Metformin suppresses hypoxia-induced migration via the HIF-1α/VEGF pathway in gallbladder cancer *in vitro* and *in vivo*

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Abstract. Hypoxia plays a crucial role in cancer development and progression. Overexpression of hypoxia-inducible factor-1α (HIF-1α) has been demonstrated in a hypoxic microenvironment in various tumor types. Metformin has been identified as an antitumor drug in various tumor types. However, its role in cellular migration in a hypoxic microenvironment, and the associated regulatory mechanism, have yet to be fully elucidated. The present study aimed to investigate the clinical significance of HIF-1α, and its biological role, in gallbladder cancer (GBC). Furthermore, the role of metformin in cellular migration, and its underlying mechanism in GBC, were also identified. Real-time quantitative polymerase chain reaction analysis and immunohistochemistry experiments revealed that HIF-1α was significantly upregulated in GBC tissues. HIF-1α overexpression was closely associated with lymph node metastasis and tumor-lymph node-metastasis (TNM) stages. HIF-1α was able to promote cell migration in a hypoxic microenvironment by overexpressing vascular endothelial growth factor (VEGF) in GBC-SD cells, an effect which was partly reversed by small-interfering RNA HIF-1α (siHIF-1α) and 2-methoxyestradiol. Further experiments demonstrated that metformin inhibited hypoxia-induced migration via HIF-1α/VEGF *in vitro*. In addition, metformin suppressed GBC growth and downregulated the expression of HIF-1α and VEGF in a GBC-SD cell xenograft model. Taken together, these results suggest that HIF-1α may contribute to tumor migration via the overexpression of VEGF in GBC, while metformin is able to inhibit tumor migration by targeting the HIF-1α/VEGF pathway.

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Introduction

Gallbladder cancer (GBC) is the fifth most common cancer among the various types of gastrointestinal malignancies, and the most commonly occurring malignant tumor of bile tract cancer (BTC) worldwide (1). Currently, the major therapeutic options include surgical resection, radiation therapy and chemotherapy, and R0 complete surgical resection is the only effective treatment (2). Although new improvements have been made in the diagnosis and therapy of GBC, leading to better prognoses, only 30% of GBC patients are considered to be suitable candidates for surgery, and the overall 5-year survival rate is <5% (3). Additionally, post-operative recurrence and metastasis also serve a crucial role in the progression of GBC. Therefore, identifying early diagnostic markers and novel therapeutic targets is urgently required.

Hypoxia exerts a crucial role in the tumor microenvironment, particularly in solid tumors (4). Accumulating evidence shows that hypoxia plays a key role in tumorigenesis, including angiogenesis and migration (5-7). Hypoxia-inducible factor-1α (HIF-1α) is sensitive to cellular oxygen levels, and is stabilized under conditions of hypoxia such that it may activate its target genes (8), particularly vascular endothelial growth factor (VEGF) (9). The role of VEGF in hypoxia-induced angiogenesis has been extensively studied in numerous types of cancer, including GBC (10). The microenvironment in numerous types of solid tumors is affected under hypoxic conditions, and results in the overexpression of HIF-1α (11,12). Therefore, targeting the expression of HIF-1α may represent a novel strategy for cancer therapy.

Metformin, which is a member of the biguanide family, is predominantly used for the treatment of type 2 diabetes by increasing insulin sensitivity (13). Recently, epidemiologists discovered that diabetic patients who were treated with metformin had a lower risk, and lower incidence, of multiple types of cancer (14,15). Furthermore, accumulating evidence has demonstrated the antitumor effect of metformin on various cancer types, including biliary tract cancer (BTC) (16). HIF-1α is a heterodimeric transcription factor, comprising α and β subunits, that is associated with glucose uptake and angiogenesis (17). Guimaraes *et al* (18) demonstrated that metformin was able to inhibit HIF-1α and led to an increase in cell death in squamous cell carcinoma. Zhou *et al* (19) showed that metformin could inhibit the expression of HIF-1α via inhibition of the enhanced cellular oxygenation capability in hepatocellular carcinoma HepG2 cells. However, the role of metformin in cellular migration in a hypoxic microenvironment in GBC-SD cells, and its associated mechanism, have yet to be fully elucidated.

In the present study, it was demonstrated that HIF-1α is overexpressed in GBC tissues, and that HIF-1α overexpression is correlated closely with lymph node metastasis and tumor-lymph node-metastasis (TNM) stages. These results suggested that hypoxia promoted cell migration and increased the expression of HIF-1α and VEGF. Furthermore, it was shown that metformin reversed hypoxia-induced migration by targeting the HIF-1α/VEGF pathway. In conclusion, the present study demonstrated that HIF-1α may contribute towards tumor migration via overexpression of VEGF in GBC, while metformin is able to inhibit tumor migration by targeting the HIF-1α/VEGF pathway.

Materials and methods

Patient tissue samples. The present study was approved by the Clinical Research Ethics Committee of the First Affiliated Hospital of Zhengzhou University. Tumor and adjacent non-tumorous tissues were collected from 34 patients with GBC who had undergone cholecystectomy at the First Affiliated Hospital of Zhengzhou University between June 2016 and May 2017. Normal gallbladder tissues from patients with chronic cholecystitis were included for comparison. Non-cancerous tissues were obtained from soft tissue located >1 cm from the edge of the tumor, and non-cancerous tissues were observed under a microscope by a pathologist. Tissues were immediately snap-frozen in liquid nitrogen following surgical resection and stored at -80˚C prior to analysis. Complete clinicopathological and laboratory data were collected from each subject. Patients who had received radiotherapy, chemotherapy or immunotherapy prior to surgery were excluded. As described by the American Joint Committee on Cancer (AJCC) in 2010 (20), clinical stage was classified according to the TNM staging system.

Cell culture and transfection. Human GBC-SD cells were purchased from the Shanghai Cell Bank (Chinese Academy of Sciences) and cultured in RPMI-1640 medium (HyClone™; GE Healthcare Life Sciences, Beijing, China) supplemented with 10% fetal bovine serum (FBS; GemCell™), 100 U/ml penicillin and 100 mg/l streptomycin (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) under a humidified atmosphere of 5% CO_2 at 37˚C. The small-interfering RNA HIF-1α (siHIF-1α) and the negative control were purchased from GenePharma Biotechnology (Shanghai, China). Transfection was performed using Lipofectamine 2000 reagent (Invitrogen; Thermo Fisher Scientific Inc., Waltham, MA, USA) following the manufacturer's protocol. The siHIF-1α-1 primer sequences were 5'-GCCGAGGAAGAACUAUGAATT-3' (sense) and 5'-UUCAUAGUUCUUCCUCGGCTT-3' (antisense); the siHIF-1α-2 primer sequences were 5'-GCUGAUUUGUGAACCCAUUTT-3' (sense) and 5'-AAUGGGUUCACAAAUCAGCTT-3' (antisense). The sequences of the negative control were 5'-UUCUCCGAACGUGUCACGUTT-3' (sense) and 5'-ACGUGACACGUUCGGAGAATT-3' (antisense).

Immunohistochemistry (IHC). For IHC, the slides were immersed in heated antigen retrieval solution (10 mmol/l citrate buffer, pH 6.0), and subsequently treated with 3% hydrogen peroxide (H_2O_2) for 10 min. After washing with PBS, the slides were incubated with diluted primary antibody (HIF-1α; cat. no. 20960-1-AP, 1:100 dilution; VEGF, cat. no. 19003-1-AP, 1:100 dilution; Proteintech, Wuhan, China) at 4˚C overnight and then the secondary antibody (cat. no. PV-9000, 1:500 dilution; ZSGB-BIO, Beijing, China) for 20 min at room temperature. The reaction was developed using a 3,3'-diaminobenzidine (DAB) kit (cat. no. ZLI-9017; ZSGB-BIO, 1:50 dilution in buffer). Finally, the slides were counterstained in hematoxylin prior to dehydration and mounting. Staining reactions were determined on examination under a light microscope (CX31, Olympus, Japan; original magnification, x200).

RNA extraction and real-time quantitative polymerase chain reaction (RT-qPCR). Total RNA was extracted from tissue samples using TRIzol (Invitrogen; Thermo Fisher Scientific, Inc.), according to the manufacturer's protocol. RNA was transcribed to cDNA according to the manufacturer's protocol by using the Takara PrimeScript First Strand cDNA Synthesis kit (Takara Biotechnology Co., Ltd., Dalian, China). SYBR-Green (Takara Biotechnology Co., Ltd.) was added to quantify the expression of HIF-1α, according to the protocol provided. The primers used were as follows: HIF-1α, 5'-TGCAACATGGAAGGTATTGC-3' (sense), and 5'-TTCACAAATCAGCACCAAGC-3' (antisense); and β-actin, 5'-CCTGGCACCCAGCACAAT-3' (sense), and 5'-GGGCCGGACTCGTCATAC-3' (antisense). PCR reaction conditions were performed as followed: 95˚C for 60 sec, and 40 cycles of 95˚C for 15 sec, 60˚C for 15 sec, and 72˚C for 45 sec The relative expression levels were determined using the comparative $\Delta\Delta C_q$ method (21).

Wound healing assays. GBC-SD cells were seeded at a density of $1x10^6$/ml into 6-well plates and cultured overnight at 37˚C. After the GBC-SD cells had been spread over the plates, a vertical long wound was scratched into the cells, and the cells were washed with PBS twice. Images of the cells were captured at that time-point; GBC-SD cells were pre-cultured under hypoxia for 2 h and treated with 20 mM metformin, maintaining the cells in culture under hypoxic conditions with 2% FBS for 48 h, after which time further images were captured. The wound closure ratio was calculated as follows: (wound width at 0 h-wound width at 48 h)/wound width at 0 h.

Migration assay. A total of 1x10^5 cells were suspended in 100 μl serum-free medium and added to the upper chamber of a Transwell plate, and 600 μl 10% FBS medium was added to the bottom chamber of the Transwell plate. GBC-SD cells were pre-cultured under hypoxia for 2 h, and treated with 20 mM metformin, following incubation for 24 h; those cells that had stuck to the lower surface of the membrane were washed with PBS and fixed in 100% methanol for 20 min, and then stained with 0.1% crystal violet for 10 min at room temperature. The cellular migration through the membrane was visualized using an inverted microscope (IX71, Olympus, Japan; original magnification, x100).

Western blot analysis. Whole cell lysates were extracted with RIPA buffer on ice for 20 min. Protein concentration was determined using a BCA kit assay (Beyotime Institute of Biotechnology). Each extract containing ~30 μg protein was subjected to 10% SDS-polyacrylamide gel electrophoresis (PAGE), and then transferred onto polyvinylidene difluoride (PVDF) membranes (Merck Millipore, Bedford, MA, USA). The membranes were blocked in PBS containing 5% non-fat dry milk for 1 h at room temperature, and were then incubated with the primary antibodies against HIF-1α (cat. no. 20960-1-AP; 1:300 dilution), VEGF (cat. no. 19003-1-AP; 1:800 dilution) and β-actin (cat. no. 60008-1-Ig, 1:5,000 dilution; all from Proteintech, Wuhan, China) overnight at 4˚C, followed by six washes for 5 min with PBS. The membranes were subsequently incubated with peroxidase (HRP)-conjugated goat anti-rabbit IgG (cat. no. IH-0031, 1:5,000 dilution; DingGuo BioTech Co., Ltd., Beijing, China) or peroxidase (HRP)-conjugated goat anti-mouse IgG (cat. no. IH-0011, 1:5,000, dilution; DingGuo BioTech Co., Ltd.) for 1 h at room temperature, and washed six times with PBS. Blots were detected using an enhanced chemiluminescence western blotting detection kit (Thermo Scientific™ Pierce™ BCA™ Protein assay; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol.

Experimental xenograft model. The animal protocol was approved by the Institutional Animal Care and Use Committee of the First Affiliated Hospital of Zhengzhou University. Twelve female immunodeficient BALB/c nude mice at 4 weeks of age, with an initial body weight of 16±2 g, were purchased from Vital River Laboratory Animal Technology Co., Ltd. (Beijing, China). The animals were raised under pathogen-free conditions at the Institute of Medicine, Zhengzhou University at a temperature of 25±2˚C and a relative humidity of 70±5% under controlled light conditions (12/12 h light/day cycle) and fed with standard laboratory food and water. GBC-SD cells in the exponential phase of growth were resuspended in 200 μl serum-free culture medium at a density of 5x10^6 cells, and subsequently tumor xenografts were established by subcutaneous inoculation of the GBC-SD cells into the right flank of nude mice. The mice were randomly divided into two groups (with 6 mice/group): i) The negative group, which was administered PBS; and ii) the metformin group, which was administered 350 mg/kg metformin (22,23) (intra-gastric infusion, daily). The volume of the tumors was measured every 4 days. Maximum allowable tumor volume based on ethical guidelines was <2,500 mm^3. Tumor volume was calculated using calipers and estimated according to the following formula: Tumor volume (mm^3) = (length x width^2)/2. One month afterwards, the animals were sacrificed by cervical dislocation after anesthetized by 1% pentobarbital, and the tumor tissue was removed and measured. Xenograft tumors were harvested and cut into 4-μm sections for IHC analysis.

Statistical analysis. All data are presented as the mean ± standard deviation from at least three independent experiments. Differences between groups were assessed using the Student's t-test followed by Shapiro-Wilk W test, or one-way (ANOVA) followed by Bonferroni test, or a χ^2 test. P<0.05 was considered to indicate a statistically significant difference, and analyses were performed using SPSS 17.0 software (SPSS, Inc., Chicago, IL, USA).

Results

HIF-1α is upregulated in GBC tissue. The expression of HIF-1α in 34 paired GBC and adjacent normal gallbladder tissues was investigated via qRT-PCR and IHC experiments. As showed in Fig. 1A, the results obtained demonstrated that the mRNA expression of HIF-1α was higher in GBC tissues compared with that observed in the adjacent normal gallbladder tissues through RT-PCR experiments. As showed in Fig. 1B, the mRNA expression of HIF-1α in lymph node metastasis cancer tissues was higher (N1/2) than that in non-lymph node metastasis cancer tissues (N0), which suggested that HIF-1α was upregulated in GBC tissues at the mRNA level, particularly in lymph node metastasis cancer tissues. Moreover, we also analyzed the HIF-1α protein expression levels through using IHC. As showed in Fig. 1C-E, expression of HIF-1α was higher in GBC tissues compared with that in the adjacent normal gallbladder tissues and chronic cholecystitis tissues, which was consistent with the PCR results. Furthermore, the association between HIF-1α and clinicopathological features was also analyzed. As shown in Table I, aberrant expression of HIF-1α was associated with lymph node metastasis and TNM stage. These results indicate that HIF-1α may serve an important role in progression of GBC.

Hypoxia induces cell migration and increases the expression of HIF-1α and VEGF. GBC-SD cells were cultured under conditions of hypoxia (94% N_2, 5% CO_2, 1% O_2) or normoxia. Wound healing and Transwell assays were performed to detect the ability of the cells to metastasize. As shown in Fig. 2A, compared with the normoxia treatment group, treatment with hypoxia significantly promoted the ability of cells to migrate. Fig. 2B also shows the presence of 234.4±17.7 migrated cells per high-power field in the hypoxia group, which was significantly higher when compared with that in the normoxia treatment group (147.4±11.7). To further elucidate the molecular mechanism involved in cell migration, the expression of HIF-1α, VEGF, β-catenin and p-Akt was investigated using western blotting. As shown in Fig. 2C, following the culture of GBC-SD cells under conditions of hypoxia for 12, 24 and 48 h, the expression level of HIF-1α and VEGF in GBC-SD cells was increased, while the expression level of β-catenin and p-Akt exhibited no obvious changes. These data indicate that hypoxia may increase cell migration by activating HIF-1α and VEGF.

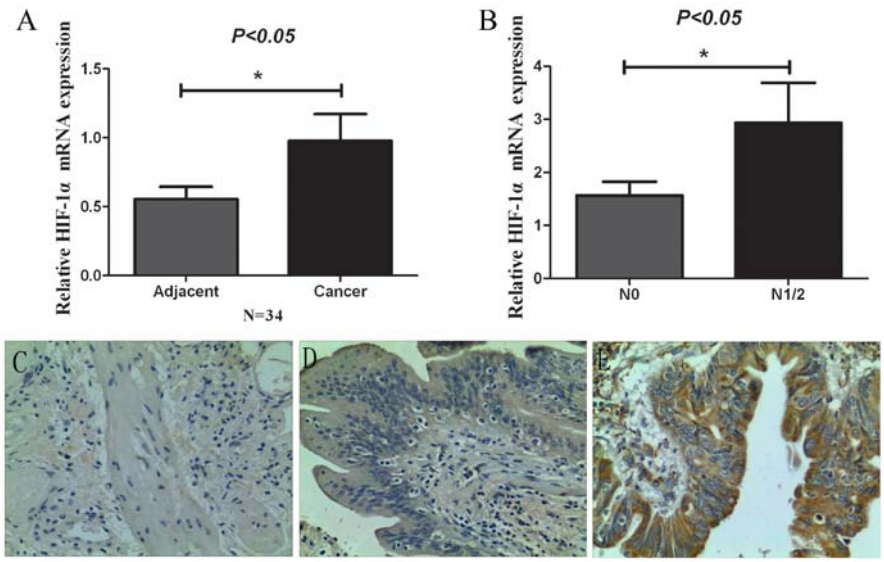

Figure 1. HIF-1α is upregulated in GBC tissues. (A) Expression of HIF-1α in adjacent non-tumorous and GBC tissues was detected by real-time quantitative polymerase chain reaction analysis. (B) Expression of HIF-1α in non-lymph node metastasis tissue (N0) and lymph node metastasis tissue (N1/2) as detected by quantitative real-time polymerase chain reaction analysis. (C) Chronic cholecystitis tissue, (D) adjacent non-tumorous tissue and (E) GBC tissue. Original magnification, x200. HIF-1α, hypoxia-inducible factor-1α; GBC, gallbladder cancer. *P<0.05 vs. Adjacent or N0.

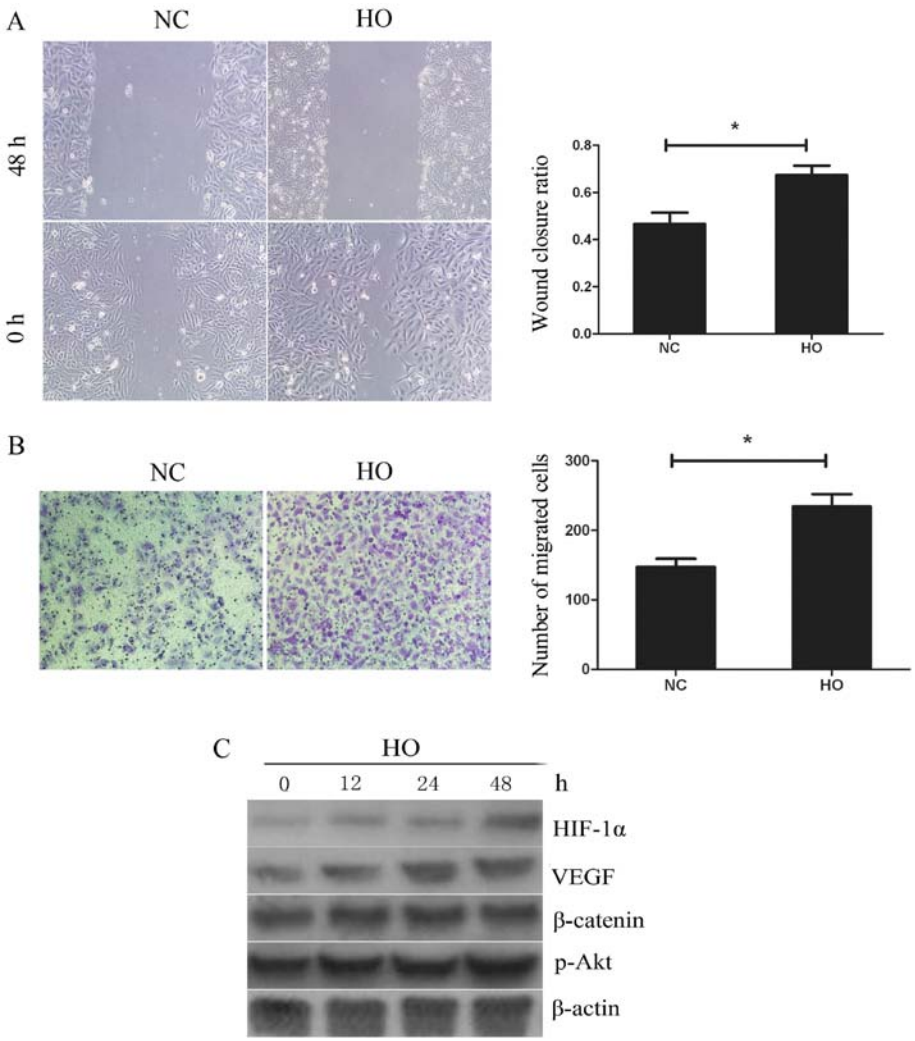

Figure 2. HO induces GBC cell migration and upregulates the expression of HIF-1α and VEGF. (A) The influence of HO on cell migration by wound closure. (B) The influence of hypoxia on cell migration by Transwell assays. (C) Analyses of HIF-1α, VEGF, β-catenin and p-Akt following treatment with hypoxia in GBC-SD cells by western blot analysis. Data shown represent the mean ± standard deviation from triplicate measurements. *P<0.05 vs. NC. Original magnification, x100. HO, hypoxia; NC, negative control; HIF-1α, hypoxia-inducible factor-1α; GBC, gall-bladder cancer; VEGF, vascular endothelial growth factor.

Table I. Association between HIF-1α expression and the clinicopathological features of the GBC cases.

Variables	No. of cases	HIF-1α expression		P-value
		Low	High	
Age (years)				0.692
<60	10	4	6	
≥60	24	7	17	
Sex				0.714
Female	17	6	11	
Male	17	5	12	
Histological grade				1.000
Well and moderate	24	8	16	
Poor	10	3	7	
N status				0.024
N0	21	10	11	
N1/2	13	1	12	
Stone				0.580
No	30	9	21	
Yes	4	2	2	
Tumor size (cm)				0.271
<5	23	9	14	
≥5	11	2	9	
Clinical stage				0.002
I-II	9	7	2	
III-IV	25	4	21	

GBC, gall bladder cancer.

Downregulation of HIF-1α by siRNA and 2-methoxyestradiol (2-ME), an HIF-1α inhibitor, suppresses hypoxia-induced migration and downregulates the expression of VEGF. The ability of the GBC-SD cells to migrate was investigated using wound healing and Transwell assays. According to the data in Fig. 3C, HIF-1α was effectively inhibited by siRNA HIF-1α (siHIF-1α). As shown in Fig. 3A and B, following downregulation of the expression of HIF-1α by specific inhibitor siHIF-1α, the wound closure ratios in the hypoxia treatment (HO) group and the HO+siHIF-1α treatment group were 0.679±0.053 and 0.335±0.009, respectively. Furthermore, the numbers of migrating cells in the aforementioned treatment groups were 221.6±10.1 and 116.2±12.2, respectively. Therefore, it was observed that the wound closure ratio and the number of migrating cells in the HO+siHIF-1α treatment group were significantly decreased compared with the HO treatment group. Subsequently, to examine whether HIF-1α is involved in hypoxia-induced migration in GBC-SD cells, the ability of the cells to migrate following downregulation of the expression of HIF-1α by siHIF-1α was investigated. As shown in Fig. 3D, siHIF-1α effectively reversed the increased expression of HIF-1α and VEGF induced by hypoxia.

It is noteworthy that similar results were obtained in subsequent experiments, which explored the effect of the downregulation of HIF-1α by 2-ME under conditions of normoxia (NC group) or hypoxia (Fig. 4A and B). The wound closure ratios in the hypoxia treatment (HO) group and the HO+2-ME group were 0.633±0.022 and 0.463±0.045, respectively. In addition, the numbers of migrating cells in the aforementioned treatment groups were 203.8±7.0 and 124.0±5.2, respectively. The expression of HIF-1α and VEGF was clearly downregulated by 2-ME (Fig. 4C), the results of which were consistent with those of the inhibition of cell migration. These results further corroborated the finding that hypoxia promotes cell migration in GBC-SD cells through activation of the HIF-1α/VEGF pathway.

Metformin inhibits hypoxia-induced migration via downregulation of the expression of HIF-1α and VEGF. GBC-SD cells were pre-cultured under conditions of hypoxia for 2 h, and subsequently treated with 20 mmol/l metformin for 24 or 48 h. As shown in Fig. 5A, the wound closure ratios in the hypoxia (HO) treatment group and the hypoxia in combination with metformin (Met+HO) group were 0.695±0.040 and 0.224±0.074, respectively. These data indicated that treatment with metformin and hypoxia resulted in a lower wound closure ratio compared with the hypoxia treatment group. In addition, the numbers of migrating cells in the aforementioned treatment groups were 185.8±10.2 and 113.4±8.6, respectively. The numbers of migrating cells in the Met+HO group were fewer compared with the HO treatment group (Fig. 5B). These results indicated that treatment with metformin significantly inhibited hypoxia-induced migration. To investigate the potential mechanism, the expression levels of HIF-1α and VEGF were also investigated. As shown in Fig. 5C, treatment with metformin decreased the expression of HIF-1α and VEGF. These data suggest that HIF-1α and VEGF signaling pathway may be involved in metformin-suppressed cell migration of the GBC-SD cells.

Metformin inhibits GBC cell growth and downregulates the expression of HIF-1α and VEGF in vivo. Metformin was administered in a xenograft model, generated by implanting GBC-SD cells into immunodeficient BALB/c nude mice in order to evaluate their antitumor effect. As shown in Fig. 6A and B, treatment with metformin significantly decreased the tumor growth rate and tumor weight at the endpoint of the animal experiment. Subsequently, the expression levels of HIF-1α and VEGF in the tumor xenograft tissues were detected by IHC. As shown in Fig. 6C, the expression levels of HIF-1α and VEGF were decreased in tumor tissues treated with metformin compared with the PBS group, the results of which are consistent with those obtained *in vitro*.

Discussion

It is well known that GBC greatly threatens human health due to the high rate of migration and recurrence, and the 5-year survival rate is less than 5% (3,24). Rapid tumor progression and difficulty in detecting early stage cancer are major obstacles in offering potentially curative treatments. Therefore, it is urgent that reliable tumor markers for early diagnosis are

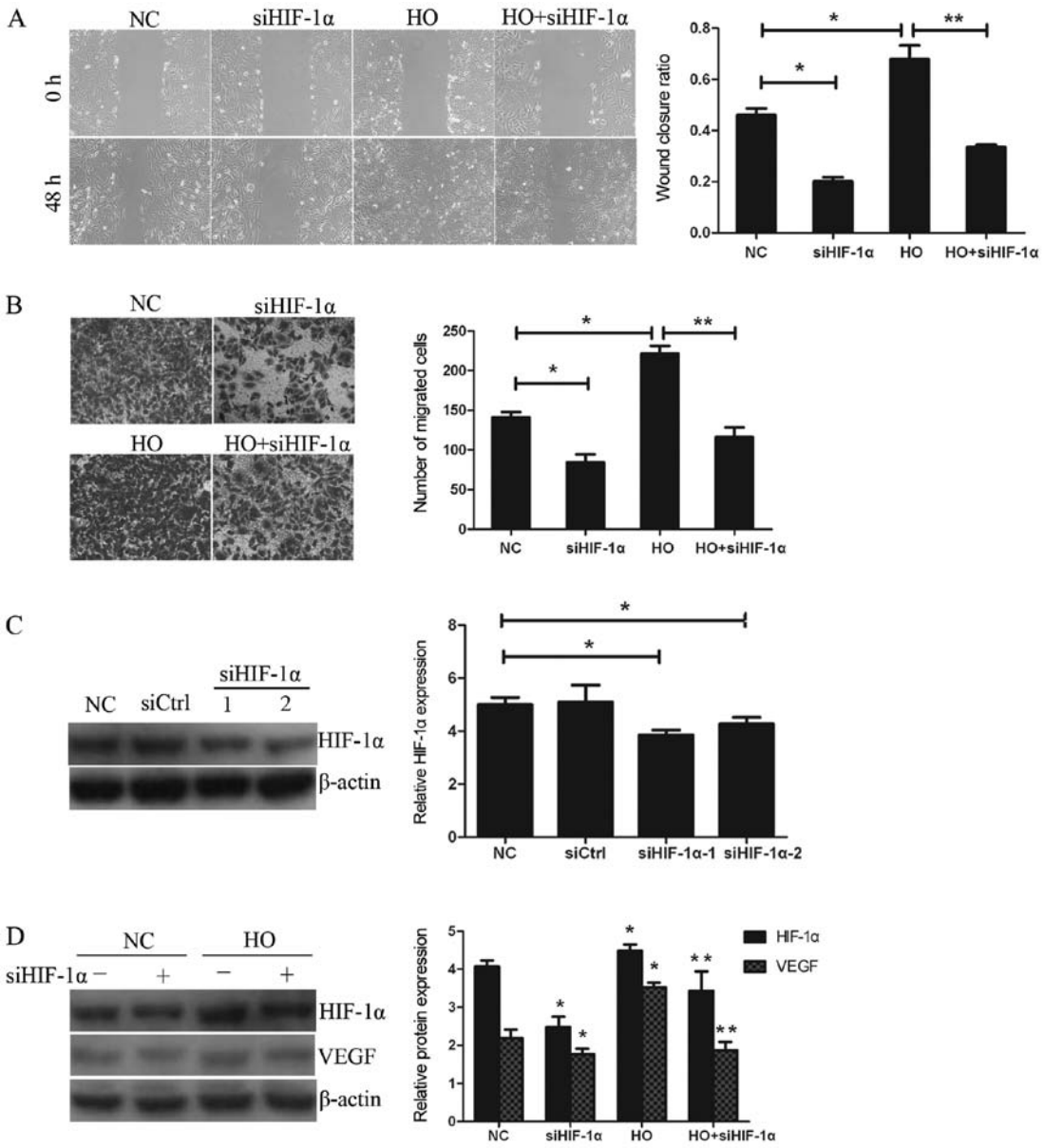

Figure 3. siHIF-1α inhibits HO-induced cell migration and downregulates the expression of HIF-1α and VEGF. (A) The influence of HO on cell migration by wound closure. (B) The influence of HO on cell migration by Transwell assays. (C) The silencing efficiency of HIF-1α in GBC-SD cells via western blot analysis. (D) Analysis of HIF-1α and VEGF following treatment with HO in either the absence or the presence of siHIF-1α in GBC-SD cells, as assessed by western blotting. Data represent the mean ± standard deviation from triplicate measurements. *P<0.05 vs. NC. **P<0.05 vs. HO. Original magnification, x100. HO, hypoxia; NC, negative control; HIF-1α, hypoxia-inducible factor-1α; GBC, gallbladder cancer; VEGF, vascular endothelial growth factor; siHIF-1α, small-interfering RNA HIF-1α.

found and that an effective drug for patients with GBC is developed. In the present study, it has been demonstrated that HIF-1α was upregulated in 23 out of 34 GBC tissues compared with that in adjacent non-tumor tissues and chronic cholecystitis tissues, according to the RT-qPCR and IHC experiments. Further experiments indicated that HIF-1α overexpression is significantly associated with both lymph node metastasis and TNM stage in GBC tissues. These results indicated that HIF-1α exerts an important role in promoting GBC progression.

Hypoxia is frequently observed in numerous types of solid tumors, including GBC (25), and also plays a crucial role in cancer progression (26). As part of a solid tumor, tumor cells may take advantage of their biological capacity to adapt to hypoxia to become even more aggressive (27). Additionally, the hypoxic microenvironment inside solid tumors limits the effectiveness of interventional embolization therapy and cytotoxic drugs. HIF-1α, the key regulatory factor in a hypoxia-influenced microenvironment, may translocate to the nucleus and induce the transcription of numerous downstream target genes (17,28). In the present study, treatment with hypoxia significantly promoted the ability of cell migration in GBC-SD cells, according to the results of the wound healing and Transwell assays. The subsequent experiments also demonstrated that the expression levels of HIF-1α and VEGF were upregulated under a hypoxic condition, while the expression levels of HIF-1α and VEGF were present at a low level under normoxic condition. The HIF-1 complex is composed of two protein subunits: HIF-1β, which is constitutively expressed, and HIF-1α, which is not present

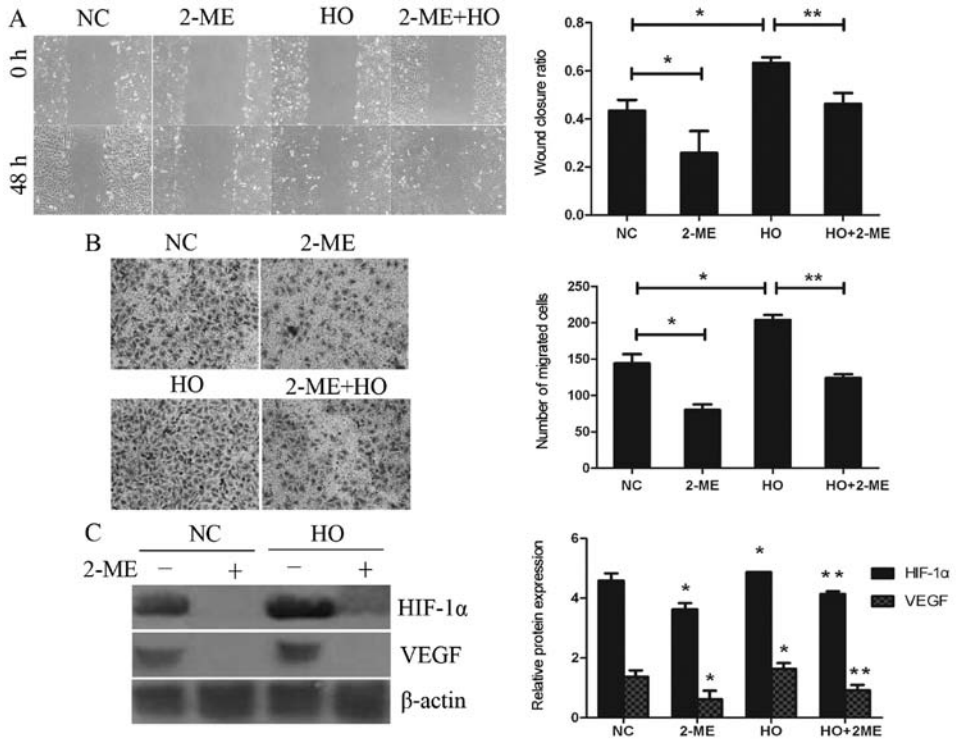

Figure 4. 2-ME inhibits HO-induced cell migration and downregulates the expression of HIF-1α and VEGF. (A) The influence of HO on cell migration by wound closure. (B) The influence of HO on cell migration by Transwell assays. (C) Analysis of HIF-1α and VEGF following treatment with HO in either the absence or the presence of 2-ME in GBC-SD cells by western blotting. Data represent the mean ± standard deviation from triplicate measurements. *P<0.05 vs. NC. **P<0.05 vs. HO. Original magnification, x100. 2-ME, 2-methoxyestradiol; HO, hypoxia; NC, negative control; HIF-1α, hypoxia-inducible factor-1α; GBC, gallbladder cancer; VEGF, vascular endothelial growth factor.

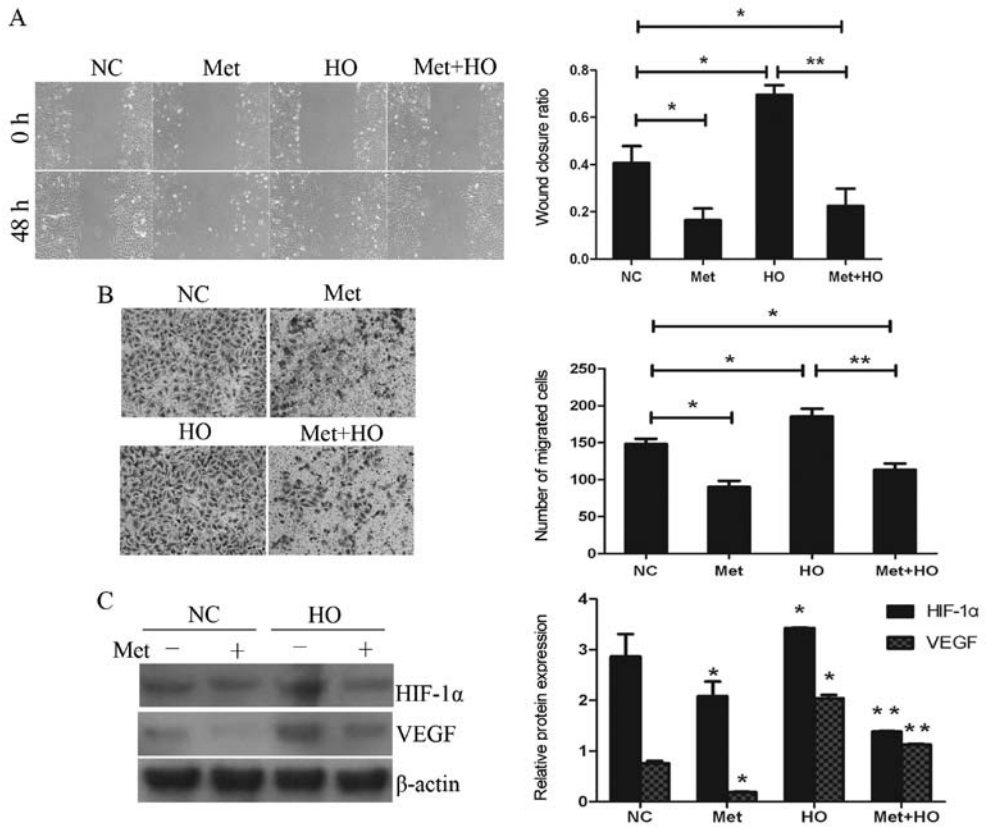

Figure 5. Metformin inhibits HO-induced cell migration and downregulates the expression of HIF-1α and VEGF. (A) The influence of HO on cell migration by wound closure. (B) The influence of HO on cell migration by Transwell assays. (C) Analysis of HIF-1α and VEGF following treatment with HO in either the absence or the presence of Met in GBC-SD cells by western blotting. Data represent the mean ± standard deviation from triplicate measurements. *P<0.05 vs. NC. **P<0.05 vs. HO. Original magnification, x100. Met, Metformin; HO, hypoxia; NC, negative control; HIF-1α, hypoxia-inducible factor-1α; GBC, gallbladder cancer; VEGF, vascular endothelial growth factor.

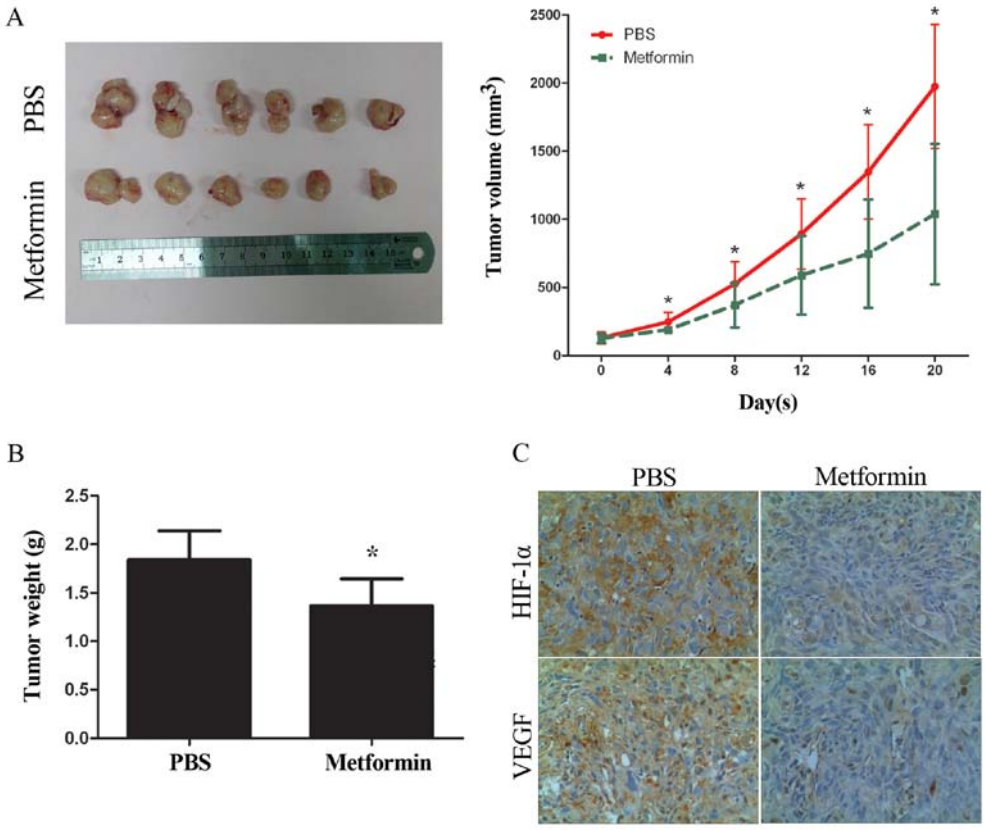

Figure 6. Metformin inhibits tumor growth in GBC-SD xenografts. (A) Growth curves are presented of tumor volumes in BALB/c nude mice following treatment with PBS or metformin. (B) Tumor weight was measured in each group at the endpoint of the animal experiment. (C) An immunohistochemistry assay was performed to detect the protein expression of HIF-1α and VEGF in tumor tissues. Original magnification, x200. *P<0.05 vs. negative control (PBS). PBS, phosphate-buffered saline; HIF-1α, hypoxia-inducible factor-1α; VEGF, vascular endothelial growth factor.

in normal cells but induced under hypoxic conditions. The HIF-1α subunit is continuously synthesized and degraded under normoxic conditions, while it accumulates rapidly following exposure to low oxygen tensions (29). Herein, GBC-SD cells are gallbladder cancer cells, not normal cells and previous studies also showed that HIF-1α was present at a low level in normoxia in GBC-SD cells (25). To demonstrate the underlying mechanism of hypoxia-induced migration, siHIF-1α was applied to the GBC-SD cells. It is noteworthy that siHIF-1α significantly reversed hypoxia-induced migration, and downregulated the expression levels of HIF-1α and VEGF. Similar results were also obtained using 2-ME, a HIF-1α-specific inhibitor. These data indicated that hypoxia promoted cell migration in GBC-SD cells through activation of the HIF-1α/VEGF signaling pathway.

Accumulating evidence has confirmed that metformin may inhibit cell migration or potentiate the effect of chemotherapeutic agents (30-32). Numerous studies have also demonstrated that metformin regulates the AMP-activated protein kinase and extracellular signal-regulated kinase pathways, and reverses drug resistance (33,34). In the present study, it was first demonstrated that metformin inhibited cellular migration, and downregulated the expression of HIF-1α and VEGF in GBC-SD cells. In addition, it was also shown that metformin reversed hypoxia-induced migration by targeting the HIF-1α/VEGF pathway. The present study also investigated further the role of metformin in GBC-SD cell migration *in vivo,* according to an experimental xenograft model. GBC-SD cells were injected into BALB/c nude mice, and it was shown that metformin markedly decreased the tumor growth rate and tumor weight at the endpoint of the xenograft model experiment. Furthermore, the expression levels of HIF-1α and VEGF in the tumor xenografts treated with metformin were decreased according to the results of the IHC experiment, which was consistent with our study *in vitro*.

In conclusion, the results of the present study revealed that HIF-1α is upregulated in GBC tissue, and that the aberrant expression of HIF-1α is closely associated with lymph node metastasis and TNM stage. Additional experiments demonstrated that hypoxia induced the migration of GBC-SD cells by increasing the expression levels of HIF-1α and VEGF. Furthermore, metformin reversed the increases in the hypoxia-induced cellular migration by targeting HIF-1α/VEGF. Thus, the data of the present study provide evidence that HIF-1α plays a vital role in GBC growth and migration, and may serve as a therapeutic target for GBC.

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Availability of data and materials

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

Authors' contributions

JY, KC, LQ, HT and RL performed the experiments; WZ and CZ designed the study; JY and RL prepared the study and wrote the manuscript. All authors read and approved the final manuscript and agreed to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

Ethics approval and consent to participate

The present study was approved by the Clinical Research Ethics Committee of the First Affiliated Hospital of Zhengzhou University. The animal protocol was approved by the Institutional Animal Care and Use Committee of the First Affiliated Hospital of Zhengzhou University.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

References

1. Hundal R and Shaffer EA: Gallbladder cancer: Epidemiology and outcome. Clin Epidemiol 6: 99-109, 2014.
2. Wang J, Narang AK, Sugar EA, Luber B, Rosati LM, Hsu CC, Fuller CD, Pawlik TM, Miller RC, Czito BG, *et al*: Evaluation of adjuvant radiation therapy for resected gallbladder carcinoma: A multi-institutional experience. Ann Surg Oncol 22 (Suppl 3): S1100-S1106, 2015.
3. Zhu AX, Hong TS, Hezel AF and Kooby DA: Current management of gallbladder carcinoma. Oncologist 15: 168-181, 2010.
4. Riffle S, Pandey RN, Albert M and Hegde RS: Linking hypoxia, DNA damage and proliferation in multicellular tumor spheroids. BMC Cancer 17: 338, 2017.
5. Ji RC: Hypoxia and lymphangiogenesis in tumor microenvironment and metastasis. Cancer Lett 346: 6-16, 2014.
6. Lin YJ, Shyu WC, Chang CW, Wang CC, Wu CP, Lee HT, Chen LJ and Hsieh CH: Tumor hypoxia regulates forkhead Box C1 to promote lung cancer progression. Theranostics 7: 1177-1191, 2017.
7. Xu W, Zhou W, Cheng M, Wang J, Liu Z, He S, Luo X, Huang W, Chen T, Yan W and Xiao J: Hypoxia activates Wnt/β-catenin signaling by regulating the expression of BCL9 in human hepatocellular carcinoma. Sci Rep 7: 40446, 2017.
8. Unwith S, Zhao H, Hennah L and Ma D: The potential role of HIF on tumour progression and dissemination. Int J Cancer 136: 2491-2503, 2015.
9. Chen MC, Hsu WL, Hwang PA and Chou TC: Low molecular weight fucoidan inhibits tumor angiogenesis through downregulation of HIF-1/VEGF signaling under hypoxia. Mar Drugs 13: 4436-4451, 2015.
10. Kawamoto M, Onishi H, Ozono K, Yamasaki A, Imaizumi A, Kamakura S, Nakano K, Oda Y, Suminoto H and Nakamura M: Tropomyosin-related kinase B mediated signaling contributes to the induction of malignant phenotype of gallbladder cancer. Oncotarget 8: 36211-36224, 2017.
11. Valsecchi R, Coltella N, Belloni D, Ponente M, Ten Hacken E, Scielzo C, Scarfo L, Bertilaccio MT, Brambilla P, Lenti E, *et al*: HIF-1α regulates the interaction of chronic lymphocytic leukemia cells with the tumor microenvironment. Blood 127: 1987-1997, 2016.
12. Gao T, Li JZ, Lu Y, Zhang CY, Li Q, Mao J and Li LH: The mechanism between epithelial mesenchymal transition in breast cancer and hypoxia microenvironment. Biomed Pharmacother 80: 393-405, 2016.
13. Wang J, Gao Q, Wang D, Wang Z and Hu C: Metformin inhibits growth of lung adenocarcinoma cells by inducing apoptosis via the mitochondria-mediated pathway. Oncol Lett 10: 1343-1349, 2015.
14. Chung HH, Moon JS, Yoon JS, Lee HW and Won KC: The relationship between metformin and cancer in patients with type 2 diabetes. Diabetes Metab J 37: 125-131, 2013.
15. Hall C, Stone RL, Gehlot A, Zorn KK and Burnett AF: Use of metformin in obese women with type i endometrial cancer is associated with a reduced incidence of cancer recurrence. Int J Gynecol Cancer 26: 313-317, 2016.
16. Liu Y, Wang Z, Li M, Ye Y, Xu Y, Zhang Y, Yuan R, Jin Y, Hao Y, Jiang L, *et al*: Chloride intracellular channel 1 regulates the antineoplastic effects of metformin in gallbladder cancer cells. Cancer Sci 108: 1240-1252, 2017.
17. He C, Wang L, Zhang J and Xu H: Hypoxia-inducible microRNA-224 promotes the cell growth, migration and invasion by directly targeting RASSF8 in gastric cancer. Mol Cancer 16: 35, 2017.
18. Guimaraes TA, Farias LC, Santos ES, de Carvalho Fraga CA, Orsini LA, de Freitas Teles L, Feltenberger JD, de Jesus SF, de Souza MG, Santos SH, *et al*: Metformin increases PDH and suppresses HIF-1α under hypoxic conditions and induces cell death in oral squamous cell carcinoma. Oncotarget 7: 55057-55068, 2016.
19. Zhou X, Chen J, Yi G, Deng M, Liu H, Liang M, Shi B, Fu X, Chen Y, Chen L, *et al*: Metformin suppresses hypoxia-induced stabilization of HIF-1α through reprogramming of oxygen metabolism in hepatocellular carcinoma. Oncotarget 7: 873-884, 2016.
20. Ma F, Wang SH, Cai Q, Zhang MD, Yang Y and Ding J: Overexpression of LncRNA AFAP1-AS1 predicts poor prognosis and promotes cells proliferation and invasion in gallbladder cancer. Biomed Pharmacother 84: 1249-1255, 2016.
21. Livak KJ and Schmittgen TD: Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta CT}$ method. Methods 25: 402-408, 2001.
22. Brodowska K, Theodoropoulou S, Meyer Zu Hörste M, Paschalis EI, Takeuchi K, Scott G, Ramsey DJ, Kiernan E, Hoang M, Cichy J, *et al*: Effects of metformin on retinoblastoma growth in vitro and in vivo. Int J Oncol 45: 2311-2324, 2014.
23. Rico M, Baglioni M, Bondarenko M, Laluce NC, Rozados V, André N, Carré M, Scharovsky OG and Menacho Márquez M: Metformin and propranolol combination prevents cancer progression and metastasis in different breast cancer models. Oncotarget 8: 2874-2889, 2017.
24. Wang SH, Zhang WJ, Wu XC, Zhang MD, Weng MZ, Zhou D, Wang JD and Quan ZW: Long non-coding RNA Malat1 promotes gallbladder cancer development by acting as a molecular sponge to regulate miR-206. Oncotarget 7: 37857-37867, 2016.
25. Sun W, Shen ZY, Zhang H, Fan YZ, Zhang WZ, Zhang JT, Lu XS and Ye C: Overexpression of HIF-1α in primary gallbladder carcinoma and its relation to vasculogenic mimicry and unfavourable prognosis. Oncol Rep 27: 1990-2002, 2012.
26. Batmunkh E, Shimada M, Morine Y, Imura S, Kanemura H, Arakawa Y, Hanaoka J, Kanamoto M, Sugimoto K and Nishi M: Expression of hypoxia-inducible factor-1 alpha (HIF-1alpha) in patients with the gallbladder carcinoma. Int J Clin Oncol 15: 59-64, 2010.
27. Wilson WR and Hay MP: Targeting hypoxia in cancer therapy. Nat Rev Cancer 11: 393-410, 2011.
28. Song Y, Zheng S, Wang J, Long H, Fang L, Wang G, Li Z, Que T, Liu Y, Li Y, *et al*: Hypoxia-induced PLOD2 promotes proliferation, migration and invasion via PI3K/Akt signaling in glioma. Oncotarget 8: 41947-41962, 2017.

29. Salceda S and Caro J: Hypoxia-inducible factor 1α (HIF-1α) protein is rapidly degraded by the ubiquitin-proteasome system under normoxic conditions. Its stabilization by hypoxia depends on redox-induced changes. J Biol Chem 272: 22642-22647, 1997.
30. Cheng K and Hao M: Metformin inhibits TGF-β1-induced epithelial-to-mesenchymal transition via PKM2 relative-mTOR/p70s6k signaling pathway in cervical carcinoma cells. Int J Mol Sci 17: E2000, 2016.
31. Yu T, Wang C, Yang J, Guo Y, Wu Y and Li X: Metformin inhibits SUV39H1-mediated migration of prostate cancer cells. Oncogenesis 6: e324, 2017.
32. Tian Y, Tang B, Wang C, Sun D, Zhang R, Luo N, Han Z, Liang R, Gao Z and Wang L: Metformin mediates resensitivity to 5-fluorouracil in hepatocellular carcinoma via the suppression of YAP. Oncotarget 7: 46230-46241, 2016.
33. Ling S, Xie H, Yang F, Shan Q, Dai H, Zhou J, Wei X, Song P, Zhou L, Xu X and Zheng S: Metformin potentiates the effect of arsenic trioxide suppressing intrahepatic cholangiocarcinoma: Roles of p38 MAPK, ERK3, and mTORC1. J Hematol Oncol 10: 59, 2017.
34. Harada K, Ferdous T, Harada T and Ueyama Y: Metformin in combination with 5-fluorouracil suppresses tumor growth by inhibiting the warburg effect in human oral squamous cell carcinoma. Int J Oncol 49: 276-284, 2016.