found to be obviously upregulated in hepatocytes (8-10). In addition, there are reports describing how GP73 is secreted to serum (11). Recent studies suggest that GP73 is a reliable biomarker for the early diagnosis of HCC, and the sensitivity and specificity may be superior to currently used biomarker α fetoprotein (AFP) (12-14). Yet, this remains controversial, as other studies found that serum GP73 levels in patients with liver cirrhosis (LC) were significantly higher than those in patients with HCC; having a lower diagnostic value for HCC (15,16). The decreased survival and severe epithelial abnormalities in the liver and kidneys of a GP73 C-terminal truncated transgenic mouse model helped to determine the physical role of GP73 in epithelial cell function in these organs (17). Sun *et al* showed that an elevated level of GP73 protein is strongly associated with augmented tumor invasion and metastasis, while the exact mechanism of elevated GP73 and tumor metastasis remains largely unknown (18). GP73 was also found to be negatively correlated with E-cadherin and positively correlated with vimentin in tissues, thus it may be associated with EMT in HCC (19).

In the present study, we demonstrated that GP73 enhanced HCC cell invasion by inducing EMT and this may promote the metastasis of HCC. High expression of GP73 was also found in HCC tissues with metastasis. Kaplan-Meier survival analysis showed that the survival of patients with high GP73

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Abbreviations: GP73, Golgi protein 73; HCC, hepatocellular carcinoma; EMT, epithelial-mesenchymal transition; LC, liver cirrhosis; AFP, α-fetoprotein

Key words: invasion, metastasis, GP73, hepatocellular carcinoma, epithelial-mesenchymal transition

expression was significantly poorer than the survival of those with low GP73 expression, indicating it is a candidate target for HCC therapy.

Materials and methods

Cell culture. Immortalized normal human liver cell line L02 and HCC cell lines (Hep3B, HepG2, Huh7, SMMC7721, MHCC97L, MHCC97H, HCCLM3) were used in our study. L02, Hep3B, HepG2, SMMC7721 and Huh7 cells were purchased from the Chinese Academy of Sciences (Shanghai, China). MHCC97L, MHCC97H and HCCLM3 cells were established in the Liver Cancer Institute, Zhongshan Hospital. The cells were all cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) at 37˚C with 5% CO_2.

Lentivirus construction and transfection. The GP73-RNA interference lentiviral vector was constructed by GeneChem Co., Ltd. (Shanghai, China). The double-stranded oligonucleotides targeted to GP73 mRNA targeting coding sequence (5'-AGGGAATGACAGAAACATA-3') was annealed and inserted into the shRNA expression vector pGV115-GFP. The cDNA encoding GP73 was amplified by reverse transcription polymerase chain reaction (RT-PCR) and cloned into the pGV218-GFP vector (the cells stably expressed GP73 shRNA/GP73 proteins). Cells only expressing vectors (Mock cells) were used for the negative control. The lentivirus was generated and harvested (Shanghai GeneChem Co., Ltd.). Then, the lentivirus was transfected into targeted cells with a multiplicity of infection (MOI) of 10 to 50 (optimal MOI is 20).

Transwell migration and invasion assays. Cell migration and invasion assays were performed using a 24-well Transwell (8.0-μm pore size; Millipore, USA) precoated without or with Matrigel (BD Biosciences, USA). SMMC7721, MHCC97H or HCCLM3 cells ($5x10^4$) were suspended in 1.5 ml serum-free DMEM and transferred into the inside chamber of a 24-well cell culture insert with a 8.0-μm pore size. An amount of 600 μl media with 20% FBS was added into the outside well. After incubation for 24 h, the cells remaining on the upper side of the filters were cleaned with cotton-tipped swabs. Cells on the lower surface of the membrane were fixed with methanol and subjected to Giemsa staining. The cells on the underside of the filters were counted in five randomly selected fields (at x200 magnification), and the average cell number per view was calculated. All experiments were performed in triplicate.

Western blot analysis. Twenty-two HCC tissues and their paired adjacent non-tumor tissues were collected from patients undergoing resection at the Liver Cancer Institute, Zhongshan Hospital. The tissues were used for western blot analysis. General characteristics regarding these 22 HCC patients are described in Table I. The study was approved by the Research Ethics Committee of Zhongshan Hospital, and informed consent was obtained from each patient.

Protein concentrations were determined using the bicinchoninic acid (BCA) method. Aliquots (20 μg) of proteins were loaded and resolved by 10% SDS-PAGE and transferred to PVDF membranes using a Bio-Rad SemiDry apparatus. After being blocked for non-specific binding sites, the membranes were incubated with the indicated primary antibodies: anti-GP73 (1:200 dilution; Santa Cruz Biotechnology, Santa Cruz, CA, USA), E-cadherin (1:200 dilution; Abcam, Hong Kong), N-cadherin (1:100 dilution; Invitrogen, Carlsbad, CA, USA) and GAPDH (1:10,000 dilution; Kang-Cheng, Shanghai, China) overnight at 4˚C, followed by HRP-conjugated secondary antibodies for 1 h at room temperature. After washing three times in Tris-buffered saline with 0.1% Tween-20 (TBST), immunoreactive protein bands were visualized using an enhanced chemiluminescence (ECL) detection system (GE Healthcare, Piscataway, NJ, USA).

Table I. General characteristics of the HCC patients whose tissues were used for western blot analysis.

Characteristics	HCC[c]
No. of individuals	22
Gender [male n (%)/female n (%)]	16 (72.7%)/6 (27.3%)
Mean age (years)	52±11
Edmondson-Steiner grade	I (n=10), II (n=12)
HBV DNA (copy)[a] [mean (range)]	$2.3x10^4$ ($1.3x10^3$-$1.7x10^5$)
AFP (ng/ml)[b] [mean (range)]	12,354.6 (3.2-70,321)
$HbsAg^+$ (%)	100
AST (U/l) [mean (range)]	130.4 (16-1,630)
ALT (U/l) [mean (range)]	110.3 (14-1,120)

[a]HBV DNA was detected using fluorescent quantitative PCR (FQ-PCR) and had a detection limit of sensitivity of ~$1x10^3$ genome equivalents per ml. [b]AFP (α-fetoprotein) was determined using standard kits (Abbott Labs) and 20 ng/ml was considered the upper limit of the normal. [c]HCC diagnosis was confirmed by ultrasound imaging and biopsy. AST, aspartate aminotransferase; ALT, alanine aminotransferase.

Table II. General characteristics of the HCC patient whose tissues were used for the immunohistochemical tissue microarray.

Characteristics	HCC[c]
No. of individuals	138
Gender [male n (%)/female n (%)]	103 (74.6%)/35 (25.4%)
Mean age (years)	55±13
Edmondson-Steiner grade	I (n=78), II (n=60)
HBV DNA (copy)[a] [mean (range)]	$1.8x10^4$ ($1.0x10^3$-$1.3x10^5$)
AFP (ng/ml)[b] [mean (range)]	10,345.6 (3.2-70,321)
$HbsAg^+$ (%)	100
AST (U/l) [mean (range)]	134.1 (18-1,567)
ALT (U/l) [mean (range)]	120.7 (16-1,256)

[a]HBV DNA was detected with fluorescent quantitative PCR (FQ-PCR) and has a detection limit of sensitivity of ~$1x10^3$ genome equivalents per ml; [b]AFP (α-fetoprotein) was determined using standard kits and 20 ng/ml was considered the upper limit of the normal; [c]HCC diagnosis was confirmed by ultrasound imaging and biopsy. AST, aspartate aminotransferase; ALT, alanine aminotransferase.

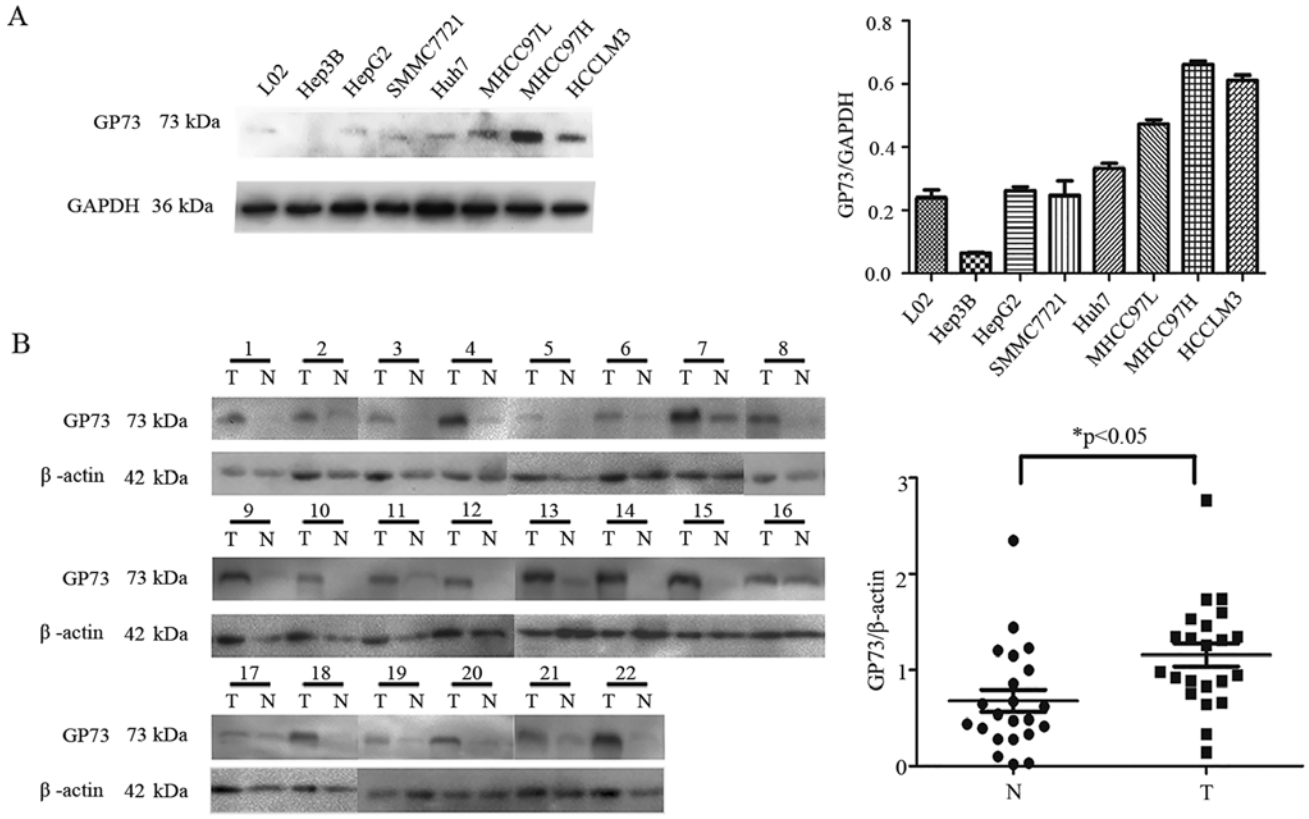

Figure 1. Expression of GP73 is upregulated in human HCCs. (A) Expression of GP73 in a human normal liver cell line (L02) and seven HCC cell lines was examined by western blot assay. (B) Representative western blotting showing the expression of GP73 in tumor tissues (T) and paired non-cancerous tissues (N) from 22 HCC patients. Compared with the paired non-cancerous tissues, the expression of GP73 in the tumor tissues was significantly increased ($^{*}p<0.05$).

Cell immunofluorescence assay. For immunofluorescence staining, cells grown on glass coverslip were fixed in 4% paraformaldehyde and permeabilized using 0.5% Triton X-100. Non-specific binding sites were blocked with normal goat or rabbit serum. The cells were then incubated with the primary antibodies against GP73 (1:50 dilution), E-cadherin (1:100 dilution), N-cadherin (1:100 dilution) overnight at 4˚C. After thorough washing, the cells were then incubated with Alexa-Fluor 555 anti-mouse IgG (1:1,000 dilution; Cell Signaling Technology, Danvers, MA, USA) or anti-goat IgG (1:1,000 dilution; Abcam). Finally, the cells were washed and stained with DAPI. Images were captured using a Leica fluorescence microscope.

Immunohistochemical analysis of tissue microarrays. Tissue samples of 48 HCC patients with or without metastasis were obtained from the Department of Hepatobiliary Surgery, The First Affiliated Hospital of Guangxi Medical University (Nanning, China). HCC diagnosis was based on World Health Organization criteria. Ethical approval was obtained from the Research Ethics Committee of The First Affiliated Hospital of Guangxi Medical University, and written informed consent was obtained from each patient. Tissue microarrays were constructed using formalin-fixed, paraffin-embedded tissue samples. Primary antibody against GP73 (1:50 dilution) and donkey anti-goat secondary antibody was used for immunohistochemical staining. Then, the integrated optical density of the tissue microarray derived from 48 HCC patients was evaluated by IPP software (Image-Pro Plus 5.1).

Staining for GP73 in the tissue microarray of 90 HCC patients was assessed using a previously described scoring method (20). The 90 HCC samples were obtained from the Liver Cancer Institute, Zhongshan Hospital. Ethical approval was obtained from the Research Ethics Committee of Zhongshan Hospital, and written informed consent was obtained from each patient. The staining intensity was scored on a scale of 0 to 3 as negative, weak, medium and strong, respectively. The stained area, which was calculated as the percentage of positively stained cells relative to the total cells, was scored on a scale of 0 to 4: 0 (0%), 1 (1-25%), 2 (26-50%), 3 (51-75%) and 4 (76-100%). The overall score was calculated by multiplying the intensity score and the staining area score. Samples were categorized into four grades: an overall score equal to 0 was graded as '-'; an overall score equal to 1, 2, 3 or 4 was graded as '+'; an overall score equal to 5, 6, 7 or 8 was graded as '++'; an overall score equal to 9, 10, 11 or 12 was graded as '+++'. The stained tissue sections were analyzed by two pathologists without any knowledge regarding the patient clinical information. Based on the immunohistochemical grades, the patients were divided into two groups: the high expression group, which included patients with grades '++' or '+++', and the low expression group, including patients graded as '-' or '+'. General characteristics of these HCC patients for the tissue microarray are described in Table II.

Statistical analysis. Statistical analysis was performed with SPSS 15.0 for Windows (SPSS, Chicago, IL, USA). Data are presented as the mean ± SD unless otherwise indicated. The

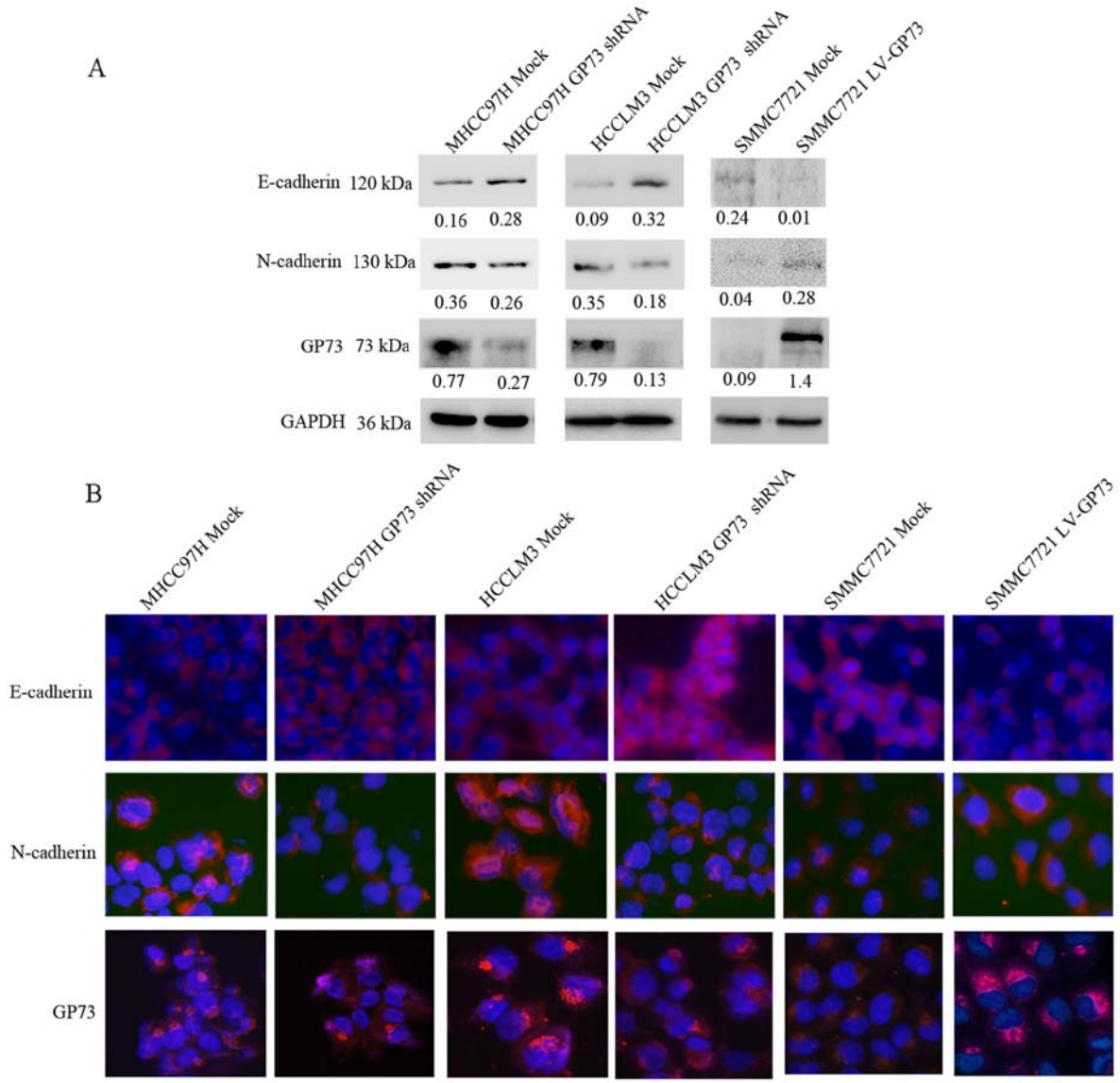

Figure 2. GP73 is correlated with EMT. (A) Expression levels of an epithelial marker (E-cadherin) and a mesenchymal marker (N-cadherin) were determined by western blot analysis in GP73-depleted MHCC97H and HCCLM3 cell lines and the GP73-overexpressing SMMC7721 cell line. (B) The expression of an epithelial marker and a mesenchymal marker in relative cell lines was detected by immunofluorescence staining (magnification, x200). Knockdown of GP73 expression may disrupt the EMT process in MHCC97H and HCCLM3 cells. Overexpression of GP73 induced EMT in the SMMC7721 cells. The ratio values were calculated using Quantity One software.

Student's t-test (two-tailed) was used to compare two groups of parametric variants, and Spearman's Rho test or Chi-square test was used to analyze non-parametric variants. $p<0.05$ was considered to indicate a statistically significant result.

Results

GP73 is correlated with cellular EMT. Expression levels of GP73 in various human cell lines (L02, Hep3B, HepG2, Huh7, SMMC7721, MHCC97L, MHCC97H and HCCLM3) were investigated. As shown in Fig. 1A, increased expression of GP73 was observed in HCC cell lines with high metastatic potential (MHCC97L, MHCC97H, HCCLM3) compared with the levels in the low or non-metastatic cell lines (L02, Hep3B, HepG2, Huh7, SMMC7721). To elucidate the effects of GP73 knockdown and overexpression on HCC cell behavior, lentiviral-mediated shRNA was used to knock down the expression of GP73 in the MHCC97H and HCCLM3 cells, while a GP73 cDNA expression vector was introduced into the SMMC7721 cells for overexpression of GP73. GP73 expression in the transfected cells was confirmed by western blotting (Fig. 2A). To examine whether GP73 promotes EMT, cellular morphology was observed. Knockdown of GP73 led to marked morphological changes from a mesenchymal-like phenotype to an epithelial-like phenotype in the MHCC97H and HCCLM3 cells (Fig. 3A and B). While in the SMMC7721 cells, overexpression of GP73 altered cells from an epithelial-like phenotype to a mesenchymal-like phenotype (Fig. 3C). Western blot and cell immunofluorescence analyses were used to detect expression of EMT markers in the GP73-shRNA-treated MHCC97H and HCCLM3 cells and the GP73-overexpressing SMMC7721 cells. In the GP73-shRNA-

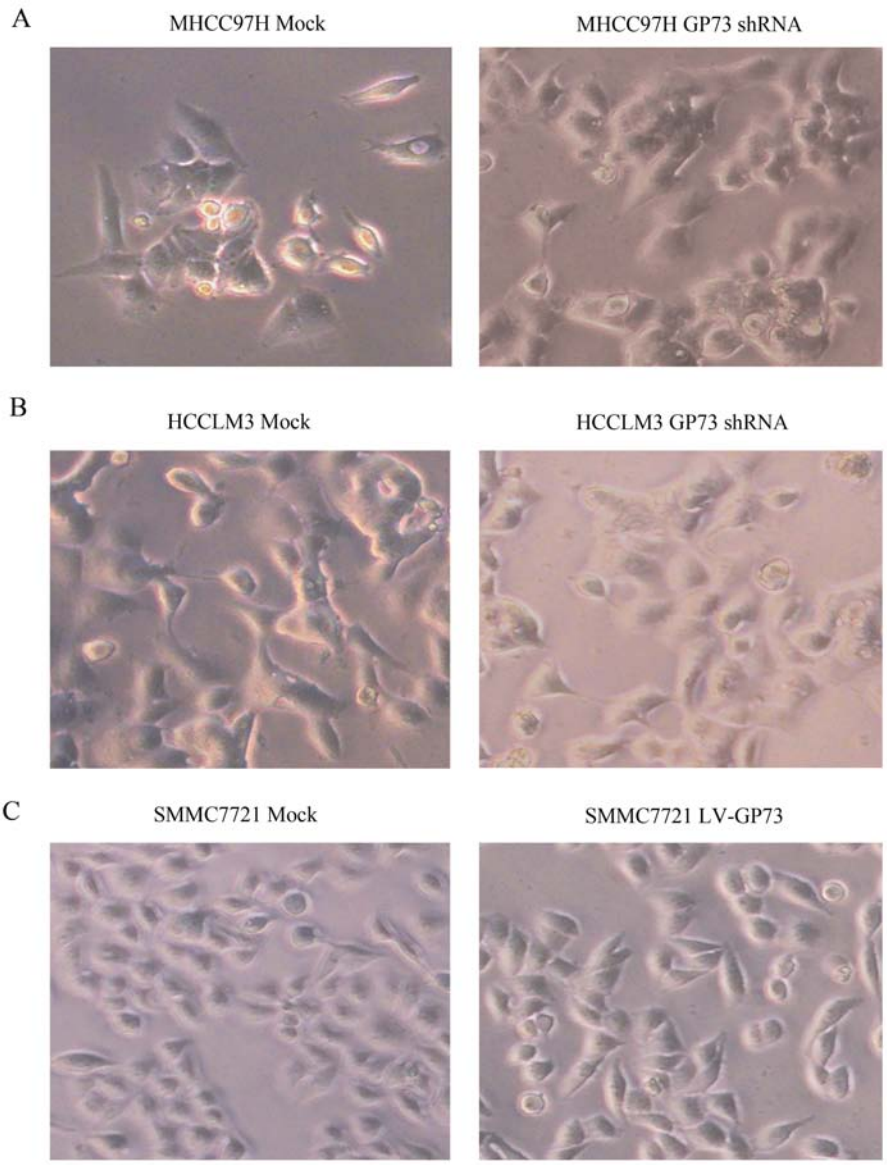

Figure 3. Morphological changes in HCC cells after knockdown and overexpression of GP73 (magnification, x200). (A) Morphological change from a mesenchymal-like phenotype to an epithelial-like phenotype in MHCC97H GP73 shRNA cells compared with MHCC97H Mock cells (B) Morphological change from a mesenchymal-like phenotype to an epithelial-like phenotype in HCCLM3 GP73 shRNA cells compared with HCCLM3 Mock cells. (C) Overexpression of GP73 altered an epithelial-like phenotype into a mesenchymal-like phenotype. [The cells stably expressed GP73 shRNA/GP73 proteins. Cells only expressing vectors (Mock cells) were used as the negative control].

treated cells, the expression of E-cadherin (an epithelial marker) was increased and the expression of N-cadherin (a mesenchymal marker) was decreased. In contrast, the protein level of E-cadherin was downregulated and N-cadherin was upregulated in the GP73-overexpressing cells (Fig. 2). These findings indicated that GP73 was involved in EMT in HCC cell lines.

Knockdown of GP73 expression inhibits cell motility and invasion. We also examined whether the change in GP73 expression affects the proliferation, migration and invasion of the HCC cells using the transfected (knockdown or overexpression of GP73) cell lines. MHCC97H Mock and HCCLM3 Mock cells (cells transfected with scrambled shRNA) and MHCC97H GP73 shRNA and HCCLM3 GP73 shRNA cells (cells transfected with GP73 shRNA), grew at similar rates (Fig. 4A and B). The same results (Fig. 4C) were obtained for the GP73-overexpressing SMMC7721 cells, indicating that GP73 was not required for the proliferation of HCC cells. Cell migration using a Transwell assay chamber was also assessed. Cells that migrated into the lower compartment of the migration chamber were fixed and then stained with Giemsa. Effective knockdown of GP73 in both MHCC97H and HCCLM3 cells markedly decreased cellular motility, as the number of migrated cells was significantly less than that for the MHCC97H Mock and HCCLM3 Mock cells. The invasive activity caused by knockdown of GP73 was determined using invasion chamber assays with Matrigel. Consistent with the cell migration results, knockdown of GP73 significantly decreased cell invasion capacity compared

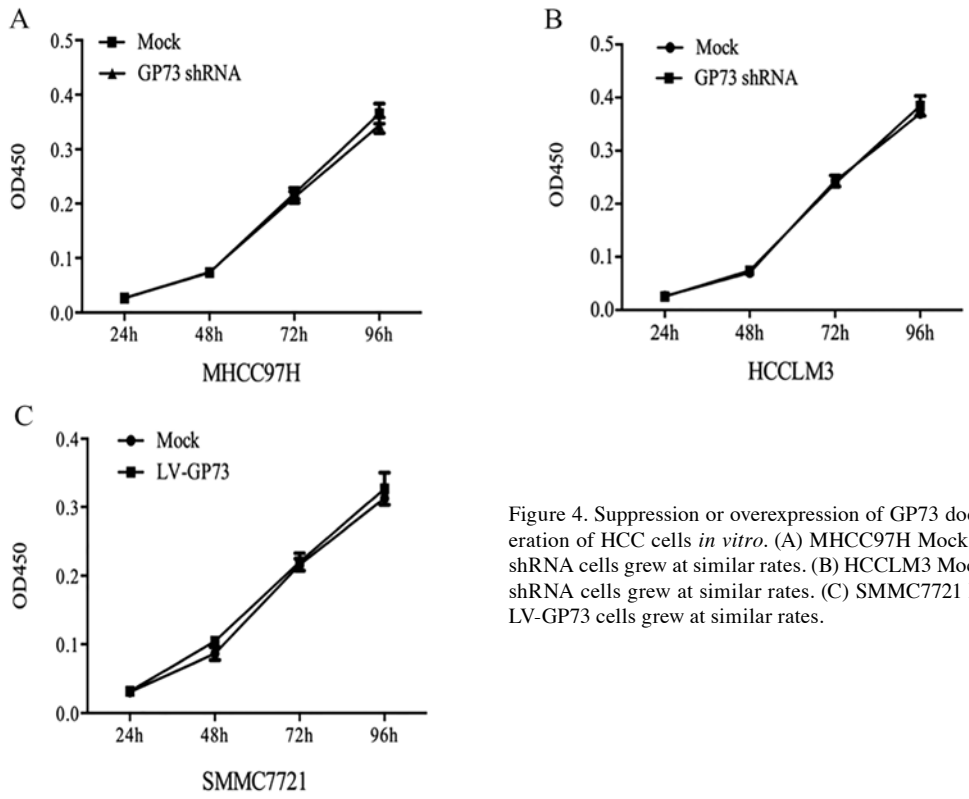

Figure 4. Suppression or overexpression of GP73 does not affect the proliferation of HCC cells *in vitro*. (A) MHCC97H Mock and MHCC97H GP73 shRNA cells grew at similar rates. (B) HCCLM3 Mock and HCCLM3 GP73 shRNA cells grew at similar rates. (C) SMMC7721 Mock and SMMC7721 LV-GP73 cells grew at similar rates.

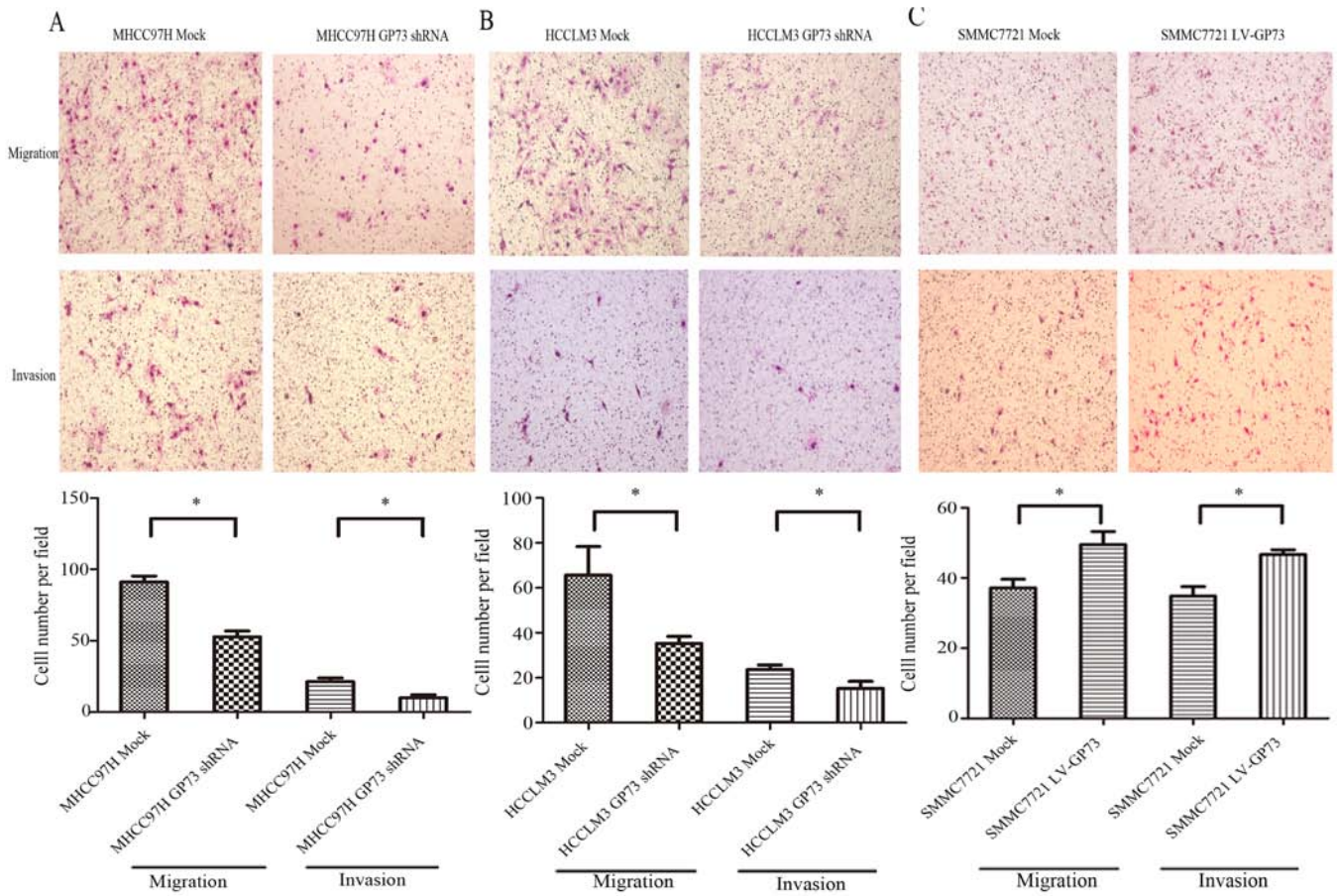

Figure 5. GP73 regulates the migration and invasion of HCC cells. The number of migratory and invasive cells in the HCC cells [(A) MHCC97H Mock vs. MHCC97H GP73 shRNA, (B) HCCLM3 Mock vs. HCCLM3 GP73 shRNA, (C) SMMC7721 Mock vs. SMMC7721 LV-GP73] as determined by migration and invasion assays. The migratory and invasive properties of the cells were analyzed as described in Materials and methods. Error bars represent SD. $^{*}p<0.05$.

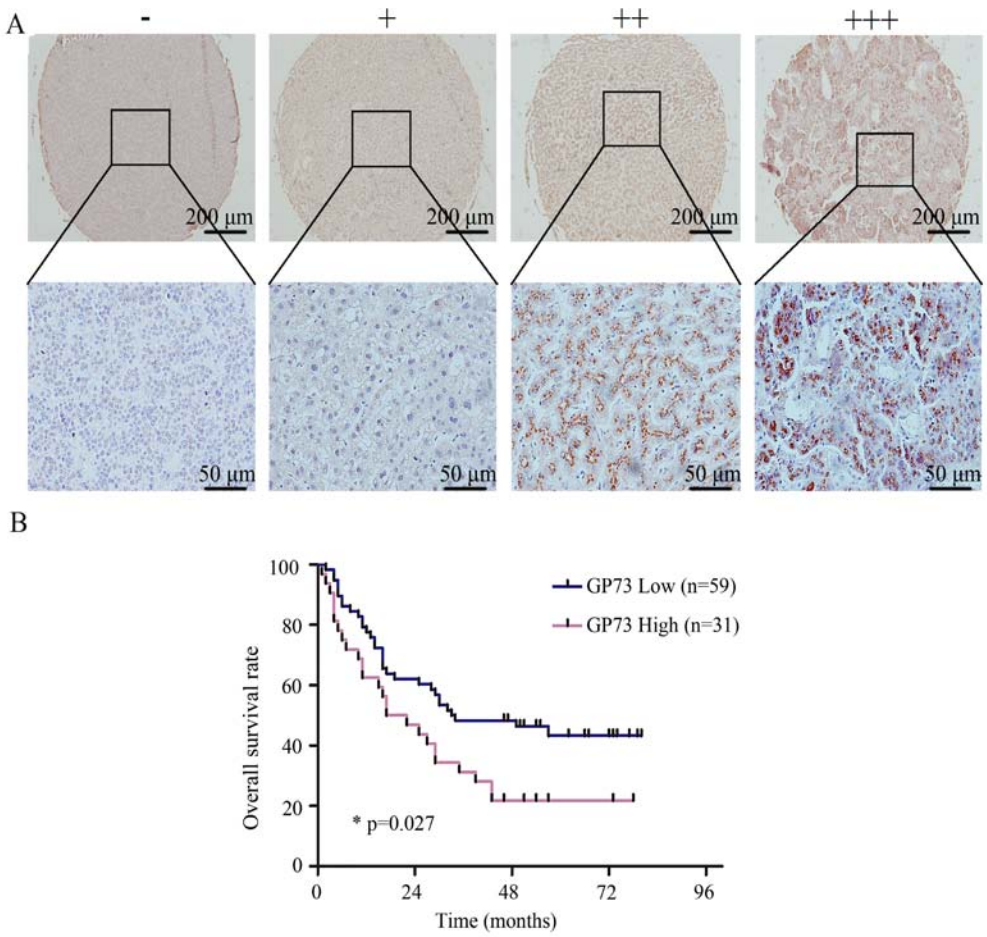

Figure 6. GP73 expression is correlated with the survival of HCC patients. (A) Representative low and high GP73 expression as shown by immunohistochemistry. (B) Kaplan-Meier analysis of overall survival in HCC patients with low GP73 expression (n=59) or high GP73 expression (n=31) using SPSS 13.0.

with the mock cells (Fig. 5A and B). Taken together, these results indicated that knockdown of GP73 in MHCC97H and HCCLM3 cells inhibited cell migratory and invasive abilities.

Overexpression of GP73 enhances cell motility and invasion. Cell migration and invasion abilities in the SMMC7721 LV-GP73 cells (cells overexpressing GP73) were assessed. The results showed that overexpression of GP73 markedly increased cell migratory and invasive abilities, as the numbers of migrated SMMC7721 LV-GP73 cells were significantly higher compared to the SMMC7721 Mock cells (cells transfected with an empty vector) (Fig. 5C). Consistent with the results of GP73 knockdown in the MHCC97H and HCCLM3 cells, GP73 overexpression in SMMC7721 cells enhanced cell invasion and metastasis.

High expression of GP73 is correlated with poor survival and metastasis of HCC patients. GP73 was significantly increased in the HCC tissues compared with the paired non-cancerous tissues in 22 patients by western blot analysis (Fig. 1B). Furthermore, HCC tissues from 90 patients with survival information from an 80-month follow-up period were collected for production of a tissue microarray. The typical images of negative and positive staining of GP73 are shown in Fig. 6A. Kaplan-Meier survival analysis showed that the survival of patients with high GP73 expression was significantly poorer than the survival of those with low GP73 expression (Fig. 6B). The GP73 level in the HCC tissues derived from the patients with metastasis was obviously increased in comparison with that in the HCC tissues from patients without metastasis by immunohistochemical analysis, indicating the possible role of GP73 in HCC metastasis and its aberrant expression was indicative of poor outcomes in HCC (Fig. 7).

Discussion

Previous studies have demonstrated the upregulation of GP73 in liver diseases (21,22). In addition, upregulation of GP73 has been reported in Alzheimer's disease (23), Wilson's disease (24), prostate cancer (25-27), renal cell cancer (28) and lung cancer (29). A number of studies show that serum GP73 levels in patients with liver disease are markedly upregulated (13,14,30), suggesting that GP73 may play an important role in liver disease. Hu *et al* showed that the transmembrane domain with a positively charged residue in the cytoplasmic N-terminal tail was necessary to support its Golgi localization (31). However, the function of GP73 remains unclear.

A previous study showed that expression of GP73 may be associated with enhanced tumor invasion and metastasis (18). In contrast, it was reported that GP73 expression had no

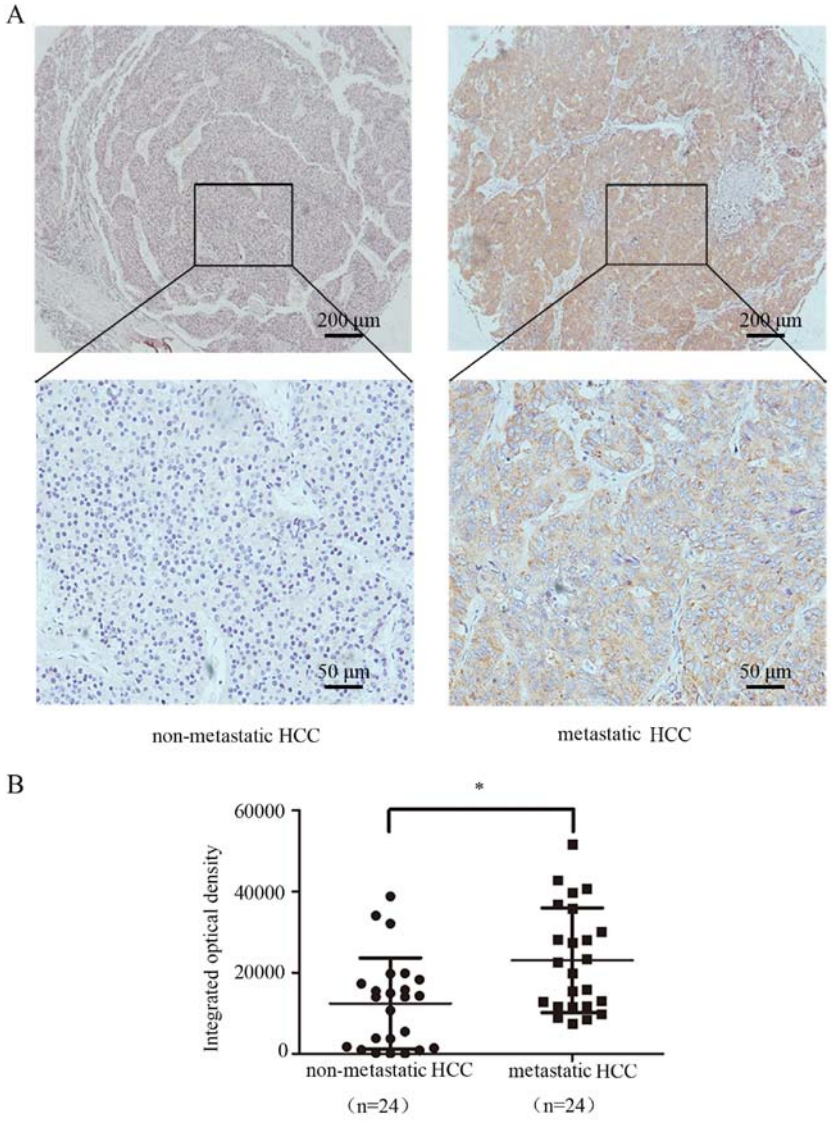

Figure 7. Immunohistochemical analysis of GP73 was performed using a tissue microarray containing 48 HCC tissues (24 non-metastatic HCC tissue dots and 24 metastatic HCC tissue dots). (A) Tissues were stained with goat anti-GP73 antibody and visualized with a donkey anti-goat secondary antibody. (B) Compared with primary HCC, the expression of GP73 in HCC specimens with metastasis was significantly increased. The integrate optical density was evaluated by IPP software (Image-Pro Plus 5.1). $^{*}p<0.05$.

relation with HCC metastasis (33). Different reagents used in various studies may have led to such discrepancy (34). Whether GP73 really plays a role in HCC metastasis is of great significance. Recently, GP73 was found to be associated with EMT markers in HCC (19). EMT plays a pivotal role in tumor metastasis. Evidence suggests that EMT could give rise to carcinoma cell metastasis (35-38). Although numerous factors have been identified to participate in EMT (4,39), whether GP73 promotes cancer metastasis via EMT remains unclear. To investigate whether GP73 is related with EMT in HCC progression, stable cell lines were established by recombinant lentiviruses for knockdown and overexpression of GP73. The results showed that the mesenchymal marker, N-cadherin, was upregulated in the SMMC7721 LV-GP73 cells, whereas the epithelial marker, E-cadherin, was decreased. These results were further confirmed by immunofluorescence staining analysis of the cultured cells. Opposite results were obtained for the MHCC97H GP73 shRNA and HCCLM3 GP73 shRNA cells. A significant regression of EMT features was observed; a gain in the expression of epithelial marker, E-cadherin and a loss in the expression of mesenchymal marker, N-cadherin. Thus, GP73 was found to be involved in EMT in the HCC cell lines.

As EMT is a process involved during cancer metastasis, cell migratory and invasive abilities were also explored. The results showed that MHCC97H GP73 shRNA and HCCLM3 GP73 shRNA cells had significantly reduced cell migration and invasion capabilities, while SMMC7721 LV-GP73 cells exhibited increased cell migratory and invasive abilities. The proliferation ability of these cells was not affected. Thus, we conclude that GP73 was responsible for HCC invasion. This finding may be in agreement with the results of Sun *et al* which showed that a higher GP73 expression level was detected in tumors with a larger load or stronger invasiveness, indicating that the overexpression of GP73 protein may be involved in the progression of HCC (18). These data indicated that GP73 may promote HCC metastasis via, at least partially, induction of EMT in HCC cell lines. However, the mechanisms of how

GP73 influences the progression of HCC and EMT remain unclear. Since GP73 is a Golgi transmembrane protein, it is unlikely whether GP73 is directly involved in the signaling pathways inducing EMT. We suspect that GP73 interacted with other important proteins directly in the EMT pathways to play its role in the process. Related reports are largely limited. Further studies should focus on this issue in order to explain the detailed mechanism of GP73 in HCC EMT.

In the present study, GP73 expression in the HCC tissues was higher than the level in the paired non-cancerous tissues. The survival of patients with high GP73 expression was significantly poorer than the survival of those with low GP73 expression, which was in accord with a study of Chen *et al* (32). GP73 expression in the HCC tissues with metastasis and the primary tumors was then detected. HCC tissues with metastasis had higher GP73 expression, and thus strong expression of GP73 in tumor tissues was correlated with metastasis and poor survival. Hence, GP73 may be a potential biomarker for HCC prognosis and a candidate target for HCC therapy. However, the sample size for this study was limited and more stratified samples should be considered for further study.

In conclusion, the present data indicated that GP73 may play an important role in HCC metastasis by EMT induction. High expression of GP73 was also found in HCC tissues with metastasis, and the survival of patients with high GP73 expression was significantly poorer than the survival of those with low GP73 expression. Taken together, the function of GP73 is of potential value for understanding tumor metastasis and it is a candidate target for HCC therapy.

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