Hypoxia regulates the expression and localization of CCAAT/enhancer binding protein α by hypoxia inducible factor-1α in bladder transitional carcinoma cells

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Abstract. Hypoxia inducible factor-1α (HIF-1α) is overexpressed in various types of solid tumor in humans, including bladder cancer. HIF-1α regulates the expression of a series of genes, which are involved in cell proliferation, differentiation, apoptosis, angiogenesis, migration and invasion and represents a potential therapeutic target for the treatment of human cancer. Despite extensive investigation of the effects of HIF-1α in the progression and metastasis of bladder cancer, the possible regulatory mechanisms underlying the effects of HIF-1α on bladder cancer cell proliferation and differentiation remain to be elucidated. It has been suggested that the transcription factor CCAAT/enhancer binding protein α (C/EBPα) acts as a tumor suppressor in several types of cancer cell, which are involved in regulating cell differentiation, proliferation and apoptosis. The present study confirmed that, in bladder cancer cells, the expression and localization of C/EBPα was regulated by hypoxia through an HIF-1α-dependent mechanism, which may be significant in bladder cancer cell proliferation and differentiation. The 5637 and T24 bladder cancer cell lines were incubated under normoxic and hypoxic conditions. The expression levels of HIF-1α and C/EBPα were detected by reverse transcription-quantitative polymerase chain reaction, western blotting and immunofluorescence analysis. The results revealed that, under hypoxic conditions, the protein expression levels of HIF-1α were markedly upregulated, but the mRNA levels were not altered. However, the mRNA and protein levels of C/EBPα were significantly reduced. The present study further analyzed the subcellular localization of C/EBPα, which was markedly decreased in the nuclei under hypoxic conditions. Following HIF-1α small interference RNA silencing of HIF-1α, downregulation of C/EBPα was prevented in the bladder cancer cells cultured under hypoxic conditions. In addition, groups of cells treated with 3-(5'-hydroxymethyl-2'-furyl)-1-benzylindazole, which inhibits the expression of HIF-1α in hypoxia, contributed to the inhibited expression of HIF-1α and enhanced expression of C/EBPα in hypoxic bladder cancer cells. These results suggested that C/EBPα was a downstream effector regulated by HIF-1α in hypoxic bladder cancer cells and that this regulatory pathway may represent a potential therapeutic target in the treatment of bladder cancer.

Introduction

CCAAT/enhancer binding protein α (C/EBPα) is a uniquely multifunctional transcription factor, which binds with the promoters of target genes and C/EBP family homodimers or heterodimers to regulate the transcription of target genes (1). In addition to its transcriptional activity, C/EBPα also promotes differentiation and suppresses proliferation in numerous cell types (2). These antiproliferative and pro-apoptotic characteristics of C/EBPα provide it with a tumor suppressive function in multiple tissues and it has been observed that downregulation of C/EBPα expression or C/EBPα gene mutations are associated with several human malignancies, including acute myeloid leukemia (3), lung cancer (4), breast cancer (5), hepatocellular carcinoma (6), head and neck squamous cell carcinoma (7) and skin cancer (8). Thus, C/EBPα has been recognized as a potential tumor suppressor and therapeutic target in human cancer.

As malignancy develops, the majority of types of solid tumor are characterized by hypoxic environments, which accelerate progression and metastasis (9). Bladder carcinoma is one of the common types of solid tumor associated with a hypoxic environment (10). The key hypoxic-responsive regulator of gene expression is hypoxia inducible factor-1 (HIF-1), which is a heterodimeric protein composed of a constitutively expressed β subunit and an inducible α subunit (11). Under normoxic conditions, HIF-1α mRNA is translated, but its protein is rapidly degraded (11). However, under hypoxic conditions, HIF-1α protein becomes increasingly stabilized and its degradation is

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prevented, facilitating the regulation of the expression of a series of genes, which are involved in cell proliferation, differentiation, apoptosis, angiogenesis, migration and invasion (12). The overexpression of HIF-1α has been identified in patients with bladder carcinoma, and the expression of HIF-1α is correlated with the progression and recurrence of bladder carcinoma (13). Consequently, in addition to being a diagnostic biomarker, HIF-1α may be a novel therapeutic target in bladder carcinoma. Although the involvement of HIF-1α in the progression and metastasis of bladder cancer has previously been reported (14), the potential regulatory mechanisms of HIF-1α on the proliferation and differentiation of bladder transitional carcinoma cancer cells has remained to be elucidated.

Based on the above observations, the present study hypothesized that HIF-1α regulated the expression of C/EBPα in hypoxic bladder cancer cells and was involved in the regulation of the proliferation and differentiation of bladder cancer cells.

Materials and methods

Cell culture. The 5637 and T24 human bladder cancer cell lines were obtained from the American Type Culture Collection (Manassas, VA, USA). The cells were grown in RPMI-1640 (Gibco Life Technologies, Grand Island, NY, USA) with 10% fetal bovine serum. The cultures were maintained at 37°C under a humidified 5% CO_2 atmosphere.

Hypoxic treatment. Hypoxia was achieved by placing 50% confluent 5637 and T24 cells in an oxygen control incubator (Heal Force, Shanghai, China), which was flushed with a mixture of 1% O_2, 94% N_2 and 5% CO_2. The 5637 and T24 cells were incubated at 37°C for 48 h under the hypoxic conditions (1% O_2, 5% CO_2 and 94% N_2).

Cell treatment with HIF-1α inhibitor. The HIF-1α inhibitor 3-(5'-hydroxymethyl-2'-furyl)-1-benzylindazole (YC-1) was purchased from Sigma-Aldrich (St. Louis, MO, USA) and resuspended in dimethyl sulfoxide (DMSO; Sigma-Aldrich). YC-1 was used at a final concentration of 50 μmol/l. The 5637 and T24 cells were incubated at 37°C for 12 h under hypoxic conditions (1% O_2, 5% CO_2 and 94% N_2). YC-1 or DMSO was added to RPMI-1640 medium prior to incubation for 12 h under hypoxic conditions. The final volume of YC-1 or DMSO in the medium was ≤0.5% (v/v).

Small interference RNA (siRNA) transfection. The HIF-1α siRNA was transiently transfected into bladder cancer cell lines using X-tremeGENE siRNA transfection reagent (Roche Diagnostics, Indianapolis, IN, USA). The cells were transfected with HIF-1α siRNA for 12 h, following 24 h hypoxia. The HIF-1α siRNAs (GenePharma, Shanghai, China) used are listed in Table I.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total RNA was extracted from the bladder cancer cell lines using TRIzol reagent (Invitrogen Life Technologies, Carlsbad, CA, USA). First-strand cDNA was then synthesized using a PrimeScript RT reagent kit (Perfect Real-Time; Takara Biotechnology, Co., Ltd., Dalian, China). RT-qPCR was performed using a SYBR Premix Ex Taq™ II

(Takara Biotechnology, Co., Ltd.) in a CFX96 Real-Time PCR Detection system (Bio-Rad Laboratories, Inc., Hercules, CA, USA) and the results were normalized against β-actin as an internal control. The PCR cycling conditions were as follows: initial denaturation step at 95°C for 30 sec, followed by 40 cycles at 95°C for 5 sec and 60°C for 30 sec. The PCR primers that were used are listed in Table I.

Western blotting. The bladder cancer cells were washed with phosphate-buffered saline (PBS), composed of 140 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4 (pH 7.4), and lysed in radioimmunoprecipitation assay buffer (Thermo Fisher Scientific, Waltham, MA, USA) containing complete protease inhibitor cocktail tablets (Roche, one tablet/10 ml aqueous buffer). The protein concentrations were quantified using a bicinchoninic acid protein assay kit (Thermo Scientific; Pierce BCA Protein Assay Kit; cat. no. 23227). All proteins were separated by 10% SDS-PAGE and transferred onto nitrocellulose membranes (Pall Life Science, Port Washington, NY, USA). The membranes were incubated overnight at 4°C with the following primary antibodies: monoclonal rabbit anti-human C/EBPα antibody (1:500; Abcam; cat. no. ab40761), monoclonal mouse anti-human HIF-1α antibody (1:500; Abcam; cat. no. ab1) and monoclonal mouse anti-human β-actin antibody (1:1000; Cell Signaling Technology, Beverly, MA, USA; cat. no. 3700). Following washing with TBST, composed of 20 mM Tris, 140 mM NaCl and 0.5% Tween-20 (pH 7.6), the membranes were incubated with horseradish peroxidase-conjugated secondary antibodies (goat anti- rabbit/mouse immunoglobulin G; 1:2,000; Cell Signaling Technology; cat. no. 7074/7076) for 1 h at room temperature. Protein expression was assessed using enhanced chemiluminescent reagents (Pierce Biotechnology, Inc.).

Immunofluorescence analysis. The 5637 and T24 cells were fixed for 20 min at room temperature with 4% formaldehyde, permeabilized for 10 min at room temperature with 0.2% Triton X-100, blocked for 1 h at room temperature with 5% BSA and incubated overnight at 4°C with monoclonal rabbit anti-human C/EBPα (Abcam). The cells were subsequently incubated for 1 h at room temperature with Cy3-conjugated goat anti-rabbit immunoglobulin G (Invitrogen Life Technologies). The cellular nuclei were counterstained with 4',6-diamidino-2-phenylindole (Roche Diagnostics) and the cells were detected using a Nikon Eclipse Ti-S fluorescence microscope (Nikon Corp., Tokyo, Japan)..

Statistical analysis. All experiments were performed at least three times. The data are expressed as the mean ± standard error of the mean and were analyzed using SPSS 19.0 (IBM SPSS, Armonk, NY, USA) and GraphPad Prism 5 software (GraphPad Software, Inc., San Diego, CA, USA). Statistical evaluations were determined using Student's two-tailed unpaired t-test. P<0.05 was considered to indicate a statistically significant difference.

Results

Hypoxia induces changes in the gene expression of HIF-1α and C/EBPα in bladder cancer cells. To confirm whether hypoxia regulated the expression of C/EBPα in bladder cancer cells, the

Table I. Primer and siRNA sequences.

Gene	Sequence (5'-3')	Experimental use
C/EBPα	ATTGGAGCGGTGAGTTTG TTGGTGCGTCTAAGATGAG	RT-qPCR
HIF-1α	CATCTCCATCTCCTACCCACA CTTTTCTGCTCTGTTTGGTG	RT-qPCR
β-actin	TCCCTGGAGAAGAGCTACGA AGCACTGTGTTGGCGTACAG	RT-qPCR
siRNA control	UUCUCCGAACGUGUCACGUTT ACGUGACACGUUCGGAGAATT	RNAi
HIF-1α siRNA	GCCUCUUUGACAAACUUAATT UUAAGUUUGUCAAAGAGGCTT	RNAi

siRNA, small interference RNA; C/EBPα, CCAAT/enhancer binding protein α; HIF-1α, hypoxia inducible factor-1α; RT-qPCR, reverse transcription-quantitative polymerase chain reaction; RNAi, RNA interference.
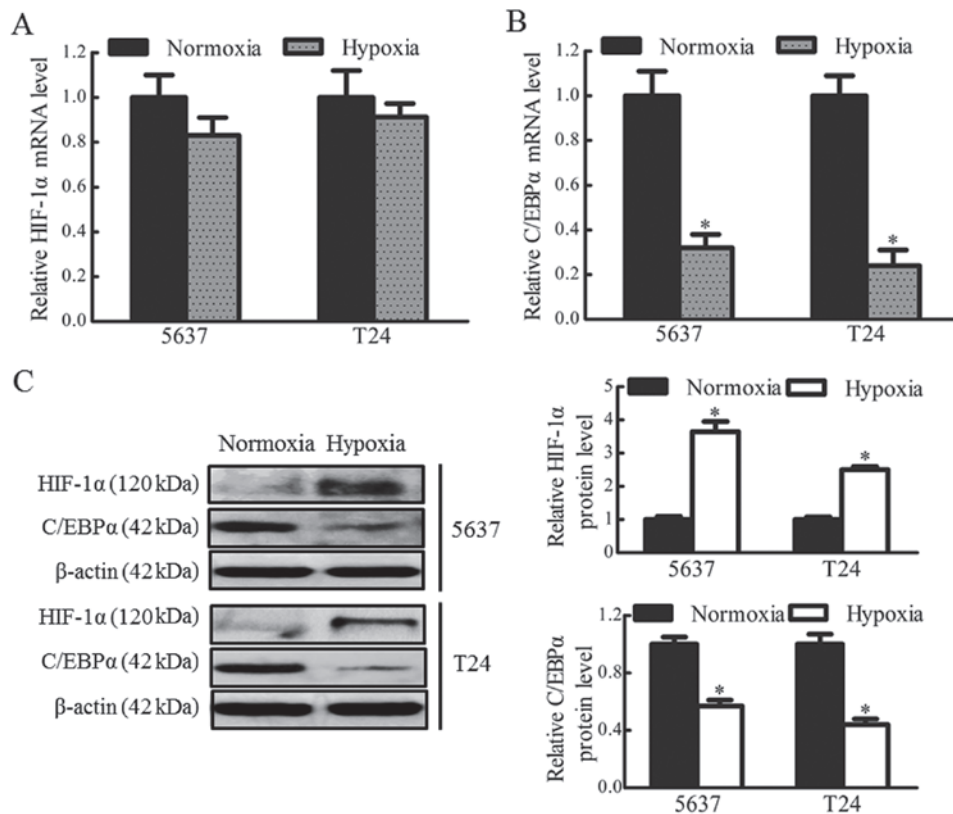

Figure 1. Hypoxia alters the gene expression levels of HIF-1α and C/EBPα in 5637 and T24 bladder cancer cells. (A and B) mRNA expression levels of HIF-1α and C/EBPα in normoxia and hypoxia were analyzed by reverse transcription-quantitative polymerase chain reaction. β-actin was used as an internal control. (C) HIF-1α and C/EBPα protein levels under normoxia and hypoxia were analyzed by western blotting. β-actin was used as an internal control. Values are expressed as the mean ± standard error of the mean; $^*P<0.05$; n=3. mRNA, messenger RNA; HIF-1α, hypoxia inducible factor-1α; C/EBPα, CCAAT/enhancer binding protein α.

mRNA and protein expression levels of HIF-1α and C/EBPα in the 5637 and T24 cells under hypoxic and normoxic conditions were determined. No significant differences were observed in the expression levels of HIF-1α mRNA between the hypoxic and normoxic groups (Fig. 1A). Low protein expression levels of HIF-1α were observed in the 5637 and T24 cells under normoxic culture conditions. However, the protein levels of HIF-1α were markedly upregulated (~3-fold increase) in the 5637 and T24 cells under hypoxic conditions, while the mRNA and protein levels of C/EBPα were significantly reduced, by 70 and 50% in the 5637 and T24 cells, respectively, under hypoxic conditions (P<0.05; Fig. 1B and C). These data indicated that the downregulation of C/EBPα may be associated with the stable expression of HIF-1α in hypoxic bladder cancer cells.
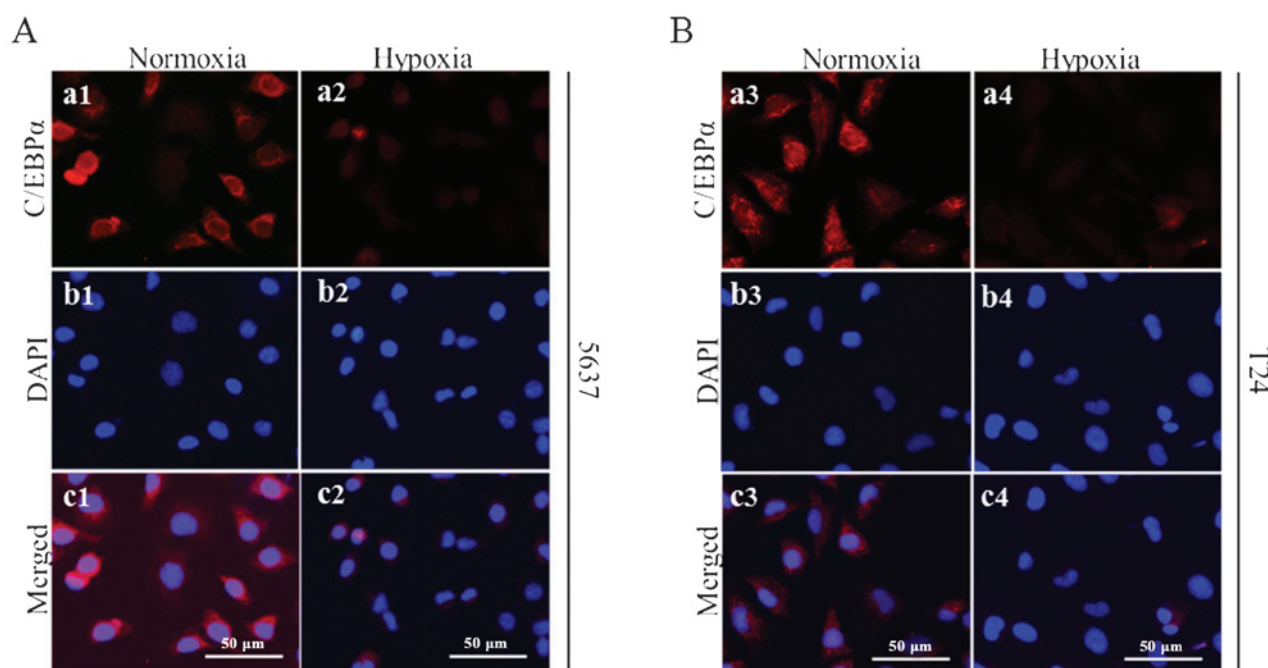
Figure 2. Hypoxia reduces the expression of C/EBPα in 5637 and T24 bladder cancer cells. Subcellular localization and protein expression levels of C/EBPα in hypoxic and normoxic bladder cancer cells. Immunofluorescence analysis of (a) C/EBPα (red) subcellular localization and (b) visualization of nuclei (DAPI, blue) in (A) 5637 and (B) T24 cells cultured under normoxic and hypoxic conditions. (Magnification, x100; scale bar, 50 µm). C/EBPα, CCAAT/enhancer binding protein α.

Hypoxia reduces the expression of C/EBPα in bladder cancer cells. To further evaluate the differences in the expression of C/EBPα in hypoxia, the present study also analyzed the subcellular localization and protein levels of C/EBPα in the 5637 and T24 cells under hypoxic and normoxic culture conditions. The protein expression and subcellular localization of C/EBPα was detected using an immunofluorescence assay. Under normoxic culture conditions, the protein expression of C/EBPα was marked in the nucleus and cytoplasm of the bladder cancer cells (Fig. 2). However, under hypoxic conditions, the protein expression of C/EBPα was markedly decreased in the cytoplasm and, in particular, the nuclei of the 5637 and T24 bladder cancer cells. These data suggested that hypoxia reduced the nuclear localization of C/EBPα protein in the bladder cancer cells.

siRNA-mediated silencing of HIF-1α enhances the expression of C/EBPα in hypoxic bladder cancer cells. In order to further confirm that HIF-1α regulated the expression of C/EBPα in hypoxic bladder cancer cells, the 5637 and T24 cells were transfected with HIF-1α siRNA or scrambled siRNA (siRNA control) prior to culture under hypoxic conditions. Transfection with the HIF-1α siRNA led to a significant reduction in the mRNA and protein expression levels of HIF-1α, by ~80 and ~50%, respectively (Fig. 3A and B). Silencing of HIF-1α may, therefore, prevent the downregulation of C/EBPα in hypoxic bladder cancer cells. Following treatment with HIF-1α siRNA, the mRNA and protein levels of C/EBPα were rescued in the 5637 and T24 cells, exhibiting a 1.4 and 1.5-fold increase, respectively (Fig. 3B and C). These results suggested that HIF-1α was involved in the downregulation of C/EBPα in bladder cancer cells under hypoxic culture conditions.

HIF-1α inhibitor YC-1 enhances the expression of C/EBPα in hypoxic bladder cancer cells. YC-1 has been reported to be a novel anticancer drug targeting HIF-1α, which may also inhibit the expression of HIF-1α (15,16). Therefore, the present study investigated whether bladder cancer cells treated with YC-1 exhibited alterations in the expression of HIF-1α and C/EBPα under hypoxic conditions. The 5637 and T24 cells were treated with YC-1 (50 µmol/l) for 12 h under hypoxic conditions. The results revealed that YC-1 reduced the mRNA (~50%) and protein (~50%) expression of HIF-1α under hypoxic conditions (Fig. 4A and B). YC-1 also prevented the downregulation of C/EBPα in the hypoxic bladder cancer cells. The mRNA and protein levels of C/EBPα were enhanced ~1.8 and 2-fold, respectively, following treatment with YC-1 in the 5637 and T24 cells (Fig. 4B and C). These data confirmed that YC-1 inhibited the expression of HIF-1α and enhanced the expression of C/EBPα in hypoxic bladder cancer cells.

Discussion

Hypoxia is one of the most fundamental and common biological phenomena found in various types of solid tumor, including bladder cancer (17). As a response to the hypoxic environment, HIF-1α is activated in cancer cells, and contributes to cellular adaptation and survival (12). A number of genes, which have been implicated in angiogenesis, invasion, metastasis, cell proliferation, differentiation and apoptosis, are regulated by HIF-1α (18). The roles of HIF-1α in tumor angiogenesis, invasion and metastasis have previously been investigated, whereas the molecular regulatory mechanisms underlying the involvement of HIF-1α in tumor cell proliferation and differentiation have remained to be fully elucidated. Previous studies have demonstrated that, in breast cancer cells, hypoxia induces
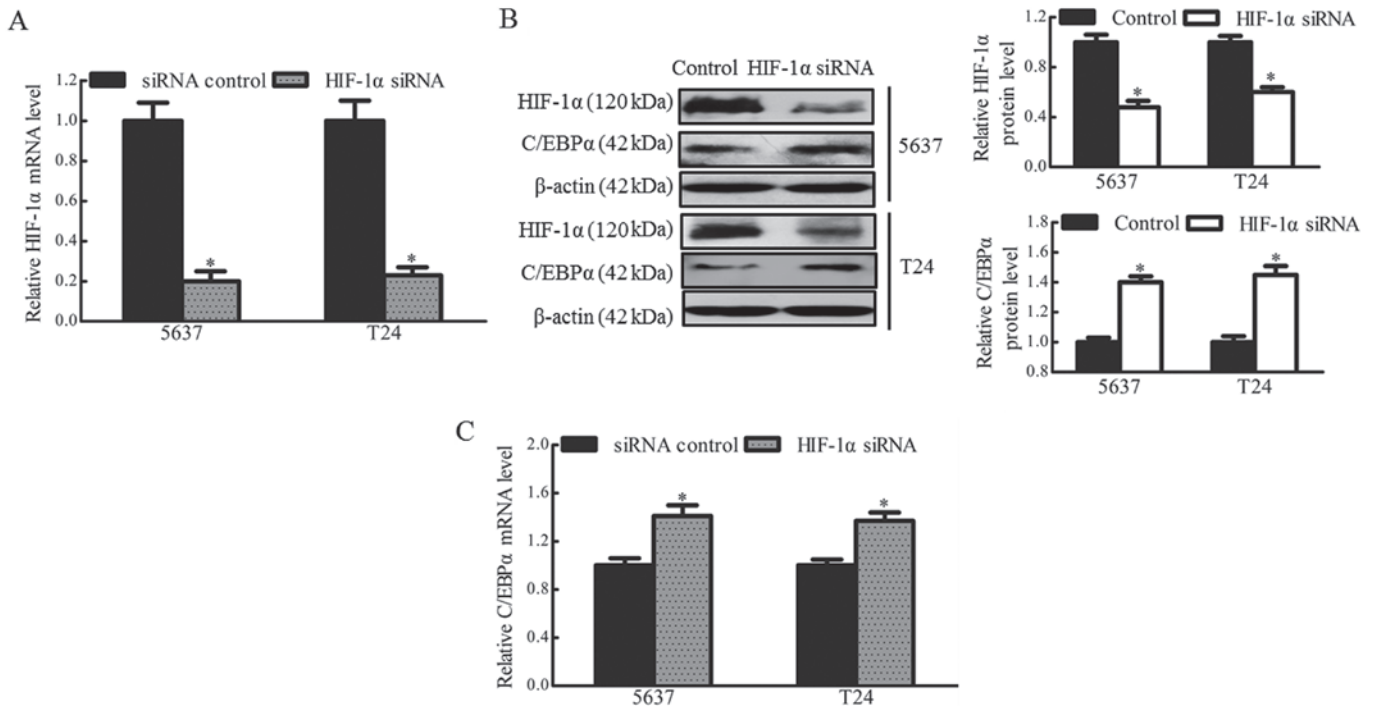
Figure 3. HIF-1α restores the expression of C/EBPα in hypoxic bladder cancer cells. Following treatment of 5637 and T24 cells with HIF-1α siRNA (A and B) mRNA levels of HIF-1α and C/EBPα were analyzed by quantitative polymerase chain reaction and (C) protein levels of HIF-1α and C/EBPα were analyzed by western blotting. β-actin was used as an internal control. Values are expressed as the mean ± standard error of the mean; *P<0.05; n=3. mRNA, messenger RNA; HIF-1α, silencing hypoxia inducible factor-1α; C/EBPα, CCAAT/enhancer binding protein α; siRNA, short interference RNA.
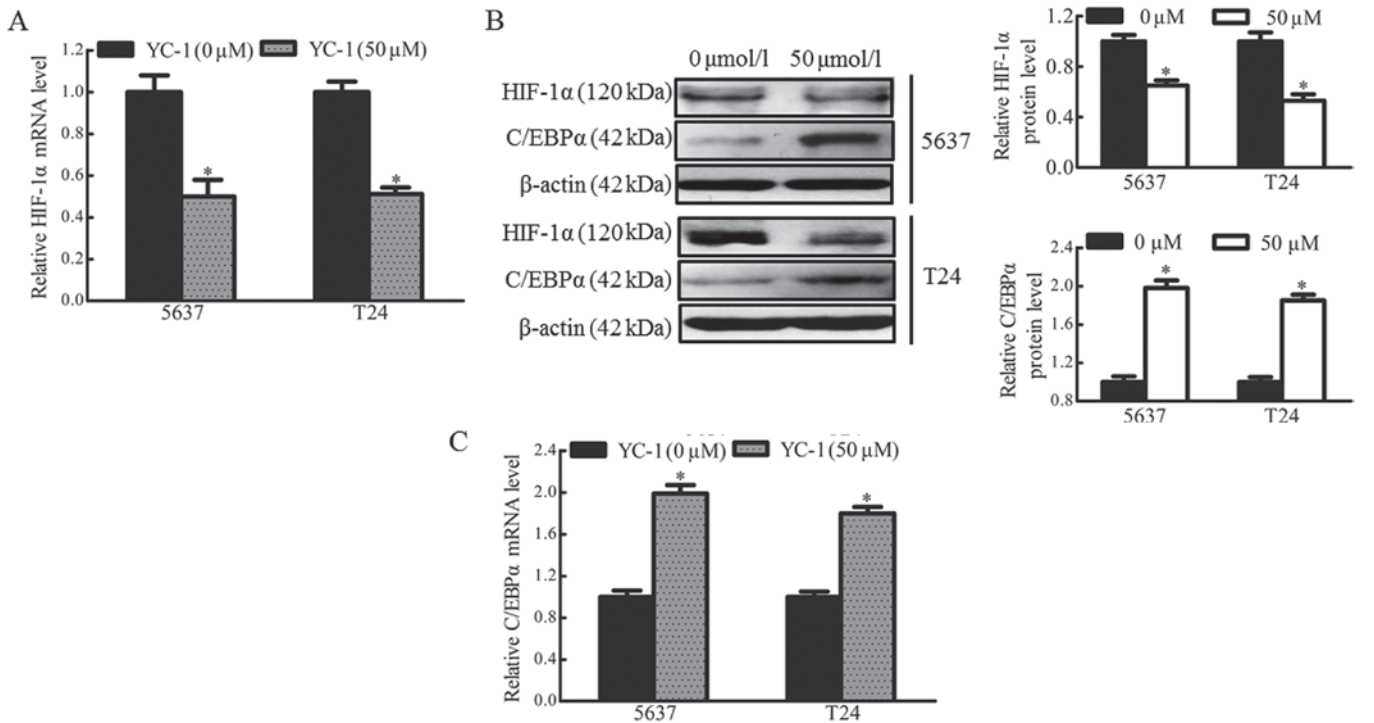

Figure 4. HIF-1α-specific inhibitor YC-1 enhances the expression of C/EBPα in hypoxic bladder cancer cells. Following treatment of 5637 and T24 cells with YC-1 (50 µmol/l) (A and B) mRNA levels of HIF-1α and C/EBPα were analyzed by quantitative polymerase chain reaction and (C) protein levels of HIF-1α and C/EBPα were analyzed by western blotting. β-actin was used as an internal control. Values are expressed as the mean ± standard error of the mean; *P<0.05; n=3. mRNA, messenger RNA; HIF-1α, silencing hypoxia inducible factor-1α; C/EBPα, CCAAT/enhancer binding protein α; YC-1, 3-(5'-hydroxymethyl-2'-furyl)-1-benzylindazole.

downregulation of the expression of C/EBPα (19,20), which indicated that hypoxia modulates breast cancer cell proliferation and differentiation, potentially through downregulation of C/EBPα. However, to the best of our knowledge, there is no current evidence indicating that hypoxia regulates the expression and subcellular localization of C/EBPα in bladder

carcinoma. In the present study, hypoxia was found to down-regulate the expression and nuclear localization of the tumor suppressor C/EBPα through an HIF-1α-dependent mechanism in bladder transitional carcinoma cells. Therefore, HIF-1α may be involved in regulating the differentiation and prolifera-tion of bladder cancer cells by controlling the expression of C/EBPα.

C/EBPα inhibits proliferation and induces differentiation in several cell types through protein-protein interactions (21-25). Several mechanisms for these effects have been proposed, including stabilization of p21 (21,22), repression of E2F (23), direct inhibition or degradation of Cdk2 and Cdk4 (24) and interaction with the SWI/SNF chromatin remodeling complex (25). Therefore, C/EBPα, through multiple mecha-nisms, functions as a tumor suppressor in tumorigenesis, however, further investigation is required to determine the exact function and mechanism of C/EBPα in bladder cancer cell differentiation and proliferation.

The functional activity of C/EBPα is regulated by modu-lation of its intracellular localization (26). C/EBPα requires localization in the nuclear region to exert its capacity for transcriptional activation and its biological function (19). Previous studies have demonstrated that C/EBPα is local-ized in the nucleus in normal epithelial cells; however, in cancer cells, C/EBPα is localized in the nucleus and cytoplasm (27). The present study also found that C/EBPα exhibited mixed nuclear and cytoplasmic localization in the bladder cancer cells. However, hypoxia markedly decreased the expression of C/EBPα in the nuclear region of the 5637 and T24 cells (Fig. 2B). The reduction of C/EBPα within the bladder cancer cell nuclei under hypoxia may contribute to a decrease in the biological activity of this protein. Thus, hypoxia regulated the biological effects of C/EBPα by modulating the nuclear localization of C/EBPα.

YC-1 is reported to be a novel antitumor agent, which decreases the expression of HIF-1α, inhibiting cell proliferation and inducing apoptosis in hypoxic bladder cancer cells (15,16). However, the antitumor mechanism of YC-1 in bladder cancer remains to be fully elucidated. The present study observed that YC-1 inhibited the expression of HIF-1α and prevented the downregulation of C/EBPα in hypoxic bladder cancer cells. These findings indicated that YC-1 may exert antitumor effects through the HIF-1α and C/EBPα pathway in hypoxic bladder cancer cells.

In conclusion, the present study demonstrated that, in bladder cancer cells, hypoxia downregulated the expression and nuclear localization of C/EBPα, a process which was medi-ated by HIF-1α. Furthermore, the HIF-1α specific inhibitor, YC-1, rescued the downregulation of C/EBPα under hypoxic conditions. Future investigations using bladder cancer tissue specimens to analyze the association between the expression of HIF-1α and C/EBPα and clinical and pathological parameters and patient survival rates are required to evaluate C/EBPα as a potential therapeutic target in bladder cancer.

Acknowledgements

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