

Elevated GPC3 level promotes cell proliferation in liver cancer

SHANSHAN WANG^{1,2}, NING CHEN³, YUHAN CHEN³, LIN SUN⁴, LI LI¹ and HUI LIU⁴

¹Beijing You'An Hospital Affiliated to Capital Medical University, Beijing Institute of Hepatology;

²Beijing Precision Medicine and Transformation Engineering Technology Research Center of Hepatitis and Liver Cancer;

³Department of Gastrointestinal and Hepatology; ⁴Department of Pathology,

Beijing You'An Hospital Affiliated to Capital Medical University, Beijing 100069, P.R. China

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Abstract. The aim of the present study was to investigate the biological role of glypican 3 (GPC3), and to identify its mechanism and clinical significance in the carcinogenesis of liver cancer. A total of 114 patients with liver cancer were involved. Their clinical data, hematoxylin and eosin-stained and Antigen Ki-67 protein (Ki-67) and GPC3 immunohistochemically-stained liver cancer tissue sections were analyzed to evaluate the correlation between the liver cancer proliferation, differentiation and GPC3 expression. Fluorescence microscopy, western blotting, MTT and reverse transcription quantitative polymerase chain reaction (RT-qPCR) assays were performed in HepG2 and HLE cell lines to investigate the potential mechanisms of action. Among the 114 patients with liver cancer enrolled in the present study, 12 exhibited well-differentiated liver cancer, of which 6 (50%) were positive for GPC3. A total of 30 cases exhibited poorly differentiated liver cancer; 26 (87%) of these expressed GPC3 and 11 cases (37%) demonstrated strong positive expression levels. The other 72 liver cancer cases were moderately differentiated; 75% (54/72) of these expressed GPC3 and 12.5% (9/72) exhibited strong positive expression levels. There was a significant association between the levels of GPC3 expression and liver cancer differentiation ($\chi^2=16.306$, $P=0.008$). Ki-67 staining as the criteria of the liver cancer cell proliferation index also indicated a cross correlation between liver cancer proliferation and GPC3 levels. Among the 39 liver cancer samples with a cell proliferation index $<5\%$, only 2.6% (1/39) exhibited strong positive GPC3 staining, but of the 16 cases with a high cell proliferation index $>50\%$, 6 exhibited strong GPC3 staining (37.5%). The difference of cell proliferation indexes between cancer cells were well, moderate and poorly differentiated, and was markedly significant ($\chi^2=26.334$, $P=0.002$), and suggested

that liver cancer cell proliferation was positively correlated with GPC3 expression ($r=0.316$, $P=0.001$). Consistently, *in vitro* analysis indicated that GPC3 promoted HepG2 and HLE cell growth, which was more apparent in HepG2 cells. The RT-qPCR results indicated that GPC3 promoted proliferation through the Hedgehog (Hh) pathway in HepG2 cells, but not in HLE cells. In the present study, it was demonstrated that patients with liver cancer with higher GPC3 levels exhibited poorer differentiation and higher proliferation levels. *In vitro* GPC3 may promote liver cancer cell lines proliferation through the Hh pathway.

Introduction

Glypican 3 (GPC3) is a 60 kDa cell-surface protein that belongs to the glypican family, and its gene is located at chromosome Xq26 (1). Glypicans are cell surface proteoglycans that are associated with the outer leaflet of the plasma membrane by a glycosyl phosphatidyl inositol anchor (2,3). This gene is expressed in a tissue-specific manner, exhibits its peak expression during embryonic tissue development and is downregulated in mature tissues (4). Glypicans are expressed predominantly during development, suggesting that they serve a role in morphogenesis (5,6).

Glypican 3 mutations have been identified as the genetic defects associated with Simpson Golabi-Behmel syndrome (SGBS), which is characterized by overgrowth, dysplasia and multiple congenital anomalies, and by an increased prevalence of Wilm's tumors, nephroblastoma and hepatoblastoma (7,8), malignant melanoma (9), ovarian cancer (10) and testicular germ cell tumors (11). The role of this protein in different types of cancer has not yet been well-defined. The Antigen Ki-67 protein (Ki-67) is an antigen associated with active cell proliferation (12). Higher levels of Ki-67 expression in tumor tissues were identified to be associated with a higher mitotic activity (12).

By the end of 2012, liver cancer is the second most frequently diagnosed cancer among the men in less development countries (13). China has observed an increasing incidence of liver cancer, and this type of malignant tumor has become the estimated second most common cause of cancer-associated mortality in China (14). GPC3 was first identified as a potential biomarker of liver cancer since its level increased significantly in the serum of patients with liver cancer in comparison with

Correspondence to: Dr Hui Liu, Department of Pathology, Beijing You'An Hospital Affiliated to Capital Medical University, 8 Xi TouTiao, You'An Men Wai, Fengtai, Beijing 100069, P.R. China
E-mail: liuhuier@126.com

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healthy donors and patients with benign liver tumors (15). The role GPC3 serves in liver cancer is controversial and its precise mechanism of action and clinical significance remains uncharacterized.

In the present study, the correlation between GPC3 expression levels in 114 patients with liver cancer and the levels of cancer cell differentiation and proliferation were retrospectively analyzed. The *in vitro* effects of GPC3 on liver cancer cells growth were observed, to verify the assumption that elevated GPC3 is a significant risk factor in liver cancer mortality due to its capability of promoting growth in tumor cells.

Materials and methods

Subjects. A total of 114 patients with histopathologically-confirmed liver cancer were recruited for the present study from Beijing You'An Hospital Affiliated to Capital Medical University (Beijing, China). All the specimens were obtained via surgical resection or liver transplantation procedures. The patient ages ranged from 19-67 years (mean age, 51.2 years), and including 97 males and 17 females. The diagnosis of liver cancer was performed following the WHO Classification of Tumors of the Digestive System (16). All the tissue samples were reviewed by 2 independent experienced pathologists (Department of Pathology, Beijing You'An Hospital Affiliated to Capital Medical University, Beijing, China). The study protocol was approved by the Ethical Committee of Beijing You'An Hospital. Written informed consent was obtained from all patients.

Immunohistochemical (IHC) staining. Liver cancer specimens were fixed in 10% neutral-buffered formalin for >24 h at room temperature. Paraffin-embedded tissue blocks were cut into 4- μ m sections for immunohistochemical staining following the tissues being handled by dehydrating, clearing, dipping wax and embedding. The expression levels of GPC3 and Ki-67 in tissues were assessed by IHC staining with monoclonal anti-GPC3 and anti-Ki-67 antibodies (catalog nos., sc-390587 and sc-23900; dilution, 1:100 and 1:200; both Santa Cruz Biotechnology, Inc., Dallas, TX, USA). A non-immune mouse IgG antibody (catalog no. ZM-0491; dilution, 1:50; OriGene Technologies, Inc., Beijing, China) was used as negative control reagent for each specimen. In brief, tissue sections were de-paraffinized in xylene at room temperature for 15 min and rehydrated in 100, 95, 85, 70 and 50% ethyl alcohol for 5 min at each concentration at room temperature, and heat-induced epitope retrieval was performed in a 10 mmol/l citrate buffer (pH 6.0) at 92°C for 15 min. Then, endogenous peroxidase was blocked with 3% H₂O₂ followed by incubation with primary antibodies overnight at 4°C. The tissues were then incubated for additional 60 min at room temperature on the second day with a biotin-free horseradish peroxidase-labeled sheep anti-mouse IgG secondary antibody (catalog no., ZDR-5307; dilution, 1:500; OriGene Technologies, Inc.). Staining was performed with 3,3'-diaminobenzidine (ZLI-9019, dilution concentrations: 1:20; OriGene Technologies, Inc.) at room temperature for 5 min.

GPC3 staining was considered positive when brown granules were located in the cytoplasm, membrane or canaliculi (17). The results were evaluated according to the proportion and staining intensity of positive cells. The proportions of positive

cells were scored as: 0, <5; 1, 5-25; 2, 26-50; and 3, >50%. The staining intensities were scored as: 0, no staining; 1, weak; 2, intermediate; and 3, strong. Next, the results of multiplying the proportion scores by the intensity scores were classified into 4 grades as follow: -, 0-1; +, 2-4; ++, 5-7; and +++, >8. Positive Ki-67 staining was indicated by brown granules located in nucleus. In order to determine the proliferation index, 500 tumor cells and the number of Ki-67-positive cells were counted at x400 magnification in several dense positive cell areas. The cell proliferation indexes were evaluated according to the percentage of the number of positive cells/500 tumor cells, as follows: -, <5; +, 5-25; ++, 26-50; and +++, >50%. All results were reviewed by light microscopy (x200 magnification) by two blinded, experienced pathologists, as previously stated.

Cell lines and treatment. The GPC3-producing HepG2 cell line, demonstrated to be a hepatoblastoma cell line (18) and the non-GPC3-producing hepatocellular carcinoma HLE cell line were cultured in Dulbecco's modified Eagle's medium (Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 10% w/v fetal bovine serum (Thermo Fisher Scientific, Inc.) at 37°C in a humidified atmosphere of 5% CO₂. HepG2 and HLE cells were gifts from the Department of Biochemistry and Molecular Biology, Peking University Health Science Center (Beijing, China).

GPC3 (10088-H08H; Sino Biological, Inc. Beijing, China) was dissolved in PBS to final concentrations of 0.01, 0.1, 1 and 10 mg/ml, which were then used to treat the 2 cultured cell lines. PBS, which served as the solvent of GPC3, was used as a control.

Western blotting. Western blotting was performed for the analysis of expression of GPC3 in HepG2 and HLE cells. Briefly, cells were lysed in RIPA lysis buffer (Beyotime Institute of Biotechnology, Beijing, China) and 40 μ g protein was utilized for each western blot. BCA assay was used to determinate the protein concentrations (Beyotime Institute of Biotechnology). Electrophoretic transfer of proteins was performed from 10% SDS-PAGE gels onto nitrocellulose membranes. Membranes were blocked by immersion in 5% non-fat milk (w/v)/PBS for 1 h at room temperature, and then incubated at 4°C overnight with anti-GPC3 (dilution, 1:500; catalog no., sc-390587) and β -actin monoclonal antibodies (dilution, 1:1,000; catalog no., sc-130656; both Santa Cruz Biotechnology, Inc., Dallas, TX, USA). Subsequent to rinsing with PBST for three times, membranes were incubated at 37°C with a horseradish peroxidase-conjugated IgG secondary antibody for 1 h. Immunocomplexes were visualized by incubation of the membranes with the Enhanced Chemiluminescence kit (catalog no., 32109, Thermo Fisher Scientific, Inc.) at room temperature for 1 min according to the manufacturer's protocol.

Immunofluorescence. Localization of GPC3 in HepG2 and HLE cells was observed at x1,000 magnification. Immunofluorescence staining was performed by a routine staining method (19). Mouse anti-human GPC3 and secondary goat anti-mouse antibody conjugated with fluorescence rhodamine (TRITC) (sc-362277, dilution 1:200) against GPC3 were purchased from Santa Cruz Biotechnology, Inc. Nuclei were

counterstained with DAPI (100 $\mu\text{g/ml}$) at room temperature for 1 min. Intracellular localization of proteins in each group were observed, and images were captured with a fluorescence microscope (magnification, x1,000).

MTT and Cell Counting Kit-8 (CCK-8) assays. To assess the effect of GPC3 on cell proliferation, MTT colorimetric assays and analyses were performed with the CCK-8 kit (Dojindo Molecular Technologies, Inc., Kumamoto, Japan). HepG2 and HLE cells were seeded into 96-well plates at a density of 5×10^3 cells/well in 200 μl medium, and then treated with various concentrations of GPC3 (0, 0.01, 0.1, 1 and 10 mg/ml). Following 24 h incubation at 37°C, MTT solution was added to the wells, followed by incubation at 37°C for 1 h. The medium was removed carefully, and dimethyl sulfoxide was added to dissolve the blue formazan in the living cells. Absorbance was measured at 570 nm for MTT assay and 450 nm for CCK-8 with an ELISA reader. The percentage of cell proliferation for each treatment was calculated as [(A570 sample-background)/(A570 control-background)] x 100.

Reverse transcription quantitative polymerase chain reaction (RT-qPCR). The RNeasy Mini kit (Qiagen GmbH, Hilden, Germany) was used to isolate total RNA from cultured HepG2 and HLE cells. cDNA was then synthesized by reverse transcribing 1 mg extracted RNA using a SuperScript II First-stand Synthesis System (Invitrogen; Thermo Fisher Scientific, Inc.). SYBR Green (QPK-201; Toyobo Life Science, Osaka, Japan) was used to detect the double-stranded DNA products during the qPCR assay. The mRNA content was normalized to the housekeeping gene GAPDH. All primer sequences for RT-qPCR are summarized in Table I. The reaction conditions were: 95°C for 5 min, then followed by 40 cycles at 95°C for 10 sec and 55°C for 40 sec with ABI 9500 sequence detection system. The RT-qPCR results were normalized to GAPDH according to the $2^{-\Delta\Delta C_t}$ method (20).

Statistical analysis. All data were statistically analyzed using SPSS software version 17.0 (SPSS, Inc., Chicago, IL, USA). The χ^2 test was performed to analyze the association between GPC3 and cell differentiation or cell proliferation. Correlation analysis was performed using Spearman's rank test. The *in vitro* results of multiple observations were presented as the mean \pm standard deviation of at least three separate experiments, and analyzed using a one-way analysis of variance followed by a Least Significant Difference test to compare the treatment and control groups. $P \leq 0.05$ was considered to indicate a statistically significant difference.

Results

Association between GPC3 expression and the differentiation of liver cancer. Among the 114 patients with liver cancer, 12 cases were well-differentiated. Of these, 50% (6/12) expressed GPC3, with 1+ expression in 2 cases, 2+ expression in 2 cases and 3+ expression in 2 cases. A total of 72 patients with liver cancer were moderately differentiated, and 75% (54/72) expressed GPC3. Of these, there were 32 cases with + expression (44.44%), 13 cases with ++ expression (18.06%) and 9 cases with +++ expression (12.50%). Of the other 30 patients

Table I. Sequences of oligonucleotides used as primers for reverse transcription quantitative polymerase chain reaction.

Gene name	Primer sequences
SHH	
Sense	5'-ACAGCGACTTCCTCACTTTCTCT-3'
Antisense	5'-CCGCGTCTCGATCACGTAGA-3'
GLI1	
Sense	5'-TTCCTACCAGAGTCCCAAGT-3'
Antisense	5'-CCCTATGTGAAGCCCTATTT-3'
PTCH	
Sense	5'-GGCAGGAGGAGTTGATTG-3'
Antisense	5'-CGTACATTTGCTTGGGAGT-3'
SMO	
Sense	5'-AGCTTCCGGGACTATGTGC-3'
Antisense	5'-GCTCGGGCGATTCTTGAT -3'
GAPDH	
Sense	5'-TGAAGGTCGGAGTCAACGGA-3'
Antisense	5'-CCTGGAAGATGGTGTATGGGAT-3'

All primers were provided by Beijing Biomed Co., Ltd. (Beijing, China; <http://www.biomed168.com>). SHH, Sonic hedgehog; GLI1, Zinc finger protein GLI1; PTCH, Protein patched homolog 1; SMO, Smoothed homolog.

Table II. Association between GPC3 expression and the differentiation in liver cancer samples.

Degree of differentiation	GPC3				Total
	-	+	++	+++	
Well-differentiated	6	2	2	2	12
Moderately-differentiated	18	32	13	9	72
Poorly-differentiated	4	6	9	11	30

GPC3, glypican 3; (-), positive area <5%; (+), positive area 5-24%; (++) , positive area 25-49%; and (+++), positive area >50%.

with liver cancer with poor differentiation, 6 cases exhibited + expression (20.00%), 9 cases exhibited ++ expression (30.00%) and 11 cases exhibited +++ expression (36.67%). These results demonstrated a statistical significance between GPC3 expression and liver cancer differentiation ($\chi^2=16.306$, $P=0.008$), and there was a significant correlation between GPC3 expression and liver cancer differentiation ($r=0.302$, $P=0.01$): The poorer the differentiation stage, the higher the level of the GPC3 expression (Table II; Figs. 1 and 2A).

GPC3 expression is significantly correlated with the expression of Ki-67. Ki-67 IHC staining usually represents the cell proliferation index (12). Among the 39 patients with liver cancer that were Ki-67-negative, 21 cases (53.85%) expressed GPC3, but only 1 (2.56%) case was strong positive (+++).

Table III. GPC3 expression is significantly associated with the expression of Ki-67.

Ki-67 expression	GPC3				Total
	-	+	++	+++	
-	18	13	7	1	39
+	2	13	11	8	34
++	3	6	9	7	25
+++	2	5	3	6	16

GPC3, glypican 3. (-), positive area <5%; (+), positive area 5-24%; (++) , positive area 25-49%; and (+++) , positive area >50%.

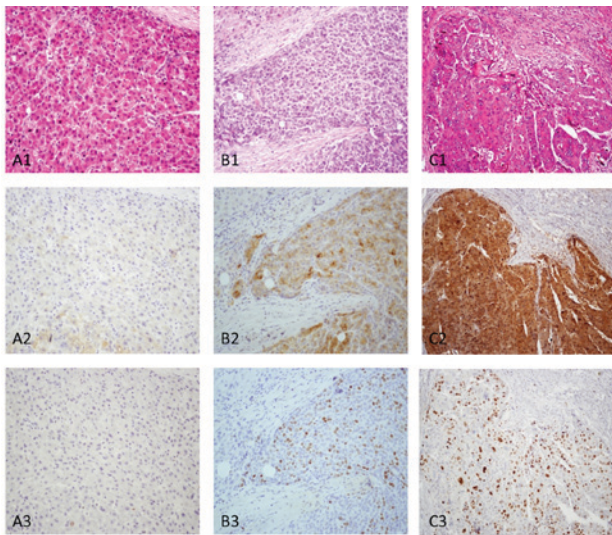


Figure 1. Association between GPC3 expression and the differentiation of liver cancer. (A) Well-differentiated liver cancer tissue and the same field. (A1) The tumor cells demonstrated a thin trabecular pattern with mild atypical cells (HE at magnification, x200). (A2) GPC3 was absent (GPC3 immunostaining at magnification, x200). (A3) Positive cells were <5% (Ki-67 immunostaining at magnification, x200). (B) Moderately-differentiated liver cancer. (B1) Tumor cells arranged in trabeculae measuring ≥ 3 cells-thick (HE at magnification, x200). (B2) Tumor tissue indicated moderate staining with GPC3 (GPC3 immunostaining at magnification, x200). (B3) Positive cells were $\sim 30\%$ (Ki-67 immunostaining, x200). (C) Poorly differentiated liver cancer and same field. (C1) The tumor tissue exhibited a solid pattern with marked atypical cells (HE at magnification, x200). (C2) Tumor tissue indicated strong staining with GPC3 (GPC3 immunostaining at magnification, x200). (C3) Positive cells were >50% (Ki-67 immunostaining at magnification, x200). HE, hematoxylin and eosin; GPC3, glypican 3; Ki-67, antigen Ki-67.

There was 94.12% (32/34) positive expression for GPC3 in liver cancer samples with low-grade cell proliferation (Ki-67+), 76% (19/25) of samples with intermediate-grade cell proliferation (Ki-67++) were positive for GPC3 and 87.5% (14/16) of samples with high-grade cell proliferation (Ki-67+++) exhibited GPC3 expression. The numbers of liver cancer samples with marked GPC3 expression with low, intermediate and high grades of cell proliferation were 23.53 (8/34), 28 (7/25) and 37.5% (6/16), respectively. There was a marked positive correlation between GPC3 and cell proliferation ($r=0.316$, $P=0.01$). The results indicated that cancer cell

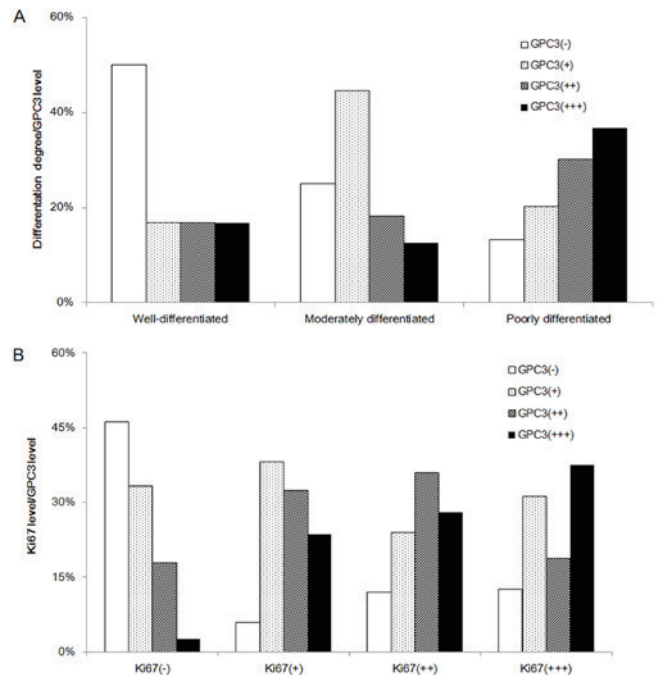


Figure 2. GPC3 expression is significantly correlated with the level of differentiation and expression of Ki-67. (A) The correlation between GPC3 expression and liver cancer differentiation. GPC3 was expressed at an increased level in poorly differentiated liver cancer compared with well-differentiated and moderately differentiated liver cancer samples. (B) The correlation between GPC3 expression and liver cancer proliferation. The higher the rate of proliferation, the more marked the expression of GPC3. GPC3, glypican 3; Ki-67, antigen Ki-67; GPC3, glypican 3.

proliferation was positively correlated with the expression of GPC3 ($r=0.316$, $P=0.001$; Table III; Figs. 1 and 2B).

Verification of the effect of GPC3 on cell proliferation in HepG2 and HLE cells. To additionally analyze the potential effect of GPC3 on cell growth, cell culture-based assays were performed with the HepG2 and HLE hepatoma cell lines. As expected, the western blotting results indicated that GPC3 was expressed in the HepG2 cells, but not in the HLE cells (Fig. 3A). Morphological images captured by the fluorescence microscope additionally confirmed that GPC3 was present in the HepG2 cells and present throughout the cytoplasm, but not in the HLE cells (Fig. 3B). CCK-8 and MTT assays were performed to verify the effect of GPC3 on tumor growth. Following treatment of the HepG2 and HLE cells with various concentrations of GPC3 (0, 0.01, 0.1, 1 and 10 mg/ml) for 24 h, the CCK-8 (Fig. 3C) and MTT (Fig. 3D) results were similar, demonstrating that the most effective GPC3 dosage in promoting growth in the HepG2 and HLE cells was 0.1 mg/l.

GPC3 promote HepG2 cells proliferation through the Hedgehog (Hh) pathway. As aforementioned, GPC3 was able to stimulate HepG2 and HLE cell proliferation. The precise mechanism of how GPC3 promotes cell proliferation remains unclear. Based on the aforementioned experimental results, HepG2 cells were treated with 0.1 mg/ml GPC3 for 24 h. RT-qPCR results indicated that GPC3-treated HepG2 cells exhibited an increase in the mRNA expression of the Hh

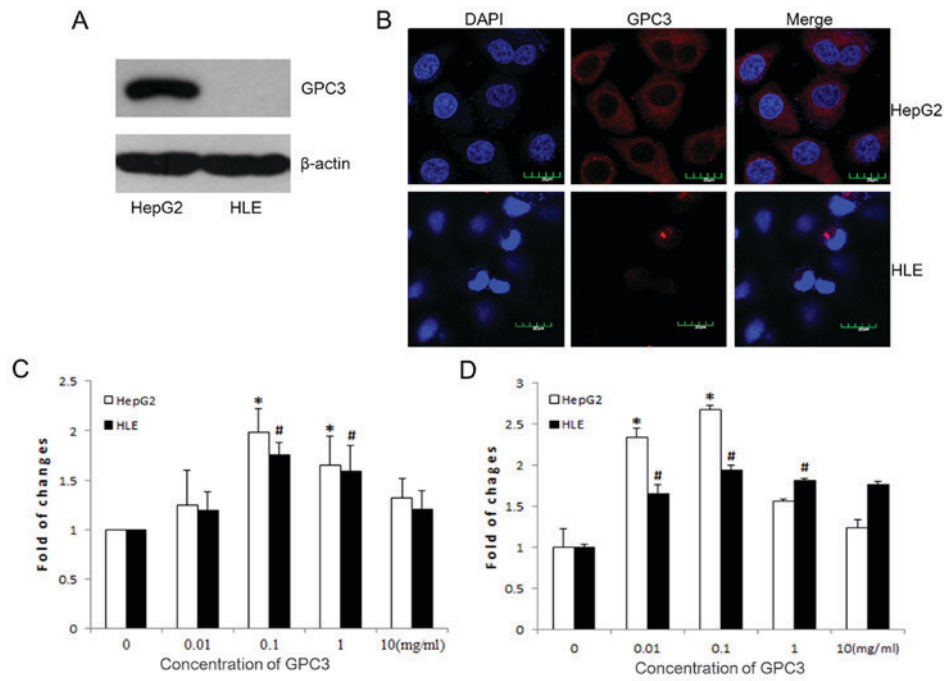


Figure 3. Proliferation effects of GPC3 in HepG2 and HLE cells. (A) Western blotting was used for the analysis of the expression of GPC3 in HepG2 and HLE cells. (B) Expression and localization of GPC3 was analyzed with fluorescence microscopy in HepG2 and HLE cells (magnification, x1,000). Different concentrations (0, 0.01, 0.1, 1 and 10 mg/ml) of GPC3 were analyzed. The proliferation of HepG2 and HLE cells was evaluated using (C) Cell Counting Kit-8 and (D) MTT assays at 24 h. The differences in proliferation in HepG2 and HLE cells were analyzed using SPSS 16 software. * $P < 0.05$ vs. HepG2 cells; # $P < 0.05$ vs. HLE cells. GPC3, glypican 3.

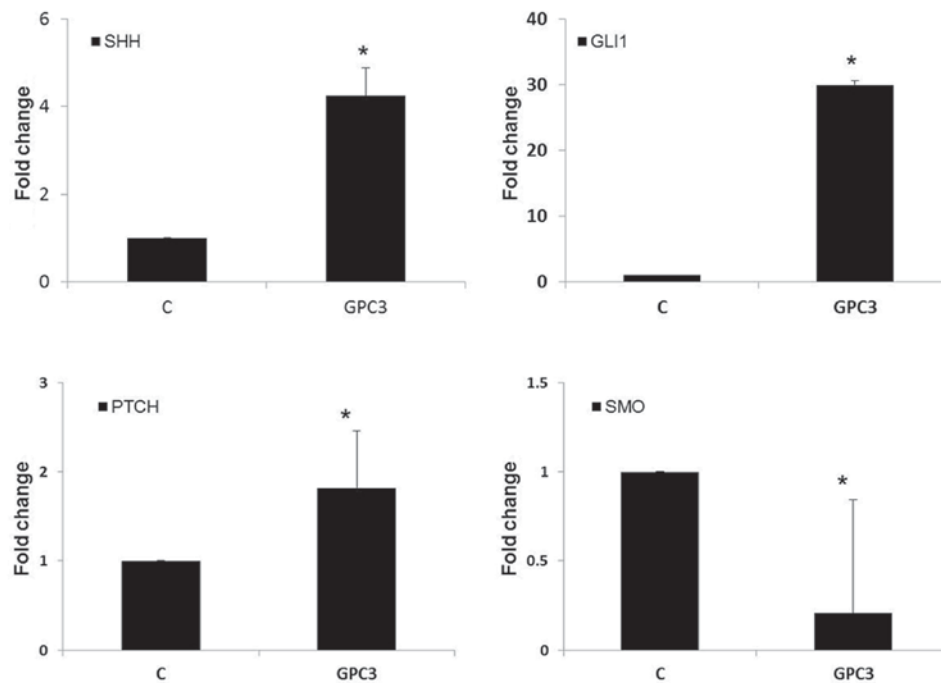


Figure 4. GPC3 promotes HepG2 cells proliferation through the Hedgehog pathway. The effects of 0.1 mg/ml GPC3 on the expression of the SHH, GLI1, PTCH and SMO genes in HepG2 cells analyzed with reverse transcription polymerase chain reaction. The differences were analyzed using a t-test with SPSS16 software. * $P < 0.05$. GPC3, glypican 3; SHH, sonic hedgehog protein; GLI1, zinc finger protein GLI1; PTCH, protein patched homolog 1; SMO, smoothed homolog; GPC3, glypican 3; C, control.

signal pathway members Sonic hedgehog protein, Zinc finger protein GLI1 and Protein patched homolog 1 (PTCH), and the negative regulator of Hh signaling, Smoothed homolog, was

downregulated (Fig. 4). These results suggested that GPC3 promoted HepG2 cell proliferation by stimulating the Hh signaling pathway.

Discussion

In view of its high mortality rate and its prevalence in several countries including China, liver cancer is a malignancy of global importance. The prognosis of patients with liver cancer is generally poor, with a 5-year survival rate of <10-15% (21). It has been demonstrated that GPC3 mRNA and protein were overexpressed in patients with liver cancer compared with healthy people and patients with benign liver lesions (22). The role that GPC3 serves in liver cancer is controversial, but the present study focused on data from clinical specimens, supporting the hypothesis that GPC3 may promote liver cancer cell proliferation through the Hh signaling pathway.

GPC3 is expressed ubiquitously in the embryo, but the expression level is reduced in adults (23). The overexpression of GPC3 has been detected in a number of human malignancies, including liver cancer, melanoma and ovarian clear-cell carcinoma (24). GPC3 has an important role in cell proliferation, differentiation, adhesion and migration, and its function in tumorigenesis is tissue-dependent (25). Previous studies indicated that GPC3 protein expression was increased with lower degrees of tumor differentiation in lung squamous cell carcinoma (26,27). Similar results were also identified in liver cancer: Suzuki *et al* (25) identified that GPC3 was preferentially stained in poorly differentiated liver cancer when compared with the well-differentiated liver cancer samples. The present study identified that poorly differentiated liver cancer was more likely to exhibit high expression of GPC3 ($r=0.295$, $P=0.001$), that 30 liver cancer cases were poorly differentiated in the total 114 specimens and that 36.67% (11/30) liver cancer samples exhibited strong positive GPC3 (3+) staining; an additional previous study suggested that GPC3 exhibited a significant correlation with levels of differentiation in liver cancer only, but did not correlate with tumor size (28).

Loss-of-function mutations in GPC3 cause SGBS, an overgrowth syndrome also involving multiple embryonal neoplasia. The study of Valsechi *et al* (29) suggested that GPC3 reduces the rate of cell proliferation through cell cycle arrest during the G1 phase in renal cell carcinoma. GPC3 may also inhibit breast cancer cells growth *in vitro* (30). In the present study, it was identified that 39/114 cases of liver cancer were negative for Ki-67 staining, of which 18/39 (50%) cases were also negative for GPC3. Only 1/39 cases were strong positive for GPC3 (3+). Statistical analysis suggested that the expression of Ki-67 was positively correlated with the expression of GPC3 ($r=0.316$, $P=0.001$).

GPC3 is frequently upregulated in liver cancer, but its mechanism is largely unclear and is currently debated (25). One of the more well-studied pathways associated with the biological functions of GPC3 is the Wnt signaling pathway. GPC3 stimulates liver cancer cell growth *in vitro* and *in vivo* by increasing autocrine or paracrine canonical Wnt signaling (31). A previous study suggested that the overexpression of GPC3 in Huh7 and SK-HEP-1 liver cancer cell lines effectively inhibited cell proliferation through induction of apoptosis (32). An additional previous study indicated that GPC3 suppressed cell growth in ovarian clear cell carcinoma (CCC) cells via the insulin-like growth factor II signaling pathway. Previous data also suggested that GPC3 has the potential to become a novel

therapeutic target for patients with ovarian CCC (33). However, an additional study indicated that the knockdown of GPC3 inhibits Huh7 cells proliferation through the downregulation of Yes-associated protein, which is a key effector molecule in the Hippo pathway (34). It has also been indicated that GPC3 binds Hh at the cell membrane and competes with PTCH, suggesting that GPC3 regulates embryonic growth, perhaps by inhibiting the Hh signaling pathway (4). The significance of GPC3 in cell proliferation is of particular interest. In the present study, the results demonstrated that exogenous GPC3 may promote HepG2 and HLE hepatoma cell proliferation, and that it is possible that HepG2 cells expressing GPC3 are more sensitive to GPC3 compared with HLE cells, thereby exhibiting increased rates of proliferation. Additional experimental results suggested that exogenous GPC3 may promote HepG2 cell proliferation by stimulating Hh signaling.

Taken together, the results of the present study emphasize the significance and importance of GPC3 level in liver cancer differentiation and proliferation. This function of GPC3 is consistent with the observations of the present study, in that patients with liver cancer with higher GPC3 levels appeared to exhibit poorer levels of differentiation when compared with patients with lower GPC3 levels. It appears that GPC3 is not only a marker for diagnosis; it is also a growth factor in tumor progression. Nonetheless, additional clinical studies are required to confirm the function and molecular mechanism of GPC3 in liver cancer.

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Availability of data and materials

All data generated and analyzed during this study are included in this published article.

Authors' contributions

WS contributed significantly in the collection and analysis all the data; CN performed the *in vitro* experiment; CY and LL undertook collection of patient information; SL and LH were responsible for the pathological diagnosis; LH was responsible for this project and wrote the manuscript.

Ethics approval and consent to participate

This study was approved by the Beijing You'An Hospital, Capital Medical University, Human Research Protection

Program (Beijing, China). Patients provided written informed consent.

Consent for publication

Written informed consent was obtained from patients for their inclusion in the present study and the publication of any accompanying data.

Competing interests

The authors declare that they have no competing interests.

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