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Full Length Research Paper

Hypoxia-inducible factor -1α in prostate cancer: Expression and significance

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To study the expression and significance of hypoxia-inducible factor -1 alpha (HIF-1α) in prostate cancer, successfully established recombinant expression plasmids pcDNA3.1-HIF-1α and pll3.7-siHIF-1α were transfected into PC-3 prostate cancer cells using Fermentas transfection reagents. Stable HIF-1α expression cell lines were selected using G418 selective culture. Protein and mRNA expressions of HIF-1α, MMP-2 and MMP-9 were assayed by Western blot and RT-PCR. Cell growth was evaluated using the MTT assay growth curve. The ability of invasion of prostate cancer cells was assayed in Transwell chambers. Compared to other four groups, HIF-1α mRNA expression was not obviously upregulated in pcDNA3.1-HIF-1α-PC-3 cells, while it was obviously downregulated in pll3.7-siHIF-1α-PC-3 cells. MMP-2 and MMP-9 mRNA were significantly upregulated in pcDNA3.1-HIF-1α-PC-3 cells. HIF-1α, MMP-2 and MMP-9 protein were upregulated in pcDNA3.1-HIF-1α-PC-3 cells by Western blot, while they were downregulated in pll3.7-siHIF-1α-PC-3 cells. The abilities of proliferation and invasion of prostate cancer cells were statistically significantly enhanced in pcDNA3.1-HIF-1α-PC-3 cells (*P*□0.05). Proliferation and invasion of PC-3 prostate cancer cells are promoted by HIF-1α, which provides a foundation for the research on HIF-1α in the pathogenesis of prostate cancer and suggests that HIF-1α gene may be a good target for anti-tumor therapy.

Key words: Hypoxia-inducible factor-1alpha, prostate cancer, expression, significance.

INTRODUCTION

Tumor cells have a strong ability of proliferation, so anoxia occurs in tumor proliferation. Tumor cells have a series of protective response to adapt to oxygen deficit environment such as increased activity of glycolytic enzymes and strong expression of vascular endothelial growth factor (VEGF) to promote neovascularization and resiliency to anoxia and thus achieve survival and development chance. Hypoxia-inducible factor-1alpha (HIF-1α) plays a major role in this process. It is a major transcription factor in anoxia response of tumor cells that is highly expressed in solid tumors (Brahimi-Horn and Pouyssegur, 2006). Additionally, there is indication that HIF-1α regulates transcription and translation of downstream target genes relating to tumor invasion, migration and proliferation (Krishnamachary et al., 2003). In this study, the successfully constructed HIF-1α over expression plasmid pcDNA3.1-HIF-1α and interference vector pll3.7-siHIF-1α were transfected into prostate cancer cells PC-3 using Fermentas transfection reagents. After cell lines expressing HIF-1α were screened, matrix metalloproteinase (MMP-2, MMP-9) expression was detected in order to identify the effect of HIF-1α on invasion of human prostate cancer cells *in vitro* and to investigate its relation with the pathogenesis of prostate cancer.

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MATERIALS AND METHODS

Recombinant expression plasmids pcDNA3.1-HIF-1α and pll3.7-siHIF-1α were established by our research group. PC-3 human prostate cancer cells and *Escherichia coli* DH5α were obtained from the School of Life Sciences of Xiamen University (Xiamen, Fujian, China).

Enzymes and reagents

Rabbit anti-human HIF-1α antibody and mouse anti-human MMP-2

and MMP-9 antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, California, USA). Goat anti-mouse and rabbit anti-human IgG secondary antibodies were obtained from Boster (Wuhan, Hubei, China). Ferments transfection reagents were purchased from Fermentas (Burlington, Ontario, Canada). Total RNA extraction kit, Gibco 1640 fetal bovine serum (FBS) and Gibco 1640 culture medium were purchased from Invitrogen (Carlsbad, California, USA). RT-PCR kit was obtained from Takara Bio (Otsu, Japan). ECL Western Blotting Substrate Kit was purchased from Thermo Fisher Scientific (Rockford, Illinois, USA). Transwell chambers were obtained from Corning Life Sciences (Corning, New York, USA). All experiment reagents were imported or domestic analytical reagents.

Transfection of pcDNA3.1-HIF-1α and pll3.7-siHIF-1α, and selection of stable HIF-1α expression cell lines

Recombinant expression plasmids pcDNA3.1-HIF-1α and pll3.7-siHIF-1α were extracted, purified and transfected into PC-3 prostate cancer cells using Fermentas transfection reagents as per manufacturer's instructions. After prostate cancer cells were cultured for 24 h, the transfection efficiency was observed. When the transfection efficiency reached 50 to 60%, prostate cancer cells were subcultured in a 10 cm plate. After prostate cancer cells confluence reached 80 to 90%, the culturing solution was discarded and the cells were rinsed in PBS and added with 400 μg/ml G418 selective culture. According to the color of culture medium and cell growth, selective culture was changed every 4 days. After 14 days, resistant clones appeared. Monoclonal clone was selected later to prepare cell suspension that was diluted using culture medium to 1 cell/10 μL. 150 μL culture medium and 10 μL cell suspension were added to each well of a 96-well plate. When the wells were gradually filled, the cell suspension was transferred to a 48, 24, 12 and 6-well plate for further culturing. G418 selective culture was added for cell selection. Prostate cancer cells obtained were cultured for proliferation, and renamed as pcDNA3.1-HIF-1α-PC-3, pcDNA3.1-PC-3, pll3.7-siHIF-1α-PC-3 and pll3.7-PC-3 cells.

Assay of proliferation and invasion of cells

Cell culturing

PC-3, pcDNA3.1-HIF-1α-PC-3, pcDNA3.1-PC-3, pll3.7-siHIF-1α-PC-3 and pll3.7-PC-3 cells were cultured using Gibco 1640 culture medium containing 10% FBS and 1% double-antibodies. When the cells were placed in the plate, the cell density was adjusted to 2×10^5/ml, and inoculated into 12-well plates in four groups (Groups I to IV) each including PC-3, pcDNA3.1-HIF-1α-PC-3, pcDNA3.1-PC-3, pll3.7-siHIF-1α-PC-3 and pll3.7-PC-3 cells. Each well was added with 150 μmol/L $CoCl_2$ (simulating the anoxia environment inside the tumor). The plates were finally put in a culturing box with 37°C and 5% CO_2.

HIF-1α protein, MMP-2 protein and MMP-9 protein expressions by Western blot

The aforementioned cells were cultured until logarithmic growth phase. Prostate cancer cells in Group I were collected and lysised rapidly to extract total cell protein. Following quantification by the bicinchoninic acid assay (BCA), 60 μg/well was loaded to undergo 10% SDS-polyacrylamide gel electrophoresis. Protein products were transferred to membranes and added with 5% milk for blocking at room temperature for 1 h. Antibodies against HIF-1α (1:1000), MMP-2 (1:200), MMP-9(1:500) and β-actin (1:1000) were added for hybridization. Primary antibody was added for placement at rocking bed at 4°C overnight. Membranes were rinsed for 10 min x 3. Then secondary antibody was added for placement at rocking bed at 4°C overnight. Membrane rinsing was repeated for 10 min x 3. ECL Western blotting substrate kit was used for coloration and exposure.

mRNA expressions of HIF-1α, MMP-2 and MMP-9 by RT-PCR

The aforementioned cells were cultured until logarithmic growth phase. Prostate cancer cells in Group II were collected and lysised using the Trizol method to extract total RNA. The total RNA was reversely transcripted to cDNA. PCR amplification was performed for the 3'-non-translation region of targeted genes. Primers used included: HIF-1α sense 5'- CAAAACACACAGCGAAGC-3', antisense 5'-TCAACCCAGACATATCCACC-3′; MMP-2 sense 5'-GCTGCGCTTTTCTCGAATC-3′, antisense 5'-TGAGAATCTCCCCCAACAC -3′; MMP-9 sense 5'-TCAGGGAGACGCCCATTTC -3′, antisense 5'-CGGTCGTCGGTGTCGTAGTT -3′. Amplification products underwent 2% agar electrophoresis and were scanned for gray values. The ratio of RT-PCR products was calculated as [HIF-1α (MMP) mRNA gray value/internal inference GAPDH mRNA gray value] × 100%.

Effect of HIF-1α on growth of prostate cancer cells by the MTT assay

The aforementioned cells were cultured until logarithmic growth phase. Prostate cancer cells in Group III were collected and inoculated into a 96-well plate. Five parallel wells were designated for each type of prostate cancer cells. Each well was added with 150 μmol/L $CoCl_2$ for culturing for 24, 48, 72 and 96 h. 20 μl MTT was added for culturing at 37°C for 4 h. Supernatants in each well were discarded and 150 μl DMSO was added for shaking for 10 min. The absorption value at 595 nm [D(595)] was determined using ELISA reader. The values were averaged for each type of prostate cancer cells to plot the cell growth curve.

Ability of invasion of prostate cancer cells in Transwell chambers

Matrigel matrix was dissolved at 4°C to be placed overnight and diluted at 1:4 using precooled Gibco 1640 culture medium. 100 μg solution was added to the upper chamber of Transwell and serum-free culture medium containing fibronectin was added to the lower chamber of Transwell. The Transwell chambers were incubated in the culturing box at 37°C for 4 to 5 h for gelling. After gel matrix was hydrolyzed using serum-free Gibco 1640 culture medium, Transwell chambers were placed at room temperature for 2 h. Prostate cancer cells in Group IV were inoculated in the 12-well plate of Transwell. Two parallel wells were designated for each type of prostate cancer cells. Each well was added with 150 μmol/L $CoCl_2$ to be incubated in a culturing box at 37°C for 24 h. Gel matrix and cells that did not penetrate the polycarbonate membrane was erased using the cotton swab. 70% methanol was added to the lower chamber for fixation for 30 min. Prostate cancer cells were stained using 0.1% crystal violet and rinsed in PBS twice. The cells penetrating the polycarbonate membrane was calculated under the microscope. The number of cells penetrating the membrane indicated the ability of cell invasion.

Statistical analysis

The experiments aforementioned were repeated 3 times. Results

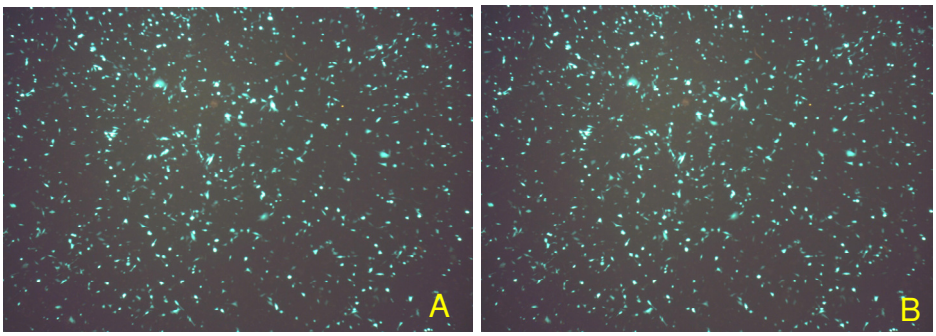

Figure 1. Expression of green fluorescent protein at 48 h after plasmids were transfected into PC-3 prostate cancer cells. A: pcDNA3.1-HIF-1α-PC-3 and B: pll3.7-siHIF-1α-PC-3.

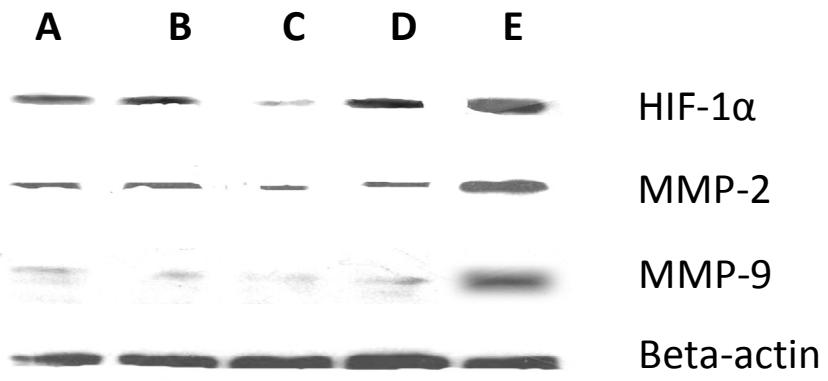

Figure 2. Protein expressions of HIF-1α, MMP-2 and MMP-9 in five types of prostate cancer cells by Western blot. A: PC-3, B: pll3.7-PC-3, C: pll3.7-siHIF-1α-PC-3, D: pcDNA3.1-PC-3 and E: pcDNA3.1-HIF-1α-PC-3.

were analyzed by SPSS11.5 statistical package and data were expressed by mean ± standard deviation (x ± s). Cell proliferation data were analyzed by single factor analysis of variance (One-way ANOVA), the number of cell invasion was analyzed by t test and a statistically significant difference was considered if P was <0.05.

RESULTS

Transfection efficiency of prostate cancer cells

Identified pcDNA3.1-HIF-1α and pll3.7-siHIF-1α plasmids were transfected into prostate cancer cells. Tranfection efficiency of prostate cancer cells was evaluated at 48 h (Figure 1).

Protein expressions of HIF-1α, MMP-2 and MMP-9

HIF-1α, MMP-2 and MMP-9 protein expressions were detected using Western blot in PC-3, pcDNA3.1-PC-3, pcDNA3.1-HIF-1α-PC-3, pll3.7-PC-3 and pll3.7-siHIF-1α-PC-3 cells. There was no statistically significant difference in HIF-1α, MMP-2, and MMP-9 protein expressions between PC-3, pcDNA3.1-PC-3 and pll3.7-PC-3 cells. HIF-1α, MMP-2 and MMP-9 proteins were upregulated in pcDNA3.1-HIF-1α-PC-3 cells compared to other three types of prostate cancer cells. These proteins were down-regulated in pll3.7-siHIF-1α-PC-3 cells compared to other types with empty plasmids (P□0.05) (Figure 2).

mRNA expressions of HIF-1α, MMP-2 and MMP-9

mRNA expressions of HIF-1α, MMP-2, and MMP-9 were detected using RT-PCR in PC-3, pcDNA3.1-PC-3, pcDNA3.1-HIF-1α-PC-3, pll3.7-PC-3 and pll3.7-siHIF-1α-PC-3 cells. There was no statistically significant difference in HIF-1α mRNA, MMP-2 mRNA, and MMP-9 mRNA expressions between PC-3, pcDNA3.1-PC-3 and pll3.7-PC-3 cells. HIF-1α mRNA was not significantly expressed in pcDNA3.1-HIF-1α-PC-3 cells. These mRNAs were downregulated in pll3.7-siHIF-1α-PC-3 cells compared to other four types of prostate cancer cells (P<0.01) (Figure 3).

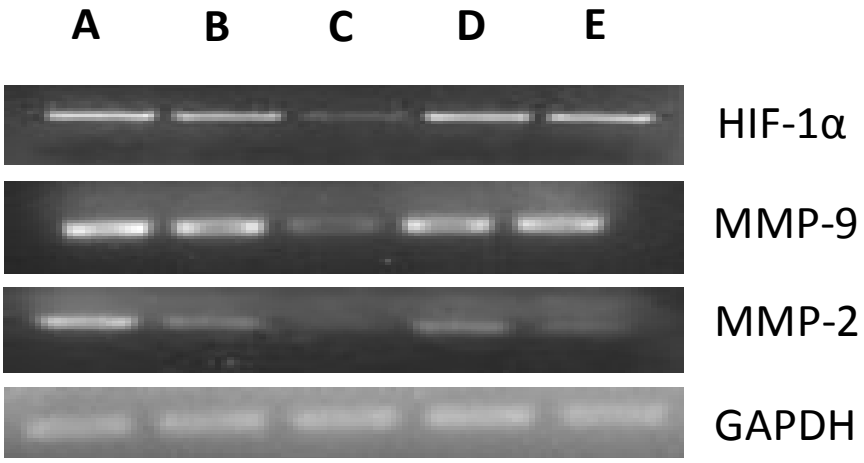

Figure 3. mRNA expressions of HIF-1α, MMP-2 and MMP-9 in five types of prostate cancer cells. A: pcDNA3.1-HIF-1α-PC-3, B: pcDNA3.1-PC-3, C: pll3.7-siHIF-1α-PC-3, D: pll3.7-PC-3 and E: PC-3.

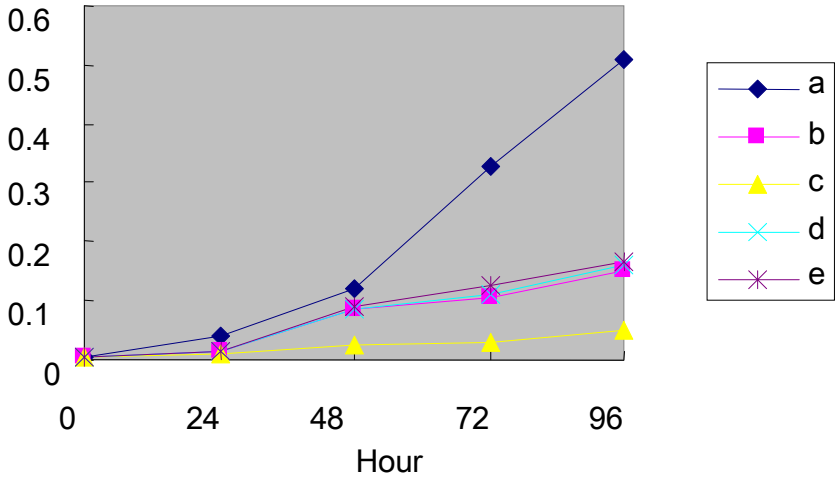

Figure 4. Growth curve of five types of prostate cancer cells. a: pcDNA3.1-HIF-1α-PC-3, b: pcDNA3.1-PC-3, c: pll3.7-siHIF-1α-PC-3, d: pll3.7-PC-3 and e: PC-3.

MTT assay and cell count

The prostate cancer cells were cultured and observed for 5 days. Average D (595) and cell count for five parallel wells at different time points for five types of prostate cancer cells were used to plot cell growth curve and for cell numeration. There was no statistically significant difference in cell growth and cell count between PC-3, pcDNA3.1-PC-3 and pll3.7-PC-3 cells. Compared to PC-3, pcDNA3.1-PC-3 and pll3.7-PC-3 cells, cell proliferation increased evidently in pcDNA3.1-HIF-1α-PC-3 cells, the effect was particularly significant at 72 h. Prostate cancer cell proliferation was inhibited in pll3.7-siHIF-1α-PC-3 cells and the proliferation difference gradually increased as time elapsed, compared to these three types of prostate cancer cells (P□0.01). Cell proliferation was faster in pcDNA3.1-HIF-1α-PC-3 cells than PC-3, pcDNA3.1-PC-3 and pll3.7-PC-3 cells (Figure 4).

Invasion ability of prostate cancer cells

The number of cells was calculated and averaged for each type of prostate cells using Transwell assay. The number of cells penetrating the membrane was 149.5□7.0, 48.25□4.57, 9.5□1.73, 61.25□6.07, and 63.5□4.2 for pcDNA3.1-HIF-1α-PC-3, pcDNA3.1-PC-3, pll3.7-siHIF-1α-PC-3, pll3.7-PC-3 and PC-3 cells respectively. No statistically significant difference was noted in the number of cells penetrating the membrane

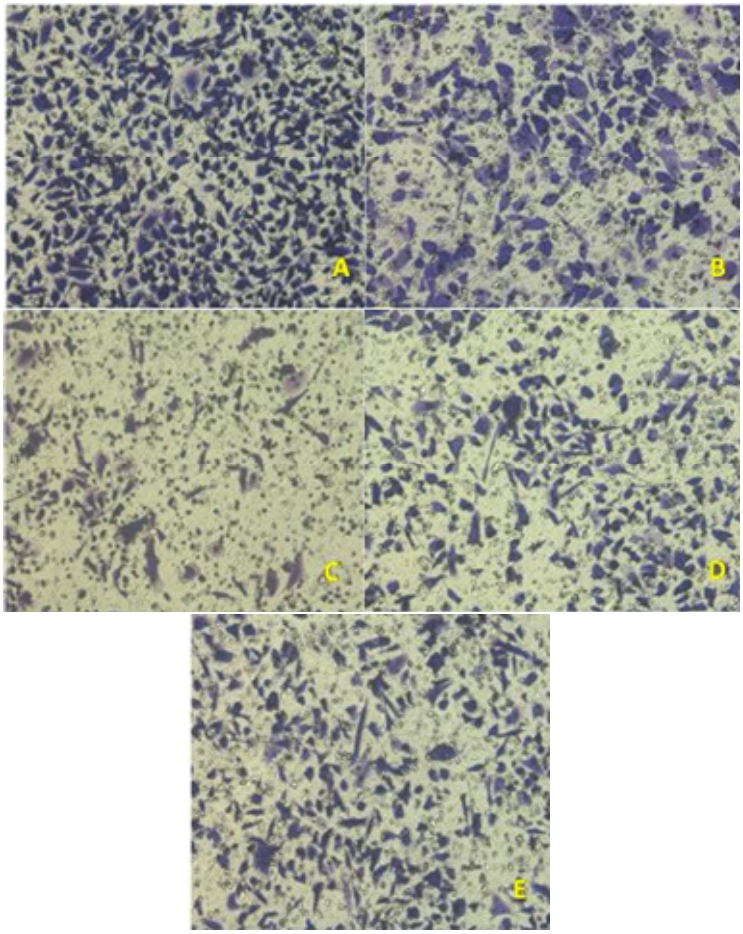

Figure 5. Ability of invasion of prostate cancer cells (X 200). A: pcDNA3.1-HIF-1α-PC-3, B: pcDNA3.1-PC-3, C: pll3.7-siHIF-1α-PC-3, D: pll3.7-PC-3 and E: PC-3.

between PC-3, pcDNA3.1-PC-3, and pll3.7-PC-3 cells ($P > 0.05$). Compared to these three cells types, the number of cells penetrating the membrane was statistically significantly higher ($P < 0.05$) for pcDNA3.1-HIF-1α-PC-3 cells, while cells that penetrated the membrane decreased statistically significantly for pll3.7-siHIF-1α-PC-3 cells ($P < 0.05$), suggesting HIF-1α promotes the ability of invasion of prostate cancer cells (Figure 5).

DISCUSSION

HIF-1 was firstly reported as a nuclear factor binding to the hypoxia response element (HRE) of erythropoietin (EPO) gene as induced by anoxia. It is a nuclear transcription factor derived in anoxia environment. HIF-1 consists of α and β subunits. The β subunit is a regulatory unit that has an important function in the activity of transcription. The α subunit is a regulatory unit that is regulated by anoxia or low sugar. It is easily degraded at a normal level of oxygen. It is stable in low-oxygen environment and transits to the nucleus to form a dimer with the β subunit. HIF- lα promotes transcription and expression and suitability of tumor cells in anoxia environment to achieve chance of survival and growth through binding to HRE of downstream target genes. One study suggests that high expression of HIF- lα is closely correlated with poor prognosis of tumor patients (Zagorska and Dulak, 2004). Zhong et al. (2004) found that HIF-1α was highly expressed in 11 of 14 damage areas of high-grade prostate intraepithelial neoplasma through immunohistochemistry of samples from 10 cases of radical prostatectomy, whereas it was lowly expressed in normal prostate tissues and prostate hyperplasia tissues, indicating that HIF- lα is highly expressed in prostate cancer and closely correlated with onset and development of prostate cancer. Researchers' opinions differ regarding whether anoxia affects expression of HIF-lα at the transcription level or at the protein level. Some studies report that HIF- lα is apparently regulated in anoxia environment and its upregulation may change the activity of downstream genes. However, one study

showed that HIF- lα mRNA expression was not increased in anoxia environment compared to oxygen-rich environment, but its protein expression was upregulated apparently, suggesting anoxia may regulate HIF- lα expression at the protein level (Stroka et al., 2001).

In our study, HIF- lα mRNA was not apparently expressed in pcDNA3.1-HIF-1α-PC-3 cells compared to pll3.7-siHIF-1α-PC-3 cells and other types of prostate cells, while its protein expression was higher than the other cell types. In our study, cell proliferation was evidently increased in pcDNA3.1-HIF-1α-PC-3 cells, while it was slowed down in pll3.7-siHIF-1α-PC-3 cells, indicating HIF- lα promotes cell proliferation. Tumor cell growth necessitates easy access of nutrients. It depends on import of nutrients and oxygen and export of metabolites out of tumor cells to provide a foundation for tumor cell survival and growth. Thus tumor neovascularization is a prerequisite for tumor cell growth. A major regulator for this process is VEGF that promotes vascular endothelial cell proliferation, vascular permeability and support for vascularization. Numerous studies show that high expression of VEGF is regulated by local oxygen level and anoxia promotes transcription of stability of VEGF mRNA and upregulation of VEGF receptors, strengthening biological effects of VEGF. When anoxia is corrected, VEGF mRNA expression can be reversed. Consequently, low oxygen plays an important role in neovascularization, whereas HIF-1α is a major factor that regulates cellular oxygen metabolism: rapid growth of local tumor tissues leads to anoxia and subsequent high expression of HIF-1α. The current body of literature indicates that HIF-1α can increase supply of blood, oxygen, and energy and correct anoxia through inducing expression of VEGF and glycolysis related enzymes (Yasuda et al., 2004) to indirectly promote cell growth and proliferation. Liotta and Stetler-Stevenson (1991) proposed a three-step hypothesis for tumor invasion consisting of decreased adhesion, matrix degradation and strengthened migration. Extracellular matrix (ECM) is a material basis for internal stability of cellular microenvironment. Its change is coregulated by matrix metalloproteinases (MMPs) and tissue inhibitors of matrix metalloproteinases (TIMPs). Invasion and migration of tumor result from interaction between tumor cells and ECM. Gelatinases are an important type of MMPs that play a major role in onset and development of tumor, neovascularizaiton and tumor invasion and migration. Including gelatinase A (MMP-2) and gelatinase B (MMP-9), they mainly hydrolyze glutin, type IV, V, and VII collagens, fibronectin and elastin, degrade ECM, and hydrolyze as well tight junction proteins and attachment proteins that maintain a normal endothelial barrier to facilitate invasion and migration of tumor cells. In addition, MMP-2 is also considered as a predictive factor for poor prognosis of tumor patients (Hilska et al., 2007).

HIF-1α reportedly binds to HRE of MMP-2 gene to promote its expression. Krishnamachary et al. (2003) found that oxygen deprivation could not induce expression of MMP-2 in HIF-1-/- embryonic stem cells, while MMP-2 was overexpressed in HIF-1α+/+ embryonic stem cells. In this study, protein expressions of MMP-2 and MMP-9 and the number of cells penetrating the membrane were higher in pcDNA3.1-HIF-1α-PC-3 cells than PC-3, pcDNA3.1-PC-3 and pll3.7-PC-3 cells, while they were statistically significantly lower in pcDNA3.1-HIF-1α-PC-3 cells. In conclusion, HIF-1α can promote proliferation and invasion of tumor cells mainly through upregulation of MMP-2 and MMP-9 proteins. Our findings suggest that HIF-1α is correlated with onset and development of prostate cancer. The current high fatality of prostate cancer results from late admission of patients when they are already at the moderate or advance stage. Surgery cannot even achieve favorable efficacy at this stage. HIF-1α, as a tumor anoxia response factor may be an effective target in a novel approach for treatment of prostate cancer.

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