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ARTICLE

GPC3 in Liver Cancer Exosomes: Dual Regulation of Growth in Normal and Hepatocellular Carcinoma Cells through the Wnt/β-catenin Signaling Pathway

Qi Wang, Ping Sun, Hui Huang, Qiqi Zhang, Xueying Sun, Zhidong Wang, Chunwen Pu

Dalian Public Health Clinical Center, Dalian, Liaoning Province 116037, China

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Abstract

Exosomes have emerged as key regulators of cellular growth in both normal and abnormal conditions. Tumor cell-secreted exosomes play a significant role in modulating the growth behavior of both normal and tumor cells. In this study, we investigated the effects of glypican-3 (GPC3) on cell growth and apoptosis in HepG2 cells, L02 cells, and GPC3-knockdown HepG2 cells (HepG2-shGPC3). Various concentrations of GPC3 were administered to these cell lines, and cell growth and apoptosis were assessed using CCK8 assays and flow cytometry. Additionally, exosomes derived from HepG2 cells (Exo) and GPC3-knockdown HepG2 cells (shGPC3-Exo) were separately employed to treat L02 and HepG2 cells. Cell growth was monitored using a CCK8 kit, while cell cycle progression and apoptosis were evaluated using flow cytometry. The expression levels of GPC3/WNT3A/β-catenin signaling proteins were determined by Western blotting. Our findings reveal a bidirectional regulation of GPC3 between normal and hepatocellular carcinoma (HCC) cells, which constitutes the novelty of this research. Treatment of L02 cells and HepG2 cells with GPC3 resulted in cell cycle arrest at the G0/G1 phase in L02 cells, accompanied by inhibited cell growth and enhanced apoptosis. In contrast, GPC3 treatment appeared to promote the growth of HepG2 cells. Knockdown of GPC3 effectively inhibited cell growth and promoted apoptosis in HepG2 cells. Furthermore, we observed the presence of GPC3 in both L02 and HepG2 exosomes, with significantly higher levels detected in HepG2 exosomes. Importantly, treatment with HepG2 exosomes (Exo) inhibited the growth and promoted apoptosis in L02 cells, consistent with the effects of GPC3 treatment. Notably, shGPC3-Exo exerted a similar impact on L02 cells as HepG2 exosomes (Exo), albeit with reduced efficacy. Conversely, shGPC3-Exo displayed a promoting effect on HepG2 cell growth. Thus, GPC3 in exosomes plays a role in regulating the growth of both L02 and HepG2 cells. Further investigations demonstrated that GPC3 in liver cancer exosomes modulates the proliferation and apoptosis of L02 and HepG2 cells through the Wnt/β-catenin signaling pathway. Our study highlights the inhibitory effect of GPC3 in liver cancer cell-derived exosomes on the growth of normal liver cells and its promotion of apoptosis through the modulation of the Wnt/β-catenin signaling pathway. These findings shed light on the influence of GPC3 in tumor microenvironment exosomes on normal cells during the progression of liver cancer.

Corresponding authors: Zhidong Wang, Dalian Public Health Clinical Center, Dalian, Liaoning Province 116037, China. 893356662@qq.com; Chunwen Pu, Dalian Public Health Clinical Center, Dalian, Liaoning Province 116037, China. 2295050337@qq.com

Introduction

Liver cancer ranks fifth in global cancer incidence, with 905,700 new cases and a high mortality rate of 90% reported in 2020 (World Health Organization, 2020). Hepatocellular carcinoma (HCC) is the most prevalent type of liver cancer, accounting for approximately 90% of cases (Llovet et al., 2016). HCC patients are frequently diagnosed at advanced stages, resulting in poor survival rates (Cao et al., 2012). Early diagnosis and treatment are crucial for improving the survival outcomes of liver cancer patients (Kudo et al., 2014). Therefore, the identification of novel diagnostic markers for liver cancer is of utmost importance.

Exosomes are membrane-bound vesicles released into the extracellular environment through fusion of multivesicular bodies with the cell membrane. Tumor-derived exosomes carry signaling molecules derived from tumor cells, playing a significant role in modulating the behavior of both normal and tumor cells and reshaping the tumor microenvironment (Meckes,2015; Tkach et al., 2016). Tumor cell-derived exosomes facilitate cell-cell communication, influencing the uncontrolled growth, invasion, and metastasis of tumor cells (Le et al., 2014; Costa-Silva et al., 2015). Furthermore, the contents of exosomes from cancer patients provide opportunities for early cancer diagnosis (Melo et al., 2015; Corcoran et al., 2011).

Glypican-3 (GPC3), a heparan sulfate proteoglycan, is involved in the regulation of cell proliferation, adhesion, and migration (Skog et al., 2008; Haruyama et al., 2016; Sung et al., 2003;Baumhoer et al., 2008). Elevated expression of GPC3 is observed in over 70% of HCC cases, often correlating with liver cancer metastasis. GPC3 promotes HCC growth by stimulating the classic Wnt/β-catenin signaling pathway, while knockdown of GPC3 expression inhibits HCC cell proliferation and induces apoptosis (Capurro et al.,2005). Autophagy has also been found to attenuate the growth of HepG2 cells through the inhibition of GPC3/Wnt/β-catenin signaling (Hu et al, 2018). Additionally, GPC3 expression is associated with poor prognosis in HCC patients (Shirakawa et al., 2009). Consequently, GPC3 serves as a suitable biomarker and prognostic factor for HCC, and targeted inhibition of GPC3 signaling may help control the proliferation and metastasis of HCC cells (Hippo et al., 2004).

Previous studies have demonstrated significantly higher expression of GPC3 in serum exosomes of HCC patients compared to patients with hepatitis B and healthy individuals (Di et al., 2020). This study explores the impact of GPC3 in HCC-derived exosomes on the growth of HCC cells and associated signaling pathways. Moreover, it provides evidence for the potential effects of GPC3 within hepatocarcinoma cell-derived exosomes on normal liver cells. These findings contribute to the understanding of the relationship between the growth of normal cells within the liver cancer microenvironment and the development of liver cancer.

Methods

1. Cell Culture

HepG2 cell variants were cultured in MEM medium (Procell, Cat# PM150410) supplemented with 0.4 mg/ml G418 and 10% FBS. L02 cells were cultured in RPMI-1640 medium supplemented with 1% P/S and 10% FBS. All cells were incubated at 37°C with 5% CO2.

2. GPC3 Protein

GPC3 protein was obtained from CUSABIO (CUSABIO, CSB-AP005371HU).

3. Exosome Isolation

Exosomes were isolated at 4°C. The collected cell culture medium was subjected to sequential centrifugation steps. First, the medium was centrifuged at 300 g for 10 minutes to remove cell pellets. The supernatant was then subjected to centrifugation at 2,000 g for 4 minutes and 10 minutes to eliminate dead cells. Next, the supernatant was centrifuged at 10,000 g for 30 minutes to remove cell debris. Finally, the supernatant was subjected to ultracentrifugation at 100,000 g for 70 minutes to obtain exosomes.

4. Transmission Electron Microscopy Assay

Exosomal particles were suspended in PBS and a 10 μl aliquot was placed on a copper mesh for 1 minute. Excess liquid was absorbed using filter paper. Subsequently, 10 μl of 2% uranyl acetate was dropped onto the copper mesh for 1 minute. The mesh was air-dried at room temperature, and the samples were observed using a JEM-2100 Plus transmission electron microscope operating at 80 kV-120 kV.

5. ZetaView Analysis

The exosome pellet was washed with 30 mL of 1×PBS, resuspended in 250 μL of 1×PBS, and stored at -80°C. Nanoparticle Tracking Analysis (NTA) was performed using a ZetaViewPMX 110 instrument (Particle Metrix, Meerbusch, Germany) and the accompanying ZetaView8.04.02 software at Shanghai VivaCell. This analysis allowed for the measurement of exosome size.

6. Enzyme-Linked Immunosorbent Assay (ELISA) Analysis

A GPC3 ELISA Kit (CUSABIO, Cat# CSB-E11333h) was employed to measure GPC3 levels following the manufacturer's instructions. CUSABIO's human GPC-3 was used as the standard. All experiments were performed in triplicate and repeated three times.

7. Lentivirus, Plasmid, Cell Transfection, and Infection

The lentivirus genOFFTM st-h-GPC3 (CGACACCCTTT-GCTGGAAT), containing GPC3-targeting shRNA, was designed and constructed by Siwega (Cat# REVG005). HepG2 cells were infected with the lentivirus using hU6-MCS-CBh-gcGFP-IRES-puromycin GV493. After 5 days of infection, cells were extracted, and RNA was collected for PCR detection.

8. Quantitative Real-Time PCR

Total RNA, from both cells and exosomes, was extracted using TRIzol. First-strand cDNA synthesis was performed using the iScript cDNA Synthesis Kit (Bio-Rad, Cat# 1708890) with random primers. Real-time PCR (qPCR) was conducted using the CFX96 real-time PCR detection system (Bio-Rad, Cat# CFX Connect) and the iTaqTM universal SYBR Green Supermix (Bio-Rad, Cat# 1725124). The mRNA content was normalized to the housekeeping gene GAPDH. The primer sequences used for RT-qPCR are summarized in Table I. The reaction conditions included an initial denaturation at 95°C for 5 minutes, followed by 40 cycles of denaturation at 95°C for 10 seconds and annealing/extension at 60°C for 30 seconds. The RT-qPCR results were normalized to GAPDH using the 2-ΔΔCq method. The experiment was repeated three times.

9. Cell Growth Assay

L02 cells, HepG2 cells, and HepG2-shGPC3 cells were seeded in 96-well plates at a density of 3-5×10^4 cells/well. They were then treated with various concentrations of GPC3 or exosomes (Exo). After incubation at 37°C for 48 hours, 10 μL of CCK-8 solution (Beyotime Biotechnology, Cat# C0038) was added to each well, and the mixture was thoroughly mixed to ensure uniform color distribution. Following another 4-hour incubation, the absorbance was measured directly at 450 nm. The percentage of surviving cells for each treatment was calculated as (OD value of the test group / OD value of the control group) × 100%. The experiment was repeated three times.

10. Flow Cytometry Analysis

Cells were seeded in a 6-well plate at a density of 1×10^5 cells/well and treated with purified GPC3 protein or exosomes derived from HepG2 cells or HepG2-shGPC3 cells. After 24 hours of treatment, the Annexin V/PI detection kit (BD Biosciences, Cat# KGA1030-100) and cell cycle staining kit (MultiSciences, Cat# CY2001-O) were used to assess cell apoptosis and cell cycle distribution, respectively. Data were analyzed using BDAccuri® software. The experiment was repeated three times.

11. Western Blot Analysis

Samples were lysed in RIPA lysis buffer (including protease and phosphatase inhibitors) and centrifuged (12,000 rpm, 5 min, 4°C). The supernatant was collected and stored at -80°C. Protein concentrations were determined using the Bradford assay. Samples were resolved by 12% SDS-PAGE and transferred to nitrocellulose membranes (MILLIPORE, Cat# IPVH00010). After blocking with 5% skim milk in PBST (PBS containing 0.1% Tween20), the membranes were incubated with specific antibodies. Subsequently, the membranes were incubated with peroxidase-conjugated secondary antibodies and treated with an Electro-Chemi-Luminescence reagent (Bio-Rad, Cat# 170-5060). Antibodies used included CD63 (1:1000, abcam, Cat# ab216130), TSG101 (1:1000, abcam, Cat# ab83), GPC3 (1:1000, Bioss, Cat# bs-1112R), WNT3A (1:1000, HuaBio, Cat# EM1706-27), β-Catenin (1:1000, HuaBio, Cat# ER0805), and GAPDH (1:10000, abcam, Cat# ab181602). RIPA lysis buffer was prepared by mixing 790 μL RIPA (Beyotime, P0013D), 100 μL protease inhibitor (10 ×), 100 μL phosphatase inhibitor (10 ×), and 10 μL PMSF (Beyotime, ST505). The experiment was repeated three times.

12. Statistical Analysis

The data were presented as mean ± SEM. Statistical analyses were performed using GraphPad Prism software. Student's t-test (two-tailed) or one-way ANOVA followed by Tukey's multiple comparisons test were employed for data analysis.

Results

1. Exosome purification and verification

Previous findings indicated elevated expression of GPC3 in liver cancer tissues compared to normal tissues (Wang et al., 2015). To confirm the presence of exosomes in the cell culture medium, nanoparticle tracking analysis (NTA) was performed using ZetaViewPMX 110 to observe the particle size of exosomes. Transmission electron microscopy analysis revealed the presence of vesicular structures with a diameter of approximately 50-150 nm, consistent with the established

Table 1. qPCR primer sequence

Primer	Sequence(5' to 3')
Hu-GAPDH -F	ACAACTTTGGTATCGTGGAAGG
Hu-GAPDH -R	GCCATCACGCCACAGTTTC
Hu-GPC3-F	GAAACAGTCAGCAGGCAAC
Hu-GPC3-R	GAAGCACACCACCGAGA

morphological characteristics of exosomes (Yuan et al., 2015) (Figure 1A). Western blot analysis confirmed the presence of exosomal markers CD63 and TSG101 (Baranyai et al., 2015) (Figure 1B), indicating the successful isolation of exosomes from L02 and HepG2 cell lines. Additionally, Western blot analysis was conducted to verify the expression of GPC3 in the exosomes derived from L02 and HepG2 cell lines. Consistent with the comparison of GPC3 expression levels in the two cell lines, the expression of GPC3 in liver cancer cell line HepG2 exosomes was higher than that in normal liver cell line L02 exosomes (Figure 1C, D).

2. The effect of GPC3 on growth and apoptosis of normal cells

Previous studies have demonstrated the promoting effect of GPC3 on the development of liver cancer. To investigate whether GPC3 exerts different effects on the growth of normal liver cells compared to liver cancer cells, we treated L02 and HepG2 cells with varying concentrations of GPC3 (0, 0.25, 0.5, 1, 1.5, and 2 mg/mL) to assess its impact on cell growth. The addition of GPC3 resulted in the inhibition of L02 cell growth, whereas it promoted the growth of HepG2 cells. Notably, the effect of GPC3 exhibited a dose-dependent pattern, with the most pronounced effect observed at a concentration of 1 mg/mL (Figure 2A). Annexin V/PI staining, analyzed by flow cytometry, revealed an increase in apoptotic L02 cells following treatment with GPC3 at a concentration of 1 mg/mL. In contrast, no significant difference in apoptosis was observed between GPC3-treated and untreated HepG2 cells (Figure 2B). Subsequently, we investigated whether GPC3 influences cell growth through cell cycle regulation. As depicted in Figure 2C, the addition of GPC3 resulted in an increased percentage of L02 cells in the G0/G1 phase, indicative of G0/G1 cell cycle arrest. Conversely, the percentage of HepG2 cells in the G0/G1 phase decreased. These findings suggest that GPC3 can inhibit the growth of normal cells by enhancing cell apoptosis and inducing cell cycle arrest in the G0/G1 phase. In contrast, GPC3 reduces cell cycle arrest in HCC cells, promoting their growth, and does not significantly impact cell apoptosis.

In order to investigate the role of GPC3 in the growth of HCC cells, an expression vector carrying short hairpin RNA (shRNA) targeting GPC3 was transfected into HepG2 cells. Following transfection, the mRNA levels of GPC3 in HepG2 cells were reduced compared to normal HepG2 cells, confirming successful GPC3 knockdown (Figure 3A). Subsequently, the growth of HepG2 cells was assessed, revealing inhibition of cell growth upon GPC3 knockdown (Figure 3B). Flow cytometry analysis of Annexin V/PI stained cells further demonstrated an increase in apoptotic cells in HepG2 cells with GPC3 knockdown (Figure 3C). Additionally, cell cycle analysis showed an increased percentage of HepG2 cells in the G0/G1 phase following GPC3 knockdown, indicative of G0/G1 cell cycle arrest (Figure 3D). Collectively, these results indicate the involvement of GPC3 in cell growth regulation in HCC cells.

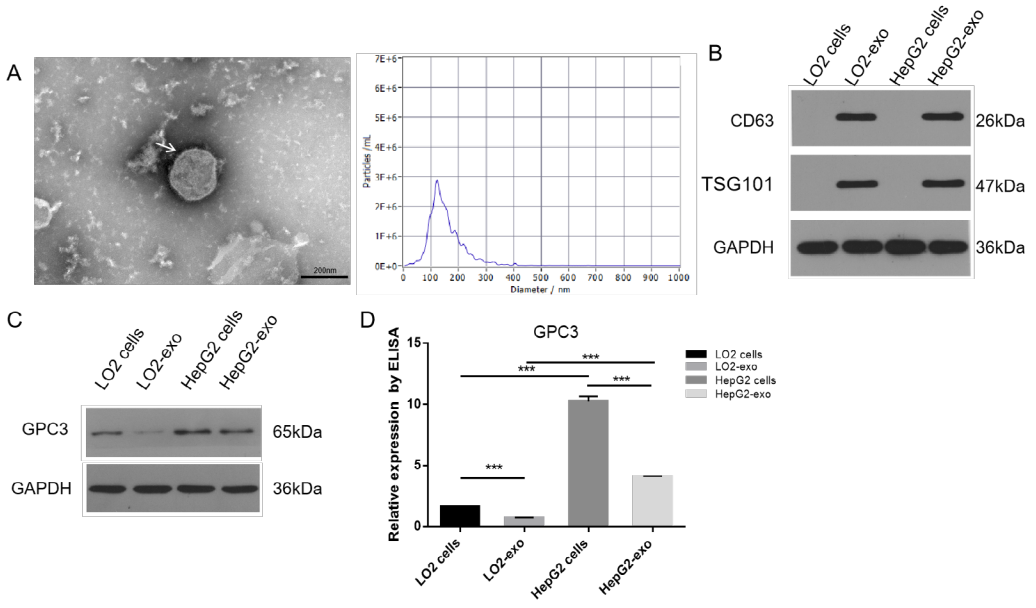

Figure 1. Characterization of exosomes derived from L02 and HepG2 cells

(A) Electron microscopy analysis of exosomes displaying vesicular structures with a diameter of approximately 200 nm. Scale bars represent 200 nm. (B) Western blot analysis confirming the presence of exosomal markers CD63 and TSG101 in the exosome-rich medium. (C) Western blot analysis assessing the expression of GPC3 in cells and exosomes derived from L02 and HepG2 cells. (D) ELISA analysis quantifying the expression of GPC3 in cells and exosomes derived from L02 and HepG2 cells. Statistical analysis indicated significant differences (***$P < 0.001$, n = 3) in GPC3 expression. Note: Error bars represent standard deviation.

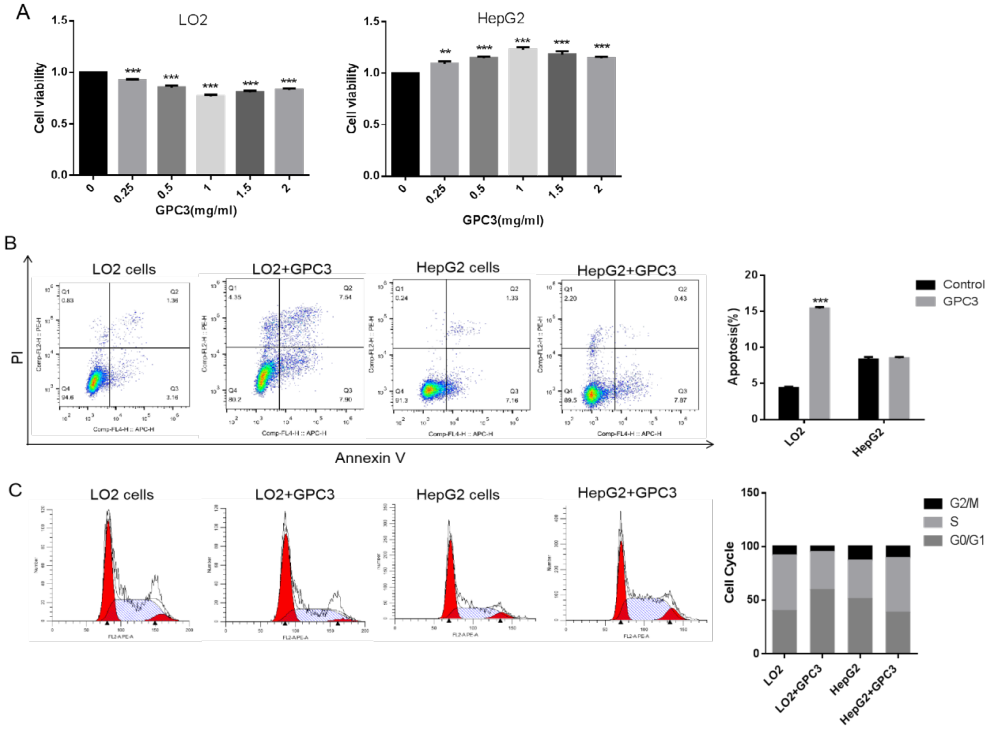

Figure 2. The effect of GPC3 on the growth of L02 and hepG2 cells

(A) Cell growth activity of L02 and HepG2 cells was assessed following treatment with different concentrations of GPC3. (B) FITC-Annexin V/PI analysis was performed to evaluate apoptotic cells in L02 and HepG2 cells treated with GPC3. Annexin V fluorescence analysis demonstrated an increase in apoptotic cells in GPC3-treated L02 cells. (C) Cell cycle analysis showed changes in the G0/G1 population of L02 and HepG2 cells treated with GPC3. GPC3 treatment resulted in an increased percentage of L02 cells in the G0/G1 phase, while the G0/G1 population decreased in HepG2 cells. Data are presented as mean ± standard error. Statistical significance was determined as $*P < 0.05$ (compared with 0 mg/mL), $**P < 0.01$, $***P < 0.001$ (compared with 0 mg/mL), n = 3. Control refers to untreated cells, L02+GPC3 indicates GPC3-treated L02 cells, and HepG2+GPC3 represents GPC3-treated HepG2 cells.

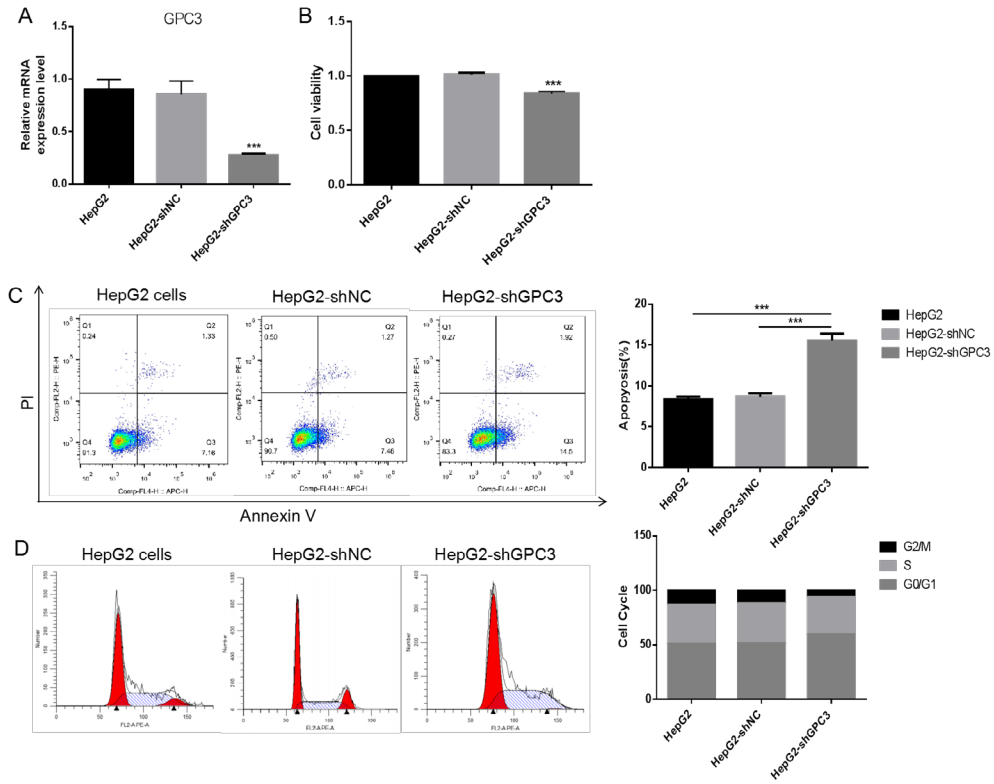

Figure 3. Knockdown of GPC3 suppresses the growth of HCC cells

Measurement of GPC3 mRNA expression in HepG2 cells transfected with GPC3 shRNA using fluorescence-based quantitative PCR (qPCR). (B) Assessment of cell growth in HepG2 cells with downregulated GPC3 expression. (C) FITC-Annexin V/PI analysis of apoptotic cells in HepG2 cells with GPC3 expression knocked down. (D) Cell cycle analysis of HepG2 cells with GPC3 expression knockdown. Data are presented as mean ± standard error. Non-specific control refers to HepG2 cells transfected with non-targeting shRNA (HepG2-shNC), while HepG2-shGPC3 represents HepG2 cells with GPC3 expression downregulation. Statistical significance was determined as $***P < 0.001$, n = 3.

3. GPC3 in HCC exosomes inhibits the growth of normal hepatocytes

Previous studies have reported the presence of GPC3 protein in serum exosomes derived from HCC patients, which correlates with GPC3 expression in HCC tissues (Di et al., 2020). Therefore, we sought to investigate the effect of GPC3 from liver cancer-derived exosomes on normal liver cells. Both L02 and HepG2 cells were treated with purified exosomes derived from HepG2 cells (Exo) or purified exosomes derived from HepG2 cells with downregulated GPC3 expression (shGPC3-Exo). The GPC3 content in shGPC3-Exo was lower compared to Exo (Figure 4A).

Cells cultured without any exosomes (0 μg/mL) served as the control group. Following the addition of Exo, the growth of L02 cells was significantly inhibited in a concentration-dependent manner (Figure 4B, left). Similarly, shGPC3-Exo also exerted an inhibitory effect on the growth of L02 cells, albeit with reduced potency compared to the Exo group at equivalent concentrations. Interestingly, shGPC3-Exo promoted the growth of HepG2 cells in a dose-dependent manner (Figure 4B, right). These results suggest that the effect of HepG2 exosomes on the growth of normal cells may be influenced by the concentration of GPC3.

Flow cytometry analysis of Annexin V/PI staining revealed a significant increase in the number of apoptotic L02 cells following treatment with 100 μg/mL Exo or 100 μg/mL shGPC3-Exo compared to the control group (Figure 4C, left). The Exo group also exhibited a higher number of apoptotic cells compared to the shGPC3-Exo treatment group. In contrast, the number of apoptotic cells in the HepG2 cell group did not show significant changes after the addition of shGPC3-Exo (Figure 4C, right). Furthermore, cell cycle analysis demonstrated that upon treatment with either Exo or shGPC3-Exo, L02 cells exhibited an increase in the G0/G1 population and a decrease in the G2/M population compared to the control group, indicating G0/G1 cell cycle arrest (Figure 4D, left). In HepG2 cells, the addition of shGPC3-Exo resulted in a decrease in the G0/G1 population (Figure 4D, right). These findings suggest that GPC3 in HepG2 cell-derived exosomes promotes apoptosis in L02 cells and may induce apoptosis by inhibiting the cell cycle of L02 cells.

Taken together, these results indicate that GPC3 present in HCC exosomes may promote the apoptosis of normal hepatocytes, as well as inhibit cell cycle progression and cell growth.

4. GPC3 in HCC exosomes modulates the Wnt/β-Catenin signaling pathway in normal hepatocytes

The Wnt/β-catenin signaling pathway plays a crucial role in the promotion of HepG2 cell growth and is implicated in the occurrence, progression, and metastasis of liver cancer. Existing evidence suggests that GPC3 is involved in Wnt/β-catenin signaling in liver cancer (Gao et al, 2011). Thus, our investigation aimed to determine whether GPC3 present in HepG2 cell-derived exosomes activates the Wnt/β-catenin signaling pathway.

Firstly, we compared the expression levels of GPC3 and components of the Wnt/β-catenin signaling pathway in cells and exosomes. Western blot analysis revealed significantly lower levels of GPC3, Wnt3a, and β-catenin proteins in L02 cells compared to HepG2 cells. Similarly, the expression levels of these proteins in L02 cell-derived exosomes were also lower than those in HepG2 cell-derived exosomes (Figure 5A, B).

Further investigations demonstrated that GPC3 content in L02 cells significantly increased upon GPC3 treatment, accompanied by elevated levels of Wnt3a and β-catenin proteins. Upon the addition of HepG2 cell-derived exosomes (Exo), the content of GPC3, Wnt3a, and β-catenin proteins in L02 cells also increased significantly. Conversely, treatment with exosomes derived from HepG2 cells with downregulated GPC3 expression (shGPC3-Exo) led to increased GPC3, Wnt3a, and β-catenin protein content in L02 cells compared to the control group, although the protein levels were lower than those observed with Exo treatment (Figure 5C, D). These findings indicate that GPC3 present in HepG2 cell-derived exosomes can enhance the content of GPC3, Wnt3a, and β-catenin proteins in L02 cells. Additionally, in our study involving HepG2 cells, we observed a significant decrease in GPC3, Wnt3a, and β-catenin protein content upon GPC3 knockdown. Conversely, GPC3 addition to HepG2 cell cultures resulted in a substantial increase in GPC3 and β-catenin protein content, along with an increase in Wnt3a levels (Figure 5C, D). Importantly, shGPC3-Exo treatment led to increased GPC3, Wnt3a, and β-catenin levels in HepG2 cells compared to the control group.

These findings collectively suggest that GPC3 and Wnt/β-catenin signaling protein levels are significantly lower in normal liver cells compared to liver cancer cells. Moreover, GPC3 present in liver cancer cell-derived exosomes can modulate the Wnt/β-catenin signaling protein levels in normal liver cells by increasing the levels of GPC3.

Discussion

Liver cancer remains a significant global health burden, with a high mortality rate and limited treatment options. The tumor microenvironment and communication between tumor and normal cells play crucial roles in tumor progression and metastasis (Seehawer et al., 2018). Exosomes, vesicles released by tumor cells, carry specific markers and signaling molecules

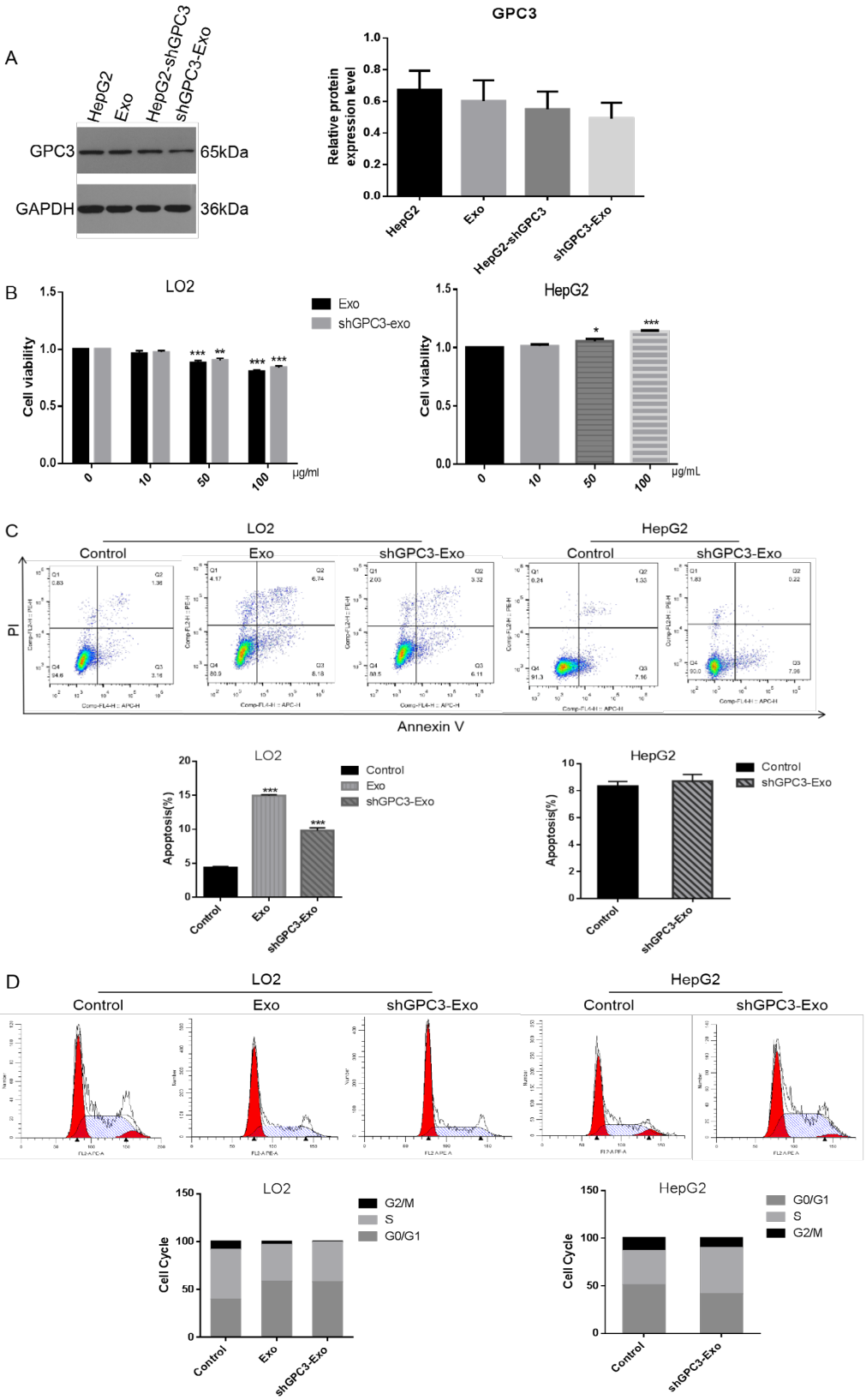

Figure 4. The effect of GPC3 in liver cancer exosomes on the growth of L02 and HCC cells

(A) Western blot analysis to determine the expression level of GPC3 protein in HepG2 cells and exosomes derived from HepG2 cells with or without GPC3 knockdown. (B) Assessment of cell growth activity in L02 and HepG2 cells treated with exosomes derived from HepG2 cells with or without GPC3 knockdown. Statistical significance indicated as $^{*}P < 0.05$, $^{**}P < 0.01$ (compared with 0 μg/mL), $^{***}P < 0.001$ (compared with 0 μg/mL), n = 3. (C) FITC-Annexin V/PI analysis performed in L02 and HepG2 cells treated with exosomes. (D) Cell cycle analysis showing changes in the G0/G1 population of L02 and HepG2 cells treated with exosomes. Statistical significance indicated as $^{*}P < 0.05$, $^{**}P < 0.01$ (compared with Control), $^{***}P < 0.001$ (compared with Control), n = 3. Data are presented as mean ± standard error. Control represents untreated cells, Exo indicates purified exosomes from HepG2 cells, and shGPC3-Exo refers to purified exosomes from HepG2 cells with downregulated GPC3 expression.

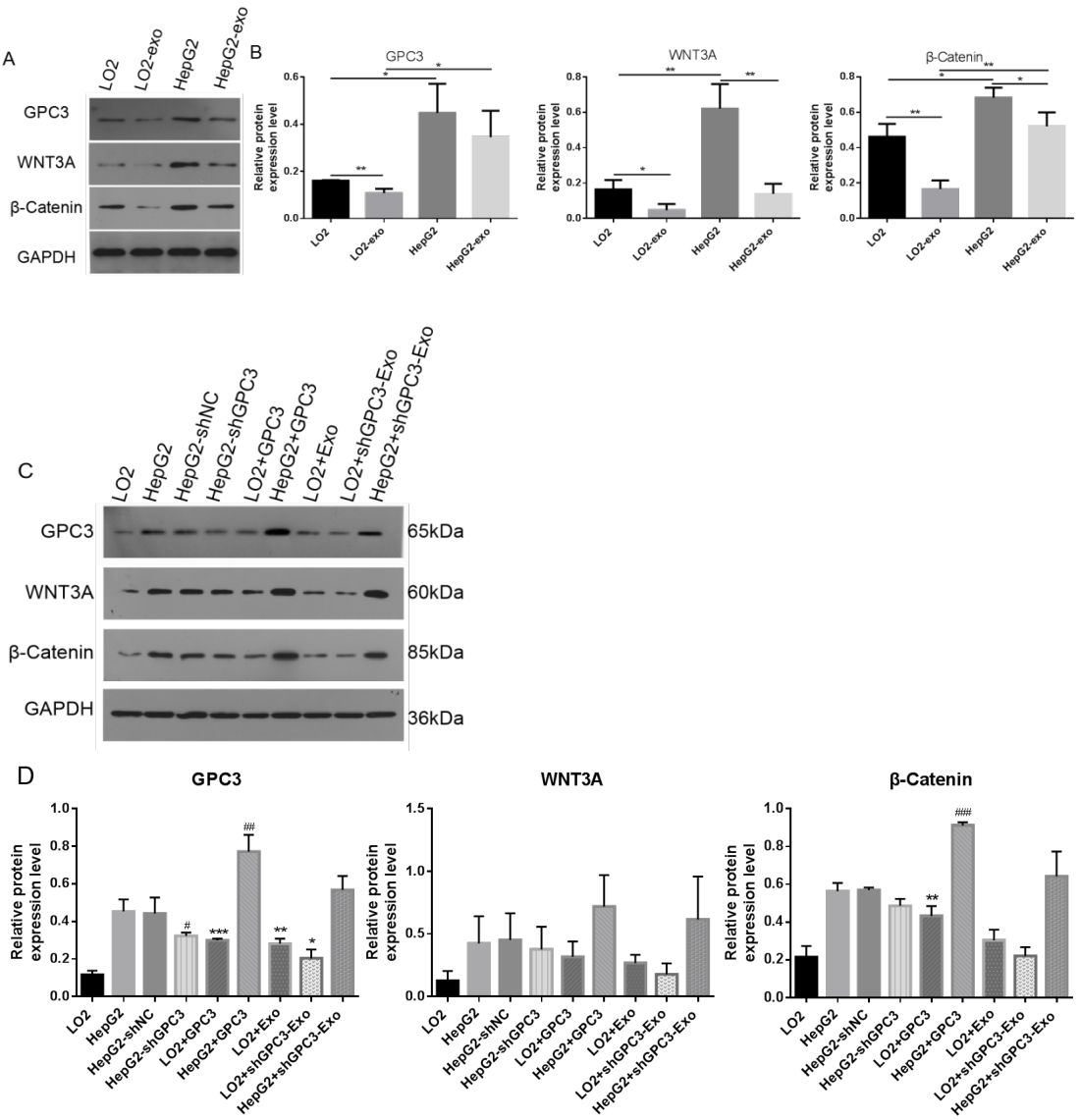

Figure 5. Exosomal GPC3 modulates the growth of normal hepatocytes and HCC cells via Wnt/β-catenin signaling

(A) Western blot analysis determined the expression levels of GPC3 in L02 and HepG2 cells, as well as in exosomes. Statistical significance indicated as $*P < 0.05$, $**P < 0.01$, $***P < 0.001$, n = 3. (B) Quantification of GPC3 expression levels in L02 and HepG2 cells, as well as in exosomes, based on the Western blot analysis. Statistical significance indicated as $*P < 0.05$, $**P < 0.01$, $***P < 0.001$, n = 3. (C) Western blot analysis to assess the expression levels of GPC3, WNT3A, and β-catenin in L02 cells treated with GPC3, exosomes derived from HepG2 cells (Exo), and exosomes derived from HepG2 cells with downregulated GPC3 expression (shGPC3-Exo). Statistical significance indicated as $*P < 0.05$ (compared with L02), $**P < 0.01$ (compared with L02), $***P < 0.001$ (compared with L02), n = 3. (D) Western blot analysis to assess the expression levels of GPC3, WNT3A, and β-catenin in HepG2 cells treated with GPC3 and exosomes derived from HepG2 cells with downregulated GPC3 expression (shGPC3-Exo). Statistical significance indicated as $\#P < 0.05$ (compared with HepG2), $\#\#P < 0.01$ (compared with HepG2), n = 3. Data are expressed as mean ± standard error. L02+GPC3 indicates L02 cells treated with GPC3. L02+Exo indicates L02 cells treated with exosomes derived from HepG2 cells. L02+shGPC3-Exo indicates L02 cells treated with exosomes derived from HepG2 cells with downregulated GPC3 expression. HepG2+GPC3 indicates HepG2 cells treated with GPC3. HepG2+shGPC3-Exo indicates HepG2 cells treated with exosomes derived from HepG2 cells with downregulated GPC3 expression.

that influence the growth and invasive behavior of tumor cells. In this study, we focused on the role of Glypican-3 (GPC3), a tumor-specific marker, in liver cancer exosomes and its impact on normal liver cells.

Previous studies have reported an elevated expression of GPC3 in liver cancer tissues and its correlation with disease progression. Furthermore, increased levels of GPC3 have been detected in the blood and serum exosomes of liver cancer patients, suggesting its secretion into the circulation. Our in vitro experiments confirmed the expression of GPC3 in normal liver cells, hepatocellular carcinoma (HCC) cells, and their respective exosomes. Importantly, the content of GPC3 in HCC cell-derived exosomes was significantly higher than that in normal liver cell-derived exosomes, indicating a potential role of GPC3 in the communication between tumor and normal cells.

We observed that GPC3 exhibited a promoting effect on HCC cell growth, consistent with previous findings. Interestingly, we made the novel discovery that GPC3 inhibits the growth of normal hepatocytes by inducing cell cycle arrest and promoting

apoptosis. These findings suggest that GPC3 may contribute to HCC cell growth by influencing the apoptosis and growth of normal liver cells, ultimately facilitating the development of liver cancer. Moreover, we found that GPC3 activation stimulated the expression of the canonical Wnt/β-catenin signaling pathway in normal liver cells. The activation of this signaling pathway has been implicated in the progression of HCC, emphasizing the importance of GPC3 in HCC pathogenesis.

Exosomes are known to transmit information through their cargo contents, and tumor-derived exosomes have been shown to create a pre-metastatic microenvironment that promotes tumor cell metastasis (Peinado et al., 2016; Yu et al., 2017). We investigated whether GPC3 in liver cancer exosomes could exert its effects on normal liver cells. We found that the optimal concentration of liver cancer exosomes (100 μg/mL) significantly inhibited the growth of normal liver cells, induced cell cycle arrest, and promoted apoptosis. Importantly, the inhibitory effects were diminished when GPC3 content in liver cancer exosomes was reduced, indicating the involvement of GPC3 in mediating the growth inhibition and apoptosis of normal liver cells. These results highlight the significant disparity in GPC3 content between liver cancer cell-derived exosomes and normal liver cell-derived exosomes, with GPC3 in liver cancer exosomes exerting inhibitory effects on normal liver cell growth and promoting apoptosis, thereby potentially contributing to HCC development.

The activation of the Wnt signaling pathway is a common molecular event in HCC progression, and previous studies have demonstrated the involvement of GPC3 in Wnt/β-catenin signaling (Wu et al., 2016). In our study, we confirmed the presence of GPC3 and Wnt/β-catenin signaling proteins in exosomes derived from normal hepatocytes and HCC cells. However, the levels of these proteins in exosomes were significantly lower than those in the cells themselves. Importantly, GPC3 in HCC cell-derived exosomes promoted the expression of Wnt/β-catenin pathway proteins in normal liver cells. Knockdown of GPC3 in HCC cell-derived exosomes (shGPC3-Exo) resulted in reduced expression of Wnt/β-catenin pathway proteins in normal liver cells compared to the control group (Exo). These findings suggest that GPC3 in HCC cell-derived exosomes can modulate the Wnt/β-catenin pathway in normal liver cells. This implies that HCC cells in the tumor microenvironment can influence the growth of normal hepatocytes through GPC3-containing exosomes, thereby promoting HCC development.

In conclusion, our study demonstrates the enrichment and expression of GPC3 in liver cancer exosomes. GPC3 in liver cancer cell-derived exosomes not only regulates the cell cycle and apoptosis of HCC cells but also inhibits the growth of normal liver cells and influences the expression of Wnt/β-catenin pathway proteins in these cells. Future studies using relevant animal models are warranted to validate our findings. Nonetheless, our results suggest that GPC3 in liver cancer cell-derived exosomes may serve as a biological indicator to explore the impact of the liver cancer microenvironment on normal cells, thereby contributing to the development of HCC.

Abbreviations

GPC3, glypican-3; Exo, Exosomes; shGPC3-Exo,HepG2 exosomes that have silenced GPC3

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of the data and materials

The datasets and the reagents used in this study are available from the corresponding author upon reasonable request.

Competing Interests

The authors have declared that no competing interest exists.

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Authors' contributions

ZDW, CWP and YZ proposed and guided this research. QW, HH, PS, QQZ and XYS performed the experiments. QW and CWP collected and analyzed the data. QW, HH, PS, QQZ, XYS and AJS contributed to the design of the study and further drafts. ZDW, CWP and YZ revised the manuscript. All authors read and approved the final version of the manuscript.

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