

# BANCR positively regulates the HIF-1 $\alpha$ /VEGF-C/VEGFR-3 pathway in a hypoxic microenvironment to promote lymphangiogenesis in pancreatic cancer cells

SHAOLONG HAO<sup>1</sup>, WEI HAN<sup>1</sup>, YU JI<sup>1</sup>, HAO SUN<sup>2</sup>, HAOWEI SHI<sup>1</sup>,  
JIHONG MA<sup>3</sup>, JAMES YIP<sup>4</sup> and YUCHUAN DING<sup>4</sup>

<sup>1</sup>Department of General Surgery, <sup>2</sup>Central Laboratory Department and <sup>3</sup>Health Care Ward, Beijing Luhe Hospital, Capital Medical University, Beijing 101149, P.R. China; <sup>4</sup>Department of Research and Development Center, John D. Dingell VA Medical Center, Detroit, MI 48201, USA

Received December 21, 2021; Accepted August 9, 2022

DOI: 10.3892/ol.2022.13542

**Abstract.** The aim of the present study was to explore the effects of BRAF-activated non-protein coding RNA (BANCR) on pancreatic microlymphangiogenesis in pancreatic cancer (PC) and its molecular mechanism under hypoxic conditions. Reverse transcription-quantitative PCR (RT-qPCR) was used to detect the expression of BANCR in SW1990 and PANC-1 PC cell lines under normoxic and hypoxic conditions. Subsequently, the expression of BANCR in the PC cells was knocked down using small interfering RNAs (siRNAs). Western blotting and RT-qPCR analyses were performed to detect the expression of hypoxia-inducible factor (HIF-1 $\alpha$ ), VEGF-C and VEGFR-3 in the transfected cells. In addition, the transfected PC cells were co-cultured with human lymphatic endothelial cells and the lymphatic microvessel density (MLVD) was detected under normal and hypoxic conditions. Furthermore, HIF-1 $\alpha$  expression in the PC cells was knocked down using siRNAs, and VEGF-C and VEGFR-3 mRNA expression in the HIF-1 $\alpha$  knockdown cells was detected using RT-qPCR. The results showed that the expression of BANCR in the SW1990 and PANC-1 PC cell lines was significantly higher than that in human pancreatic duct endothelial cells. Additionally, the expression of BANCR was significantly increased in PC cells under hypoxic conditions compared with normoxic conditions. The MLVD of PC cells under hypoxic conditions was significantly higher compared with that under normoxic conditions, and the MLVD in the si-BANCR group was lower than that in the si-NC group, indicating that si-BANCR downregulated MLVD. These results indicate that

BANCR positively regulated the expression of HIF-1 $\alpha$  in PC cells at the transcriptional and translational levels. Finally, the expression levels of VEGF-C and VEGFR-3 in PC cells were significantly reduced when BANCR or HIF-1 $\alpha$  expression was knocked down. In conclusion, the results demonstrate that the expression of BANCR in PC cells was significantly increased under hypoxic conditions and suggest that BANCR promoted tumor cell lymphangiogenesis by upregulating the HIF-1 $\alpha$ /VEGF-C/VEGFR-3 pathway, which plays an important role in the process of PC lymph node metastasis.

## Introduction

Pancreatic cancer (PC) is a malignant tumor of the digestive system that is challenging to diagnose and treat, exhibits a high degree of malignancy and is associated with poor prognosis (1). According to the literature, the 5-year survival rate of PC is <8% (2), and >90% of PC cases are pancreatic ductal adenocarcinoma (3). As the onset of PC is often missed, patients are frequently diagnosed in the first instance with metastatic or advanced cancer, which limits the clinical treatment options. Lymph node metastasis, as the primary pathway and an early event in PC, is an important factor that influences the clinical stage, treatment and prognosis of patients with PC (4). Clinical studies have shown that the incidence of lymphatic invasion by cancer cells is 3-5-fold higher than that of vascular invasion (5). Although the lymph node metastasis of PC is important clinically, the molecular mechanism that induces PC cells to separate from the primary focus, invade lymphatic vessels and metastasize to regional lymph nodes is unclear. Therefore, it is of great value to explore the molecular mechanism of lymph node metastasis of PC to improve the clinical diagnosis, treatment and prognosis of patients with PC.

Lymphatic system vessels are the primary channels through which cancer cells spread from a local tumor to lymph nodes and then the lymphatic circulation to distant sites (6). Lymphangiogenesis is considered to be the most critical and rate-limiting step in the development of lymph node metastasis by malignant tumor cells (7). Due to the lack of specific molecular markers for lymphatic endothelial cells,

*Correspondence to:* Dr Wei Han, Department of General Surgery, Beijing Luhe Hospital, Capital Medical University, 82 Xinhua South Road, Tongzhou, Beijing 101149, P.R. China  
E-mail: dr-han@mail.ccmu.edu.cn

**Key words:** pancreatic cancer, long non-coding RNA, hypoxic microenvironment, lymphangiogenesis

the study of lymph node metastasis has been overshadowed by the study of vascular system invasion. However, since specific markers for lymphatic endothelial cells have been discovered, these now provide a basis for further study of the mechanism of lymph node metastasis. VEGF-C has been shown to be an important growth factor in the lymphangiogenesis of solid malignant tumors, which promotes lymphangiogenesis and microlymphangiogenesis by binding to VEGFR-3. The VEGF-C and VEGFR-3 axis has been shown serve a central role in the initiation of lymphangiogenesis (8). A study by Ochi *et al* (9) showed that the expression levels of VEGF-C and VEGFR-3 were significantly correlated with each other in patients with pancreatic carcinoma. The study also demonstrated that VEGFR-3 combined with VEGF-C to stimulate the formation of lymphatic vessels in pancreatic carcinoma, which induced the formation of new lymphatic capillaries and increased the risk of lymph node metastasis. The aforementioned studies indicate that VEGF-C/VEGFR-3 in PC tissues is an important pathway that induces the formation of lymphatic vessels and lymph node metastases. However, the regulatory mechanism regulating the VEGF-C/VEGFR-3 pathway in PC has not been determined previously, to the best of our knowledge. Therefore, the present study investigated the potential regulatory role of BRAF-activated non-protein coding RNA (BANCR) and hypoxia-inducible factor (HIF)-1 $\alpha$  in the VEGF-C/VEGFR-3 pathway in PC by knocking down their expression.

Studies have shown that lncRNAs are abnormally expressed in several types of human malignant tumors and participate in the proliferation, invasion and metastasis of tumor cells (10). Previous studies by Jiang *et al* (11) and Shen *et al* (12) have demonstrated the important role of the long non-coding RNA (lncRNA) BANCR in the tumor lymph node metastasis of breast and colorectal cancer, respectively. BANCR was first reported by Flockhart *et al* (13), who identified it in melanoma cells in 2012. It is a ~693-bp lncRNA that is present on chromosome 9 and specifically activated by a mutation in the BRAF<sup>V600E</sup> gene; its expression is upregulated in melanoma cells and it plays an important role in the promotion of lymph node metastasis. With increased interest in BANCR, subsequent studies found that in addition to melanoma, BANCR also serves a key role in other types of tumors (14). Studies have detected the upregulated expression of BANCR in gastric cancer (15), colorectal cancer (12), hepatocellular carcinoma (16), esophageal squamous cell carcinoma (17), osteosarcoma (18) and thyroid cancer (19,20), and shown BANCR to be significantly associated with a poor prognosis, tumor cell proliferation, invasion and local lymph node metastasis. Notably, a study by Wu *et al* (4) demonstrated that the expression of BANCR was upregulated in PC tissues and cell lines, namely PANC-1 and SW1990, and found that BANCR upregulation was closely associated with lymph node metastasis in patients with PC. In addition, the study demonstrated that interfering with the expression of BANCR effectively inhibited the proliferation and invasion of PC cells.

The present study aimed to verify the important role of BANCR in PC proliferation, invasion and lymph node metastasis. The expression levels of BANCR in human PC cells were compared with those in normal human pancreatic cells, and the role and molecular mechanisms of BANCR in the

lymphangiogenesis of PC were determined for the first time, to the best of our knowledge.

A hypoxic microenvironment is common for the occurrence and development of malignant tumors. In particular, a hypoxic microenvironment is important in the induction of highly invasive PC, and is established due to a poor blood supply to the rapidly proliferating cells (21); it is also a key factor underlying the high degree of malignancy and poor curative outcomes (22). A previous study on hypoxia focused on tumor angiogenesis and drug resistance (23); however, little is known regarding the mechanism of tumor lymphangiogenesis and lymph node metastasis. The activation of HIF is the key molecular characteristic of tumor cell changes occurring in a hypoxic microenvironment, which is closely associated with the occurrence, development, invasion and metastasis of tumors (24). HIF has three subtypes: HIF-1, -2 and -3, of which HIF-1 is the most important subtype in hypoxic tumor cells and is widely expressed in a variety of human tumors (25). HIF-1 is composed of HIF-1 $\alpha$  and HIF-1 $\beta$ , and the former is the key transcription factor in the hypoxic response, due to its important role in the regulation of hypoxic gene expression and in the signal transduction network (26). Previous studies have shown that the upregulated expression of HIF-1 $\alpha$  in PC is associated with tumorigenesis and progression (27,28). Liu *et al* (29) confirmed the high expression of HIF-1 $\alpha$  in PC and its association with lymph node metastasis and TNM staging. In the present study, the expression levels of HIF-1 $\alpha$  in PANC-1 and SW1990 PC cell lines were assessed, and the effect of knocking down the expression HIF-1 $\alpha$  was evaluated. Furthermore, the ability of BANCR to regulate the expression of HIF-1 $\alpha$  was investigated, and the effect of HIF-1 $\alpha$  on the transcription and translation of VEGF-C in PC cells was explored. The role of BANCR in the regulation of HIF-1 $\alpha$  was thereby revealed, and the potential value of the BANCR/HIF-1 $\alpha$ /VEGF-C/VEGFR-3 pathway in the lymphangiogenesis and lymph node metastasis in PC was determined.

## Materials and methods

**Cell lines and culture conditions.** The PC cell lines PANC-1 and SW1990, immortalized human pancreatic ductal epithelial cells (HPDCs) and human lymphatic tube endothelial cells (HDLECs) were purchased from Guangzhou Genio Biotech Co., Ltd. The SW1990 and HPDC cells were cultured in DMEM (MilliporeSigma), the PANC-1 cells were cultured in RPMI-1640 (HyClone; Cytiva) and the HDLECs were cultured in Endothelial Cell Medium (ScienCell Research Laboratories, Inc.), each supplemented with 10% FBS (Thermo Fisher Scientific, Inc.) and 1% antibiotics (penicillin-streptomycin; Thermo Fisher Scientific, Inc.). Culture was performed under normoxic or hypoxic conditions in a humidified incubator at 37°C. The normoxic conditions were 20% O<sub>2</sub>, 5% CO<sub>2</sub> and 75% N<sub>2</sub>, and the hypoxic conditions were 1% O<sub>2</sub>, 5% CO<sub>2</sub> and 94% N<sub>2</sub>. Cells in the logarithmic growth stage were selected for subsequent experiments.

**Cell transfection and grouping.** PC cell lines in the logarithmic growth stage were divided into two groups after digestion. Lipofectamine™ 3000 (Thermo Fisher Scientific,

Table I. Primer sequences.

Primer name	Forward (5'-3')	Reverse (5'-3')
GAPDH	TCAAGAAGGTGGTGAAGCAGG	TCAAAGGTGGAGGAGTGGGT
BANCR	GAGCCTTGCCAGTTCCATTT	TGCAGAGGTGAGATTCAGGT
HIF-1 $\alpha$	ACTTCTGGATGCTGGTGATTTG	GCTTCGCTGTGTGTTTGTCT
VEGF-C	CTACAGATGTGGGGGTTGCT	GATTGGCAAACTGATTGTGAC
VEGFR-3	CTCTGACCTAGTGGAGATCCTG	CTTCGGTGATATGTAGAGCTGTG

BANCR, BRAF-activated non-protein coding RNA; HIF, hypoxia-inducible factor.

Inc.) was used to transfect the PC cell lines with a small interfering (si)RNA BANCR knockdown plasmid (TIANpure Mini Plasmid Kit II; Tiangen Biotech Co., Ltd.) and negative control plasmid (si-NC) to establish the si-BANCR and si-NC groups, respectively. In brief, PC cells at 80% confluence were plated in a 12-well plate. A total of 100  $\mu$ l Opti-MEM was used to dilute the Lipofectamine™ 3000 (4  $\mu$ l) and vector/pcDNA (6  $\mu$ l; 100 ng/ $\mu$ l siRNA/plasmid), incubated at room temperature for 5 min, mixed gently and then incubated for a further 20 min at 37°C. Next, the cell medium was replaced with Opti-MEM (700  $\mu$ l/well; Thermo Fisher Scientific, Inc.), and the aforementioned transfection mixture was added to each well. After 8 h at 37°C, the medium was replaced with standard supplemented medium, and after transfection for 48 h, the transfected cells were used for subsequent experiments. The si-BANCR sequence was 5'-GGUGTGGCGUCTUGCUUTT-3'. The si-NC sequence was 5'-GGCCGGUTTCCUUTTCUGCG-3'. In a subsequent experiment, the PC cell lines were transfected with the si-HIF-1 $\alpha$  sequence 5'-CTGATGACCACACAACTTGA-3' to establish a HIF-1 $\alpha$  knockdown group using the aforementioned transfection protocol. Transfection success was evaluated by the detection of green fluorescent protein and reverse transcription-quantitative PCR (RT-qPCR) as shown in Figs. S1 and S2.

**Lymphangiogenesis experiments.** PC cells stably transfected with si-BANCR or si-NC were digested and HDLECs were added to establish a mixed cell suspension. The mixed cell suspension (PC cells:HDLEC cells, 1:1; 7.5x10<sup>3</sup> cells/well) was seeded on a Matrigel basement membrane (BD Biosciences) coating in 96-well plates at 100  $\mu$ l/well, with 3 wells per condition, and the cells were cultured under the aforementioned normoxic or hypoxic conditions. The formation of microlymphatic vessels was observed after culturing for 12 h.

**Detection of MLVD.** The lymphatic vessel distribution was observed using a low magnification inverted fluorescence microscope (x100) and assessed in a double-blinded manner by two pathologists. Subsequently, 3 hot-spot areas (areas that appear to have a high MLVD density) were selected and the positive structure of each area was observed using a high-power field of view (x400). Finally, 5 regions were randomly selected for counting the number of vessels, and the maximum value was selected as the MLVD of each hot-spot area for statistical analysis.

**RT-qPCR.** A TRIzol® RNA extraction kit (Invitrogen; Thermo Fisher Scientific, Inc.) was used to extract total RNAs from the cells according to the manufacturer's instruction. The RNA was reverse transcribed into cDNA using PrimeScript RT Master Mix (Takara Biotechnology Co., Ltd.) at 37°C for 15 min. The relative expression levels of BANCR, HIF-1 $\alpha$ , VEGF-C and VEGFR-3 in each group were detected. RT-qPCR was performed in strict accordance with the instructions of the SYBR® Primx Ex Taq™ (Tli RNaseH Plus) (cat. no. RR420A; Takara Bio, Inc.), with GAPDH as the internal reference gene in a reaction system of 20  $\mu$ l. The thermocycling conditions were as follows: Pre-denaturation at 95°C for 5 min; followed by 38 cycles of denaturation at 95°C for 30 sec, annealing at 65°C for 30 sec and extension at 72°C for 30 sec; and a final extension step of 72°C for 8 min. Primers were designed based on the following human gene sequences in NCBI GeneBank: BANCR (NC\_000009.12), HIF-1 $\alpha$  (NC\_000014.9), VEGF-C (NC\_000004.12), VEGFR-3 (NC\_000005.10) and GAPDH (NC\_000012.12). The primers were synthesized by Bio-Engineering Co., Ltd., and their sequences are provided in Table I. Quantitative analysis of relative gene expression data used the 2<sup>- $\Delta\Delta C_q$</sup>  method, and GAPDH was used as the internal reference control (30).

**Western blotting.** The transfected cells were examined by western blotting. Total protein was extracted under different treatment conditions using RIPA buffer (cat. no. R0010; Beijing Solarbio Science & Technology Co., Ltd.) and protein concentration was quantified using the BCA method (Thermo Fisher Scientific, Inc.). A total of 20  $\mu$ g protein/lane was loaded for electrophoresis. SDS-PAGE on a 10% gel was performed at a constant voltage of 100 V for 40 min. After electrophoresis, the electroporation apparatus was used to transfer the resolved proteins to a PVDF membrane using a constant current of 250 mA for 2 h. The membranes were subsequently blocked at room temperature for 1 h with 5% skimmed milk/TBS-0.1% and Tween-20 (TBST) solution. Primary antibodies against HIF-1 $\alpha$  (cat. no. ab2185; 1:500; Abcam), VEGF-C (cat. no. 22601-1-AP; 1:1,000; ProteinTech Group, Inc.) and VEGFR-3 (cat. no. Ab27278; 1:1,000; Abcam) were used. A GAPDH antibody (cat. no. ab9485; 1:3,000; Abcam) was also used to detect GAPDH as an internal reference. The membrane was incubated overnight with the primary antibodies at 4°C. After washing the film with TBS-0.1% and Tween-20 (TBST) three times, the film was incubated with the secondary antibody (Alexa Fluor® 568; cat. no. ab175473; 1:5,000; Abcam)

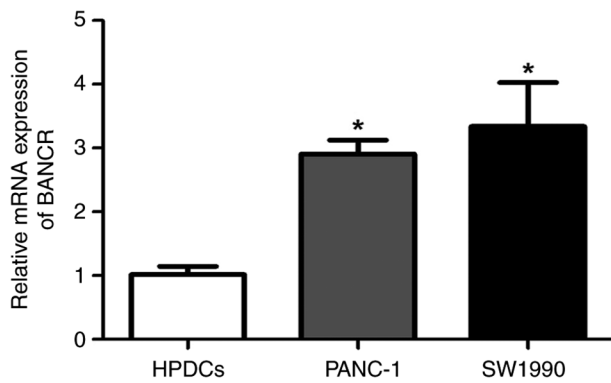


Figure 1. BANCR mRNA expression in PANC-1 and SW1990 pancreatic cells and HPDCs. \* $P < 0.05$  vs. HPDCs. BANCR, BRAF-activated non-protein coding RNA; HPDCs, human pancreatic ductal epithelial cells.

at room temperature for 1 h. TBST was used to wash the films again three times, after which the signals were developed and visualized using an ECL reagent (Thermo Fisher Scientific, Inc.). A CanoScan Lide 120 scanner (Canon, Inc.) was used to scan the film for densitometric analysis. Densitometric analysis was performed using ImageJ 1.48 (National Institutes of Health).

**Statistical analysis.** SPSS version 19.0 (IBM Corp) was used for statistical analysis. The relative expression levels of BANCR, HIF-1 $\alpha$ , VEGF-C and VEGFR-3 in PC cells and MLVD values are expressed as the mean  $\pm$  standard deviation. The relative expression levels of BANCR in HPDCs and the PANC-1 and SW1990 cell lines were analyzed by one-way ANOVA followed by Tukey's multiple comparison test. Differences between two groups were compared using an independent samples Student's t-test.  $P < 0.05$  was considered to indicate a statistically significant difference.

## Results

**BANCR expression is upregulated in PANC-1 and SW1990 cells.** RT-qPCR was used to detect the relative expression of BANCR in the HPDCs and the PANC-1 and SW1990 PC cell lines. The results showed that the expression of BANCR in the PANC-1 and SW1990 cells was significantly upregulated compared with that in the HPDCs ( $P < 0.05$ ). No significant difference in the expression of BANCR was detected between the PANC-1 and SW1990 cells (Fig. 1).

**BANCR expression is upregulated in PANC-1 and SW1990 cells under hypoxic conditions.** RT-qPCR was used to detect the expression of BANCR in PANC-1 and SW1990 cells under normoxic and hypoxic conditions. The results showed that the expression of BANCR was significantly higher under hypoxic conditions compared with normoxic conditions ( $P < 0.05$ ; Fig. 2).

**BANCR increases the MLVD of PC cells under hypoxic conditions.** BANCR expression was knocked down in PANC-1 and SW1990 cells, after which the cells were co-cultured with HDLECs under normoxic and hypoxic conditions. In

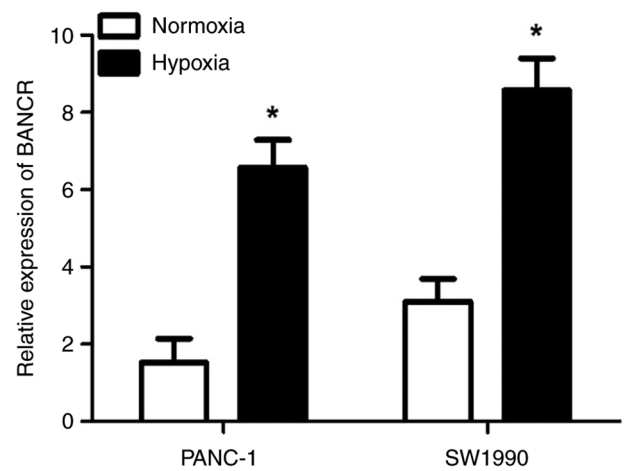


Figure 2. Relative BANCR expression in PANC-1 and SW1990 pancreatic cancer cells under normoxic and hypoxic conditions. \* $P < 0.05$  vs. the respective normoxia group. BANCR, BRAF-activated non-protein coding RNA.

the PANC-1 cell line, the MLVD values in the si-NC group and the si-BANCR group increased significantly under hypoxic conditions compared with those in cells grown under normoxic conditions (Fig. 3A and B). In addition, the degree of MLVD was reduced significantly following the knockdown of BANCR expression in PANC-1 cells (si-BANCR: hypoxia vs. normoxia:  $9.133 \pm 3.925$  vs.  $5.8 \pm 3.189$ , respectively,  $P < 0.05$ ; si-NC: hypoxia vs. normoxia:  $61.6 \pm 12.53$  vs.  $18.13 \pm 6.128$ , respectively,  $P < 0.05$ ). Similar results were observed in the SW1990 cells (si-BANCR: hypoxia vs. normoxia:  $9.2 \pm 4.411$  vs.  $4.933 \pm 2.604$ , respectively,  $P < 0.05$ ; si-NC: hypoxia vs. normoxia:  $59.0 \pm 14.89$  vs.  $18.2 \pm 6.45$ , respectively,  $P < 0.05$ ; Fig. 3C and D).

**HIF-1 expression is upregulated by BANCR at the transcriptional level.** SW1990 and PANC-1 cells transfected with si-BANCR or si-NC were cultured under hypoxic conditions. Western blotting was used to detect the relative protein expression levels of HIF-1 $\alpha$  in each group, and the results are shown in Fig. 4A and B. The relative protein expression of HIF-1 $\alpha$  in the si-BANCR group was significantly lower compared with that in the si-NC group in each cell line (SW1990:  $0.646 \pm 0.200$  vs.  $1.630 \pm 0.453$ , respectively,  $P < 0.05$ ; PANC-1:  $0.815 \pm 0.195$  vs.  $1.838 \pm 0.228$ , respectively,  $P < 0.05$ ). RT-qPCR was used to detect the relative mRNA expression levels of HIF-1 $\alpha$  in the si-BANCR and si-NC groups and the results were consistent with the protein results (SW1990:  $1.076 \pm 0.430$  vs.  $2.253 \pm 0.950$ ,  $P < 0.05$ ; PANC-1:  $1.248 \pm 0.228$  vs.  $2.309 \pm 0.426$ ,  $P < 0.05$ ; Fig. 4C).

**BANCR and HIF-1 $\alpha$  upregulate the expression of VEGF-C and VEGFR-3.** RT-qPCR and western blotting were used to detect the relative protein and mRNA expression levels of VEGF-C and VEGFR-3 in SW1990 cells transfected with si-BANCR, si-HIF-1 $\alpha$  or si-NC under hypoxic conditions. The relative protein and mRNA expression levels of VEGF-C and VEGFR-3 the si-BANCR group were significantly lower compared with those in the si-NC group ( $P < 0.05$ ; Fig. 5A-C). The relative protein and mRNA expression levels of VEGF-C and VEGFR-3 in the si-HIF-1 $\alpha$  group were also decreased



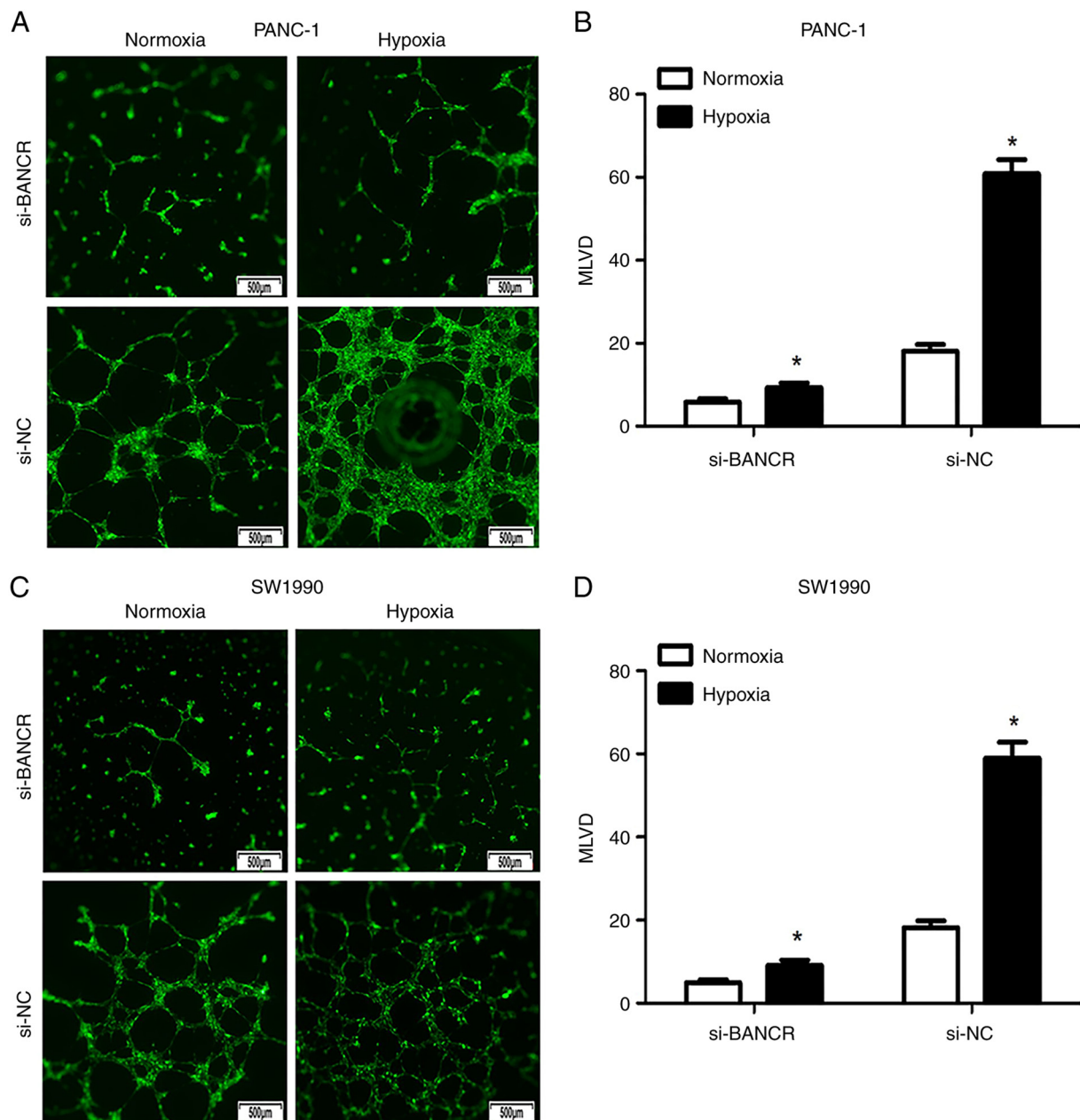


Figure 3. Effect of BANCR knockdown on MLVD in PANC-1 and SW1990 pancreatic cancer cells under normoxic and hypoxic conditions. (A) Representative images (magnification, x100) and (B) quantitative analysis of MLVD in si-BANCR and si-NC transfected PANC-1 cells under normoxic and hypoxic conditions. (C) Representative images (magnification, x100) and (D) quantitative analysis of MLVD in si-BANCR and si-NC transfected SW1990 cells under normoxic and hypoxic conditions. \* $P < 0.05$  vs. the respective normoxia group. BANCR, BRAF-activated non-protein coding RNA; MLVD, lymphatic microvessel density; si, small interfering; NC, negative control.

significantly compared with those in the si-NC group ( $P < 0.05$ ; Fig. 5D-F). These results indicate that BANCR may upregulate VEGF-C and VEGFR-3 by regulating HIF-1 $\alpha$  expression.

## Discussion

PC exhibits a high degree of malignancy and is associated with a poor prognosis. The majority of patients with PC are first diagnosed with middle- to late-stage cancer, and this limits the clinical curative effects of treatments. Despite advancements in surgery, radiotherapy and chemotherapy, the prognosis of patients with PC has not substantially improved in the past 20 years (1). Lymph node metastasis is the primary pathway and an early event

in PC metastasis, which affects the clinical stage, treatment and prognosis of patients with PC (4). Previous studies on the lymph node metastasis of PC have focused on clinical high-risk factors and their correlations; however, less research has been performed to determine the molecular mechanisms underlying lymph node metastasis. In the present study, the molecular mechanism underlying lymph node metastasis in PC was preliminarily explored at the cellular level. PANC-1 and SW1990 cell lines were chosen for use in the study as they are the most widely used representative PC cell lines, which are easy to cultivate and use in lymphangiogenesis experiments and also exhibit high BANCR expression levels. The present study demonstrated that under hypoxic conditions, BANCR promoted the lymphangiogenesis of PC cells by a

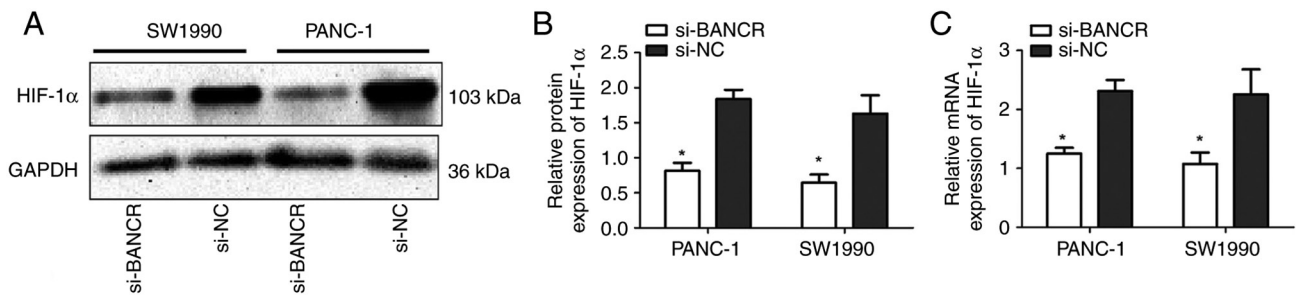


Figure 4. Effect of BANCR knockdown on HIF-1 $\alpha$  protein and mRNA expression in PANC-1 and SW1990 PC cells under hypoxic conditions. (A) Western blots and (B) quantitative analysis of HIF-1 $\alpha$  expression in PC cells. (C) HIF-1 $\alpha$  mRNA expression levels in the treated PC cells. \* $P$ <0.05 vs. the respective si-NC group. BANCR, BRAF-activated non-protein coding RNA; HIF, hypoxia-inducible factor; PC, pancreatic cancer; si, small interfering; NC, negative control.

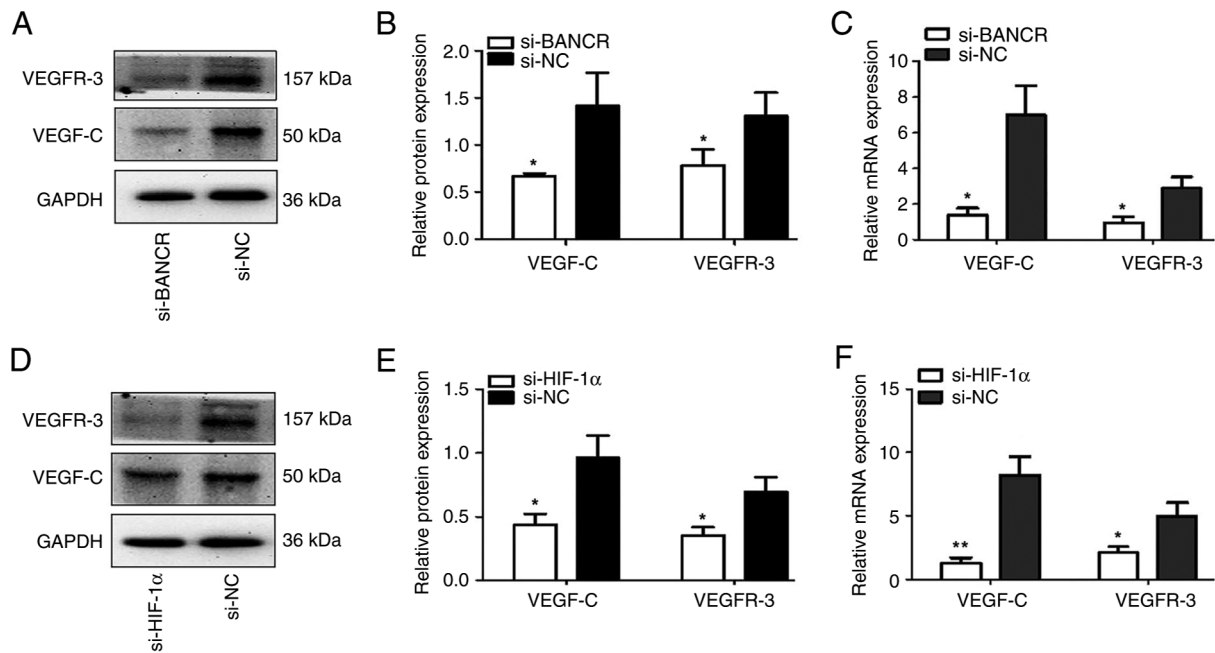


Figure 5. BANCR and HIF-1 $\alpha$  upregulate the expression of VEGF-C and VEGFR-3 in PC cells. (A) Western blots, (B) quantitative western blot analysis and (C) RT-qPCR analysis of the VEGF-C and VEGFR-3 expression levels in SW1990 PC cells transfected with si-BANCR or si-NC. (D) Western blots, (E) quantitative western blot analysis and (F) RT-qPCR analysis of the VEGF-C and VEGFR-3 expression levels in SW1990 PC cells transfected with si-HIF-1 $\alpha$  or si-NC. \* $P$ <0.05 and \*\* $P$ <0.01 vs. the respective si-NC group. BANCR, BRAF-activated non-protein coding RNA; PC, pancreatic cancer; RT-qPCR, reverse transcription-quantitative PCR; si, small interfering.

mechanism that may be associated with increased activity of an HIF-1 $\alpha$ /VEGF-C/VEGFR-3 axis.

Lymphangiogenesis is the formation of new lymphatic vessels in tissues. Liu *et al* (31) detected the presence of what they termed microlymphatics, new lymphatic vessels, or a lymphoid labyrinth in the tissues of patients with gastric or colorectal cancer, and this was significantly positively associated with lymph node metastasis in nude mice xenografts. In PC, Sipos *et al* (32) detected microlymphatics in PC tissues and an increased number of lymphatic vessels around malignant tumor tissues. Additionally, a significant association between the number of lymphatic vessels and lymph node metastasis was observed. The aforementioned studies together indicated that lymphangiogenesis is a key step in tumor lymph node metastasis. In the present study, it was shown that lymphangiogenesis in PC cells increased significantly under hypoxic conditions, and this was decreased by knocking down the expression of BANCR. These results reveal that a hypoxic

microenvironment and upregulation of BANCR expression are key factors in lymphangiogenesis of PC.

A study by Keklikoglou *et al* (33) revealed that the expression of VEGF-C was upregulated in PC and positively associated with MLVD, the Dukes stage and lymph node metastasis. VEGFR-3 was the first marker of the tyrosine-protein kinase family to be discovered and is the specific receptor for VEGF-C. Yang *et al* (34) demonstrated that downregulation of VEGFR-3 inhibits lymphangiogenesis, which further inhibits the lymphatic metastasis of bladder cancer. In addition, a review conducted by Winder and Lenz (35) described data indicating that VEGFR-3 combined with VEGF-C induces the formation of new lymphatic capillaries and increases the risk of lymph node metastasis in colon cancer. Furthermore, Ochi *et al* (9) detected a correlation between VEGF-C and VEGFR-3 expression levels in PC by analyzing the clinical and pathological data from patients; the authors concluded that VEGFR-3 combined

with VEGF-C induced the formation of new lymphatic capillaries and increased the risk of lymph node metastasis in PC. These findings indicate that the VEGF-C/VEGFR-3 pathway is important in the formation of microlymphatic vessels and lymph node metastasis in PC. However, the molecular mechanisms regulated by the VEGF-C/VEGFR-3 pathway in PC remain to be determined.

A meta-analysis showed that BANCR is upregulated in a variety of solid malignancies and is closely associated with a poor overall survival rate, lymph node metastasis and distant metastasis (36). In the present study, the expression of BANCR in SW1990 and PANC-1 cells was detected. The results showed that BANCR was upregulated in PC cells, and the upregulated expression of BANCR was significantly associated with lymphangiogenesis. Furthermore, knocking down the expression of BANCR significantly downregulated the expression of HIF-1 $\alpha$ , VEGF-C and VEGFR-3 at the transcriptional and translational levels. Based on the aforementioned results, it may be assumed that BANCR is upregulated in PC and can promote tumor lymphangiogenesis via the HIF-1  $\alpha$ /VEGF-C/VEGFR-3 pathway, which may lead to tumor lymph node metastasis.

In the present study, the effects of hypoxia on the expression of BANCR in PC cells were also investigated. The results showed that the expression of BANCR in PC cells was significantly increased under hypoxic conditions. Therefore, it is suggested that hypoxia and BANCR are closely associated with the occurrence and development of PC. Microlymphangiogenesis was also assessed, and the results showed that the MLVD of PC cells increased significantly under hypoxic conditions, and the MLVD in the negative control cells was higher than that in the cells in which BANCR was knocked down. The aforementioned study by Sipos *et al* (32) detected microlymphatics, new lymphatics or a lymphoid labyrinth in the tissues of patients with PC. Another study of tissue samples from patients with PC, conducted by Cheng *et al* (37), obtained similar results, with the observation of microlymphatics, new lymphatic vessels and/or a lymphoid labyrinth in PC tissues. The aforementioned results indicate that BANCR may promote the formation of PC microlymphatics under hypoxic conditions.

Nakajima *et al* (38) reported a significant association between the elevated expression of HIF-1 $\alpha$  and VEGF-C mRNA and lymph node metastasis in patients non-small cell lung cancer. In addition, Schoppmann *et al* (39) provided evidence that HIF-1 $\alpha$  is involved in the regulation of VEGF-C expression and lymphangiogenesis in breast cancer, and a study by Katsuta *et al* (40) in esophageal cancer presented a similar result. However, the role of HIF-1 $\alpha$  in PC is not fully understood. The activation of HIF-1 $\alpha$  is the most notable molecular tumor cell alteration that occurs under hypoxic conditions, and its abnormal expression is associated with a poor prognosis in numerous types of tumors (41). A recent study by Liu *et al* (29) reported that the increased expression of HIF-1 $\alpha$  in the tumor tissues of patients with PC is associated with tumor lymph node metastasis, late-stage tumors and a poorer predicted prognosis at first diagnosis, and revealed the high expression of HIF-1 $\alpha$  in PC tissues and its relationship with TNM stage and lymph node metastasis. The results of the present study showed an association between the expression levels of BANCR and HIF-1 $\alpha$  in PC cells. Knocking down the expression of BANCR induced a significant reduction in the expression of HIF-1 $\alpha$  in

PC cells at the transcriptional and translational levels, demonstrating the positive regulation effect of BANCR on HIF-1 $\alpha$  in PC cells.

An increase in lymphangiogenesis and the infiltration of lymphatic vessels into solid malignant tumor tissues are necessary conditions for local lymph node metastasis, and MLVD is an important quantitative index of these processes. VEGF-C has been shown to be an important growth factor in the lymphangiogenesis of solid malignant tumors, which can promote lymphangiogenesis and microlymphangiogenesis via its combination with VEGFR-3. In the present study, the expression levels of VEGF-C and VEGFR-3 were significantly reduced by knocking down the expression of BANCR. Furthermore, VEGF-C and VEGFR-3 expression levels were also significantly decreased by knocking down the expression of HIF-1 $\alpha$ . The present study revealed that the expression of BANCR was significantly increased in PC cells under hypoxic conditions, and higher levels of BANCR were associated with higher expression of components of the HIF-1 $\alpha$ /VEGF-C/VEGFR-3 axis at the transcriptional and translational levels.

In conclusion, the expression of BANCR in PC cells was significantly increased under hypoxic conditions and the upregulation of BANCR promoted lymphangiogenesis and upregulated the expression of all components of the HIF-1 $\alpha$ /VEGF-C/VEGFR-3 pathway, which plays an important role in the process of PC lymph node metastasis. These findings suggest that BANCR may be a useful biomarker and potential novel target for the diagnosis, treatment and prognostic prediction of PC.

#### Acknowledgements

Not applicable.

#### Funding

This study was supported by grants from the Science Technology Innovation Special Fund of Tongzhou area (grant nos. KJ2021CX008-22 and KJ2021CX008-24).

#### Availability of data and materials

The datasets used and/or analyzed during the present study are available from the corresponding author on reasonable request.

#### Authors' contributions

SLH, WH, YJ and HS conceived the study. HWS, JHM, JY and YCD designed the study. SLH, WH and HS analyzed the data. JY and YCD wrote the manuscript. SLH and WH edited the paper. All authors have read and approved the final manuscript. SLH and WH confirm the authenticity of all the raw data.

#### Ethics approval and consent to participate

Not applicable.

#### Patient consent for publication

Not applicable.



## Competing interests

The authors declare that they have no competing interests.

## References

- Mizrahi JD, Surana R, Valle JW and Shroff RT: Pancreatic cancer. *Lancet* 395: 2008-2020, 2020.
- Schwarz RE and Smith DD: Extent of lymph node retrieval and pancreatic cancer survival: Information from a large US population database. *Ann Surg Oncol* 13: 1189-1200, 2006.
- Chari ST, Sharma A and Maitra A: Early detection of sporadic pancreatic ductal adenocarcinoma: Problems, promise, and prospects. *Ann Intern Med* 172: 558-559, 2020.
- Wu X, Xia T, Cao M, Zhang P, Shi G, Chen L, Zhang J, Yin J, Wu P, Cai B, *et al*: LncRNA BANCER promotes pancreatic cancer tumorigenesis via modulating MiR-195-5p/Wnt/ $\beta$ -catenin signaling pathway. *Technol Cancer Res Treat* 18: 1533033819887962, 2019.
- Ran S, Volk L, Hall K and Flister MJ: Lymphangiogenesis and lymphatic metastasis in breast cancer. *Pathophysiology* 17: 229-251, 2010.
- Sundar SS and Ganesan TS: Role of lymphangiogenesis in cancer. *J Clin Oncol* 25: 4298-4307, 2007.
- Stacker SA, Williams SP, Karnezis T, Shayan R, Fox SB and Achen MG: Lymphangiogenesis and lymphatic vessel remodeling in cancer. *Nat Rev Cancer* 14: 159-172, 2014.
- Choi JU, Chung SW, Al-Hilal TA, Alam F, Park J, Mahmud F, Jeong JH, Kim SY and Byun Y: A heparin conjugate, LHBisD4, inhibits lymphangiogenesis and attenuates lymph node metastasis by blocking VEGF-C signaling pathway. *Biomaterials* 139: 56-66, 2017.
- Ochi N, Matsuo Y, Sawai H, Yasuda A, Takahashi H, Sato M, Funahashi H, Okada Y and Manabe T: Vascular endothelial growth factor-C secreted by pancreatic cancer cell line promotes lymphatic endothelial cell migration in an in vitro model of tumor lymphangiogenesis. *Pancreas* 34: 444-451, 2007.
- Kung JT, Colognori D and Lee JT: Long noncoding RNAs: Past, present, and future. *Genetics* 193: 651-669, 2013.
- Jiang J, Shi SH, Li XJ, Sun L, Ge QD, Li C and Zhang W: Long non-coding RNA BRAF-regulated lncRNA 1 promotes lymph node invasion, metastasis and proliferation, and predicts poor prognosis in breast cancer. *Oncol Lett* 15: 9543-9552, 2018.
- Shen X, Bai Y, Luo B and Zhou X: Upregulation of lncRNA BANCER associated with the lymph node metastasis and poor prognosis in colorectal cancer. *Biol Res* 50: 32, 2017.
- Flockhart RJ, Webster DE, Qu K, Mascarenhas N, Kovalski J, Kretz M and Khavari PA: BRAFV600E remodels the melanocyte transcriptome and induces BANCER to regulate melanoma cell migration. *Genome Res* 22: 1006-1014, 2012.
- Fang S, Liu Z, Guo Q, Chen C, Ke X and Xu G: High BANCER expression is associated with worse prognosis in human malignant carcinomas: An updated systematic review and meta-analysis. *BMC Cancer* 20: 870, 2020.
- Fan YH, Ye MH, Wu L, Wu MJ, Lu SG and Zhu XG: BRAF-activated lncRNA predicts gastrointestinal cancer patient prognosis: A meta-analysis. *Oncotarget* 8: 6295-6303, 2017.
- Zhou T and Gao Y: Increased expression of lncRNA BANCER and its prognostic significance in human hepatocellular carcinoma. *World J Surg Oncol* 14: 8, 2016.
- Liu ZH, Yang TX, Xu ZP, Lv J, Cao XF, *et al*: Long non-coding RNA BANCER expression in esophageal squamous cell carcinoma and its effects on cell growth and invasion. *Prog Mod Biomed* 16: 4622-4627, 2016. (In Chinese).
- Lou KX, Li ZH, Wang P, Liu Z, Chen Y, Wang XL and Cui HX: Long non-coding RNA BANCER indicates poor prognosis for breast cancer and promotes cell proliferation and invasion. *Eur Rev Med Pharmacol Sci* 22: 1358-1365, 2018.
- Zheng H, Xu J, Hao S, Liu X, Ning J, Song X, Jiang L and Liu Z: Expression of BANCER promotes papillary thyroid cancer by targeting thyroid stimulating hormone receptor. *Oncol Lett* 16: 2009-2015, 2018.
- Stojanović S, Šećemetjev S, Đorić I, Rončević J, Janković Miljuš J, Živaljević V and Iščić Denčić T: Elevated BANCER expression levels have different effects on papillary thyroid carcinoma progression depending on the presence of the BRAFV600E mutation. *Eur J Surg Oncol* 46: 1835-1842, 2020.
- Koong AC, Mehta VK, Le QT, Fisher GA, Terris DJ, Brown JM, Bastidas AJ and Viera M: Pancreatic tumors show high levels of hypoxia. *Int J Radiat Oncol Biol Phys* 48: 919-922, 2000.
- Dauer P, Nomura A, Saluja A and Banerjee S: Microenvironment in determining chemo-resistance in pancreatic cancer: Neighborhood matters. *Pancreatol* 17: 7-12, 2017.
- Kang G, Hu M, Ren H, Wang J, Cheng X, Li R, Yuan B, Balan Y, Bai Z and Huang H: VHH212 nanobody targeting the hypoxia-inducible factor 1 $\alpha$  suppresses angiogenesis and potentiates gemcitabine therapy in pancreatic cancer in vivo. *Cancer Biol Med* 18: 772-787, 2021.
- Yang QK, Wang XX, Wang Y and Ni N: Integrative analysis reveals the landscape of hypoxia-inducible factor (HIF) family genes in pan-cancer. *J Oncol* 2020: 8873104, 2020.
- Semenza GL: Targeting HIF-1 for cancer therapy. *Nat Rev Cancer* 3: 721-732, 2003.
- Harris AL: Hypoxia-a key regulatory factor in tumour growth. *Nat Rev Cancer* 2: 38-47, 2002.
- Ye LY, Zhang Q, Bai XL, Pankaj P, Hu QD and Liang TB: Hypoxia-inducible factor 1 $\alpha$  expression and its clinical significance in pancreatic cancer: A meta-analysis. *Pancreatol* 14: 391-397, 2014.
- Jin X, Dai L, Ma Y, Wang J and Liu Z: Implications of HIF-1 $\alpha$  in the tumorigenesis and progression of pancreatic cancer. *Cancer Cell Int* 20: 273, 2020.
- Liu M, Zhong J, Zeng Z, Huang K, Ye Z, Deng S, Chen H, Xu F, Li Q and Zhao G: Hypoxia-induced feedback of HIF-1 $\alpha$  and lncRNA-CF129 contributes to pancreatic cancer progression through stabilization of p53 protein. *Theranostics* 9: 4795-4810, 2019.
- Livak KJ and Schmittgen TD: Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) method. *Methods* 25: 402-408, 2001.
- Liu HD, Zhao LH, Zhang YF, Li YL, Li XD, Yang SC, Li YM and Nie CS: Morphologic features of lymphatic in periphery region of gastric carcinoma and colon carcinoma. *Ai Zheng* 24: 699-703, 2005.
- Sipos B, Kojima M, Tiemann K, Klapper W, Kruse ML, Kalthoff H, Schniewind B, Tepel J, Weich H, Kerjaschki D and Klöppel G: Lymphatic spread of ductal pancreatic adenocarcinoma is independent of lymphangiogenesis. *J Pathol* 207: 301-312, 2005.
- Keklikoglou I, Hosaka K, Bender C, Bott A, Koerner C, Mitra D, Will R, Woerner A, Muenstermann E, Wilhelm H, *et al*: MicroRNA-206 functions as a pleiotropic modulator of cell proliferation, invasion and lymphangiogenesis in pancreatic adenocarcinoma by targeting ANXA2 and KRAS genes. *Oncogene* 34: 4867-4878, 2015.
- Yang H, Kim C, Kim MJ, Schwendener RA, Alitalo K, Heston W, Kim I, Kim WJ and Koh GY: Soluble vascular endothelial growth factor receptor-3 suppresses lymphangiogenesis and lymphatic metastasis in bladder cancer. *Mol Cancer* 10: 36, 2011.
- Winder T and Lenz HJ: Vascular endothelial growth factor and epidermal growth factor signaling pathways as therapeutic targets for colorectal cancer. *Gastroenterology* 138: 2163-2176, 2010.
- Zhang G and Cai J: Evaluation of prognostic value of lncRNA BANCER in tumor patients: A systematic review and meta-analysis. *J BUON* 24: 2553-2559, 2019.
- Cheng P, Jin G, Hu X, Shi M, Zhang Y, Liu R, Zhou Y, Shao C, Zheng J and Zhu M: Analysis of tumor-induced lymphangiogenesis and lymphatic vessel invasion of pancreatic carcinoma in the peripheral nerve plexus. *Cancer Sci* 103: 1756-1763, 2012.
- Nakajima T, Anayama T, Koike T, Shingyoji M, Castle L, Kimura H, Yoshino I and Yasufuku K: Endobronchial ultrasound doppler image features correlate with mRNA expression of HIF1- $\alpha$  and VEGF-C in patients with non-small-cell lung cancer. *J Thorac Oncol* 7: 1661-1667, 2012.
- Schoppmann SF, Fenzl A, Schindl M, Bachleitner-Hofmann T, Nagy K, Gnani M, Horvat R, Jakesz R and Birner P: Hypoxia inducible factor-1 $\alpha$  correlates with VEGF-C expression and lymphangiogenesis in breast cancer. *Breast Cancer Res Treat* 99: 135-141, 2006.
- Katsuta M, Miyashita M, Makino H, Nomura T, Shinji S, Yamashita K, Tajiri T, Kudo M, Ishiwata T and Naito Z: Correlation of hypoxia inducible factor-1 $\alpha$  with lymphatic metastasis via vascular endothelial growth factor-C in human esophageal cancer. *Exp Mol Pathol* 78: 123-130, 2005.
- Jun JC, Rathore A, Younas H, Gilkes D and Polotsky VY: Hypoxia-inducible factors and cancer. *Curr Sleep Med Rep* 3: 1-10, 2017.

