Original Article

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FASN promotes gallbladder cancer progression and reduces cancer cell sensitivity to gemcitabine through PI3K/AKT signaling

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SUMMARY Lipid metabolism plays an important role in the growth and development of tumors. However, the role of lipid metabolism in gallbladder cancer (GBC) has not been clearly clarified. Here, we demonstrated that fatty acid synthase (FASN), a key enzyme in *de novo* fatty acid biosynthesis, had upregulated expression in GBC samples both at protein and mRNA levels. Analysis of clinical data indicated the association between elevated FASN expression and poorer histology grades. Furthermore, FASN activity impairment through FASN knockdown or treatment with orlistat resulted in the inhibition of cell proliferation and migration, as well as increased sensitivity to gemcitabine. Both FASN knockdown and orlistat treatment induced cell apoptosis. Mechanistically, impairment of FASN activity suppressed the activation of the PI3K/AKT signaling pathway, which led to increased cell apoptosis and sensitivity to gemcitabine. These findings were also validated through nude mouse xenograft models, thus highlighting the potential of targeting FASN as a clinical treatment strategy. Collectively, the present study underscores the crucial role of FASN in the progression of gallbladder cancer *via* the PI3K/AKT pathway.

Keywords GBC, orlistat, drug combination

1. Introduction

Gallbladder cancer (GBC) represents the most frequent malignant tumor within the biliary system (*1*), with a poor prognosis and a median survival of only 4-7 months (*2*). It was estimated that there were 115,949 cases of GBC and 84,695 deaths in 2020 (*3*). Despite surgery being the principal treatment method for gallbladder cancer, most patients are typically diagnosed during advanced stages, and as a result, are not eligible for radical resection (*4*). As a consequence, chemotherapy-based comprehensive treatment represents the primary strategy for extending the survival time of patients suffering from advanced GBC. Currently, gemcitabine (GEM) constitutes one of the principal drugs employed in first-line chemotherapy regimens for gallbladder cancer. Nonetheless, its objective response rate is a mere 30%, largely due to its low sensitivity (*5*). It is therefore urgent to explore the molecular mechanisms that underlie tumor progression and chemotherapy resistance in GBC to pave the way for the development of novel therapeutic approaches.

Fatty acid synthase (FASN) is the paramount lipogenic enzyme responsible for the terminal stages of *de novo* synthesis of fatty acids, specifically palmitate. In contrast to healthy cells, cancerous cells rely on the active production of fatty acids to construct membranes that promote cellular growth and proliferation. Additionally, palmitate can form covalent bonds with certain tumor-promoting growth factors, including Wnt, HRAS, and NRAS. These factors must undergo post-translational modification through palmitoylation to achieve correct localization and functional efficacy (*6*). While FASN is known to be overexpressed in a multitude of cancer types, its expression in healthy tissues is comparably meager. FASN activity seems to coincide with the advancement of numerous human malignancies. In human cancers, FASN is excessively upregulated and linked with unfavorable prognoses, as it sustains cancer cell growth and survival. Conversely, in the majority of normal cells, FASN is markedly repressed by adequate exogenous dietary fat supplementation (*7*). Additionally,

it has been demonstrated that overexpression of FASN contributes to cellular resistance against genotoxic drugs, including doxorubicin and cisplatin (*8,9*). Another study has indicated that FASN expression is markedly upregulated in pancreatic cancer. Furthermore, this study revealed that knockdown of FASN could reverse the cellular resistance to gemcitabine induced by overexpression of PKM2 (*10*). Although studies have identified FASN as a potential prognostic and therapeutic target in various cancers, its role in GBC remains unknown. In this study, we will examine the expression level of FASN through tissue microarray (TMA) and quantitative RT-PCR. Furthermore, we will elucidate the functional role of FASN in the progression of GBC and its impact on gemcitabine sensitivity.

Orlistat, a compound with reactive β-lactones, shows irreversible FASN inhibition ability. Currently, orlistat is registered as an obesity treatment drug in several countries. Its mechanism of action involves suppression of gastrointestinal lipases, which impairs the metabolism of lipids in the gastrointestinal lumen and prevents the absorption of up to 30% of the lipids in dietary fat (*11*). Studies indicate that orlistat shows therapeutic effects on a variety of cancers *in vivo* and *in vitro* (*12,13*). It has been reported that orlistat can reverse resistance to sorafenib in liver cancer (*14*) and cisplatin in ovarian cancer (*15*) by regulating the process of fatty acid metabolism. Given its favorable safety profile and affordability, the potential role of orlistat in cancer treatment is an area of significant clinical interest. Specifically, its role in GBC and the underlying mechanisms of action warrants further exploration.

In summary, our study affirms the significance of FASN in GBC through evaluating the correlation between FASN expression in GBC tissues and clinical features. *In vivo* and *in vitro* experiments were performed to ascertain the function of FASN in the progression of GBC and its impact on sensitivity to gemcitabine.

2. Materials and Methods

2.1. Cell lines and culture

NOZ, and the SGC-996 of human GBC cell lines, obtained from colleagues of Shanghai Key Laboratory of Biliary Tract Disease Research of Xinhua Hospital, Shanghai Jiao Tong University School of Medicine, China. These cells were routinely maintained in DMEM medium (Gibco, NY, USA) supplemented with 10% fetal bovine serum (Yeasen, Shanghai, China) and ampicillin/streptomycin (1%). All cells were cultured at 37°C, 5% CO_2. Cells were routinely verified and assured for free of mycoplasma contamination.

2.2. Patients and specimens

48 pairs of GBC tissues and adjacent normal tissues were collected for this study to verify expression of FASN with TMA. The patients received surgery between 2018 and 2021 in Xinhua Hospital of Shanghai Jiao Tong University School of Medicine (Shanghai, China). The complete medical records of 38 cases of these patients were available and the clinicopathologic features were retrospectively analyzed. All procedures followed were in accordance with the Helsinki Declaration (as revised in 2013). Informed consent was obtained from all patients for being included in the study.

2.3. Tissue microarray (TMA)

Tissue microarrays were constructed with 2 mm cores to evaluate the expression of FASN. The tissue sections were soaked in xylene for 15 min and then rehydrated through three decreased concentrations of ethanol for 2 min, and boiled in a pH 6.0 antigen retrieval solution for 20 min at 95°C. To remove endogenous peroxidase, the tissue sections were blocked with 3% hydrogen peroxide after cooling for enough time. The slides were then incubated with 10% goat serum for 60 min at room temperature. After incubation with primary and second antibodies, labeling was detected.

FASN expresses cytoplasmically. A total score (IHC score) which is obtained by multiplying proportion score (< 5%, 0; 6 to 25%, 1; 26 to 50%, 2; 51 to 75%, 3; and > 75%, 4) and intensity score (0; no staining, 1; weak intensity, 2; moderate intensity, 3; strong intensity), which represent the average intensity and proportion of positively stained cancer cells relatively. Subsequently, the 48 pairs of samples were designated as absent/low FASN expressers (IHC scores 0 to 4) or high FASN expressers (IHC scores 6 to 12) (*16*). A ratio was calculated to define the relative FASN expression of cancer (IHC scores) to the adjacent tissue (IHC scores): R = IHC score (cancer)/IHC score (paired normal tissue). A ratio greater than 2 was defined as relatively high expression. The evaluation of the immunohistochemical staining was independently performed by two experienced pathologists without knowledge of clinical data.

2.4. Plasmids, lentivirus particles and regents

The sequences of Small Interfering RNA (siRNA) were purchased from Tsingke Biotechnology Co., Ltd. (Beijing, China). siRNAs were transfected using RFect Reagent (Changzhou Biogenerating Biotechnologies corporation, Jiangsu, China) according to provided manufactures. The sequence of the siRNA is as follows: 5′-GCCGAGTACAATGTCAACATT -3′. Cells were used for further experiments at 48 h after transfection.

Short hairpin RNAs (shRNAs) against FASN were cloned into a pLKO.1 vector. HEK293T cells were co-transfected with pLKO.1 shRNA, psPAX2 and pMD2.G plasmid (gifts from Jiaxue Wu, School of

Life Sciences Fudan University, Shanghai, China) at a ratio of 4:3:1. The lentiviral particles were harvested 48 h after transfection. GBC cells were infected with recombinant lentivirus transducing units in 4 μg/mL polybrene (Beyotime, Shanghai, China). Cells were selected with puromycin for 1 week 48 after infection. The forward sequence of shRNA1 and shRNA2 are 5′-CCGGAAGCCGAGTACAATGTCAACACTCGA GTGTTGACATTGTACTCGGCTTTTTTTG -3′and 5′-CCGGAACTGCTAGGTATGGAGTTCTCTCGAG AGAACTCCATACCTAGCAGTT TTTTTG-3′.

2.5. CCK-8 assay and colony formation assay

The cell counting kit-8 (CCK-8) (Yeasen, Shanghai, China)was used to assess cell proliferation and viability. The NOZ and SGC-996 cells were seeded at a concentration of 2,000 cells/well in 100 μL in 96-well plates. To measure the IC_{50} of GEM, gradient concentrations of them were added to cells for 48 or 72 h after cells were seeded for 24 h. The concentration of DMSO in the medium was controlled below 0.1%. CCK-8 (10 μL) was added to each well, then the absorbance at 450 nm was measured after incubating at 37°C for 2 h. The IC_{50} value was calculated using GraphPad Prism 9.0.

The NOZ and SGC-996 cells were seeded at 1,000 cells/well in 6-well culture plates and grew for about two weeks. 4% paraformaldehyde was then used to fix the cells for 30 min, and 0.5% crystal violet solution was used to stain. The colony numbers (> 50 cells/colony) were manually counted.

2.6. Apoptotic assay

GBC cells were plated in 6-well plates (2×10^5 cells/well) and incubated overnight. After incubation, cells were transfected with control, FASN siRNA or treated with orlistat or gemcitabine for 48 h. Then, the apoptotic state of GBC cells was assessed by the Annexin V-FITC and PI (BD, United States, Cat number: 556547) cell staining following manufacturer's protocol. The samples were detected using flow cytometry (Cytoflex, Beckman, United States) and the results were analyzed using the CytExpert Software.

2.7. Western blot analysis

GBC cells were plated in 6-well plates (2×10^5 cells/well) and incubated overnight. After incubation, cells were transfected with control, FASN siRNA or treated with Orlistat or gemcitabine for 48 h. Then, cells were harvested and protein was extracted with radioimmunoprecipitation assay buffer (Beyotime, Shanghai, China). Protein was separated on 7.5-12.5% sodium dodecyl sulfate (SDS)-polyacrylamide gels and transferred onto 0.22 μm PVDF membrane (Merck Millipore, Burlington, MA, USA). The membranes were blocked with 5% milk (Yeason, Shanghai, China) for 1 h, incubated with the primary antibodies (1:1,000 dilution) at 4°C overnight, and incubated with the secondary antibody (1:1,000 dilution) at room temperature for 1h. Bands were visualized using ECL (Yeason, Shanghai, China). Antibody information: anti-β-actin (Abclonal, Wuhan, China, AC026); anti-FASN (Abclonal, A19050); anti-E-cadherin (Abclonal, A11509); anti-CDK-4 (Raybiotech, Georgia, USA, 144-00366); anti-cyclin-D1 (Cell Signaling, Massachusetts, Danvers, USA, #2978); anti-Bcl-2 (Proteintech, Wuhan, China, 60178-1-Ig); anti-Bax (Proteintech, 50599-2-Ig); anti-vimentin (Proteintech, 10366-1-AP); anti-PI3K (Proteintech, 20584-1-AP); anti-p-PI3K(Tyr458/Tyr199) (Cell Signaling, #4228); anti-AKT (Cell Signaling, 10176-2-AP); anti-p-AKT (Ser473) (Cell Signaling, #9271).

2.8. Xenografted animals

Four-week-old BALB/c nude mice were purchased from the Shanghai SLAC Laboratory Animal Co., Ltd. (Shanghai, China), and the study was approved by Laboratory Animal Ethical and Welfare Committee Xin Hua Hospital Affiliated to Shanghai Jiao Tong University School of Medicine. We subcutaneously administered 1×10^6 NOZ cells (NC, lv-shFASN) resuspended in 0.1 mL PBS to nude mice on the left armpit. The mice were randomly grouped into four groups ($n = 6$). Two groups (control and shFASN) received an intraperitoneal injection with vehicle (Phosphate buffered saline, PBS) and the others (control and shFASN) with gemcitabine (50 mg/kg, twice a week), and body weight was measured every 2 days. We sacrificed the mice using CO_2, removed the tumors and weighed them. The tumor volume was calculated (V) (length × width^2)/2.

2.8. Statistical analyses

All *in vitro* experiments were conducted in triplicate, and data were presented as mean ± standard deviation (SD). Differences between the two groups were evaluated using the Student's *t*-test. Depending on the total sample size and expected frequencies, the chi-square test or Fisher's exact test were chosen to assess the significance of differences of clinicopathological figures between groups. Data analyses were carried out in the GraphPad Prism 9 and SPSS version 25. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, and ****$p < 0.0001$ indicated statistical significance.

3. Results

3.1. Enhanced FASN expression is detected in GBC and involved with unfavorable clinicopathology of GBC tissue

To evaluate FASN expression in GBC, we collected

a total of 48 human GBC paired tumors and adjacent normal tissues. Tissue microarray data showed that FASN was mainly localized on the cell membrane and the cytoplasm of GBC tissues (Figure 1A). We assessed and scored staining pattern of FASN in both GBC and adjacent paracancerous gland tissue. It revealed that expression levels of FASN protein in cancer and normal tissues are both high. Of note, FASN protein expression was significantly increased in cancers compared to the matched gallbladder tissues (Figure 1B). Specifically, 40 out of 48 cases exhibited high expression of FASN (score > 4) in cancer tissues, while 20 out of 48 cases showed high expression in paracancerous tissues. RT-PCR data from another collected cohort also suggested that FASN mRNA expression levels were notably higher in gallbladder cancer tissues (14 cases) than in the adjacent normal tissues (14 cases) (Figure 1C). To investigate the correlation between FASN protein expression and clinicopathological features, 38 cases of complete medical reports were obtained and analyzed (Table 1). FASN expression was not statistically associated with age, gender, histological types, resection method, and gallstone state, but high FASN expression was significantly correlated with advanced clinical stage and nevin stage (Table 1). These results indicate upregulated FASN expression in GBC tissues and this gene's oncogenic properties in GBC.

3.2. FASN knockdown promotes apoptosis and inhibits proliferation and migration of GBC cells

After evaluating the FASN expression in GBC cell lines by western blot (Figure 2A), we selected NOZ and SGC-996 for further experiments. To further elucidate the oncogenic function of FASN, we knocked down the FASN protein expression in GBC cells by siRNA. The knockdown efficiency was confirmed by western blot (Figure 2B). The CCK8 assay and colony formation assay showed that the proliferative capacity of NOZ and SGC-996 cells were impaired following the knockdown

Table 1. Summary of patient characteristics according to FASN expression

Variable	Total number	FASN expression		*P* value
		Relatively low (R < 2)	Relatively high (R ≥ 2)	
Age (y)				1
> 65	19	9	10	
≤65	19	10	9	
Gender				1
Male	11	5	6	
Female	27	14	13	
Histological types				1
Adenocarcinoma	33	17	16	
Other[a]	5	2	3	
Clinical stage				0.003**
1-2	7	7	0	
3-4	31	12	19	
T classification				0.055
T1-T2	11	9	2	
T3-T4	27	10	17	
N classification				0.329
N0	18	10	8	
N1	10	3	7	
N2	10	6	4	
M classification				1
M0	26	13	13	
M1	12	6	6	
Nevin staging				0.008**
IV, V	31	12	19	
I, II, III	7	7	0	
Resection method				0.728
R1, R2	12	7	5	
R0	26	12	14	
Gallstone				1
(+)	18	9	9	
(−)	20	10	10	

[a]adenosquamous carcinoma, large cell neuroendocrine carcinoma, sarcomatoid carcinoma; χ^2 test was performed.

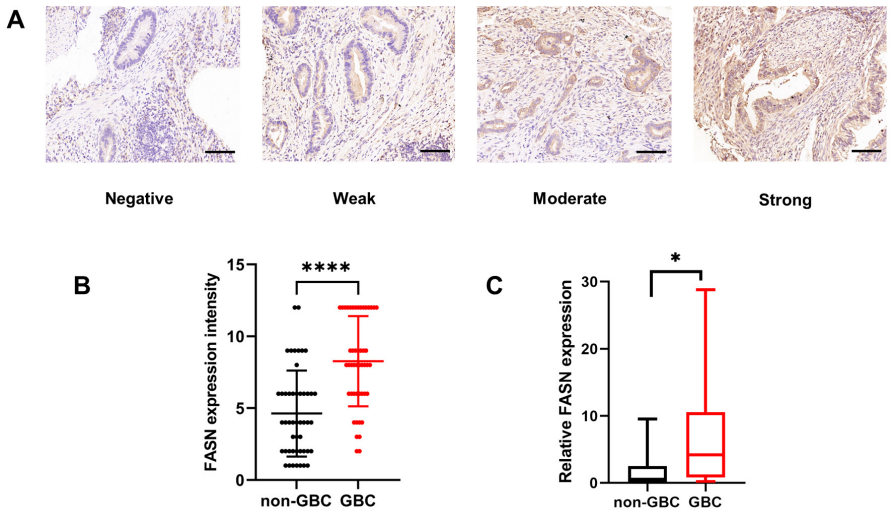

Figure 1. Enhanced FASN expression is detected in GBC and involved with unfavorable clinicopathology of GBC tissue. (**A**), Representative images of FASN expression in GBC tissues as visualized by TMA(Bar,100μm). (**B**), Expression of protein FASN in 48 paired GBC and adjacent normal tissues analyzed by TMA. (**C**), qPCR analysis of FASN mRNA expression. Data information: In (**B**), data are presented as mean ± SD. *$p < 0.05$, ****$p < 0.0001$ (Mann–Whitney U test for B, Student's-*t* test for C).

of FASN (Figures 2C and 2D). This was further proved by impaired protein levels of cyclin-D1 and CDK-4 in FASN knocking down cells (Figure 2G). Moreover, FASN knockdown significantly enhanced apoptosis in NOZ and SGC-996 cells (Figure 2E). Increased levels of apoptosis associated proteins Bax and decreased levels of Bcl-2 in FASN knockdown cells supported the results above (Figure 2G). Transwell assay and western blot assay were performed to evaluate the effect of FASN on the migration ability of GBC cells. The migrated cell numbers were significantly reduced after the knocking down of FASN (Figure 2F). Enhanced E-cadherin and reduced vimentin protein expression after knocking down FASN supported that FASN indeed promoted migration in GBC cells (Figure 2G). Taken together, these results demonstrate that FASN might promote proliferation and migration while impairing apoptosis of GBC cells.

3.3. Orlistat promotes apoptosis and inhibits proliferation and migration of GBC cells

Orlistat is a classical inhibitor of the thioesterase domain of FASN approved by the Food and Drug Administration for treating obesity, recently it has been proven to halt cell proliferation, and induce cancer cell apoptosis in various types of tumors (*12,17*). However, the role of orlistat in GBC remains unknown. To characterize the effects of orlistat on GBC and pave a way for clinical application, we first measure cell viability of NOZ/SGC-996 incubated with different concentrations of orlistat for 48 h *via* CCK-8 assay (supplementary Figure S1, *http://www.ddtjournal.com/action/getSupplementalData.php?ID=167*). Cell viability assay showed orlistat suppressed the growth of both two cell lines dose-dependently (supplementary Figure S1, *http://www.ddtjournal.com/action/getSupplementalData.php?ID=167,* Figure 3A). Also, the

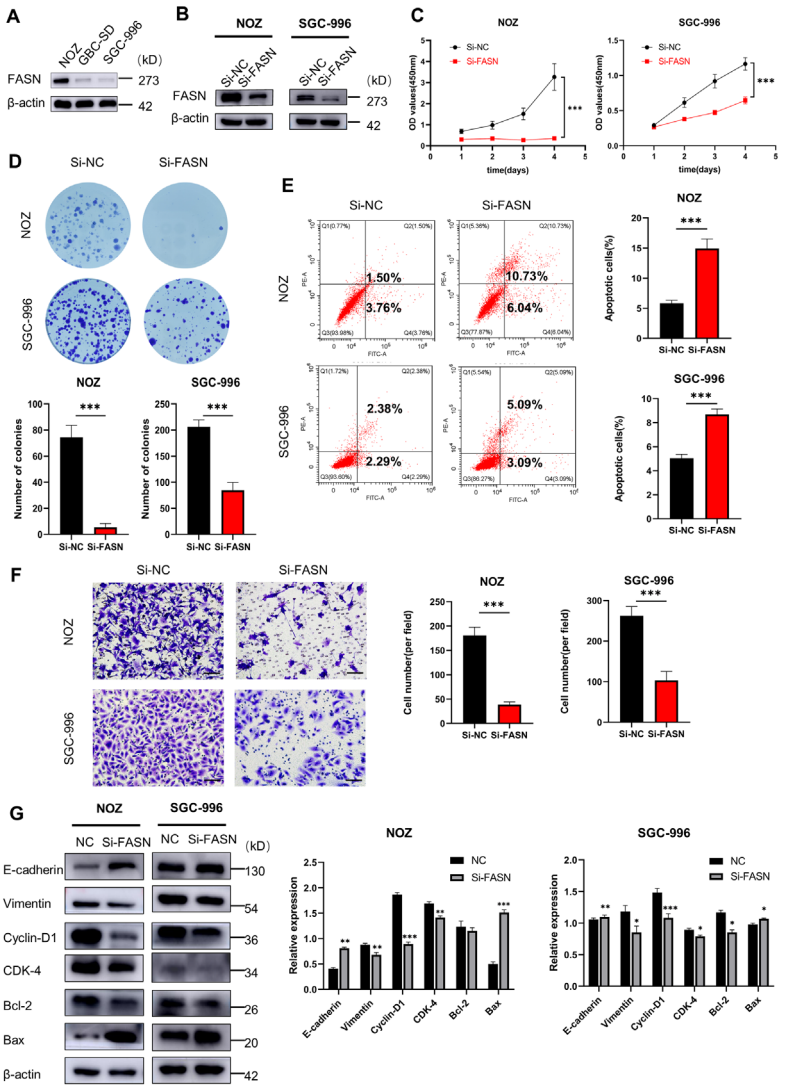

Figure 2. FASN knockdown promotes apoptosis and inhibits proliferation and migration of GBC cells. (**A**), Relative expression of FASN in three GBC cell lines. (**B**), Knockdown efficiency in NOZ and SGC-996. (**C**), Cell viability assay of FASN knockdown cell lines. (**D**), Colony formation assays and statistical analysis of two cell lines transfected with si-FASN. (**E**), Apoptosis assays and quantification of the apoptotic cell population. (**F**), Transwell assays and statistical results (Bar,100μm). (**G**), Western blotting of cell fractions in FASN knockdown cell lines of NOZ and SGC-996. Data are presented as mean ± SD. *$p < 0.05$, **$p < 0.01$,***$p < 0.001$.

number of colonies formed by GBC cells was obviously reduced after treatment by different concentrations of orlistat for 48 h (Figure 3B). Orlistat also induced NOZ and SGC-996 cell apoptosis to a degree (Figure 3C). Besides, the transwell assay for cell migration showed that the number of migrated NOZ (0, 10, 100 μM) and SGC-996 (0, 50, 100 μM) cells treated with orlistat were decreased in a dose-dependent manner (Figure 3D). We observed an increase in protein expression of Bax and E-cadherin, and a decrease in the expression of Bcl-2, cyclin-D1, CDK-4, and vimentin in NOZ/SGC-996 cells (Figure 3E), which supported and verified that orlistat treatment could induce cell apoptosis, inhibit GBC cell proliferation and migration.

3.4. Impaired activity of FASN enhances gemcitabine sensitivity in GBC

FASN was reported to mediate chemoresistance of various drugs like gemcitabine (*10*), cisplatin, and doxorubicin (*18*). Considering that orlistat indeed showed anti-cancer activity in GBC cells without killing normal cells (*18*), we further wondered whether FASN inhibition by orlistat could show a positive effect on gemcitabine sensitivity *in vitro*. Then we evaluated sensitivity changes to gemcitabine in both NOZ and SGC-996 cells in the presence of 10 μM orlistat. Compared to gemcitabine alone, combined treatment with orlistat for 72 h significantly lowered the IC_{50} of gemcitabine in NOZ (from 56.14 nM to 15.18n M) and SGC-996 (from 1,206 nM to 406.6 nM) (Figures 4A and 4B).

We then examined the effect of the combined use of gemcitabine (1 μM) and orlistat (10 μM) on protein expression associated with cancer progression. Western

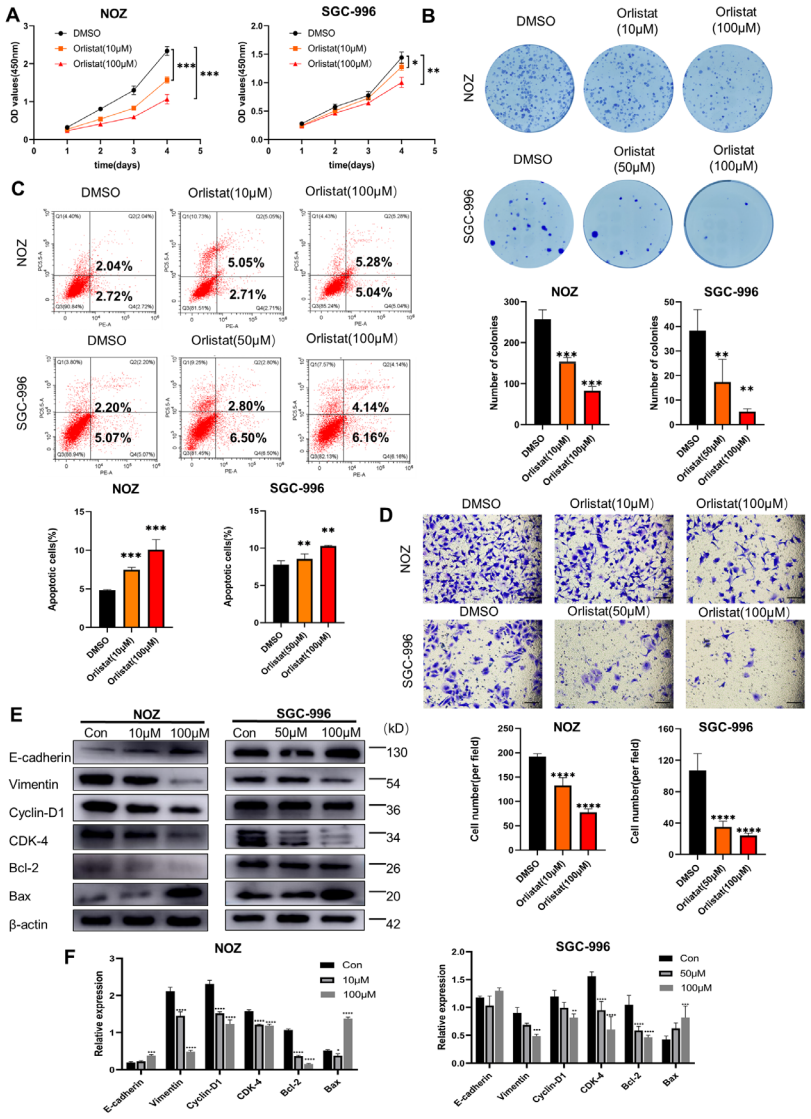

Figure 3. Orlistat promotes apoptosis and inhibits proliferation and migration of GBC cells. (**A**), Cell viability assay of cells pretreated with orlistat for 48h. (**B**), Colony formation assays and statistical analysis of two cell lines incubated with orlistat. (**C**), Apoptosis assays and quantification of the apoptotic cell population. (**D**), Transwell assays and statistical results (Bar,100μm). (**E-F**), Western blotting of cell fractions in cell lines of NOZ and SGC-996 treated with orlistat for 48h. Data are presented as mean ± SD. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$.

blot results demonstrate that the addition of orlistat could enhance the effect of gemcitabine on the expression of apoptosis-associated protein (Bax and Bcl-2), which indicated that orlistat could promote gemcitabine-induced cell death (Figures 4C-4E). Also, the combination of gemcitabine with orlistat showed synergistic decreasing expression of cyclin-D1 and CDK-4 (Figures 4C-4E). Additionally, the combination of the two agents enhanced the suppression of migration compared to treatment with gemcitabine alone (Figures 4C-4E).

Apart from that, flow cytometry apoptosis assays were conducted to evaluate the effects of FASN knockdown on gemcitabine sensitivity in GBC cells, revealing that FASN knockdown cells had a higher proportion of apoptosis (Figure 4F). Taken together, these results revealed that impairment of FASN activity could enhance the gemcitabine sensitivity in GBC cells.

3.5. FASN mediated PI3K/AKT activation involves in GBC cells sensitivity to gemcitabine

Accumulating evidence has revealed that PI3K/AKT activation participates in multi-drug resistance and neoplastic lipogenesis of various cancers. Therefore, we examined the phosphorylated forms of PI3K and AKT in NOZ and SGC-996 with downregulated FASN expression or orlistat treatment by western blot assay. It suggested that activation levels of PI3K/AKT pathway was significantly impaired by FASN activity repression, with relatively slight or no changes in the total protein expression levels (Figures 5A and 5B). Furthermore, treatment with gemcitabine or orlistat independently or in combination was conducted to assess the modulatory effect on PI3K/AKT cascade reaction. Compared to separately application or vehicle control (DMSO), the

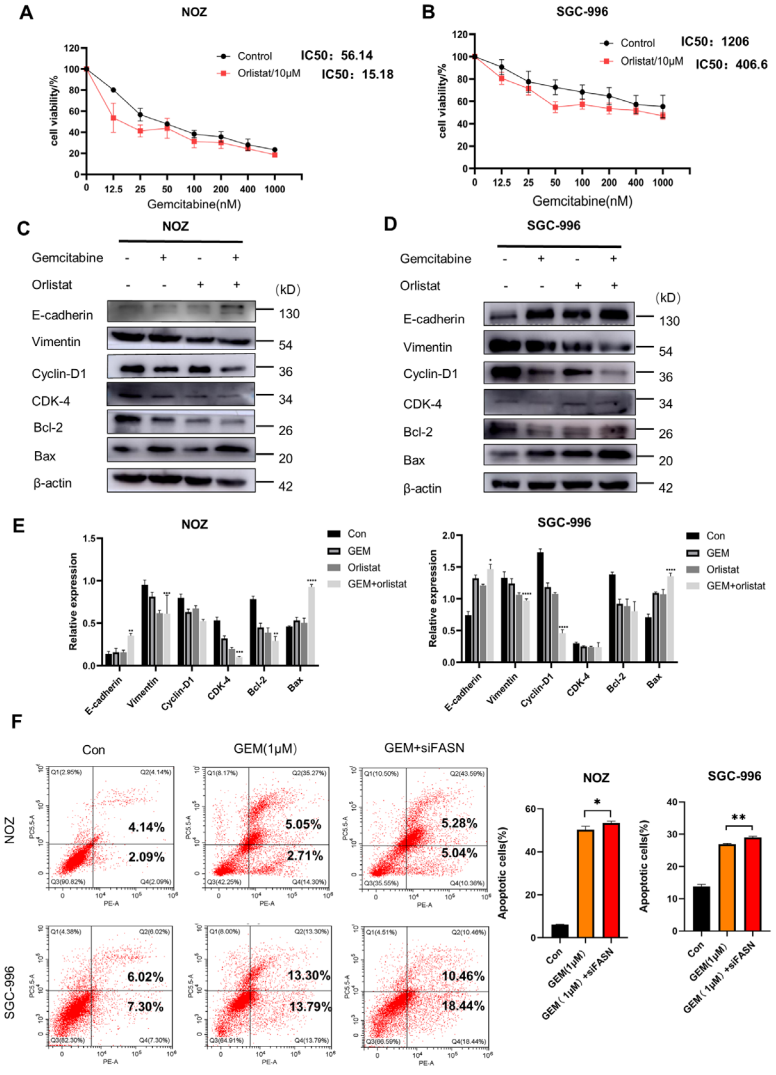

Figure 4. Impaired activity of FASN enhances gemcitabine sensitivity in GBC. (**A-B**), CCK8 assay showing the IC_{50} of gemcitabine with or without orlistat (10 μM) addition in NOZ/SGC-996 cells. (**C-E**), Western blot analysis of bax, bcl-2, cyclin D1, CDK-4, vimentin and E-cadherin in cells treated with gemcitabine (1 μM) or orlistat (10 μM) alone or in combination. (**F**), Apoptosis assays and quantification of the apoptotic cell population in GBC cells treated with gemcitabine (1 μM), siFASN, or a combination of both. Data are presented as mean ± SD. The GEM + orlistat group was compared to the GEM group, and the other groups were compared to the control group. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$. GEM: gemcitabine.

gemcitabine/orlistat simultaneous administration showed more efficiency on inhibition of PI3K/AKT signaling (Figure 5C). Taken together, FASN mediates GBC progression and gemcitabine resistance, at least, in part, through PI3K/AKT pathway.

To further validate the role of the PI3K/AKT pathway in GBC cells, a rescue experiment was conducted using SC79, an AKT agonist. The apoptotic proportions of NOZ and SGC-996 cells treated with gemcitabine or gemcitabine in combination with orlistat (100 μM) were assessed. As depicted in Figure 5D, orlistat augmented the apoptotic effect induced by gemcitabine, and pre-treatment with SC79 for one hour partially reversed the trend.

3.6. FASN knockdown inhibits tumor growth and enhances gemcitabine sensitivity in mice

To further explore the role of FASN in GBC progression and gemcitabine sensitivity *in vivo*, we developed nude mouse xenograft models to perform following experiments. FASN-silenced and matching control (lv-shFASN and lv-control) NOZ cells (Figure 6A) were subcutaneously injected into the right armpit of nude mice. These two kinds of mice were randomly divided into two groups separately and administered either gemcitabine (50 mg/kg) or PBS intraperitoneally. The tumor size and weight of mice were measured twice a week. The difference in the tumor volumes and weights between the lv-control and FASN-silenced, the lv-control treated with gemcitabine and the FASN-silenced treated with gemcitabine grouped mice were calculated. As exhibited by Figures 6B-6E, FASN knockdown could significantly abrogate tumor growth and the tumor abrogation effect of gemcitabine was significantly more evident in mice transfected with FASN-impaired cells. Immunohistochemical analyses of xenograft

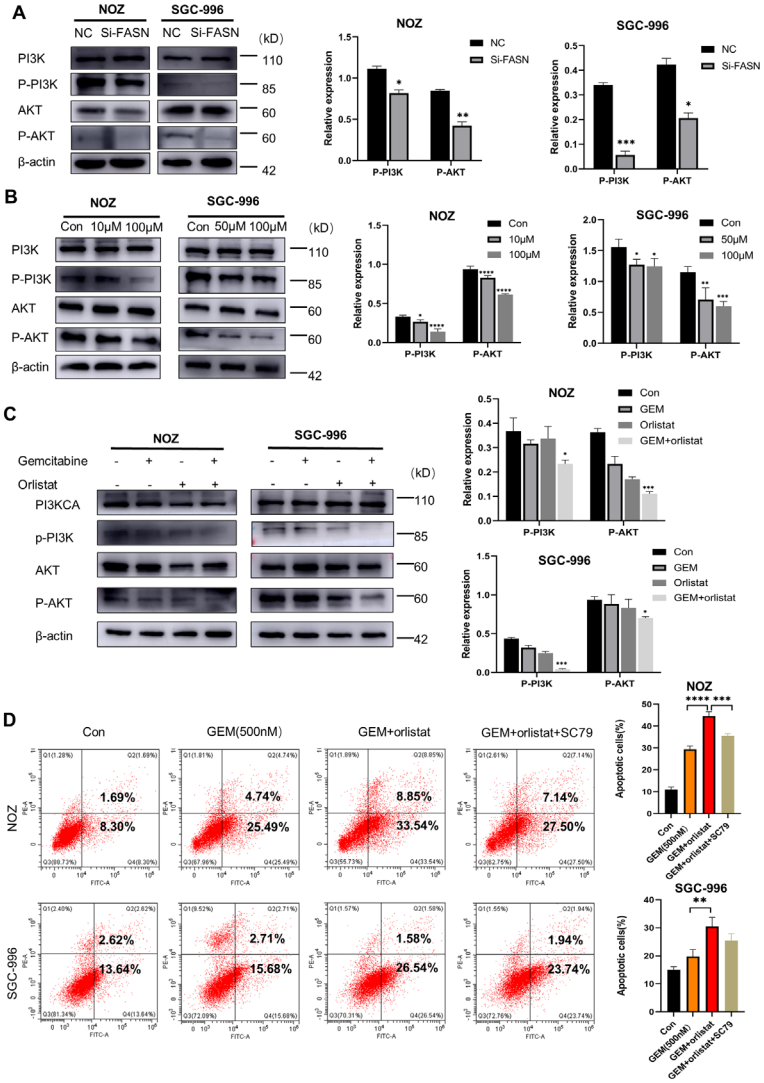

Figure 5. FASN mediated PI3K/AKT activation involves in GBC cells sensitivity to gemcitabine. (**A-B**), Western blot assay showing the expression levels of PI3K, p-PI3K, AKT, and p-AKT in FASN knockdown (**A**) and orlistat treatment (**B**) NOZ/SGC-996 cells. (**C**),Western blot analysis of PI3K, p-PI3K, AKT, and p-AKT in cells treated with gemcitabine (1μM)/orlistat (10μM) alone or in combination. (**D**), Apoptosis assays and quantification of the apoptotic cell population in cells treated with gemcitabine (500 nM) or gemcitabine in combination with orlistat (100 μM). Pretreatment with 10 μM SC79 was used to rescue the effect of orlistat. Data are presented as mean ± SD. The GEM + orlistat group was compared to the GEM group, and the other groups were compared to the control group. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, $****p < 0.0001$.

tumor tissues demonstrated that the expression level of apoptosis marker cleaved caspase 3 was elevated in the gemcitabine treatment group, and this was further enhanced by FASN knockdown (Figure 6F). Additionally, gemcitabine treatment, FASN knockdown, and their combination decreased the expression of Ki-67 (Figure 6F). Western blot analysis also showed that stable FASN knockdown downregulated p-AKT protein levels in xenograft tumor tissues (Figure 6G). Collectively, these results suggest that FASN knockdown can inhibit GBC tumor growth and improve gemcitabine sensitivity *in vivo via* the PI3K/AKT signaling pathway.

4. Discussion

Dysregulation of lipid metabolism is closely associated with the occurrence and progression of various tumors (*19-21*). Current clinical research indicates that lipid metabolism-targeted therapies exhibit promising anti-cancer effects (*19,22*). Nevertheless, there is still a relatively limited amount of research regarding lipid metabolism in GBC. Epidemiological and clinical studies have demonstrated a positive correlation between obesity and overweight status with an elevated risk of GBC (*23-25*). Bile lipidomics experiments have revealed that the abnormal composition of lipids in the bile of GBC patients is correlated with disease severity (*26*), suggesting that lipid metabolism dysfunction may serve as a hallmark for early diagnosis of GBC (*27*). α-Mangostin, a dietary xanthone, has been shown to augment the susceptibility of gallbladder cancer to gemcitabine treatment by repressing lipid biosynthesis through the targeting of AMPK/SREBP1 signaling pathways (*28*). These findings suggest that aberrant lipid metabolism may also be involved in the tumorigenesis and malignant progression of GBC. To further clarify the relationship between lipid metabolism and GBC progression, we focused on a potential target, FASN, by

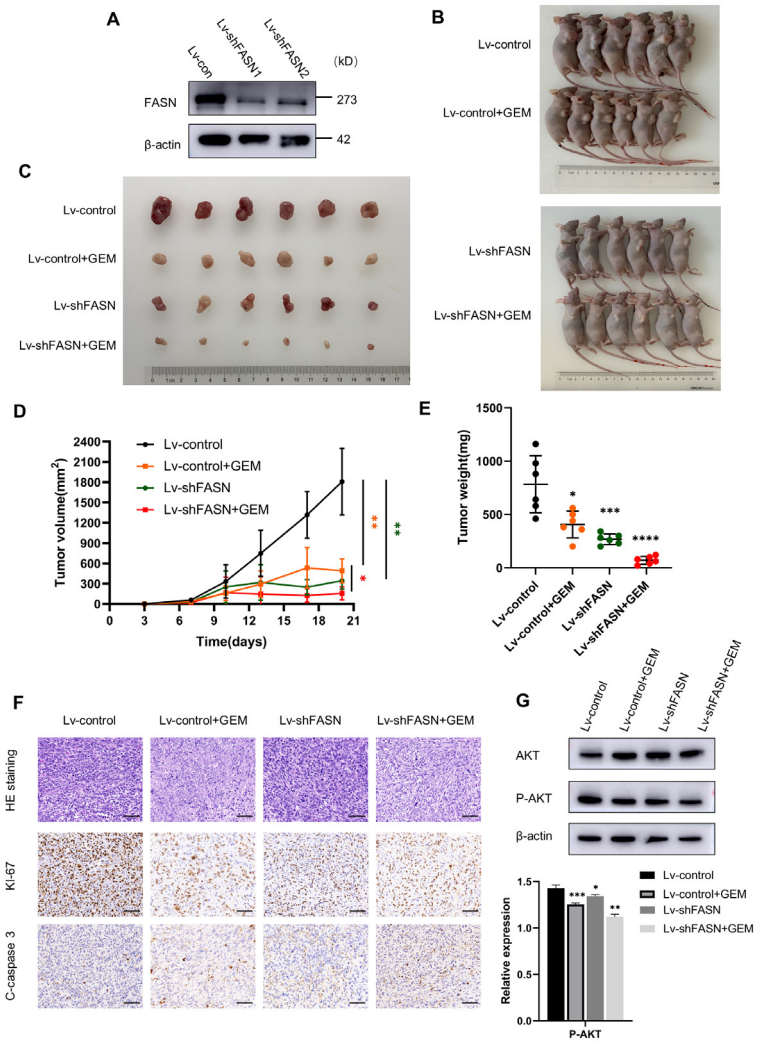

Figure 6. FASN knockdown inhibits tumor growth and enhances gemcitabine sensitivity in mice. (**A**), Validation of FASN knockdown efficiency. (**B-C**), Xenograft tumors in BALB/c nude mice. NOZ (lv-control, lv-shFASN) cells were inoculated into BALB/C nude mice. One week later, the mice were intraperitoneally injected with gemcitabine(50mg/kg) or PBS. The volumes (**D**) and weight (**E**) of tumors were measured. (**F**), IHC of Ki-67 and cleaved caspase 3 (c-caspase 3) in the tumors (Bar,100 μm). (**G**), Western blot analysis of AKT and p-AKT in four groups of xenograft tumor tissues. Data are presented as mean ± SD. The lv-shFASN + GEM group was compared to the lv-control + GEM group, and the other groups were compared to the lv-control group. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$.

exploring its expression and function in GBC.

Fatty acids are a primary source of energy for rapidly dividing tumor cells and also serve as the fundamental building blocks for cell membrane synthesis. FASN is an essential enzyme in fatty acid synthesis and plays a pivotal role in this process. Extensive research has been conducted on the role of FASN in cancer progression and carcinogenesis (*8,9,12*), but its involvement in GBC has not been reported. In this study, we demonstrate that FASN is upregulated in GBC and facilitates malignant progression and gemcitabine insensitivity. Additionally, we show that inhibition of FASN leads to cell apoptosis by regulating the levels of PI3K and AKT phosphorylation, which are crucial for the oncogenic process. Further, we reveal that apoptosis induced by FASN inhibition can be rescued by the application of an AKT activator, SC79.

Studies have shown that various types of tumors, including prostate cancer, breast cancer, ovarian cancer, colon cancer, and gastric cancer, exhibit high FASN expression and elevated FASN activity (*12*). According to a previous study, ovarian cancer tissue overexpressed FASN protein by an average of 1.8 times relative to healthy fallopian tube tissue (*29*). The expression levels of FASN were found to be associated with different grades of ovarian cancer tissues (*29*). Additionally, FASN in serum was absent or present at lower levels in both normal and non-malignant conditions, suggesting that it may be useful as a marker for early cancer detection (*30*). In our results, both FASN protein and mRNA are overexpressed in GBC tissues compared to adjacent normal tissues. Moreover, increased FASN expression is positively correlated with more advanced GBC pathological stages, which are associated with poorer clinical outcomes for patients. Since some patients are lost to follow-up and the samples collected were from 2018 to 2020, the survival data is incomplete. Additional cohorts including more patients are needed to reaffirm these conclusions.

A previous study demonstrated that knockdown or knock-out of FASN inhibited esophageal squamous cell carcinoma cell proliferation (*31*). Exogenous expression of FASN enhanced the proliferation, migration, and cell motility of SK-UT-1 *via* promoting trimethylation of H3K9 (H3K9me3) and acetylation of H3K27 (H3K27ac) in uterine leiomyosarcomas cells (*32*). In addition, the reduction of palmitate synthesis by FASN inhibition contributed to cancer cell apoptosis by disrupting cell membranes formation, repressing signaling transduction such as PI3K-AKT-mTOR and lipid biosynthesis, and suppressing gene expression such as c-Myc (*33*). Interestingly, FASN is also dispensable for the function of Treg cells and FASN knock-out in Treg inhibits tumor growth (*34*). But no documentation uncovers the role of FASN playing in GBC. The results of our *in vitro* and *in vivo* experiments clearly demonstrate that inhibition of FASN by knockdown leads to a marked reduction in GBC cell proliferation and migration while increasing apoptotic rates. Unfortunately, we encountered challenges in performing functional experiments by overexpressing FASN in GBC cells due to the large size of the protein, which can reach up to 273 kD, making it difficult to overexpress using current technologies. Therefore, we selected the FASN inhibitor orlistat to support our knockdown experiment. In the future, this part of the experiment could potentially be completed through supplementation with FASN catalytic products or the use of mutation techniques to enhance the enzyme activity of FASN (*31*).

Resistance is one of the key contributing factors to low response rate to chemotherapy in GBC. Lipid metabolism has been reported to be associated with drug resistance (*20*), FASN activity inhibition was also reported to reverse chemotherapy resistance in pancreatic cancer (*10,35*) and ovarian cancer (*29*). In our study, gemcitabine treated GBC cells show increased apoptotic rates after FASN knockdown. And in our xenograft animal experiment, gemcitabine shows more excellent therapeutic efficiency on tumor growth in mice transfected with FASN-knockdown NOZ cells. These findings suggest a possible target for GBC treatment and warrent further investigation to uncover the underlying mechanisms.

Orlistat irreversibly inhibits FASN through binding to the thioesterase domain of this enzyme. It is widely used for obesity treatment with considerable advantages in clinical safety over other inhibitors. Moreover, scores of studies have sought to verify the anticancer potential of this agent through *in vivo* and *in vitro* experiments aimed at evaluating its efficacy (*12*). Therefore, we conducted further investigations into the potential role of orlistat in GBC cells. Our findings reveal that orlistat effectively inhibits GBC cell progression, particularly in NOZ cells expressing higher levels of FASN. These results suggest that inhibitors may exhibit greater efficacy against tumors with elevated FASN activity. Thus, cancer patients exhibiting abnormally high FASN expression may represent appropriate targets for therapy. We further explored whether FASN inhibition by orlistat could enhance gemcitabine sensitivity. The CCK-8 assay demonstrates that administration of orlistat (10 μM) can indeed reduce the IC_{50} in GBC cell lines. Additionally, apoptotic assays indicate that orlistat can amplify gemcitabine-induced cell death. Nonetheless, we must acknowledge that the synergistic effect of orlistat and gemcitabine is not particularly pronounced, although we should not discount its modest effect. Considering that the side effects of orlistat are manageable, higher doses of the drug could be administered to enhance its effectiveness. In the future, a combination of orlistat and gemcitabine may improve the efficacy and safety of chemotherapy regimens for GBC. Further animal and clinical studies should be conducted to pave the way for GBC treatment.

PI3K/AKT signaling transduction is considered one of the causes of chemoresistance in various tumors. Aberrant activation of this pathway inhibits chemotherapy-induced apoptosis *via* multiple mechanisms (*36*). Phosphorylation activated AKT promotes cancer cells survival through mediating Bcl-2 and Bax expression (*37*). Cancer cells escape from apoptosis depending on Bcl-2 in a variety of tumors (*38*). In gastrointestinal stromal tumor cells, FASN knockdown resensitized drug-resistant cells to imatinib by inactivating the PI3K/AKT/mTOR signaling pathway (*39*). The present study validates that FASN knockdown and inhibition downregulate the phosphorylation level of PI3K and AKT protein without affecting expression levels of total protein. Both FASN downregulation and inhibition also induce apoptosis *via* enhancing Bax and suppressing Bcl-2 protein expression. Similarly, orlistat enhances gemcitabine-induced GBC cell death through boosting regulation of Bcl-2/Bax expression. Additionally, pretreatment with SC79, an AKT agonist, was shown to reverse the increased apoptosis of GBC cells caused by the combination treatment of orlistat and gemcitabine. Although we have conducted different experiments to confirm the role PI3K/AKT pathway plays in the function of FASN, we do not know how FASN interact with PI3K/AKT. Further mechanisms should be explored in following studies.

In conclusion, our study provides compelling evidence that FASN inactivation exerts inhibitory effects on GBC cell proliferation and migration, while promoting cell apoptosis and gemcitabine sensitivity through the PI3K/AKT pathway. This work not only identifies FASN as a promising therapeutic biomarker for GBC patients, but also highlights the potential clinical utility of orlistat, a lipid metabolism regulator, in improving the clinical outcomes of GBC patients through enhancing gemcitabine sensitivity. These findings open up a promising avenue for further research into targeting lipid metabolism in the treatment of GBC.

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