

Leptin promotes proliferation and metastasis of human gallbladder cancer through OB-Rb leptin receptor

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Abstract. Emerging evidence has shown that leptin, an adipocyte-derived cytokine that is closely associated with obesity, play a significant role in carcinogenesis and tumorigenesis. However, its impact on gallbladder cancer (GBC) remains unclear. In this study, we firstly found that leptin and its functional receptor OB-Rb were significantly co-expressed in human GBC tissues and cell lines, the content of which were higher than those in normal human gallbladder tissues. Treatment with leptin promoted the proliferation, migration and invasion of GBC cells, which were attenuated by OB-Rb shRNA. Blocking in the G2/M period of cell cycle, increasing of MMP3 and MMP9, increasing of VEGF-C/D, activation of SOCS3/JAK2/p-STAT3 pathway was demonstrated after treatment with leptin. All of these positive responses were attenuated by OB-Rb receptor shRNA. Taken together, our findings suggest that leptin promoted the proliferation, migration and invasion of GBC cells by increasing OB-Rb expression through the SOCS3/JAK2/p-STAT3 signal pathway. Targeting the leptin/OB-Rb axis could be an attractive therapeutic strategy for treatment of GBC.

Introduction

Gallbladder cancer (GBC) is a rare but highly aggressive malignancy. The lack of severe symptoms makes the diagnosis very difficult (1). Even though there are some therapies such as

cholecystectomy or radical resection, chemotherapy, or radiotherapy (2,3), they are not as effective as expected. The 5-year survival rate is extremely low (4). So far there is no systemic therapy with a satisfactory outcomes. Thus, studying novel signal molecules involved in GBC margin and metastasis may provide new effective therapeutic strategies.

Leptin, the product of the OB gene, is a 16 kDa non-glycosylated peptide hormone which is synthesized almost exclusively by adipocytes that regulates appetite and energy expenditure at the hypothalamic level (5,6). In recent years, accumulating evidence suggests that leptin plays an important role in tumorigenesis, angiogenesis and metastasis of many cancers, including breast (7), pancreatic (8), and stomach cancer (9). Previous studies have shown that leptin could activate Janus kinase 2 (JAK2) when leptin was bound to one form of the receptor, OB-Rb. Then JAK2 initiated downstream signaling including members of the signal transducers and activators of transcription (STAT) family of transcription factors (10). However, the expression of leptin and OB-Rb in GBCs has not been fully investigated, and the precise role of leptin in the development and promotion of GBC remains unknown.

In this study, we investigated the clinical implications of leptin and OB-Rb in GBC patients. Moreover, we explored the role of leptin and one form of its receptor OB-Rb in GBC cells through *in vitro* and *in vivo* studies. SOCS3/JAK2/p-STAT3 signaling pathways were also assessed and these pathways may be involved in cell migration and metastasis by leptin.

Materials and methods

Immunohistochemistry and evaluation. Forty paraffin-embedded specimens of normal gallbladder tissues and 40 specimens of gallbladder cancer (GBC) tissues were collected from January 1, 2005 to June 30, 2010 at Department of Hepatopancreatobiliary Surgery, the Second Affiliated Hospital of Kunming Medical University, Kunming, China. No patients had received chemotherapy or radiotherapy before biopsy. The prior patient's consents and approval from the Institutional Research Ethics Committee were obtained. Rabbit anti-human polyclonal leptin and OB-Rb antibody

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(Sigma, St. Louis, MO, USA) was used for immunohistochemistry assay, which was performed following the protocol of Universal SP kit (MXB Biotechnology, Fujian, China). Positive staining of leptin protein is brown in the cytoplasm, partly in the nucleus, and positive staining of OB-Rb protein is brown in the cytomembrane. The human GBC tissue sections were blindly examined and scored concurrently by two observers. The intensity of the immunostaining was scored as 0 (negative), 1 (weak), 2 (moderate), or 3 (strong). Four visual fields were selected randomly under high power lens (x400). The number of positive cells was counted and the average positive rate was calculated. The percentage of positive tumor cells was scored as '+' (<25%), '++' (26-50%), '+++ (51-75%), or '++++' (76-100%), and that without any positive cells scored as '-'.

Cell culture. The human GBC cell sublines (GBC-SD) were obtained from the Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China). The cells were cultured in Dulbecco's modified Eagle's medium/ α -modified Eagle medium (DMEM) (Gibco Life Technology, Gaithersburg, MD, USA) supplemented with 10% fetal bovine serum (FBS) (Gibco Life Technology) and maintained at 37°C in a humidified atmosphere of 5% CO₂.

shRNA synthesis and transfection. Four different template oligonucleotides targeting OB-Rb (Table II) were synthesized by Ribobio Inc. (Guangzhou, Guangdong, China), and were annealed and ligated into pGPH1/GFP/Neo plasmid. The shRNAs inserted vectors were named as pGPH-GFP-s1, pGPH-GFP-s2, pGPH-GFP-s3, pGPH-GFP-s4 and pGPH-GFP-NC, respectively.

Transfection of shRNAs was performed using Lipofectamine 2000 reagent (Invitrogen Co., Carlsbad, CA) according to the manufacturer's instructions. Briefly, GBC-SD cells were applied for OB-Rb silence, which were cultured in 6-well plates at a density of 5×10^4 cells/well. Then GBC-SD cells were subject to shR-OB-Rb and shR-Con treatment for 24, 48 and 96 h. To evaluate the infection efficiency, cells were observed under a fluorescence microscope and the percentage of GFP-positive cells was counted.

Quantitative real-time PCR analysis. Total RNA from GBC-SD cells was extracted using the TRIzol reagent (Invitrogen). Then, 2 μ g of total RNA was subjected to reverse transcription for cDNA synthesis by using MMLV (MBI Fermentas, Euromedex, Souffelweyersheim, France). Real-time PCR was performed with the manufacturer's (Kapa Biosystem, Hercules, CA, USA) instructions. The primer sequences listed below were used. A mathematical model, $2^{-\Delta\Delta CT}$ method, was used for relative quantification in real-time PCR (11). GAPDH was used as internal control gene to normalize the variability at mRNA expression levels.

Western blot analysis. The GBC-SD cell pellet was washed twice with ice-cold phosphate buffered saline (PBS) and lysed with lysis buffer (Beyotime Institute of Biotechnology, Jiangsu, China). Protein (30 mg) was loaded and separated in 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gel and transferred to polyvinylidene difluoride membranes (Millipore, Bedford, MA, USA).

The following antibodies were used to probe the alterations of protein: JAK2 (Abcam, Cambridge, UK), SOCS3 (Abcam), STAT3 and p-STAT3 (Cell Signaling Technology, Inc., Danvers, MA, USA), MMP-3 and MMP-9 (Santa Cruz Biotechnology, Santa Cruz, CA, USA), VEGF-C/D (Santa Cruz Biotechnology). GAPDH (Santa Cruz Biotechnology) was used as loading control. Signal was detected by enhanced chemiluminescence techniques (Pierce Thermo Scientific, Rockford, IL, USA).

Cell proliferation assay. Cells (2×10^4) per well were seeded into 96-well plate and incubated overnight. Then the medium was removed. Medium (100 μ l) with the final concentration of 100 nM OB-Rb shRNA was added to each well with or without leptin (250 ng/ml). Scramble shRNA or untreated cells were used as the control group. All groups were in triplicate. After 24, 48, and 72 h transfection, cell proliferation was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay (Beyotime Institute of Biotechnology).

Flow cytometry analysis. Cells (2×10^5) were seeded in 6-well plates and incubated overnight to 50-60% confluence. OB-Rb shRNA was added into the medium at a final concentration of 100 nM with or without leptin (250 ng/ml). Scramble shRNA or untreated cells were used as the control group. The cells were incubated with leptin for 24 h, then treated into single cell suspension with cold PBS. The experiment was performed following manufacturer's protocol of Annexin V-FITC Apoptosis and Cells cycle Detection kit (Beyotime Biotechnology, Jiangsu, China). Then, rates of apoptosis were analyzed with FACScan system (BD Biosciences, Franklin Lakes, NJ, USA). Each experiment was performed in triplicate, independently.

Cell migration and invasion assay. Transwell chambers and Matrigel Invasion Chambers (8 μ m pore size, Corning Inc., Corning, NY, USA) were used for cell migration and invasion assay, respectively. GBC-SD cells transfected with OB-Rb shRNA or scramble shRNA were treated with or without leptin (250 ng/ml). After 24 h, cells were detached. Then 500 μ l medium with 20% FBS was added into each lower chamber which was incubated at 37°C. Incubation periods were 2 h for migration, and 4 h for invasion. Then, the surface of the upper chamber was swabbed with cotton-tipped applicators to remove the cells that did not migrate. The lower membrane surface was fixed in methanol and stained by crystal violet. Migrating cells were counted using light microscopy (five random 100x fields per well) or a spectrophotometer. Results were calculated from three independent experiments.

Immunofluorescence. shRNA-transfected cells (2×10^5) and untreated cells were all seeded on coverslips. Cells were cultured in a 6-well plate and incubated with leptin for 24 h, then rinsed twice in PBS and fixed with methanol at -20°C for 20 min. Following fixation, the coverslips were directly washed in PBS for 5 min, followed by incubation with PBS, 0.2% Triton X-100 and 5% bovine serum albumin for 20 min at room temperature. Following rinsing with PBS, the cells were incubated with primary antibody at 4°C in a humidity

Table I. Clinical characteristics of 40 gallbladder carcinoma (GBC) patients and leptin and OB-Rb expression.

Characteristics	Leptin		P-value	OB-Rb		P-value
	Low (n=15)	High (n=25)		Low (n=17)	High (n=23)	
Age						
≥60	8	11	0.567	11	8	0.061
<60	7	14		6	15	
Gender						
Male	3	9	0.285	5	7	0.994
Female	12	16		12	16	
BMI, kg/m ²						
<30	12	1	<0.001	13	0	<0.001
≥30	3	24		4	23	
CA199, U/ml						
<35	9	1	<0.001	10	0	<0.001
≥35	6	24		7	23	
T classification (1)						
T1b/T2	14	15	0.030	16	13	0.012
T3	1	10		1	10	
N classification (1)						
N0	15	14	0.003	17	12	0.001
N1	0	11		0	11	
Tumor differentiation						
I/II	13	13	0.026	13	13	0.191
III/IV	2	12		4	10	
AJCC (1)						
I	14	10	0.001	16	8	<0.001
II	1	15		1	15	

P<0.05 was considered statistically significant. Pearson's Chi-square test and Fisher exact test (when 1<T<5) were used.

box. Primary antibodies included mouse anti-JAK2 (1:200; Abcam), rabbit anti-STAT3 or p-STAT3 (1:200; CST) and rabbit polyclonal to SOCS3 (1:200; Abcam). Coverslips were subsequently washed 3 times with cold PBS and incubated with the corresponding secondary antibodies (diluted to 1:500, Santa Cruz Biotechnology) for 2 h at room temperature in the dark, humid box. DAPI staining was then performed to identify the nuclei.

Gelatin zymography assay. Cells (2×10^5) transfected with OB-Rb shRNA or scramble shRNA were seeded in a 6-well plates and incubated for 6 h. Then leptin (250 ng/ml) was added and the cells incubated for another 24 h. After each treatment, the cells were washed twice with serum-free medium, and used for a zymogram according to the protocol of Zymography kit (Genmed, Shanghai, China).

Enzyme-linked immunosorbent assay (ELISA). VEGF-C and VEGF-D levels were detected using the ELISA kit according to the manufacturer's manual (Keygentec,

Shanghai, China). Colorimetric measurement was recorded as OD 450 readings.

Xenograft model assay. All animal procedures were previously approved by the Kunming Medical University ethics committee. Female BALB/c nu/nu mice (4-5 weeks old, 15-18 g), from Vital River Laboratory Animal Technology Co., Ltd. (Peking, China), were randomly assigned into five groups as described above: control, shR-NC, leptin (1 mg/kg), shR-OB-Rb, and shR-OB-Rb + Leptin (1 mg/kg), groups. Approximately 5×10^6 cells were suspended in 0.1 ml PBS and injected subcutaneously into each mouse. The tumors were monitored every 5 days beginning at day 5 by measuring two perpendicular diameters with a caliper. The mice were sacrificed on the 30th day after injection. The tumors were dissected and weighed.

Statistical analysis. Statistical analysis was performed with SPSS software (17.0; SPSS, Inc., Chicago, IL, USA). Values are expressed as mean \pm standard deviation (SD). The Student's t-test was used for comparisons between groups.

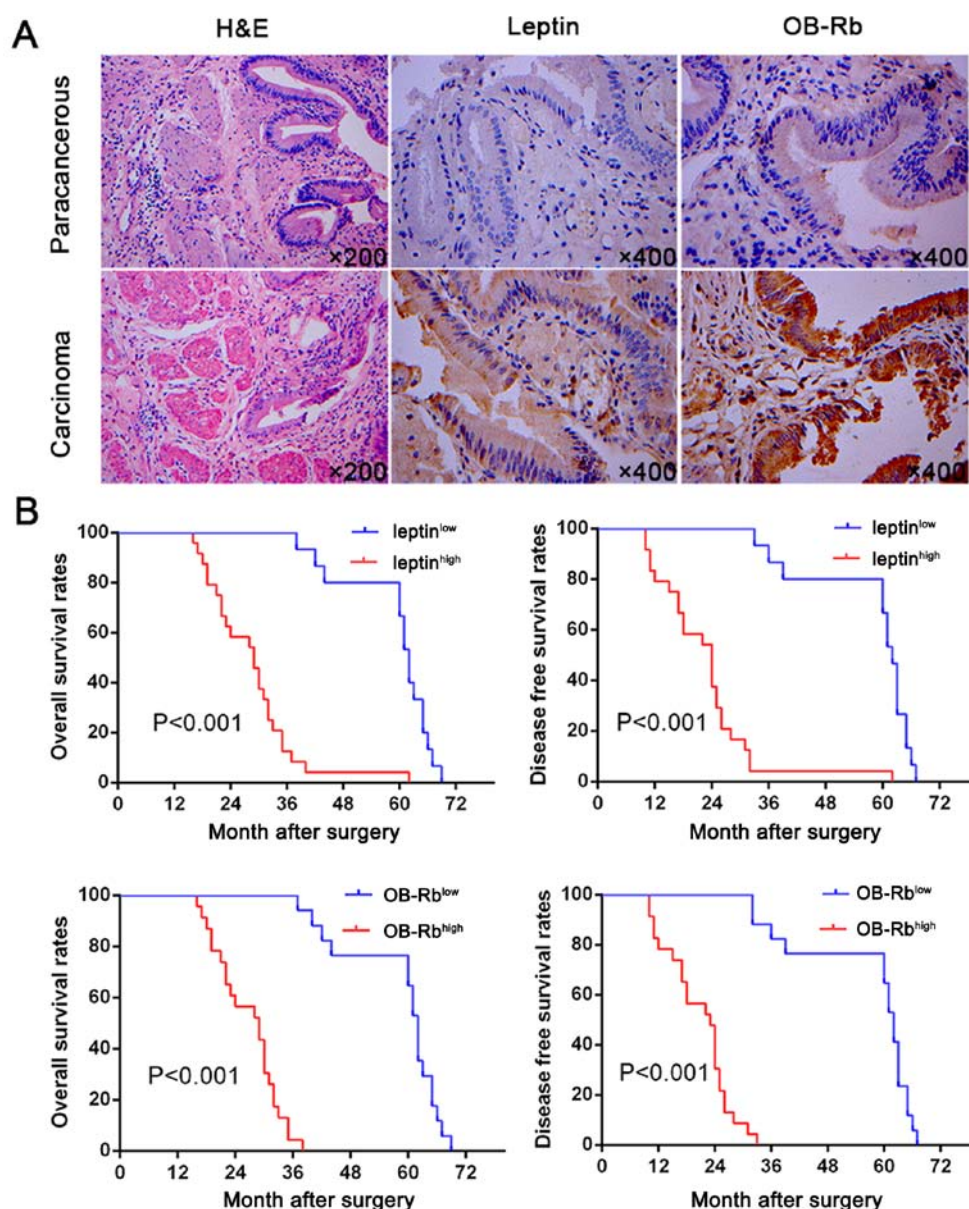


Figure 1. Leptin and OB-Rb expression in gallbladder carcinoma (GBC) tissues and their prognostic significance in GBC patients. (A) Immunohistochemistry staining of leptin and OB-Rb in human gallbladder tissues. GBC tissues exhibited significantly higher expression of leptin and OB-Rb. (B) High expression of leptin and OB-Rb was associated with a poor prognosis in 40 GBC patients. Kaplan-Meier plots show disease-free survival (DFS) and overall survival (OS) curves according to leptin and OB-Rb expression levels (low, blue line; high, red line) in the primary tumor ($P<0.001$, log-rank test).

Categorical data were analyzed by the chi-square or Fisher's exact tests. Correlation analysis was performed between leptin and OB-Rb. Cumulative recurrence and survival rates were analyzed using Kaplan-Meier's method and the log-rank test. Cox's proportional hazards regression model was used to analyze independent prognostic factors. Statistical significance was defined as P -value <0.05 .

Results

Expression levels of leptin and OB-Rb in GBC tissues. To evaluate the possible roles of leptin in GBC, we investigated the expression levels of leptin and OB-Rb in human normal gallbladder and GBC tissues by immunohistochemistry. Compared to normal gallbladder tissues, expression levels of leptin were significantly upregulated in GBC tissues

($P=0.000$). Moreover, OB-Rb density was significantly higher in GBC tissues than in normal gallbladder tissues ($P=0.001$) (Fig. 1A). In addition, a scatter plot of leptin and OB-Rb expression revealed a significantly positive correlation between leptin and OB-Rb levels in cancerous tissues ($r=0.797$, $P=0.000$). The characteristics of the study participants including age, gender, BMI, T classification, N classification, Tumor differentiation, AJCC stage are shown in Table I. Results demonstrated that GBC patients with leptin^{high} had high BMI ($P<0.001$), elevated CA199 ($P<0.001$), high T ($P=0.030$), N ($P=0.003$) classification and AJCC stage ($P=0.001$), poor differentiation ($P=0.026$). Moreover, GBC patients with OB-Rb^{high} had high BMI ($P<0.001$), elevated CA199 ($P<0.001$), high T ($P=0.012$), N ($P=0.001$) classification and AJCC stage ($P<0.001$), poor differentiation ($P=0.026$). We then analyzed the prognostic implication of leptin and OB-Rb

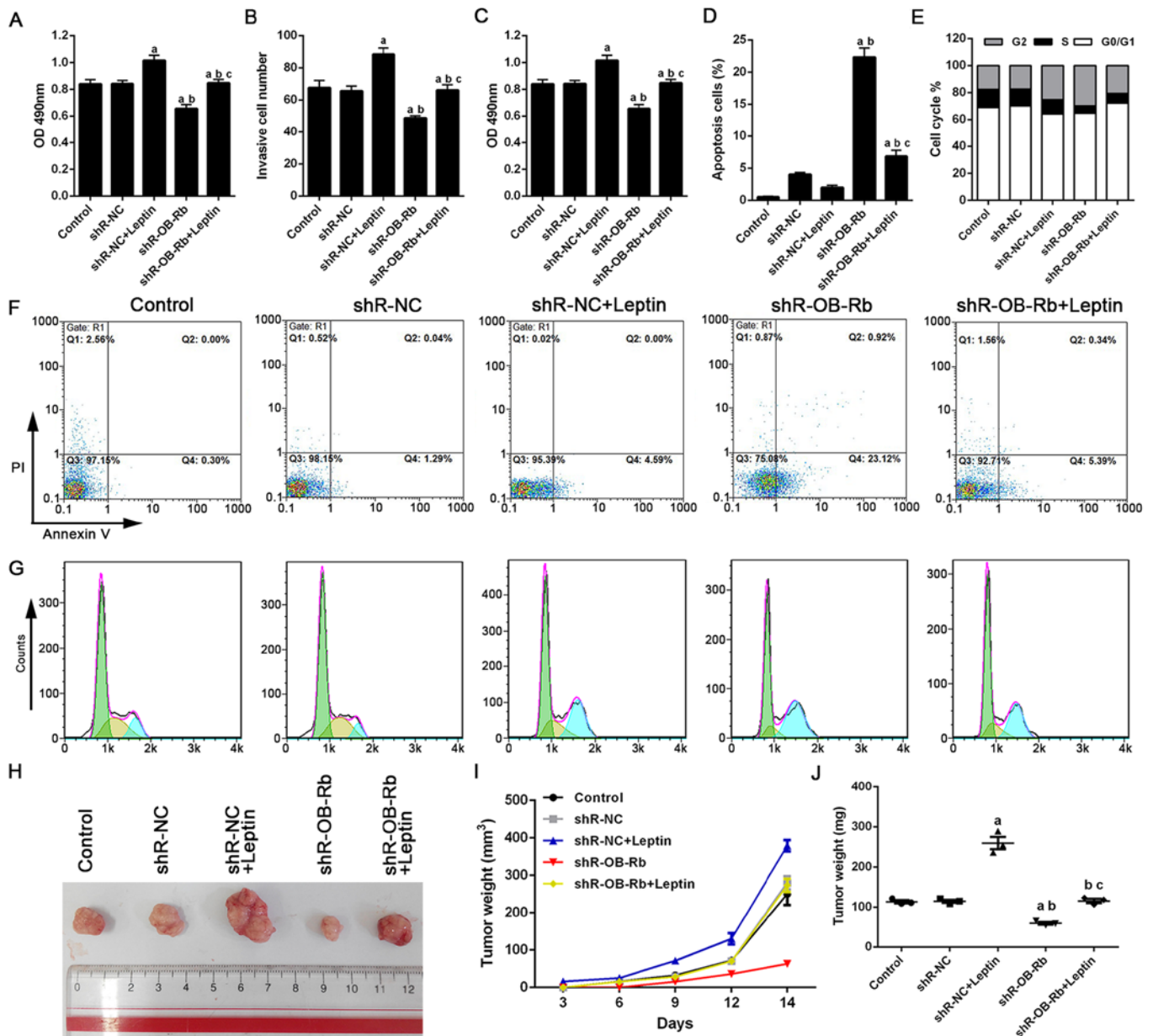


Figure 2. Effects of leptin and OB-Rb in human gallbladder carcinoma cells *in vitro* and *in vivo*. (A) Growth of GBC-SD cells was analyzed by MTT after 24 h of treatment with 40 ng/ml leptin with control shRNA or OB-Rb shRNA. (B) Migration and (C) invasion of GBC-SD cells were both analyzed by Transwell. (D and F) Cell apoptosis and (E and G) cell cycle were analyzed by flow cytometry after 24 h of incubation with 40 ng/ml leptin with control shRNA or OB-Rb shRNA. GBC-SD cells were stably infected with shR-NC or shR-OB-Rb and injected into BALB/c-nude mice. Established s.c. xenografts were treated with 1 mg/kg leptin or control saline on two consecutive days per week for 4 weeks. (H) Representative images of xenografts from different groups. (I and J) Tumor volume and tumor weight were determined. These data represent the mean \pm SD of three independent experiments. Statistical analysis used two-tailed Student's t-test (* P <0.05 vs. shR-NC group; * P <0.05 vs. shR-NC + Leptin group; * P <0.05 vs. shR-OB-Rb group).

expression. Importantly, we found that patients with leptin^{high} and OB-Rb^{high} expression had significantly worse prognosis than those with leptin^{low} and OB-Rb^{low} expression (Fig. 1B). Multivariate analysis identified leptin and OB-Rb expression as an independent predictor for disease-free survival and overall survival (OS; Table II). These results indicate that leptin and OB-Rb is likely involved in tumorigenesis and progression of GBC.

Involvement of OB-Rb receptor in leptin-mediated growth, migration and invasion of GBC-SD cells. Leptin exhibits its effects on cancer through interaction with specific

leptin receptors (OB-Rb and OB-Rs) (12). In this study, we examined the effect of leptin on GBC-SD cell growth, migration and invasion. The result from MTT assay showed leptin (40 ng/ml) significantly increased the proliferation of GBC-SD cells after 24-h incubation compared with basal values, which were significantly inhibited by shR-OB-Rb transfection (Fig. 2A).

Leptin was able to promote GBC-SD cell migration, which was significantly suppressed in GBC-SD cell transfected with OB-Rb shRNA at 24 h compared with the control group as shown in Fig. 2B. Transwell matrix penetration assay showed that leptin treatment increased the mean of GBC-SD cell

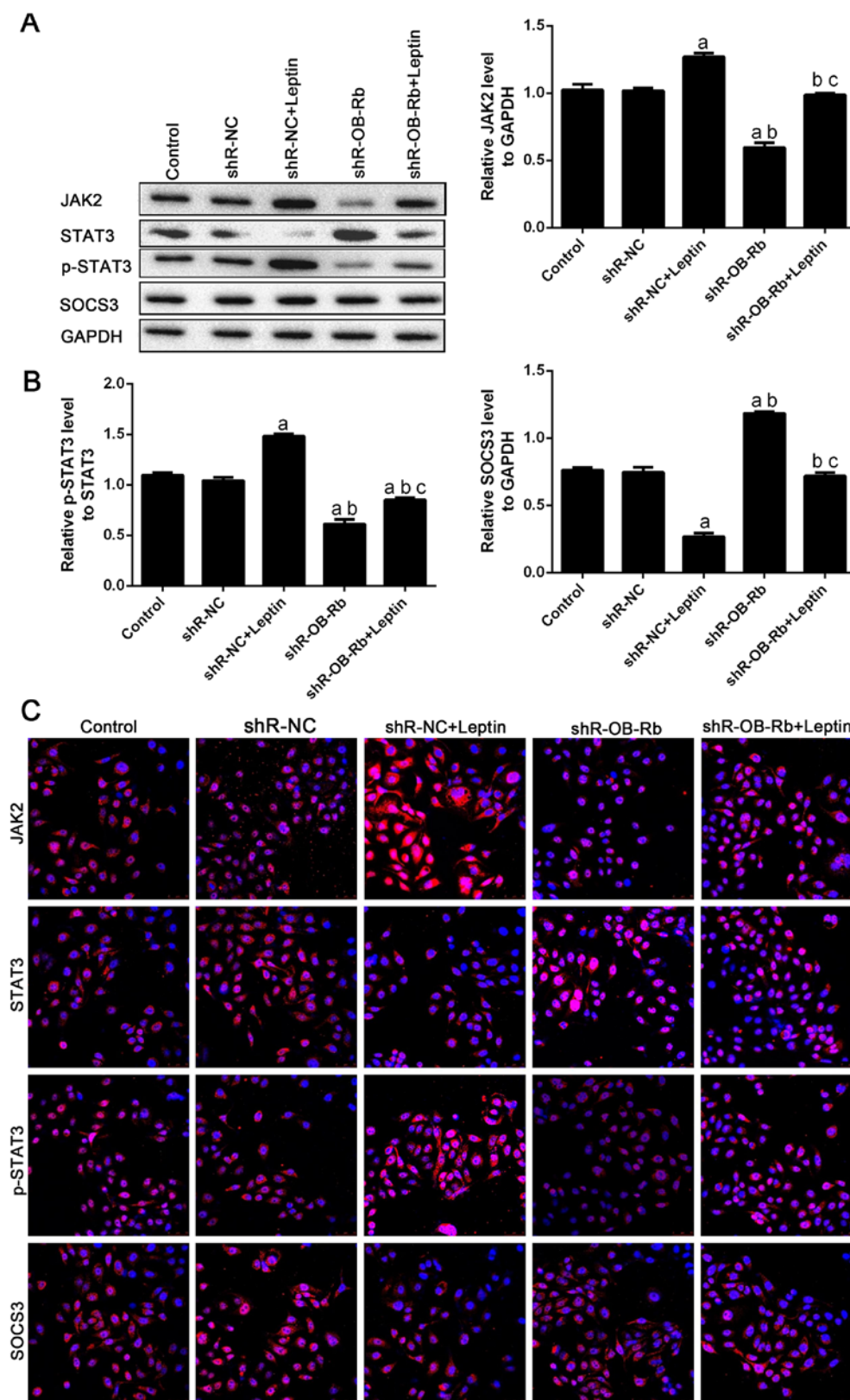


Figure 3. Leptin activates the JAK2/STAT3/SOCS3 signal pathway. (A and B) Western blot analysis of the protein expression of JAK2, STAT3, phosph-STAT3 and SOCS3 in leptin or shR-OB-Rb-transfected GBC-SD cells. (C) Immunofluorescence analysis of these protein in leptin or shR-OB-Rb-transfected GBC-SD cells. These data represent the mean \pm SD of three independent experiments. Statistical analysis used two-tailed Student's t-test (^a $P < 0.05$ vs. shR-NC group; ^b $P < 0.05$ vs. shR-NC + Leptin group; ^c $P < 0.05$ vs. shR-OB-Rb group).

invasive number, which could be repressed by transfection with OB-Rb shRNA (Fig. 2C). Flow cytometry was used to analyze cell apoptosis in GBC-SD treated with leptin or

OB-Rb shRNA for 24 h. As shown in Fig. 2D-G, leptin treatment or OB-Rb knockdown significantly induced G2/M-phase cell cycle arrest, and decreased cells number of G0/G1 and

Table II. Uni- and multivariate analyses of factors associated with survival.

Factor	OS				DFS			
	Univariate, P-value	Multivariate			Univariate, P-value	Multivariate		
		HR	95% CI	P-value		HR	95% CI	P-value
Gender (male vs. female)	0.117			NA	0.328			NA
Age, years (≥ 60 vs. < 60)	0.056			NA	0.070			NA
BMI, kg/m ² (≥ 30 vs. < 30)	0.584			NA	0.326			NA
CA199, U/ml (≥ 35 vs. < 35)	0.002	3.532	1.485-13.406	0.012	0.004	3.876	1.514-12.711	0.027
T classification (T1 vs. T2)	0.091			NA	0.242			NA
N classification (N0 vs. N1)	0.093			NA	0.167			NA
Tumor differentiation (I/II vs. III/IV)	0.782			NA	0.765			NA
AJCC (I vs. II)	0.575			NA	0.648			NA
Leptin expression (low vs. high)	0.028	2.271	1.865-18.615	0.001	0.044	2.874	1.196-19.682	0.003
OB-Rb expression (low vs. high)	0.016	3.461	2.043-18.292	0.001	0.020	2.931	1.970-11.438	0.001

Cox's proportional hazards regression model. OS, overall survival; DFS, disease-free survival; HR, hazard ratio; 95% CI, 95% confidence interval; NA, not adopted.

S-phase. OB-Rb knockdown significantly induced apoptosis in GBC-SD cell line, compared with the other groups ($P < 0.05$).

Leptin promoting growth and metastasis were retarded by OB-Rb RNAi. *In vivo*, the volumes and weight of xenograft tumors removed from nude mice which were injected with leptin and shR-NC were both higher than the other groups. In addition, they were retarded apparently in Leptin + shR-OB-Rb group after 30 days. As shown in Fig. 2H-J, the growth of xenograft tumors in shR-OB-Rb group obviously less than the other groups.

Signaling pathways of JAK2/STAT3/SOCS3 were involved in leptin stimulation. The leptin action was by signaling via JAK2 and phosphorylation of STAT3 or other pathways such as SOCS3 (13,14). In GBC-SD cells, we found that leptin increased JAK2 expression levels and STAT3 phosphorylation, and decreased SOCS3 expression levels. Such an effect was blocked by shR-OB-Rb treatment (Fig. 3A and B). The immunofluorescence experiments also confirmed similar results (Fig. 3C). These results indicate that the JAK2/STAT3/SOCS3 pathway is involved in leptin-induced migration of human GBC cells.

shR-OB-Rb downregulated MMP-3/9 activity and expression of VEGF-C/D increased by leptin. Numerous studies have mechanistically associated the invasive and metastasis ability of cancer cells with expression of VEGF factors (15) and activation of MMP family (16). To understand the mechanism by which leptin promoted the invasiveness and migration of GBC-SD cells, we investigated the expression of VEGF-C/D and activity of MMP-9 in GBC-SD cells treated with leptin and/or transfected with shR-OB-Rb. As shown in Fig. 4A, gelatin zymography assay results show leptin increases MMP-3 and MMP-9 activity in GBC-SD cells, which are attenuated by transfecting shR-OB-Rb. ELISA also confirmed similar result

in expression of VEGF-C and VEGF-D (Fig. 4B). Then we detected activity of MMP-3/9 and expression of VEGF-C/D *in vivo*, which were confirmed by western blot (Fig. 4C and D) and immunofluorescence (Fig. 4E-J) analysis. Taken together, these data suggested that leptin upregulated VEGF-C/D levels and activated MMP-3/9 *in vivo* and *in vitro*.

Discussion

Our results indicate that leptin and OB-Rb are expressed at a high level in GBC patients, compare with normal gallbladder tissue. In addition, a high level of leptin and OB-Rb is a poor prognostic marker for GBC patient. Furthermore, this study shows for the first time that leptin and OB-Rb mediates migration of human gallbladder cancer (GBC) cells. We show that *in vitro*: i) leptin stimulates growth and migration of GBC-SD cells; ii) the enhancement of GBC-SD cell growth by leptin is associated with G2/M cell cycle arrest, iii) activity of MMP-3/9 and expression VEGF-C/D is determined. iv) JAK2/STAT3/SOCS3 pathway is involved in this process. Moreover, *in vivo* v) genetic ablation of leptin-mediated signaling enhanced cancer growth in an animal model of GBC. Moreover, these effect could all be attenuated by OB-Rb receptor shRNA.

Several studies have shown strong epidemiologic evidence suggesting the existence of a close link between obesity, a clinical condition characterized by high levels of circulating leptin (17) and a multitude of cancers, such as prostate (18), mammary (19), endometrial (20), hepatocellular (21), colon (22), pancreatic (23), adenocarcinoma of esophagus (24), and cholangiocarcinoma (25). In this study, consistent data show that leptin enhanced progression of GBC. Leptin is usually related to binding with its receptor OB-Rb, which belongs to the cytokine receptor superfamily (26). It has been reported that human cancer cells expressed OB-Rb and other OB-Rs leptin receptors (27). However, the role of OB-Rb in human GBC is

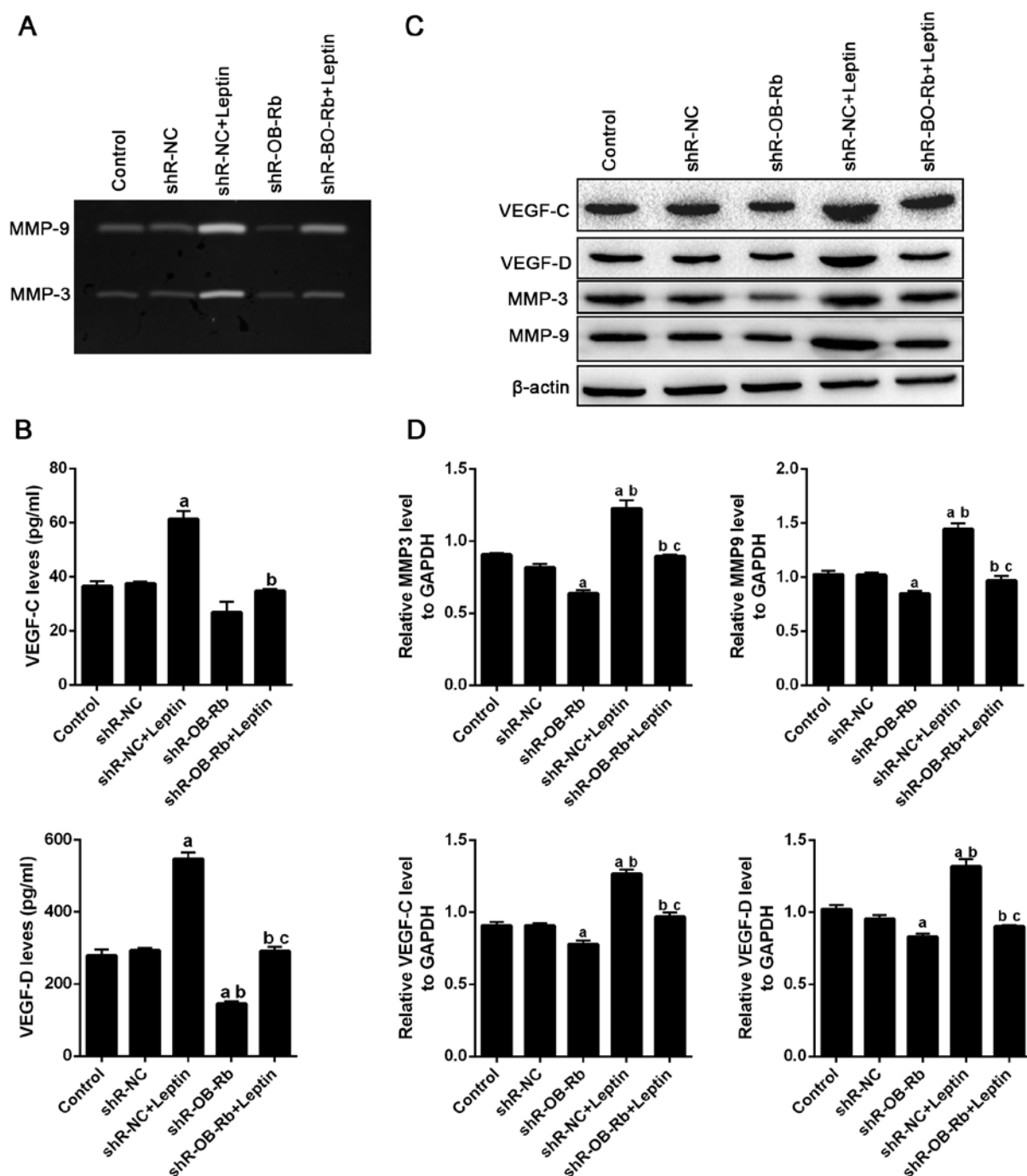


Figure 4. Leptin or OB-Rb regulates MMP3/9 and VEGF-C/D. (A) Gelatin zymography assay shown leptin upregulate expression of MMP3/9 and shR-OB-Rb reverse them. (B) Leptin promote secretion of VEGF-C/D in GBC-SD cells by ELISA assay. The protein expression of MMP3/9 and VEGF-C/D were also confirmed by western blot (C and D) analysis and in leptin or shR-OB-Rb-transfected GBC-SD cells. These data represent the mean \pm SD of three independent experiments. Statistical analysis used two-tailed Student's t-test (^a $P < 0.05$ vs. shR-NC group; ^b $P < 0.05$ vs. shR-NC + Leptin group; ^c $P < 0.05$ vs. shR-OB-Rb group).

mostly unknown. In this setting, we found that cell migration and integrin upregulation induced by leptin were attenuated by OB-Rb knockdown. Upon leptin binding, OB-Rb could activate JAK2, which in turn phosphorylated tyrosine residues in the receptor tails, leading to the recruitment and activation of STAT-3 (28). The leptin receptor, through the activation of JAK2, was also able to downregulate SOCS3 proteins and stimulate the downstream signaling pathway (13,29). Herein, we used the OB-Rb shRNA to determine its role and found

that it inhibited leptin-induced migration and JAK2/STAT-3/SOCS3 upregulation, indicating the possible involvement of OB-Rb in leptin-induced cell growth and migration in GBC-SD cells.

Human MMPs, also known as collagenase, are a matrix metalloproteinase originally identified in breast carcinomas (30). Recent studies have revealed that this enzyme was also produced by a variety of malignant tumors (31). In all of the cases, the expression of MMPs was associated with

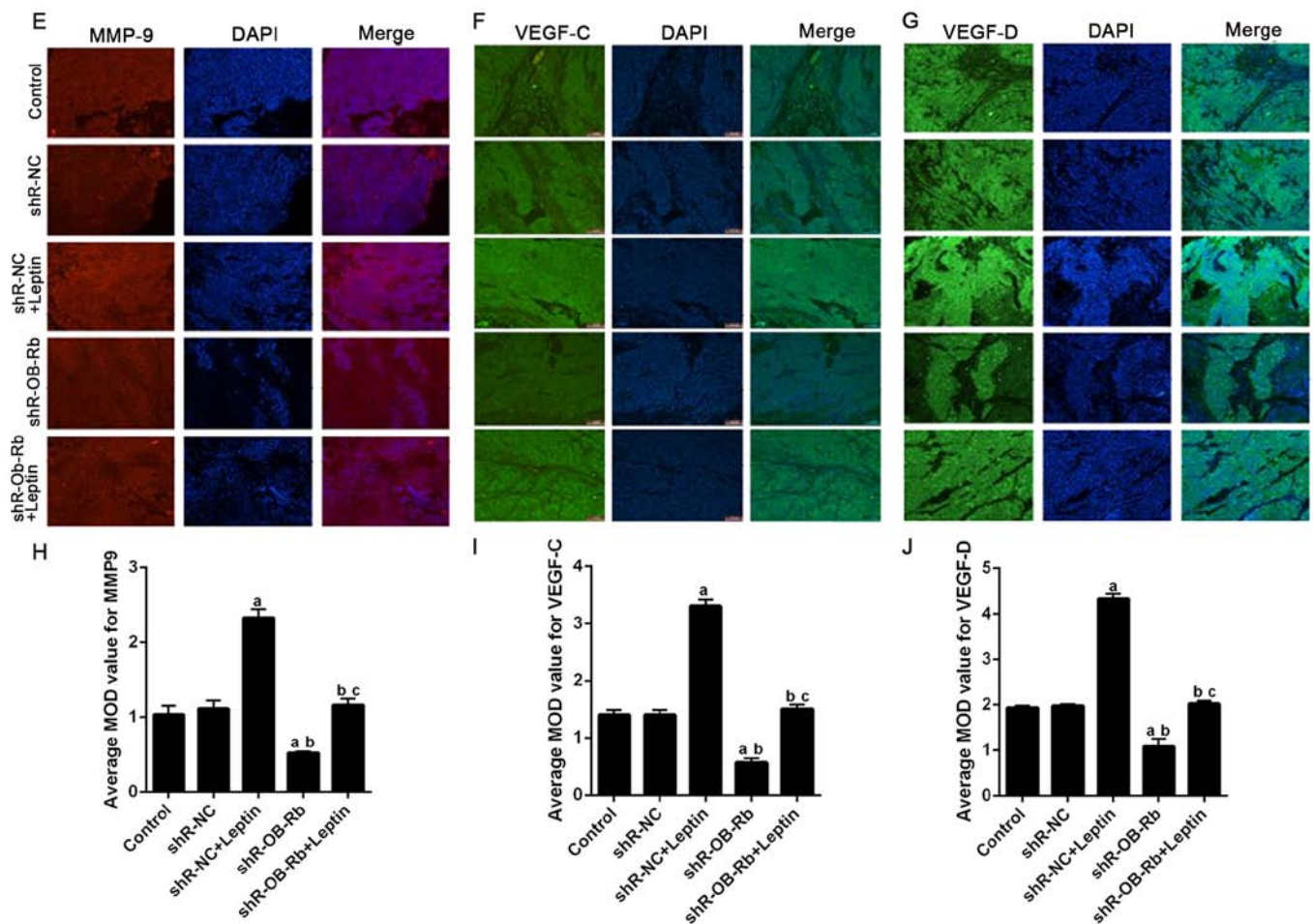


Figure 4. Continued. Leptin or OB-Rb regulates MMP3/9 and VEGF-C/D. The protein expression of MMP3/9 and VEGF-C/D were also confirmed by immunofluorescence (E-J) analysis and in leptin or shR-OB-Rb-transfected GBC-SD cells. These data represent the mean \pm SD of three independent experiments. Statistical analysis used two-tailed Student's t-test (^aP<0.05 vs. shR-NC group; ^bP<0.05 vs. shR-NC + Leptin group; ^cP<0.05 vs. shR-OB-Rb group).

aggressive tumors. GBC is known to have a high potential for invasion and metastasis. In the present study, we compared the expression levels of MMP-3 and MMP-9 in GBC cells with and without leptin and/or OB-Rb shRNA treatment. We found MMP-3/9 was activated by treating with leptin, and downregulated by OB-Rb interruption suggesting that leptin's regulation of MMPs is in a tissue-specific manner.

In addition, cell migration, and metastatic colonization must be successful with angiogenesis, which is essential for metastasized tumors in distant sites (32). Thus, we detected VEGF-C/D, which is the most important mediator of tumor angiogenesis, inducing formation of new blood vessels (33). Our study indicated that leptin could increase VEGF-C/D expression *in vivo* and *in vitro*. However, further research is necessary to clarify the mechanism underlying this process. Considering previous studies and the results described above, leptin and its receptor OB-Rb could be a potential therapeutic target for GBC.

In summary, our data showed that leptin and its receptor OB-Rb may be implicate in growth and metastasis of gallbladder carcinoma, which may involve the regulation of MMPs and VEGF family through SOCS3/JAK2/STAT3 pathways. Regulation of leptin and its receptor OB-Rb could serve as a promising intervention strategy for gene therapy of gallbladder carcinoma.

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