Original Article

# The effect of HIF-1α on glucose metabolism, growth and apoptosis of pancreatic cancerous cells

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Objectives: The aim of this study is to explore the possible role of HIF-1α in glucose metabolism, proliferation and apoptosis of pancreatic cancerous cells. Method: The pancreatic cancerous BxPC-3 cells were cultured in normoxia or hypoxia (3% $O_2$), respectively. Cell proliferation was determined by MTT assay, apoptosis was determined by Annexin V/PI staining. Expression of Pyruvate dehydrogenase kinase (PDK1), Lactate dehydrogenase (LDHA), pyruvate kinase M2 (PKM2) and citrate synthase (CS) was determined by Western-blot and Real-time PCR. Results: Under hypoxia, the expression of HIF-1α and the lactate production were increased. The expression of glucose metabolic enzymes PDK1, LDHA, PKM2 was also increased compared with that under aerobic condition. Hypoxia treatment had little effect on expression of CS. Under hypoxia, knockdown of HIF-1α inhibited the production of lactate and the expression of PDK1, LDHA and PKM2. Knockdown of HIF-1α repressed the growth of pancreatic cancer BxPC-3 cells and induced apoptosis of the cells under hypoxia. Conclusion: Under hypoxia, the expression of HIF-1α is induced, leading to the increase of glycolysis in BxPC-3 cells possibly through upregulation of the enzymes related to glycolysis. HIF-1α knockdown can inhibit the prolife ratio and promote apoptosis of pancreatic cancerous BxPC-3 cells *in vitro*.

**Key Words: HIF-1α, RNA interfering, glycolysis, pancreatic cancer, hypoxia**

## INTRODUCTION

Pancreatic cancer is a common malignant tumor in the digestive system, with high incidence and mortality worldwide.[1,2] Though in the past decades there is great improvement in terms of basic and clinical research on pancreatic cancer which plays a very important role in improving the efficacy for pancreatic cancer, the overall treatment of pancreatic cancer is not satisfactory. Therefore, the study for onset and development of pancreatic cancer is very important. Meanwhile, how to kill the malignant cells without affecting normal cells is becoming a hot topic for research.

Hypoxia is an important characteristic of microenvironment of tumor.[3] Under hypoxia, the biological characteristic of the tumor cells change significantly. On one hand, the growth of tumor cells are suppressed under hypoxia and some may even undergo apoptosis. And on the other hand, the tumor cells can adapt to the low oxygen microenvironment which may increase the malignancy of tumor. Many studies have showed that hypoxia is one factor for poor prognosis of malignant solid tumors.[4,5]

Hypoxia inducible factor-1α (HIF-1α) plays a very important role in the adaptation of tumor cells to hypoxia, and is the most critical transcription factor mediating cell hypoxia reaction. A number of studies have confirmed that HIF-1 activity is the determining factor for tumor development, and is related to invasion, metastasis and prognosis.[6-9]

Warburg and co-workers showed that, under aerobic conditions, tumor tissues metabolize approximately ten-fold more glucose to lactate in a given time than normal tissues, a phenomenon known as the Warburg effect. Though many studies have been performed since "Warburg effect" was proposed, the underlying mechanism remains unclear. It is found that HIF-1 plays an important role in glucose metabolism of tumor cells. As a master transcription factor, HIF-1α regulates transcription of many genes involved in glycolysis. The augmentation of glycolysis not only makes tumor cells adapted to the unfavorable environment, but also decreases the damage of reactive oxygen species (ROS) on cell DNA during aerobic metabolism. The augmentation of glycolysis increases the intake of glucose and the synthesis of lactate, changes the microenvironment of tumor, and promotes the invasion and metastasis of the tumor cells.

The role of HIF-1α in glucose metabolism in pancreatic cancer cells is not fully understood. In this study, we investigated the possible role of HIF-1 in pancreatic cancerous BxPC-3 cells by means of RNAi technique. We determined the effect of HIF-1α on the expression of the

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enzymes related to glucose metabolism and synthesis of lactate under aerobic and hypoxic conditions. Moreover, we determined its effects on proliferation, apoptosis and invasion of BxPC-3 cells and explored the possible mechanisms underlain.

## MATERIALS AND METHODS

***Cell culture***

Pancreatic cancerous BxPC-3 cells were obtained from the cell bank of Chinese Academic Institute, and cultured in Dulbecco's Modified Eagle Medium (DMEM) (Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal calf serum (GIBCO, Eggenstein, Germany), 10 units/ml penicillin-G, and 10mg/ml streptomycin. All groups of cells were cultured under normoxic or hypoxic conditions for 24h, respectively. For normoxic condition, cells were incubated at 37˚C in 5% $CO_2$ humidified air. For hypoxic treatment, cell culture dishes were placed into a hypoxia incubator (Kendro Laboratory Products, Newtown, CT, USA) at 3% oxygen and 5% $CO_2$ concentrations. The ethics committee of Zhongshan Hospital, Shanghai approved the protocol.

***shRNA and lentiviral plasmids construction***

The short hairpin RNA (shRNA) cassette against HIF-1α was 5'-CGCGTCCCCAAAGGACAAGTCACCACAG GATTCAAGAGATCCTGTGGTGACTTGTCCTTTTTT TTGGAAAT-3'. shHIF-1α lentivirus was generated by transfection of HEK293T cells with transducing vector and packaging vectors. After 48 hr, lentivirus particles in the medium were harvested and used for infection of BxPC-3 cells. The lentivirus that only expresses Green Fluorescent Protein (GFP) was used as a control.

***Real-Time PCR***

Cells were incubated in normoxia or hypoxia (3% $O_2$) for 24 h, and RNA was extracted using TRIZOL (Invitrogen). Reverse transcription reaction was performed using the First Strand Synthesis kit (Invitrogen). Real-time PCR was performed with ABI Prism 7900 Sequence Detection System (PE Applied Biosystems, Foster City, CA) using SYBR Green reporter dye (Invitrogen). Primer sequences are listed in Table 1 and β-actin was used for normalization. Relative expression was determined using the Ct method [Gibson U.E., Heid C.A., Williams P.M.. A novel method for real time quantitative RT-PCR, Genome Res. 6 (1996) 995–1001.] that calculates relative expression through the equation: fold value = $2^{-[\Delta\Delta Ct]}$, where $\Delta\Delta Ct = \Delta Ct$ sample - $\Delta Ct$ calibrator; $\Delta Ct$ = Ct gene of interest - Ct β-actin.

***Western blotting***

Treated cells were harvested and dissolved in RIPA buffer containing protease inhibitors. Proteins extracted from BxPC-3 cells were resolved in 10% SDS-PAGE, and blotted to PVDF membrane. Blots were probed with antibody for HIF-1α, PDK1, LDHA, PKM2, CS (all from Abcam, Cambridge, MA, USA) and for β-actin (Santa Cruz, Santa Cruz, CA). The antibodies were used at a dilution of 1:1000. Densitometric analysis was performed using ImageJ software.

***Lactate assay***

Total cell lysates were harvested and the concentration of lactate was measured by lactate kit (BioVision, Milpitas, CA, USA) according to the manufacturer's instructions.

***MTT assay***

The cells were grown in a 96-well plate (the initial cell number is $4\times10^3$/well). MTT assay was performed in the next five days. Optical density (OD) value at 570nm was obtained using ELISA reader. Growth curve was drawn using the mean value of OD every day.

***Apoptosis assay***

The cells were harvested and stained with Annexin V-FITC and propidium iodide (PI) using the annexin V-FITC apoptosis detection kit (B.D. Biosciences Pharmingen, San Jose, CA USA). The stained cells were then quantified by flow cytometry.

***Statistical analysis***

All data were presented by mean±SD, using SPSS 17.0 software for statistical analysis and one-way ANOVA and chi-square test to compare the statistical difference among groups. A *p-value of* <0.05 is regarded as statistically significant.

## RESULTS

***The expression of HIF-1α of BxPC-3 cells under normoxia and hypoxic conditions***

As shown in Figure 1, the expression of HIF-1α mRNA and protein were significantly increased in hypoxia compared to those in normoxia. Infection of the cells with shHIF-1α virus markedly decreased the HIF-1α mRNA level in both normoxia and hypoxia, and reduced the HIF-1α protein level in hypoxia.

***HIF-1α knockdown decreases the lactate production in hypoxia***

Lactate is an important product of glycolysis. This biochemical reaction is the main source of energy for cancer cells. In BxPC-3 cells cultured under hypoxic condition, lactate production increased significantly, compared with that under aerobic condition (Figure 2). Knockdown of HIF-1α had little effect on lactate production by BxPC-3

**Table 1.** Primer sequences

| Gene | Forward Primer (5'–3') | Reverse Primer (5'–3') |
|---|---|---|
| HIF-1α | cgtgttatctgtcgctttgagtc | atgtagtagctgcatgatcgtct |
| PDK1 | aaatcaccaggacagccaataca | cctcctcggtcactcatcttcac |
| LDHA | gattcagcccgattccgttacct | caccagcaacattcattccactcca |
| PKM2 | ctgtggacttgcctgctgtg | tgccttgcggatgaatgacg |
| CS | tgaagggattggtctatgaaa | tctgttgggatatgtccagtta |
| β-actin | accaactgggacgacatggagaaa | tagcacagcctggatagcaacgta |

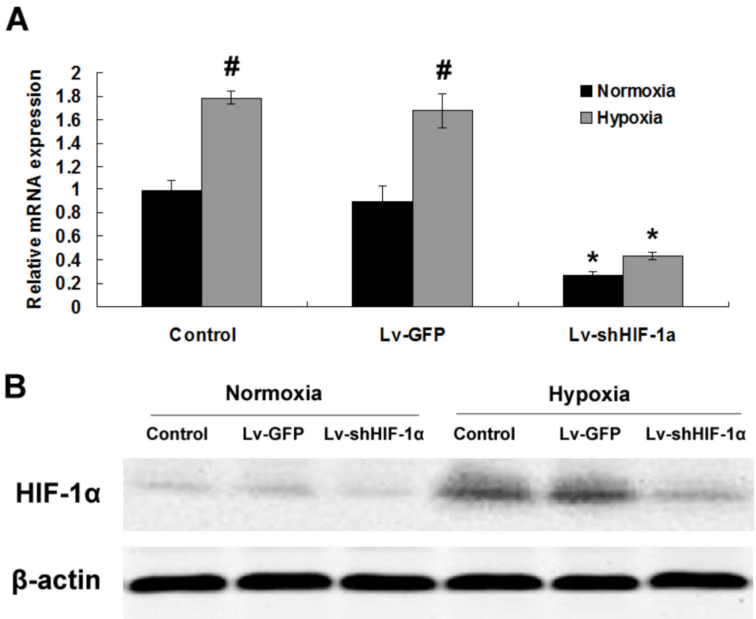


**Figure 1.** The expression of HIF-1α of BxPC-3 cells under normoxia and hypoxic conditions. A, mRNA expression of HIF-1α. The expression of HIF-1α mRNA significantly increased in hypoxia compared to those in normoxia. B, protein expression of HIF-1α. The expression of HIF-1α protein was significantly increased in hypoxia compared to those in normoxia. Infection of the cells with shHIF-1α virus markedly decreased HIF-1α mRNA concentrations in both normoxia and hypoxia, and reduced HIF-1α protein concentrations in hypoxia. * $p<0.05$ vs control; # $p<0.05$ vs normoxia.

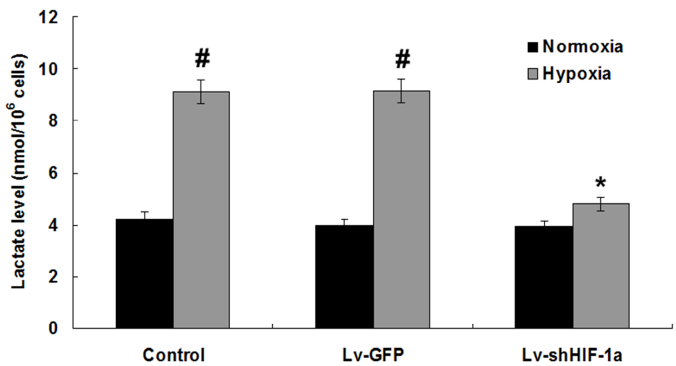


**Figure 2.** HIF-1α knockdown decreases the lactate production of BxPC-3 cells in hypoxia. Under hypoxic condition, lactate production increased significantly, compared with that under aerobic conditions. Knockdown of HIF-1α decreased the lactate production of BxPC-3 cells in hypoxia.* $p<0.05$ vs control; # $p<0.05$ vs normoxia.

cells in normoxia. However, inhibition of expression of HIF-1α repressed the production of lactate significantly in hypoxia. This result implies that HIF-1α can regulate energy metabolism of BxPC-3 cells under hypoxia.

***HIF-1α regulates the expressions of Pyruvate dehydrogenase kinase (PDK1), Lactate dehydrogenase (LDHA), pyruvate kinase M2 (PKM2) and citrate synthase (CS)***

To investigate how HIF-1α regulate energy metabolism of BxPC-3 cells, we detected the expression of some enzymes in glucose metabolism. Under hypoxic condition, the mRNA and protein concentrations of LDHA and PKM2 increased (Figure 3B, C and E). The expression of PDK1, a kinase inactivating pyruvate dehydrogenase (PDH), also increased in BxPC-3 cells under hypoxia (Figure 3A and E), indicating that the tricarboxylic acid cycle (TCA cycle) was inhibited. Knockdown of HIF-1α prevented the increase of expression of LDHA, PKM2 and PDK1 in hypoxia (Figure 3). The results suggest that the expression changes of LDHA, PKM2 and PDK1 in hypoxia are mediated by HIF-1α. As a key enzyme in TCA cycle, CS converts acetyl coenzyme A into citric acid. Hypoxia treatment or HIF-1α knockdown had little effect on expression of CS.

***Knockdown of HIF-1α influences proliferation and survival of BxPC-3 cells***

Under normoxia, knockdown of HIF-1α had little effect on proliferation of the cells (Figure 4A). Proliferation of BxPC-3 cells was inhibited under hypoxia and inhibition of HIF-1α further prevented the proliferation of the cells (Figure 4A). Moreover, we found that knockdown of HIF-1α increased apoptosis of the cells in hypoxia (Figure 4B).

**DISCUSSION**

The growth of malignant tumor cells depends upon oxygen and the nutrients. Abnormal proliferation and overgrowth of the tumor induce hypoxia inside the tumor. HIF-1 is a heterodimeric protein, composed of HIF-1α

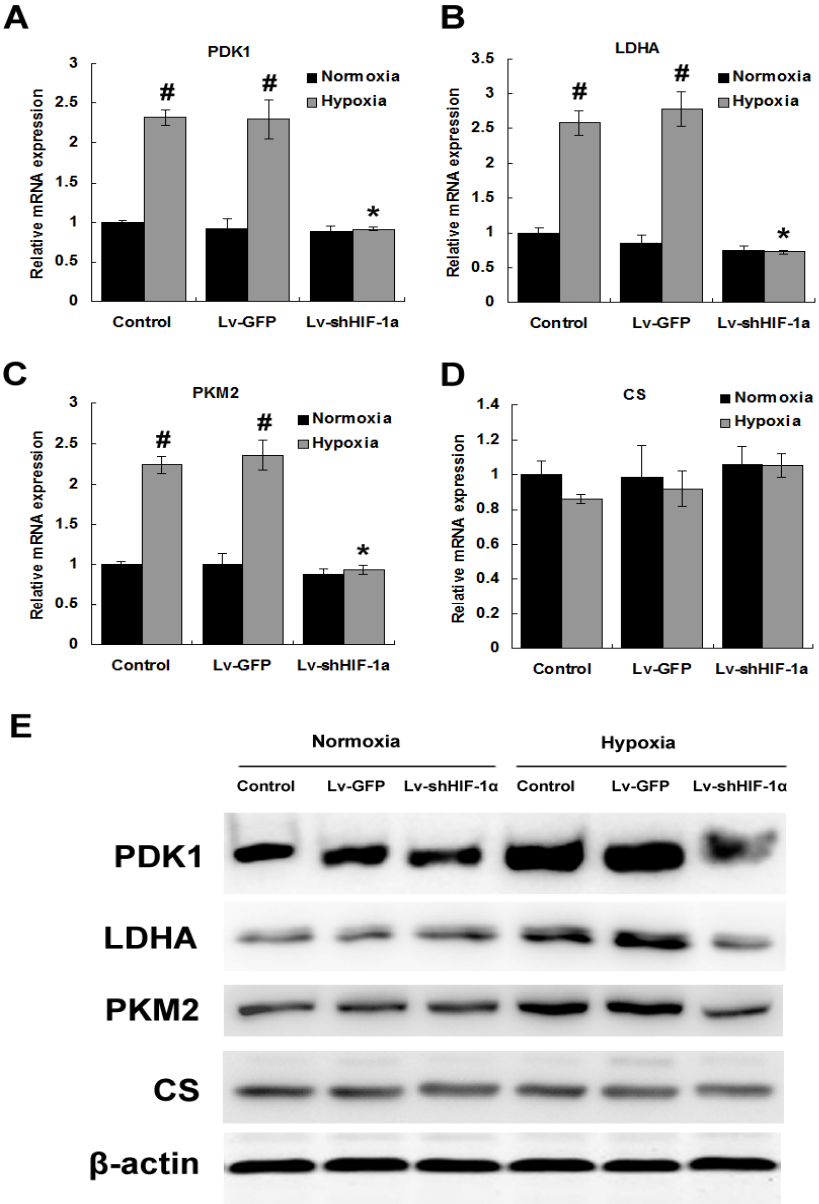


**Figure 3.** HIF-1α regulates the expressions of PDK1, LDHA, PKM2 and CS. A, the mRNA concentrations of PDK1, LDHA, PKM2 and CS. Under hypoxic conditions, mRNA concentrations of PDK1, LDHA and PKM2 increased compared with that under aerobic conditions. Knockdown of HIF-1α decreased mRNA concentrations of LDHA, PKM2 and PDK1 in hypoxia. B, protein concentrations of PDK1, LDHA, PKM2 and CS. Under hypoxic conditions, protein concentrations of PDK1, LDHA and PKM2 increased compared with that under aerobic conditions. Knockdown of HIF-1α decreased the protein concentrations of LDHA, PKM2 and PDK1 in hypoxia.* $p<0.05$ vs control; # $p<0.05$ vs normoxia.

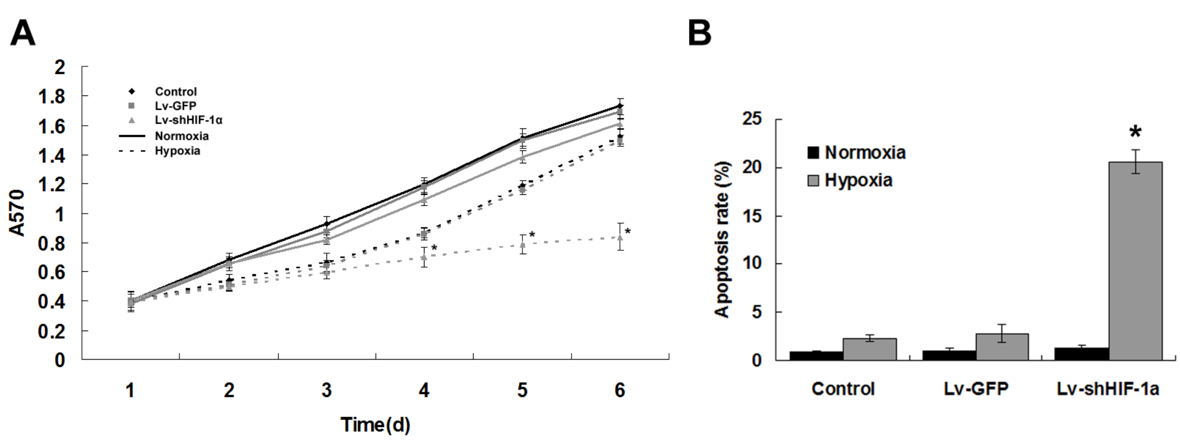


**Figure 4.** HIF-1α knockdown inhibits cell proliferation and increases apoptosis. A, Cell proliferation was determined by MTT assay. Proliferation of BxPC-3 cells was inhibited under hypoxia and inhibition of HIF-1α further prevented the proliferation of the cells. B, Cell apoptosis. Knockdown of HIF-1α increased apoptosis of the cells in hypoxia. * $p<0.05$ vs control.

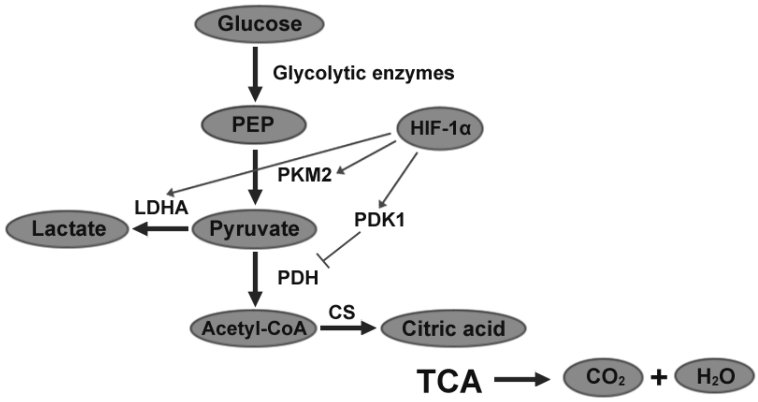


**Figure 5.** A model indicating the process by which HIF-1α regulates glucose metabolism. HIF-1α upregulates the expression of PKM2 and LDHA, which guides the direction of glucose metabolism to glycolytic. Meanwhile, HIF-1α inhibits pyruvate dehydrogenase (PDH) by upregulating PDK1, which prevent the product of acetyl coenzyme A (Acetyl-CoA) and the metabolism of glucose in tricarboxylic acid cycle (TCA cycle). Arrow indicates up-regulation; blocked line indicates inhibition.

and HIF-1β subunits.[10,11] HIF-1α is one of the most critical transcription factors mediating hypoxic response. HIF-1α concentrations increase dramatically as $O_2$ concentration declines. Under normoxic conditions, HIF-1α is subjected to ubiquitination and proteasomal degradation due to the binding of the von Hippel-Lindau (VHL) tumor suppressor protein,[12-14] which is the substrate recognition subunit of an E3 ubiquitin-protein ligase. VHL binds to HIF-1α only when the latter is hydroxylated on proline residue 402 and/or 564.[15,16] The hydroxylation reaction is performed by prolyl hydroxylases (PHDs). Under hypoxic conditions, hydroxylation, ubiquitination and degradation are inhibited, leading to the accumulation of HIF-1α. HIF-1α regulates the expression concentrations of more than 200 kinds of target hypoxia responsive genes (HRGs) and plays an important role in tumor cells energy metabolism.[17,18] It can induce glucose transporters GLUT1 and 3, hexokinase 2, 6-phosphofructokinase, and enolase 1 and increase the intake of glucose and the synthesis of lactate.[19-21] HIF-1 can induce expression of pyruvate dehydrogenase kinase 1 (PDK1), pyruvate kinase M2.[22] These enzymes are key enzymes in the process of glucose to pyruvate. In addition, HIF-1 is also a regulator of expression of lactate dehydrogenase A and monocarborxylat transporter (MCT4). All these results suggest that HIF-1 plays a critical role in glycolysis, in cancer cells.

In this study, we determined the effects of HIF-1 on expression of PDK1, LDHA, PKM2, CS and glucose metabolite lactate in pancreatic cancerous BxPC-3 cells under aerobic and hypoxic conditions. We found that the expression of glycolysis-related enzymes PDK1, LDHA and PKM2 was upregulated under hypoxic condition compared with that under aerobic condition ($p<0.05$). There is no difference in the expression of CS between aerobic and hypoxic treatments. This suggests that, under hypoxic condition, the glycolysis of tumor cells increased and the tricarboxylic acid cycle didn't change. When HIF-1α was knockdown, the formation of lactate reduced significantly, and the expression of PDK1, LDHA and PKM2 was inhibited under hypoxic condition compared with control groups ($p<0.05$). Under aerobic condition, however, HIF-1α knockdown had little effect on these. This is due to the hypoxia-induced HIF-1α that promotes the expression of PDK1, LDHA and PKM2 and increases glycolysis. Once HIF-1α was knockdown, the expression of PDK1, LDHA and PKM2 was blocked, leading to repression of glycolysis. HIF-1α knockdown has little effect on expression of CS under aerobic or hypoxic condition (Figure 5). Under hypoxia, HIF-1α is activated which upregulates the expression of the glycolysis enzymes and glycolysis of BxPC-3 cells. This may affect the biological characteristics of BxPC-3 cells.

HIF-1 plays an important role in the development of tumor, making it an ideal target for tumor therapy. The personalized therapy of tumor based on HIF-1 has not been used in clinical therapy, but this potential treatment strategy has been put into laboratory research and clinical trials.[5,23,24] Our study with pancreatic cancer cells provides more evidence that HIF-1 plays a critical role in the metabolism of cancer cells and HIF-1α is a potential cancer therapy. Further study should focus on exploring the mechanism of HIF-1 regulating "Warburg effect" in basic and clinic concentrations, and searching HIF-1 inhibitor with efficacy, safety and specificity.[25]

## ACKNOWLEDGEMENTS
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## AUTHOR DISCLOSURES
The authors declare that there are no conflicts of interest.

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# The effect of HIF-1α on glucose metabolism, growth and apoptosis of pancreatic cancerous cells

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# 探讨 HIF-1α 基因对胰腺癌细胞之葡萄糖代谢、生长和凋亡的影响

目的：探讨 HIF-1α 基因沉默对胰腺癌细胞生长和凋亡的影响；探讨 HIF－1α 对胰腺癌细胞糖代谢的调控作用。方法：胰腺癌细胞分别在有氧及缺氧($3\%O_2$)条件下培养。利用 MTT 实验绘制胰腺癌细胞生长曲线，流式细胞仪检测细胞凋亡；利用乳酸试剂盒测定乳酸含量；利用定量 PCR 和 Western Blot 方法测定丙酮酸脱氢酶激酶 1(PDK1)、乳酸脱氢酶 A(LDHA)、丙酮酸激酶 M2(PKM2)和柠檬酸合成酶(CS)的表达，观察 HIF-1α 沉默前后相关检测指标的变化。结果：缺氧条件下，HIF-1α mRNA 和蛋白的表达均明显升高；乳酸的生成、糖代谢酶 PDK1、LDHA、PKM2 mRNA 和蛋白的表达均明显升高，同有氧条件下比较，差别有统计学意义；CS mRNA 和蛋白的表达同有氧条件下比较，差别无统计学意义。缺氧条件下，HIF-1α 基因沉默后，乳酸的生成、糖代谢酶 PDK1、LDHA、PKM2 mRNA 和蛋白的表达均受到明显抑制，与空载体组比较，差别有统计学意义；CS mRNA 和蛋白的表达没有受到明显影响，与空载体组比较，差别无统计学意义。缺氧条件下，HIF-1α 基因沉默后，胰腺癌 BxPC-3 细胞的生长受到抑制，凋亡率升高，同空载体组比较，差别有统计学意义。结论：在缺氧条件下，HIF-1α 的表达明显增强，它可能通过上调其下游糖酵解相关酶的表达，增强胰腺癌细胞的糖酵解代谢；HIF-1α 沉默在体外能抑制胰腺癌 BxPC-3 细胞的生长，使其凋亡率升高。

**關鍵字： HIF-1α、 RNA干擾、糖酵解、胰腺癌、缺氧**