European Review for Medical and Pharmacological Sciences 2013; 17: 436-446

Effects of the silencing of hypoxia-inducible Factor-1 alpha on metastasis of pancreatic cancer

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Abstract. – BACKGROUND: Hypoxia plays a crucial role in the development of solid tumors. Hypoxia-inducible factor-1alpha (HIF-1alpha) is essential for this process, and has been suggested to be a target for cancer therapy. New therapeutic approaches for pancreatic cancer are needed owing to the extremely poor prognosis, in large part as a consequence of high rates of metastasis. The mechanism remains to be explored.

AIM: To illustrate the role of hypoxia-inducible factor-1alpha in pancreatic cancer metastasis and the value of the molecule as a target for pancreatic cancer therapy.

MATERIALS AND METHODS: To address this shortcoming, we used both in vitro and in vivo approaches to evaluate the overall effects of HIF-1alpha on pancreatic cancer. We used a plasmid encoding small interfering RNAs (SiRNAs) to silence HIF-1alpha expression in the Panc-1 pancreatic cancer cell line, and used a series of assays to detect changes in gene expression at the protein and mRNA levels, cell proliferation, cell apoptosis, and the abilities of cells to migrate under both hypoxia and normoxia conditions.

RESULTS: Both *in vitro* and *in vivo* analysis suggested that hypoxia significantly promotes cell proliferation and migration, resulting in metastasis. Pancreatic cancer cells in which HIF-1alpha expression was inhibited were less invasive, with reduced resistance to hypoxia, impaired migration, and reduced capacity to cause metastasis.

CONCLUSIONS: HIF-1alpha may be a dominant factor driving the metastatic progression of pancreatic cancer and can be a potent therapeutic target for the disease.

Key Words:
Tumor hypoxia, Pancreatic carcinoma, RNA interference, Metastasis.

Abbreviations

HIF-1 = hypoxia-inducible factor-1
VEGF = vascular endothelial growth factor
PCNA = proliferating cell nuclear antigen
SiRNA = small interfering RNA
ATCC = American Type Cell Collection
DMEM = Dulbecco's Modified Eagle's Medium
EGFP = enhanced green fluorescent protein
MTT = 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
DMSO = dimethyl sulfoxide
FITC = fluorescein isothiocyanate
SDS-PAGE = sodium dodecyl sulfate-polyacrylamide gel electrophoresis
HRP = horseradish peroxidase
HE = stain hematoxylin-eosin
PBS = propidium iodide phosphate buffered propidium iodide saline
XIAP = X-linked inhibitor of apoptosis protein
HRE = hypoxia reactive element

Introduction

The behavior of tumor cells is determined largely by their microenvironments, with hypoxia being a dominant feature of the microenvironment of solid tumor cells, especially in pancreatic cancer[1]. During the past two decades, several investigations have confirmed that hypoxia causes genetic instability through the misregulation of transcription factors, which in turn causes aberrant gene expression[2]. Hypoxia drives clonal selection of more invasive tumor cells, which also proliferate more rapidly than less invasive cells and are resistant to hypoxia[3]. Consequently, hypoxia is suggested to be a major cause of the poor prognosis of solid tumors, especially pancreatic cancer[4].

Hypoxia-inducible factor-1 (HIF-1) plays a key role in the response of cells to hypoxia[5]. HIF-1 is a heterodimer that comprises α subunit and β subunit[6]. As a transcription factor, HIF-1α regulates a large number of genes that are involved in tumorigenesis, metabolism, angiogenesis, oncogene expression metastasis, and so forth[7-10]. Given its importance, HIF-1α is also recognized widely as an attractive target for cancer therapies[7]. The effects of several clinically

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approved drugs, including rapamycin, trastuzumab, imatinib, and geldanamycin, have been associated with their abilities to inhibit HIF-1 activity indirectly[11]. Although these and other antineoplastic agents improve the prognosis of pancreatic cancer, their long-term effectiveness remains limited[12], which emphasizes the need for more innovative therapeutic methods. In spite of the importance of HIF-1α in the development of cancer, its particular roles in cancers of specific cell or tissue types remain to be established.

Human pancreatic cancer is associated commonly with tumor hypoxia, with lesions that are usually surrounded by a rim of cells characterized by poor tissue oxygenation, reduced blood flow, and low tissue pH[13]. As the central regulator of the adaptive response to hypoxia, HIF-1 activates the transcription of hypoxia inducible genes which regulate diverse cellular functions including metabolism, angiogenesis and tumor invasion. In pancreatic cancer, HIF-1-regulated genes, such as VEGF, have been proved comprehensively to promote angiogenesis which is known as a vital process in metastasis. Furthermore, it was found that overexpression of HIF-1α SiRNA blocks EGF-induced survivin gene up-regulation and increases apoptosis induced by the chemotherapy drug docetaxel. Meanwhile, over expressing of HIF-1α gene activates survivin gene expression and reduces the apoptotic response[14]. Survivin is a multifunctional protein whose involvement in AKT signaling pathway and regulating mitosis, apoptosis, and cell motility can provide proliferative and metastatic advantages to tumor cells[15]. Both migration and invasion require signaling events within the cell and with the extracellular matrix for navigation of tumor cells within their microenvironment and to distant sites. Recent studies have implicated the important roles of survivin in metastasis. It cooperated with XIAP to mediate cell invasion in breast cancer and activated Akt signaling pathway and upregulation of α5 integrin to enhance the migration of melanoma cells[15].

The body of research mentioned above implies the complicated multifaceted role of HIF-1α in pancreatic cancer metastasis. To explore this question further, in the present study we used both *in vitro* and *in vivo* approaches to examine the overall effects of HIF-1α on pancreatic cancer. Our findings will improve our understanding of pancreatic cancer and might open the way for promising new therapeutic strategies to improve the very poor prognosis for this disease.

Materials and Methods

Plasmid Construction

Five SiRNAs that were designed to target HIF-1α and one SiRNA designed to target a negative control sequence were ligated into the pGenesil-1 plasmid to produce the pGenesil-1-HIF-1α plasmid that carrying five sequences of the SiRNAs that targeted HIF-1α and pGenesil-1-HK plasmid, respectively. The constructs were generated by Genesil Biotechnology Co. (Wuhan, China). Expression of the SiRNAs was under the control of the hU6 promoter in pGenesil-1 (Hu6/kan/neo/EGFP). The sequences were listed Table I. We used the Basic Local Alignment Search Tool (BLAST) to search the human genome database with the SiRNA sequences to avoid nonspecific SiRNA-mediated silencing.

Cell Culture

The PANC-1 human pancreatic cancer cell line was obtained from the American Type Cell Collection (ATCC, Manassas, VA, USA). Cells were cultured medium was prepared as in ATCC-formulated Dulbecco's Modified Eagle's Medium (DMEM), to which fetal bovine serum was added at a final concentration of 10%. Cells were transfected with either plasmid Genesil-1-HIF-1α (encoded five SiRNAs that targeted HIF-1α) or plasmid Genesil-1-HK (encoded the negative control SiRNA) using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). The former cells

Table I. Five sequences of the SiRNAs that targeted HIF-1α and the negative control sequence ("‡").

Sense strand	Antisense strand
5'-AAC CGG TTG AAT CTT CAG ATA-3'	5'-TAT CTG AAG ATT CAA CCG GTT-3'
5'-AAC CTC AGT GTG GGT ATA AGA-3'	5'-TCT TAT ACC CAC ACT GAG GCC-3'
5'-AAG ACC GTA TGG AAG ACA TTA-3'	5'-TAA TGT CTT CCA TAC GGT CTT-3'
5'-AAG CCC TAA CGT GTT ATC TGT-3'	5'-ACA GAT AAC ACG TTA GGG CGG-3'
5'-AAG TCG GAC AGC CTC ACC AAA-3'	5'-TTT GGT GAG GCT GTC CGA CTT-3'
‡5'-AAA GCT TCA TAA GGC GCA TGC -3'	‡5'-GCA TGC GCC TTA TGA AGC TTT-3'

were named pG2 and the latter cells were named pG1. Cells were cultured under conditions of either humidified normoxia (5% CO_2 in air) or hypoxia (1% O_2, 5% CO_2, and 94% N_2) at 37°C.

Proliferation Assay

We used an MTT [3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide] dye reduction assay to analyze cell proliferation. Each well in a 96-well plate was seeded with 1×10^3 cells/200 µl DMEM medium, and the plate incubated for 7 days under conditions of either humidified normoxia or hypoxia at 37°C. We then added 20 µl of MTT (5 mg/ml) to each well and incubated the plate for an additional 4 h at 37°C before removing the medium and MTT. Dimethyl sulfoxide (DMSO, 150 µl/well) was added and the plate incubated for another 10 minutes. Absorbance at 490 nm was determined for the contents of each well using a spectrophotometer (Cany Precision Instruments Co., Ltd., Shanghai, China).

Flow Cytometry Assay

Pancreatic cancer cells were double-labeled with both propidium iodide and fluorescein isothiocyanate (FITC) using an Annexin V-FITC reagent kit (Genemay Inc., Shenzhen, China) in accordance with the manufacturer's instructions. In brief, we washed the cells twice with phosphate buffered saline (PBS) and modified the cell concentration such that it fell within the range of 5×10^5 cells/ml and then suspended 8×10^4 cells in 250 µl of binding buffer (It is diluted 1:4 in deionized water). We then added 5 µl of Annexin V/FITC to 195 µl of the cell suspension and incubated the mixture in the dark for 15 minutes at room temperature after mixing. The cells were then resuspended in 190 µl of binding buffer, and a solution of 10 µg/ml propidium iodide was added to the cell suspension to give a final concentration of 1 µg/ml propidium iodide. Samples were then analyzed using a flow cytometer (FACSCanto II, Becton, Dickinson and Company, Franklin Lakes, NJ, USA).

Migration Assay

The 24-well traditional transwell plates that were used for the study were purchased from Corning Company (Corning, New York, NY, USA). We added 0.6 ml of DMEM medium without fetal bovine serum to the bottom well and then 0.1 ml of the same medium to the transwell insert. We then added 0.1 ml of a suspension of pancreatic cancer cells (2×10^5 cells/ml) prepared in fresh medium without fetal bovine serum to each transwell insert compartment, and incubated the samples at 37°C for 1 hour. After removing the medium, we firstly added 0.6 ml of medium containing 20% fetal bovine serum (FBS) to the lower compartment, and then added 0.1 ml of medium without FBS to the inside compartment of each transwell insert before incubating at 37°C for 12 hours. Cells that were growing on the lower surface of the membrane were stained with hematoxylin-eosin (HE) and counted using a microscope.

Reverse Transcription-PCR

Total RNA was extracted from pancreatic cancer cells using Trizol Reagent (Invitrogen, Carlsbad, CA, USA) and used for reverse transcription polymerase chain reaction (RT-PCR) analysis in accordance with a routine procedure. The level of mRNA was estimated by semi-quantitative RT-PCR and reported as the ratio of the optical density of the product of interest relative to the optical density of the amplified product generated using oligonucleotides specific for transcripts encoding -actin. The PCR primers used were listed in Table II.

Table II. Sequences of the PCR primers.

Moleculer	bps of products		Sequences
HIF-1α	324	Sense	5'-ACTTCTGGATGCTGGTGATT-3'
		Antisense	5'-TCCTCGGCTAGTTAGGGTAC-3'
PCNA	470	Sense	5'-GAGTGGTCGTTGTCTTTC-3'
		Antisense	5'-AACTTTCTCCTGGTTTGG-3'
Survivin	175	Sense	5'-CGGCTCCTCTACTGTTTAA-3'
		Antisense	5'-CTGCCAGACGCTTCCTAT-3'
VEGF	421	Sense	5'-CCTTGCTGCTCTACCTCC-3'
		Antisense	5'-AAATGCTTTCTCCGCTCT-3'
β-actin	301	Sense	5'-TGGACATCCGCAAAGAC-3'
		Antisense	5'-AAAGGGTGTAACGCAACTA-3'

Western Blot Analysis

Monoclonal antibodies against HIF-1α, survivin, VEGF, NME1, angiopoietin-1, and PCNA were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA). Cells were treated with RIPA lysis buffer. After the protein concentration of the cellular extracts had been measured, the samples were combined with sample buffer in a 1:2 ratio and heated to 100°C for 5 minutes. The protein samples were loaded onto SDS-PAGE gels and resolved at 100 volts for approximately 100 minutes until the bromophenol blue reached the end of the gel. The proteins were blotted onto nitrocellulose hybridization transfer membrane at 400 mA for 45 minutes. The membranes were then placed in 1×PBS that contained diluted primary antibody and incubated for 1 hour. The membranes were incubated for 1 hour in 1×PBS that contained the secondary antibody diluted 1:10,000. Finally, the membranes were visualized using the enhanced chemiluminescence detection kit and the film was developed.

Tumor Models in Nude Mice

We separated 48 BALB/C nude mice randomly into eight groups when they were 3-5 weeks old and weighed 18-20 grams. We prepared four groups of suspensions each of that contains 8×10^6 cells/ml and separately injected 0.1 ml of the suspension of each group subcutaneously in four groups of each nude mouse for the xenogenous implant tumor model. In the remaining four groups of nude mice, 0.1 ml of the suspension of each group is separately injected into the tail vein of each mouse for the metastatic model. Four weeks later, subcutaneous tumors, lungs and livers were fixed in 4% paraformaldehyde. Deparaffinized sections were stained using HE stain by staff of the Pathology Department of the Medical College at Huazhong Science and Technology University. Microscopy was then used to evaluate the histomorphology of the tumor samples and extent of metastasis in the lung and liver.

TUNEL Assay

Sections that had been used to study the pathology were dewaxed and dehydrated. Before the slices were digested, they were blocked in a solution that contained 3% H_2O_2 in methanol at 15°C for 30 minutes. Apoptosis was assayed using a TUNEL kit (Promega Biotech Co., Ltd. Shanghai, China) in accordance with the manufacturer's guidelines. Brown granular nuclear staining was considered to indicate apoptosis and the rate of apoptosis was calculated as (apoptotic cell number/total cell number) × 100%.

Statistical Analysis

Statistical analysis was performed using the Graphpad Prism 5 Demo (GraphPad Software, Inc. La Jolla, CA, USA). Owing to the small number of mice used, the data obtained using the tumor models were analyzed with Fisher's exact test. All other data were analyzed using two-way ANOVA. We considered $p < 0.05$ to indicate a statistically significant difference.

Results

To determine the specific effects of HIF-1α on pancreatic cancer progression and metastasis, a plasmid (pGenesil-1-HIF-1α) that encoded five SiRNAs against HIF-1α was transfected into pancreatic cancer cells to silence the expression of HIF-1α and the cells were named pG2. Meanwhile, pancreatic cancer cells that have been transfected with the negative control plasmid pGenesil-1-HK were named pG1.

HIF-1α Silencing Causes Opposite Effects on Gene Expression at the mRNA and Protein Levels

To examine the consequences of pG2 cells, we used both RT-PCR and western blotting to investigate the expression of HIF-1α and several other proteins and mRNA transcripts. We observed that levels of HIF-1α, VEGF, PCNA, and survivin were considerably lower in pG2 cells than in pG1 cells. The differences in the levels of expression relative to control pG1 cells were all significant ($p < 0.001$, Figure 1). HIF-1α mediates tumor angiogenesis by upregulating the genes that encodes VEGF through binding to the Hypoxia Reactive Element (HRE) in the promoter of the VEGF gene during hypoxia. Consistent with this, we demonstrated that the induction of HIF-1α under hypoxia coincided with increased levels of VEGF expression in pG1 cells. In contrast, silencing of HIF-1α decreased levels of VEGF in pG2 cells ($p < 0.001$, Figure 1).

HIF-1α Inhibition Arrests Cell Proliferation

The significantly decreased levels of HIF-1α and PCNA in pG2 cells ($p < 0.001$, Figure 1) prompted us to evaluate the effects of HIF-1α on

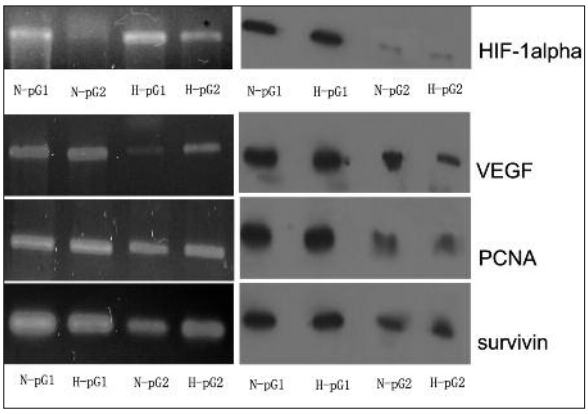

Figure 1. Expressions of HIF-1α, PCNA, and survivin by RT-PCR (*left*) and western blot (*right*). HIF-1α showed positive effect on VEGF, PCNA and survivin. When HIF-1α expression was silenced, no differential expression of each molecule was found between the hypoxia and normoxia groups (p = 0.0784-0.1862). Before HIF-1α was silenced, hypoxia statistically enhanced HIF-1α expression (p = 0.0425). H denotes hypoxia and N denotes normoxia. pG2 denotes Panc-1 cells that were transfected with plasmid Genesil-1- HIF-1α that encoded SiRNA targeting HIF-1α. pG1 denotes Panc-1 cells that were transfected with negative control plasmid which named Genesil-1- HK.

the proliferation of pancreatic cancer cells. *In vitro* analysis revealed trends of slow proliferation of pG2 cells under both normoxia and hypoxia, but there was no significance existed between normoxia and hypoxia (p = 0.1034, Figure 2). However, rates of growth of pG1 cells were significantly greater than those of pG2 cells under both normoxia and hypoxia (p < 0.001, Figure 2). Rate of growth of pG1 cells was also statistically more rapid under conditions of hypoxia than normoxia (p < 0.001, Figure 2).

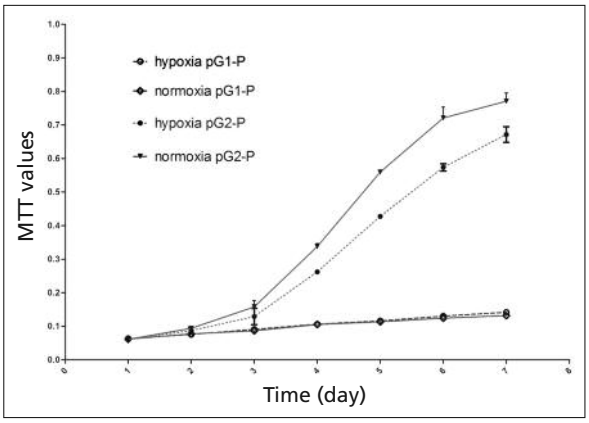

Figure 2. Proliferating curves of Panc-1. No discrepancy was found between two groups (hypoxia and normoxia) of pG2 cells in MTT assay (p = 0.1034), whereas significant differences were defined when comparing pG1 cells and pG2 cells (p < 0.001). Rate of growth of pG1 cells was also statistically more rapid under hypoxia than under normoxia (p < 0.001). The figure demonstrated stimulation effect of HIF-1α on pancreatic cancer cells. H denotes hypoxia and N denotes normoxia.

In vivo analysis indicated that pG2 cells cultured under hypoxic conditions could not form tumors after subcutaneous injection into nude mice. Injection of pG2 cells cultured under normoxic conditions resulted in a low rate of formation (16.7% tumorigenicity) of very small, slow-growing subcutaneous tumors. Only one of the six animals that received these cells developed a macroscopic tumor, which took 24 days to form and was only 0.3 cm in diameter 28 days after injection.

By contrast, pG1 cells were extremely tumorigenic. When cultured under normoxic conditions, the tumorigenicity was 66.7%; four of the six animals injected with the cells developed subcutaneous tumors with an average diameter of 0.9 cm. The mean time at which tumor formation became evident was 13 days after injection. When pG1 cells were cultured under hypoxic conditions, they showed strikingly enhanced tumorigenicity, with tumors evident in all six of the animals injected with these cells (Table III). The mean diameter of these tumors was 1.5 cm and the mean time at tumor evident formation was only 9 days. The statistical analysis of the data displayed the significant difference between the pG2 cells and hypoxic pG1 cells (p = 0.0152). Concurrently, there was no difference between normoxic pG1 cells and other groups (p = 0.0606). Because of the small number of samples in the experiment, the result needs further study.

Silencing of HIF-1α Enhances Apoptosis

Flow cytometry and TUNEL assay were used to determine the effect of HIF-1α on the apoptosis of pancreatic cancer cells. The rate of apoptosis of pG1 cells under hypoxic conditions was significantly lower than that under normoxic conditions (p = 0.017), although the difference in rates of mortality under the two conditions was not meaningful (p = 0.375). Under either normoxic or hypoxic conditions, the rate of apoptosis of pG1 cells was lower than that of pG2 cells (p < 0.001). The rates of both apoptosis and mortality for pG2 cells were higher under hypoxic conditions than under normoxic conditions (p < 0.001). The discrepancy in the rates of apoptosis between hypoxia and normoxia was not significant (p = 0.682), whereas the discrepancy between the rates of mortality was significant (p = 0.0025).

Table III. Results of xenogenous subcutaneously implant tumor models.

	pG2* hypoxia	pG1† hypoxia	pG2 normoxia	pG1 normoxia
Number of formed	0	6	1	4
Number of unformed	6	0	5	2
Mean time (days)	/	8.5	24	10.5
Mean diameter (cm)	/	1.5	0.3	0.9

The statistical analysis of the data displayed the significant difference between the pG2 cells and hypoxic pG1 cells (p = 0.0152). Concurrently, there was no difference between normoxic pG1 cells and other groups (p = 0.0606). When HIF-1α expression was inhibited (pG2 cells), there was less xenogenous tumor. To pG1 cells, hypoxia obviously stimulated cell proliferation and tumorigenicity. *pG2 denotes cells transfected with plasmid encoding siRNA (small interfering RNA) of HIF-1α (hypoxia inducible factor -1 alpha. †pG1 denotes cells transfected with plasmid encoding negative control siRNA duplex.

The rate of apoptosis was significantly higher in subcutaneous tumors formed by pG1 cells exposed to hypoxia than in tumors formed by cells cultured under normoxic conditions before injection (p = 0.0026). This result was inconsistent with the results of the *in vitro* experiments. There was extensive necrosis in tumors derived from pG1 cells exposed to hypoxic conditions (Figure 3B and C). The rates of apoptosis were very similar in the single, very small, pG2 -derived tumor and the tumors derived from pG1 cells exposed to normoxia (p = 0.8813, Figure 3D and A).

Deficiency of HIF-1α Decreases Rates of Cell Migration and Metastasis

Rates of cell migration were similar in pG2 cells that had been exposed to either hypoxic or normoxic conditions (p = 0.3052, Figure 4C and

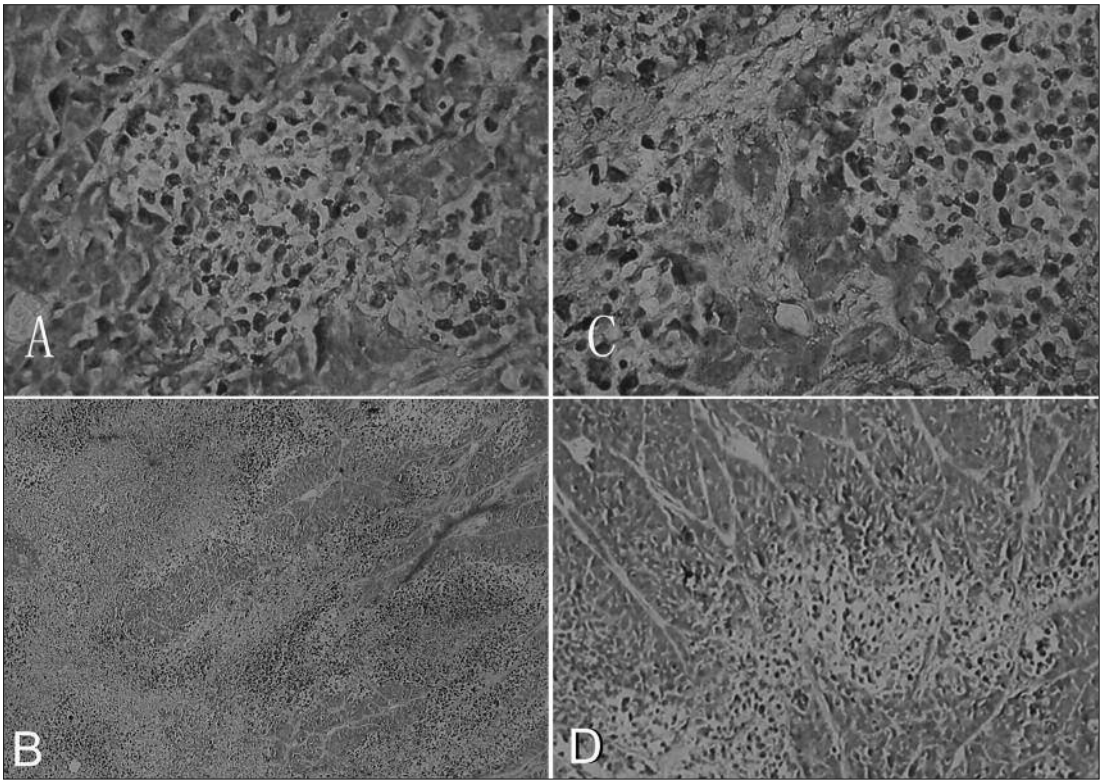

Figure 3. Tunnel assay in subcutaneous xenografts. ***A,*** Displayed the focal apoptosis in tumor of normoxia group of pG1 (H&E, 400×). ***B, C,*** Showed the diffuse apoptosis in tumors of hypoxia group of pG1 (H&E, 100× and 400× respectively). ***D,*** Showed the apoptosis in tumor derived from pG2 cells and the apoptosis rate is similar to that in tumor derived from normoxia pG1 cells (p = 0.8813) (H&E 200×). *In vitro*, regardless of whether hypoxia or not, apoptotic rate of pG1 was lower than pG2 cells (p < 0.001). However, the presented data indicated extensive apoptosis in hypoxia pG1 cell derived tumors in vivo (p = 0.0026). The puzzling result needs more research.

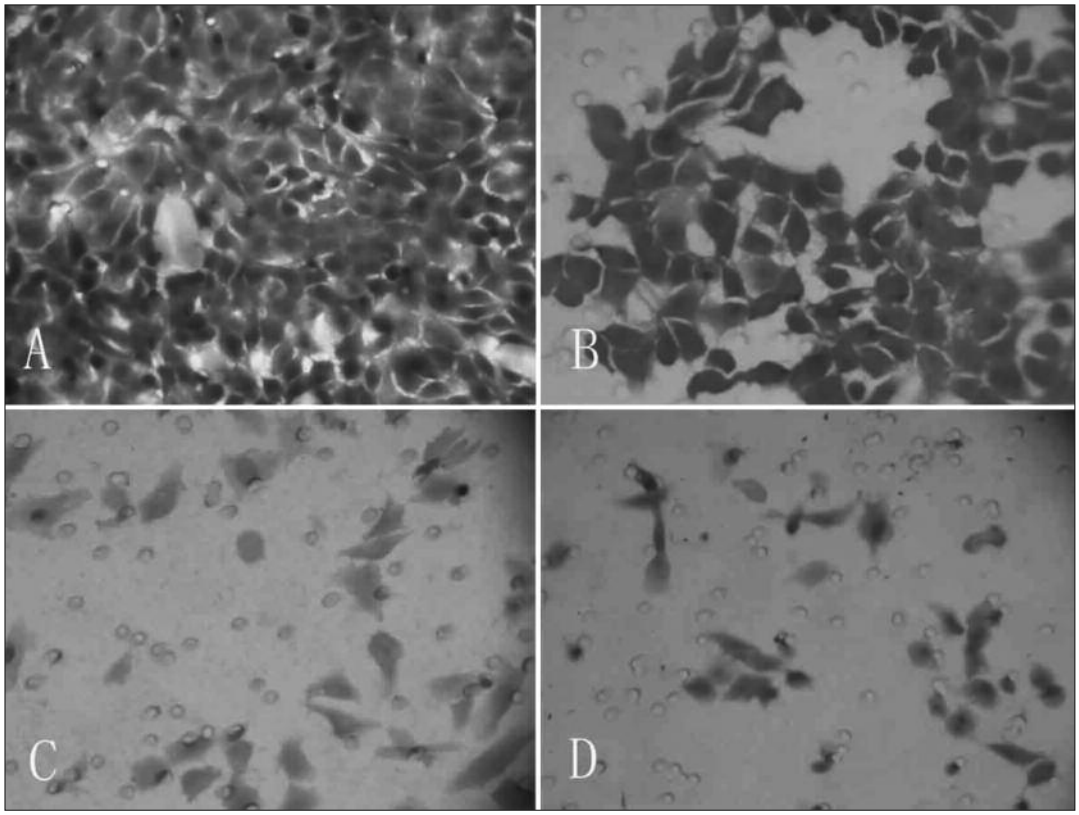

Figure 4. Cell migration ability by transwell method. The migrated amount of pG2 cells in hypoxia was similar to that in normoxia ($p = 0.3052$). The quantity of migrated pG1 cells in hypoxia were statistically more than that in normoxia ($p = 0.0014$). Either under normoxia or hypoxia, both of the migrated amount of pG1 cells were remarkably more than those of pG2 cells ($p < 0.001$). Upper pictures ***(A*** and ***B)*** showed pG1 cells. Lower pictures ***(C*** and ***D)*** showed pG2 cells. The left ***(A*** and ***C)*** was contributed to hypoxia group and the right ***(B*** and ***D)*** contributed to normoxia group (H&E, 400×).

D). In contrast, significantly more pG1 cells migrated under hypoxic conditions than under normoxic conditions ($p = 0.0014$, Figure 4A and 4B). Moreover, under both normoxic and hypoxic conditions, the number of pG1 cells that migrated was significantly more than the number of pG2 cells that migrated ($p < 0.001$). *In vivo*, the cells were injected into the tail vein and the matastases were invested. Liver metastases were observed in only three of the six animals injected with pG1 cells cultured under normoxic conditions, and these lesions were both small and sparse (3-6 lesions per animal). By contrast, metastases were evident in all six of the animals injected with pG1 cells incubated under hypoxic conditions (Table IV), and the metastatic lesions

Table IV. Results of tail vein metastasis model.

	pG2* hypoxia	pG1† hypoxia	pG2 normoxia	pG1 normoxia
Number of metastasis	0	6	0	3
Number of no metastasis	6	0	6	3
Number of lesions	0	10-20	0	3-6

Metastasis disappeared in group of pG2, whereas there was a great amount of metastasis in group of pG1 and more metastasis was found in hypoxia than in normoxia. The statistical analysis of the data displayed the significant difference between the pG2 cells and hypoxic pG1 cells ($p = 0.0022$). Meanwhile, there was no difference between normoxic pG1 cells and other groups ($p = 0.1818$). The result of tail vein models was similar to that of subcutanous models. *pG2 denotes cells transfected with plasmid encoding siRNA (small interfering RNA) of HIF-1α (hypoxia inducible factor -1 alpha). †pG1 denotes cells transfected with plasmid encoding negative control siRNA duplex.

were both larger and more numerous (10-20 lesions per animal). In animals injected with pG1 cells that had been exposed to hypoxia, the entire liver was macroscopically nodular, and the structure of the liver lobule and/or portal area was destroyed by metastasis (Figure 5). Injection of pG2 cells was not associated with metastasis in either subcutaneous tumor models or tail vein models. The statistical analysis of the data displayed the significant difference between the pG2 cells and hypoxic pG1 cells ($p = 0.0022$). Meanwhile, there was no difference between normoxic pG1 cells and other groups ($p = 0.1818$). The result of tail vein models was similar to that of subcutanous models.

Discussion

The transcription factor HIF-1 regulates the expression of many genes, including important factors involved in tumorigenesis, such as regulators of angiogenesis[16]. Tumor metastasis is dependent on cell proliferation, capillary tube formation and migration. Owing to the various effects of VEGF in the progression of ischemic-reperfusion injury and cancer, the gene that encodes VEGF is the most studied target of HIF-1. VEGF increases the density of capillary vessels to improve tissue oxygenation, which makes it crucial to tumor progression, and several studies have confirmed a direct relationship between HIF-1 and VEGF[17]. Our *in vitro* data confirmed that hypoxia leads to upregulation of the expression of HIF-1α and VEGF and it suggested that hypoxia stimulates the expression of HIF-1 in pancreatic cancer cells, which should in turn promote angiogenesis through the upregulation of VEGF. When HIF-1 expression was silenced by RNA interference, the VEGF expression was downregulated and the migration ability was inhibited in the Transwell assay. As a consequence, enhanced angiogenesis in tumors that resulted in immature neovascularization[18] would aggravate hypoxia in the local tumor environment, which in turn would enable tumor cells to promote their own proliferation and metastasis.

HIF-1α was found to play a vital role in the proliferation of pancreatic cancer cells. Although the details of the underlying mechanisms involved appear opaque, enhancement of an oxygen-independent metabolic pathway is involved[19]. Given that cell proliferation requires substantial synthesis of proteins, lipids, and nucleic acids, signals that stimulate cell proliferation must also reorganize metabolic activities to stimulate the proliferation of quiescent cells[20]. For example, increased rates of glycolysis drive the generation of energy when the partial pressure of oxygen decreases. Accordingly, the dramatic upregulation of glycolysis is an important consequence of HIF-1 upregulation[21]. It has been

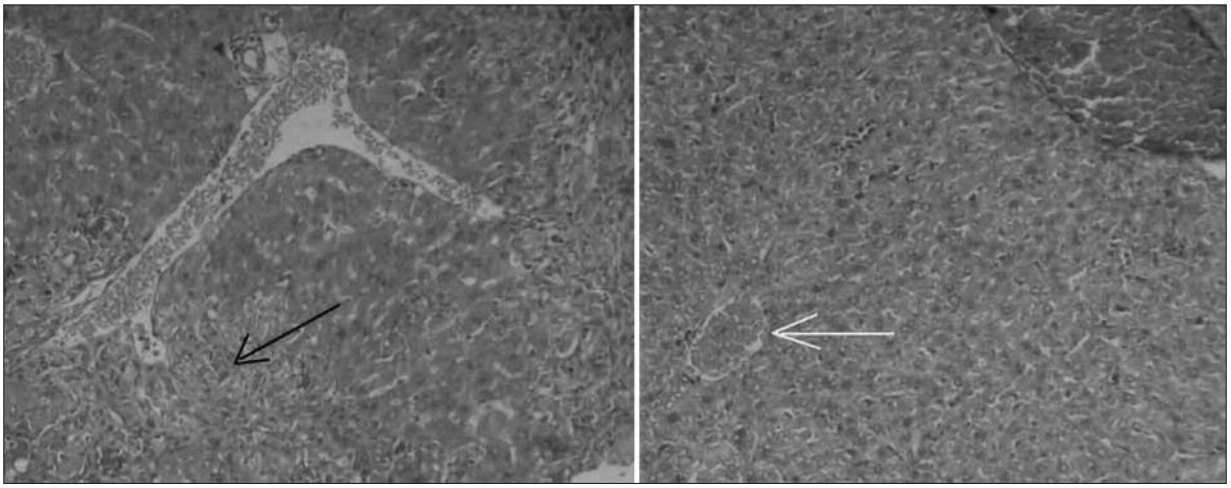

Figure 5. *In vivo* tail vein metastasis assay. The metastasis rate of normoxia pG1 group was 50% (3/6) and they were all occurred in liver. The metastatic lesions were small and exiguous (the number of liver metastatic lesions was 3-6 per mice) and no metastasis was discovered in other organs. After exposed in hypoxia, the pG1 cells exhibited increased metastasis rate, which was 100% ($p = 0.0022$, 6/6). The metastatic lesions were bigger and plentiful. The liver was macroscopically tuberculous. The structure of liver lobule and/or portal area was destroyed diffusely microscopically (the black arrow points the destroyed liver portal area). The left displays a normal liver and the right displays a metastatic liver (the white arrow points the normal liver portal area) (H&E, 400×).

reported that glioma cells are characterized by a positive feedback loop that involves pyruvic acid, lactic acid, and HIF-1 activation[22]. The analysis that involved RT-PCR and western blotting indicated that when HIF-1α expression was silenced, expression of PCNA decreased and the promotive effect of HIF-1α on cell proliferation disappeared.

In vitro, rates of growth of pG1 cells were statistically more rapid under conditions of hypoxia than normoxia. When HIF-1α expression was silenced by SiRNA, pG2 cells became vulnerable to hypoxia and grew slowly both under normoxia and hypoxia. The results of our MTT assays suggested that hypoxia and HIF-1α did indeed promote the proliferation of pancreatic cancer cells. *In vivo*, HIF-1α silenced pG2 cells cannot form tumor when they were cultured in hypoxia due to the impaired resistance to hypoxia. Even under normoxic treatment, the number of tumors derived from pG1 cells was more than pG2 cells. These results suggested irreplaceable role of HIF-1α on pancreatic cancer cell proliferation both under hypoxia and normoxia, particularly under hypoxia.

The regulation of apoptosis is another mechanism adopted by HIF-1α to enable tumor cells to adapt to hypoxic conditions[23]. Although activation of HIF-1 is usually an adaptive measure to ensure cell survival, it is likely that the induction of apoptosis by HIF-1 will differ among cardiac myocyte[24] and similarly to hypoxia, cobalt triggers the stabilization of HIF-1α, which is followed by increased expression of the pro-apoptotic factors Nip3 (nineteen KD interacting protein-3) and iNOS (inducible nitric oxide sinthase)[25]. To investigate the role of HIF-1 in the apoptosis of pancreatic cancer cells under hypoxic conditions, we cultured the pG1 and pG2 cells under either hypoxic or normoxic conditions, and then, propidium iodide and FITC were used to double-label the cells to assess the frequency of apoptosis. After HIF-1α expression was suppressed in pG2 cells, the resistance to hypoxia decreased and the extent of apoptosis increased. Furthermore, these changes were accompanied by a striking decrease in the expression of survivin, which is well-documented to be an anti-apoptotic factor and has been proposed to have unique therapeutic potential by virtue of its capacity for global inhibition of the development of cancer[26]. Although HIF-1α has been suggested to trigger apoptosis, it is regarded on the whole as a key factor responsible for conferring resistance to apoptosis[27]. Our *in vitro* data suggest that HIF-1α might cooperate with survivin to inhibit apoptosis under hypoxic conditions. Besides, the significantly increased mortality rate in hypoxia pG2 cells implied the apoptosis-independent mechanism via HIF-1α.

HIF-1α-induced increased cell proliferation and decreased cell apoptosis is the basis on which cancer metastasis can develop. Eventually, the cell migration capacity is the driving force to initiate cancer metastasis. The results of the transwell experiments indicated that the migration of pG1 cells was significantly higher under hypoxia than under normoxia, but there was no migration difference between hypoxia and normoxia pG2 cells. The migration is enhanced in pG1 cells by hypoxia and upregulated HIF-1α expression, whereas it was weakened in pG2 cells by inhibited HIF-1α expression. Immature vessel architecture and an enhanced migration under hypoxic conditions both contribute to the extremely poor prognosis that is usually associated with pancreatic cancer. *In vivo* data of tail vein metastasis model indicated that hypoxia significantly improved tumor metastasis of pancreatic cancer before HIF-1α silence, because the phenomenon was only observed in nude mouse injected with pG1 cells. What is more, there was no metastasis in nude mouse injected with pG2 cells. It might remind that inhibition of HIF-1α did completely eliminate metastasis and the molecule plays an indispensable role in pancreatic cancer metastasis. However, owing to the restricted number of the experiment, it is unclear whether metastasis was actually inhibited completely. This issue needs to be investigated further with more extensive approaches.

It should be emphasized that some of our findings are far different from those reported elsewhere. For instance, inhibition of HIF-1α under hypoxic conditions has also been reported to promote growth and metastases of subcutaneous tumors[28]. In the present study, microscopy revealed that the subcutaneous tumors obtained were characterized by a central necrotic area encompassed by an extensive apoptotic area, which was in turn circumscribed by a thin margin of pancreatic cancer cells (Figure 3B). The possibility that prolonged oxygen deficiency might promote the accumulation of proapoptotic molecules, such as Nip3, and drive cell death[30] may account for this phenomenon, although the signaling events responsible require further investigation. Molecules, such as HIF-

1α, were supposed to be released from the dead cells might act on the circumjacent cells of the marginal zone to endow the tumor with the capability to grow rapidly.

Conclusions

In the study reported herein, we investigated the specific effects of HIF-1α on proliferation, apoptosis and metastasis of pancreatic cancer cells. The eukaryotic expression vector, which can steadily express HIF-1α SiRNA, was used to silence HIF-1α expression. Both *in vitro* and *in vivo*, downregulation of HIF-1α in pG2 cells appeared to evidently restrain cell proliferation, angiogenesis, and metastasis, but raise apoptosis in hypoxic tissue. The results were dramatically reversed in pG1 cells yet. Our findings underscore the likelihood that HIF-1α is a compelling target for therapies to control pancreatic cancer metastasis and treat the disease. It is likely that realizing this goal will require greater insight into the roles of HIF-1α in regulating cellular behavior under hypoxic conditions.

Acknowledgements

All authors are very grateful to the staff of the Pathology Department of the Medical College at Huazhong Science and Technology University for their excellent technical assistance. The Authors also acknowledge Professor Chunyou Wang in Huazhong Science and Technology University for his kind assistance with the preparation of this manuscript.

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