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Original Article

A chronic obstructive pulmonary disease negatively influences the prognosis of patients with bladder urothelial carcinoma via hypoxia inducible factor-1α

Zhichao Huang[1], Ran Xu[1], Chen Lv[2], Zhaohui Zhong[1], Lei Zhang[1], Liang Zhu[1], Yongzhong Tang[3], Xiaokun Zhao[1]

[1]Department of Urology, The Second Xiangya Hospital, Central South University, Changsha 410011, Hunan, China; [2]Department of Urology, Shanghai Changhai Hospital, 168 Changhai Road, Shanghai 200433, China; [3]Department of Anesthesiology, The Third Xiangya Hospital, Central South University, 138 Tong Zhipo Road, Changsha 410013, Hunan, China

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Abstract: Objective: In this study, we investigated the relationship between the expression of hypoxia inducible factor-1α (HIF-1α) and tumor hypoxia, which is caused by chronic hypoxemic hypoxia in chronic obstructive pulmonary disease (COPD), and the prognostic value of COPD in patients with bladder urothelial carcinoma (BUC). Methods: The clinicopathological variables of 80 patients with BUC who underwent surgery were analyzed by retrospective methods. Overall survival (OS), disease-specific survival (DSS) and progression-free survival (PFS) were analyzed with clinicopathological variables including concomitant COPD, pulmonary function test (PFT), serum hemoglobin level and smoking history, using Kaplan-Meier survival analysis. The Cox proportional hazards regression model was used for multivariate analysis. The localization of HIF-1α expression was analyzed by immunohistochemistry. Results: Both the median OS and PFS of patients with COPD were shorter than the patients without COPD ($P < 0.001$). High levels of HIF-1α expression were associated with BUC of higher clinicopathological stage and histological grade ($P < 0.001$). COPD was an independent prognostic variable for OS, PFS and DSS. The clinicopathological stage was an independent prognostic variable for OS and DSS. The level of HIF-1α expression was an independent prognostic variable for PFS. Conclusions: COPD is an independent prognostic variable for BUC, and contributes to poor prognosis.

Keywords: Bladder urothelial carcinoma, chronic obstructive pulmonary disease, chronic hypoxia, hypoxia inducible factor-1α, prognosis

Introduction

Bladder cancer is the most common tumor of the urinary system, and 70-80% of bladder cancers are bladder urothelial carcinomas (BUC). Currently, the standard treatments for BUC are transurethral resection and radical cystectomy. The recurrence rate at 2 to 5 years post-surgery is 50-70%, and tumor malignancy (clinicopathological stage and histological grade) progress is observed in 10-20% of cases [1]. Therefore, investigation of the molecular mechanisms of bladder cancer and factors relevant to its progression has clinical significance when choosing the therapeutic schedule and judging prognosis.

As characteristics of tumor cells, uncontrolled proliferation and relative angiogenesis inadequacy caused by hypermetabolism can be observed in most solid tumors, resulting in relative or absolute anoxic zones within these tumors. Acute hypoxia can block cellular fission and differentiation, and even induce apoptosis, by decreasing the supply of oxygen and nutrients. Thus, hypoxia seems to inhibit the proliferation of tumor cells, and can be considered as a good target for therapy. Furthermore, tumor cells can be selected by chronic hypoxia, while surviving tumor cells can adapt to anoxic environments and hyponutrient states.

HIF-1 is a heterodimeric transcription factor expressed in chronic hypoxia. To date, the transcription and expression of more than 60 target genes has been shown to be controlled by HIF-1 [2-4]. The protein products of these genes are

involved in the processes of proliferation, invasion and metastasis, which determine tumor aggressiveness.

COPD has been defined as a disease state characterized by airflow limitation that is not fully reversible, and is a commonly and frequently encountered disease of the respiratory system. Peripheral airway obstruction, destruction of lung parenchyma and abnormal decrease of pneumoangiogram will reduce the gas exchange capacity in the lung, causing hypoxemia and histanoxia, which implies elevated levels of HIF-1 [5]. In the present study, we investigated the relationship between the expression of HIF-1α and tumor hypoxia, which is caused by chronic hypoxemic hypoxia in COPD, and the prognostic value of COPD in patients with BUC. To our knowledge, few previous studies have been performed.

Materials and methods

Patients

We obtained approval for this study from the ethics committee of our hospital, and we obtained informed consent from all participants in our study. The informed consent was written and specified in the operative consent. Eighty patients with histologically proven BUC who underwent surgeries at our institution between 2006 and 2008 were enrolled. Thirty-seven patients underwent transurethral resection of bladder tumor, 13 underwent radical cystectomy and 30 underwent partial cystectomy. After fixing in 10% formalin and paraffin-embedding, the tissue blocks were cut into 4 μm-thick serial sections. HIF-1α protein expression and localization in specimens was analyzed by immunohistochemistry. All the patients were followed up and assessed by urine cytology and cystoscopy every 3 months in the first 2 years, and every 6 months in the next 2 years, and yearly thereafter for a total of 5 years.

Assessment of COPD and pulmonary function test (PFT)

Patients were diagnosed by a pulmonologist, and those who had history and previous treatment were determined to have COPD. Patients who had no treatment or present symptoms were incidentally diagnosed by the PFT. The PFT was defined as abnormal if the forced vital capacity (FVC) and forced expiratory volume in 1 second (FEV1) were less than 80% of predicted value, and the FEV1/FVC ratio was less than 70% of the predicted value, regardless of FVC or FEV1. If the PFT results were abnormal patients were considered to have COPD.

Assessment of anemia and smoking history

Patients with anemia were diagnosed using laboratory tests. The patients were divided into two groups based on hemoglobin levels of ≤ 110 g/L or > 110 g/L. Cigarette smoking history was determined by reviewing medical records, and smoking information was recorded, including how long the patient smoked and how many packs were smoked daily. Then the patients were divided into two groups: non-smokers or current smokers. A previous study showed that past smokers who had not smoked for 10 years had a similar risk as those who had never smoked [6], thus we considered past smokers who had quit smoking more than 10 years ago as non-smokers.

Assessment of other comorbidities

Information of comorbidities was collected by reviewing medical records. Hypertension, diabetes mellitus, cardiovascular and cerebrovascular disease, and other advanced malignancies were considered to be confounding variables in the patient population. The diagnosis of comorbidities has definitive clinical evidence.

Immunohistochemistry and statistical analysis

In our study, all procedures were performed using standard protocols. Serial sections from formalin-fixed, paraffin-embedded material were de-paraffinized in 100% xylene, and rehydrated in a descending ethanol series (100%, 90%, 80%, 70% ethanol) and water. Then antigen retrieval was performed by submerging slides into 0.01 mol/L citrate buffer (PH 6.0) for 15 min at 120°C. Samples were then treated with 3% hydrogen peroxide in methanol to quench endogenous peroxidases and incubated with 10% bovine serum albumin to block nonspecific binding. The sections were then incubated overnight at 4°C with a mouse monoclonal antibody against HIF-1α (Thermo Fisher Scientific, Mab H1α67, IgG2b isotype, Cat. #MS-1164-P0, diluted 1:100). After washing in phosphate buffered saline (PBS), a subsequent reaction was carried out using an Elivision super HRP (Mouse) IHC Kit (Maixin Bio, Fujian,

Table 1. Patient characteristics

Mean age (months)	68.2 (51.0-88.0)
Gender	
Male	43
Female	37
Clinicopathological stage (n=80, %)	
Ta-T1	44 (55.0)
T2-T4	36 (45.0)
Histological grade (n=80, %)	
I	30 (37.5)
II	27 (33.8)
III	23 (28.7)
COPD (n=35, %)	35 (43.6)
Smoking history	
Never smoking	40 (50.0)
Current smoker	40 (50.0)
Other comorbidity (n=58, %)	
Hypertension	28 (35.0)
Diabetes mellitus	14 (17.5)
Cardiovascular and cerebrovascular disease	8 (10.0)
Other advanced malignancy	8 (10.0)
Number of tumor	
Single	42 (52.5)
Multiple	38 (47.5)
Size of tumor (diameter, cm)	
≤ 3 cm	48 (60.0)
> 3 cm	32 (40.0)
Mean serum hemoglobin (g/L)	110.5 (95.0-145.0)

COPD: chronic obstructive pulmonary disease.

China) at 37°C for 30 min. Then, the sections were washed three times with PBS and positive staining was revealed with DAB treatment for 5 min. Nuclei were lightly counterstained with hematoxylin. Negative controls included incubation in PBS without primary antibody. The tumor cell immunoreactivity for HIF-1α was scored and interpreted as positive according to the nuclear staining. A minimum of five randomly-selected fields throughout the whole section at ×400 magnification was examined, and 200 tumor cells were counted in each field. Then the percentage of positively-stained tumor cells was calculated to determine the expression of HIF-1α. HIF-1α protein expression was classified as follows: -, < 1%; +, < 1-10%; ++, 10-50%; +++, > 50% [7]. All the immunohistochemical results were examined by two blinded independent reviewers. Whenever differences of > 10% between reviewers occurred, these slides were rechecked and the final result was determined with the two reviewers simultaneously viewing these slides on a multihead microscope. The evaluations agreed in > 90% of the samples for all markers. All statistical analyses were performed with SPSS version 17.0 (SPSS Inc., Chicago, Illinois, USA). The significance of results was assessed using Student's *t*-tests or one-way ANOVA and chi-square tests. After diagnosis of BUC, the overall survival (OS), disease-specific survival (DSS) and progression-free survival (PFS) were analyzed with the clinicopathological variables including the presence of COPD, results of PFT, smoking history and serum hemoglobin level using the Kaplan-Meier method, and the significance of differences was assessed with the log-rank test. Associations of variables with survival analysis were further tested with univariate and multivariate analyses using the Cox proportional hazard model. For all tests, *P* values of < 0.05 were considered statistically significant.

Results

Our retrospective review identified 80 patients, and their basic characteristics are shown in **Table 1**. Sixty patients (75%) died during the follow-up, and 16 died from other comorbidities. The median OS and PFS were 37.0 and 14.5 months, respectively. HIF-1α protein immunoreactivity was present in the nuclei with or without cytoplasmic expression; cytoplasmic expression was very weak if present. The majority of positive tumors had a diffuse pattern of nuclear staining, with tumor cells near or far from the blood vessels all intensely stained, although increased HIF-1α expression was detected in tumor cells that were adjacent to necrotic regions and distal to the blood vessels in a few cases. Therefore, the staining heterogeneity within tumor-islands was not obvious in most specimens. Meanwhile, the normal urothelial tissue of some patients with COPD was positive for HIF-1α. However, compared with the tumor cells, the expression of HIF-1α was much weaker.

Table 2. Clinicopathological characteristics according to COPD

	No COPD group		COPD group		*P*
	Number	%	Number	%	
Age (years)					0.365
< 60	13	28.9	5	14.3	
60-69	14	31.1	10	28.6	
70-79	12	26.7	14	40.0	
≥ 80	6	13.3	6	17.1	
Clinicopathological stage					0.054
Ta-T1	29	64.4	15	42.9	
T2-T4	16	35.6	20	57.1	
Histological grade					0.002
I	22	48.9	8	22.9	
II	17	37.8	10	28.6	
III	6	13.3	17	48.5	
No. of tumor					0.535
Single	25	55.6	17	48.5	
Multiple	20	44.4	18	51.5	
Size of tumor(diameter, cm)					0.358
≤ 3 cm	29	64.4	19	54.3	
> 3 cm	16	35.6	16	45.7	
Pulmonary function test					< 0.001
Normal	33	73.3	11	31.4	
Abnormal	12	26.7	24	68.6	
Smoking history					0.499
Never smoking	24	53.3	16	45.7	
Current smoker	21	46.7	19	54.3	
Serum hemoglobin (g/L)					0.022
≤ 110	18	40.0	23	65.7	
> 110	27	60.0	12	34.3	
Other comorbidity					0.082
Hypertension	16	35.6	12	34.3	
Diabetes mellitus	6	13.3	8	22.9	
Cardiovascular and cerebrovascular disease	4	8.9	4	11.4	
Other advanced malignancy	2	4.4	6	17.1	
Expression of HIF-1α					< 0.001
-	22	48.9	0	0.0	
+	15	33.3	1	2.8	
++	2	4.5	22	62.9	
+++	6	13.3	12	34.3	

COPD: chronic obstructive pulmonary disease; HIF-1α: hypoxia inducible factor-1α.

Clinicopathological characteristics were not significantly different between patients with and without COPD except in regard to abnormalities in PFT, histological grade, serum hemoglobin, and expression level of HIF-1α (**Table 2**). The clinicopathological stage and histological grade had an intimate correlation with HIF-1α expression. Overexpression of HIF-1α was detected in high clinicopathological stage and histological grade BUC (**Table 3**, **Figure 1**). Smoking habit and tumor grade was not correlated in our study.

Both median OS and PFS of patients with COPD were shorter than patients without COPD ($P < 0.001$). Cases with higher expression of HIF-1α

Table 3. Chi-square test analysis of HIF-1α expression thought to be associated with clinicopathological stage and histological grade of tumor

	Clinicopatholo-gical stage		χ^2	*P*	Histological grade			χ^2	*P*
Expression of HIF-1α	Ta-T1	T2-T4			Ⅰ	Ⅱ	Ⅲ		
-	20	2	19.680	< 0.001	12	10	0	23.836	< 0.05
+	8	8			6	7	3		
++	12	12			10	6	8		
+++	4	14			2	4	12		

HIF-1α: hypoxia inducible factor-1α.

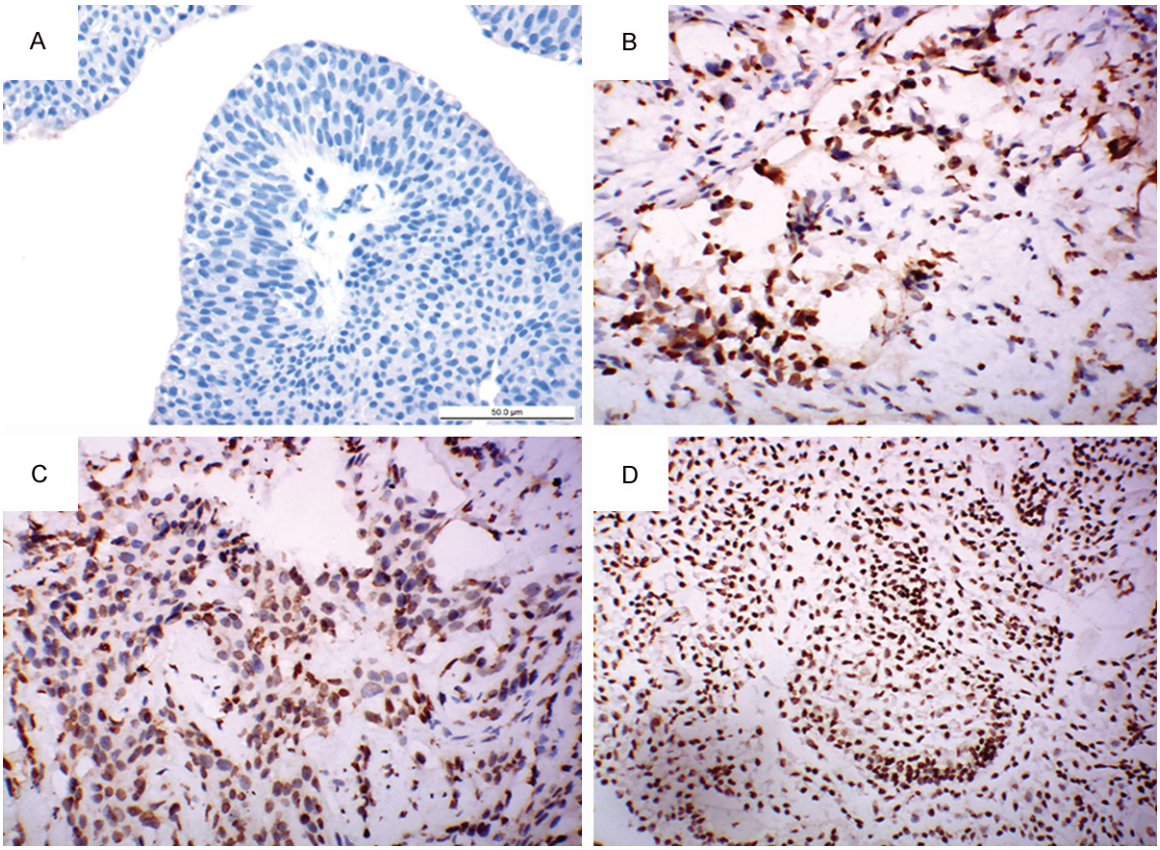

Figure 1. Representative immunohistochemical staining of HIF-1α in BUC tissues. A. Negative expression (-) of HIF-1α; B. Mild positive expression (+) of HIF-1α; C. Moderate positive expression (++) of HIF-1α; D. Severe positive expression (+++) of HIF-1α. (original magnification, ×200, immunoperoxidase stain).

had shorter OS and PFS ($P < 0.001$). **Table 4** shows the relationship between prognosis and clinical variables. **Figure 2** shows the Kaplan-Meier survival curves of 80 BUC cases stratified by COPD, and **Figure 3** shows the Kaplan-Meier survival curves of 35 COPD cases stratified for HIF-1α expression.

In the multivariate analysis, our results conclusively showed that none of the other comorbidities evaluated had a statistically significant influence on OS, PFS and DSS. Moreover, COPD was an independent prognostic variable for OS, PFS and DSS. The clinicopathological stage was an independent prognostic variable for OS and DSS. HIF-1α expression was an independent prognostic variable for PFS (**Table 5**).

Discussion

HIF-1 is a heterodimer composed of an alpha subunit regulated by O_2 and a beta subunit known as aryl hydrocarbon nuclear translocator, and is arranged in a helix-loop-helix [8]. HIF-

Table 4. Significant prognostic variables on survival by univariate analysis

	No.	Median OS (months)	*P*	Median PFS (months)	*P*
Age (years)			< 0.001		< 0.05
< 60	18	55.0		15.0	
60-69	24	50.5		15.0	
70-79	26	40.0		16.0	
≥ 80	12	21.5		9.5	
Clinicopathological stage			< 0.001		< 0.05
Ta-T1	44	57.5		14.0	
T2-T4	36	27.0		15.0	
Histological grade			<0.05		0.074
Ⅰ	30	44.0		16.0	
Ⅱ	27	45.0		16.0	
Ⅲ	23	27.0		12.0	
Number of tumor			0.875		< 0.05
Single	42	33.0		15.0	
Multiple	38	40.0		14.0	
Size of tumor (cm)			< 0.05		< 0.05
≤ 3 cm	48	43.5		15.0	
> 3 cm	32	27.0		13.5	
COPD			< 0.001		< 0.001
Presence	35	26.0		11.0	
Absence	45	50.0		17.0	
Pulmonary function test			< 0.001		0.130
Normal	44	47.5		15.0	
Abnormal	36	27.0		13.0	
Smoking history			0.138		0.847
Never smoking	40	44.5		14.0	
Current smoker	40	31.5		15.0	
Serum hemoglobin (g/L)			< 0.05		< 0.05
≤ 110	41	32.0		13.0	
> 110	39	44.0		18.0	
Expression of HIF-1α			< 0.001		< 0.001
-	22	60.0		26.0	
+	16	36.5		14.5	
++	24	32.0		12.5	
+++	18	25.0		10.0	

OS: overall survival; PFS: progression free survival; COPD: chronic obstructive pulmonary disease; HIF-1α: hypoxia inducible factor-1α.

1α contains two nuclear localization signals, located at the C-terminal (amino acids 718-721) and the N-terminal (amino acids 17-33), but only the C-terminal correlates with the nuclear accumulation of HIF-1α [9]. In the only oxygen-dependent degradation domain of HIF-1α, the hydroxylation of proline residues at positions 402 and 564 has a close relationship with the stability of the protein in normoxic conditions, and is a critical point that regulates the proteasomal degradation [8, 10-12].

In the absence of oxygen, HIF-1α prolyl hydroxylase and factor inhibiting HIF-1 are inactive, which reduces the degradation of HIF-1α and elevate HIF-1α levels [13]. Therefore, the main regulator of HIF-1α is oxygen [14]. The second most important regulators are anti-oncogenes. For example, *TP53* gene and its protein inhibit the activity of HIF-1α protein and promote its degradation [15]. The product of the von-Hippel-Lindau (*VHL*) gene also regulates the stability of HIF-1α [16]. In the presence of oxygen, *VHL* protein can bind to HIF-1α and degrade it through prolyl-hydroxylation [12, 17, 18]. Many studies have reported that expression of HIF-1α can be regulated through other pathways, such as protein-kinase B and phosphatidylinositol 3'-kinase [19]. Molecules and cytokines, such as oxygen-reactive species, tumor necrosis factor-α and angiotensin can regulate the expression of HIF-1α through the RAS/RAF1/MEK1/ERK1/2/ and/or p53/JNK signaling pathways [20-22].

When hypoxia in the internal environment of a tumor is established, HIF-1α transcription factor is activated, which accumulates in its heterodimerized form with HIF-1β. The up-regulation of HIF-1 increases vascularization with the production of vascular endothelial growth factor to provide enough oxygen and nutrients for the proliferation and differentiation of tumor cells, increased glucose transport 1 to regulate glycometabolism for providing enough energy, increased activity

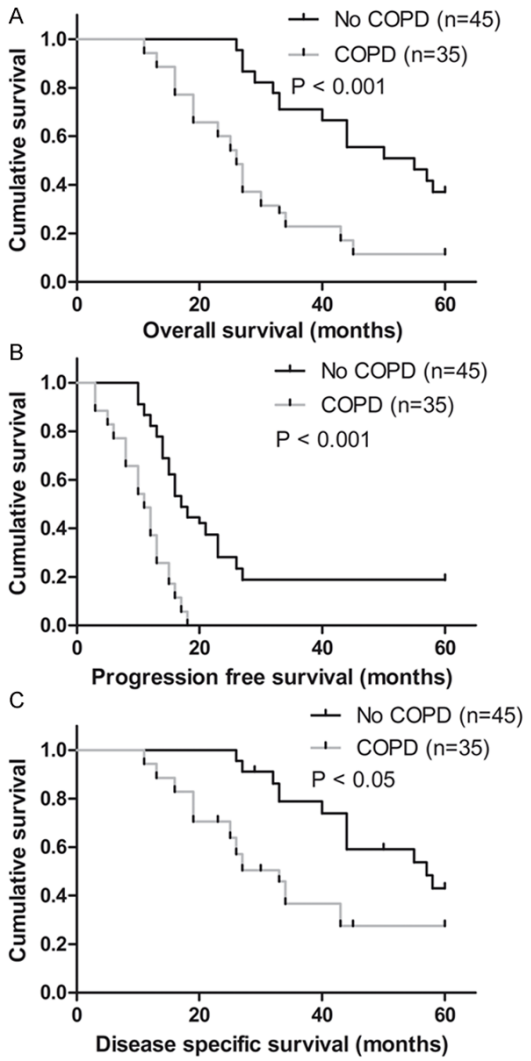

Figure 2. Kaplan-Meier survival curves of 80 BUC cases stratified by COPD. A. Overall survival (OS) by COPD; B. Progression free survival (PFS) by COPD; C. Disease specific survival (DFS) by COPD.

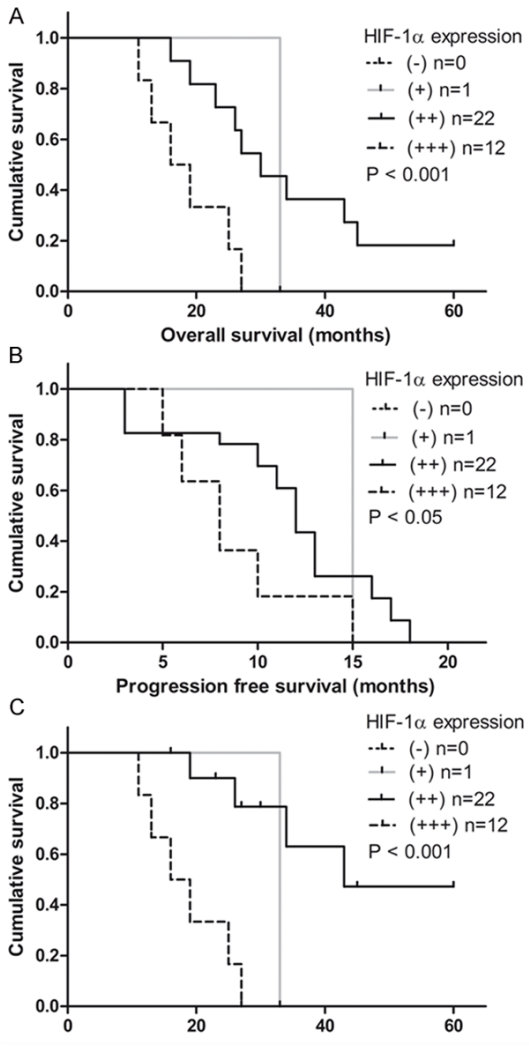

Figure 3. Kaplan-Meier survival curves of 35 COPD cases stratified for HIF-1α expression. A. Overall survival (OS) by HIF-1α expression; B. Progression free survival (PFS) by HIF-1α expression; C. Disease specific survival (DFS) by HIF-1α expression.

of carbonic anhydrase IX to keep the balance between the intracellular and extracellular PH, and even influences apoptotic genes inhibiting apoptosis of tumor cells induced by hypoxia, which induces the processes of invasion, recurrence and metastasis [23, 24]. Therefore, HIF-1α makes tumor cells more aggressive [25]. Hong J et al [26] revealed the activation of HIF-1α via nuclear factor-κB in rats with COPD. Our study also showed the expression levels of HIF-1α in patients with COPD were higher than patients without COPD, and overexpression of HIF-1α was detected in high clinicopathological stage and histological grade BUC.

There are many reasons for anemia in patients with BUC: chronic blood loss because of hematuria, hematopoietic dysplasia resulting from nutritional deficiencies, bone marrow infiltration of tumors, and so on. Anemia contributes to the decrease of peripheral serum hemoglobin levels and inefficiency of unit volume blood to combine and transport oxygen, which are all responsible for tumor hypoxia. Our research showed anemia was associated with shorter OS and PFS, but was not an independent prognostic variable. However, further study is required for a decisive conclusion.

Many previous studies in animal models and cell lines suggested that constituents in cigarette smoke may promote cancer growth by decreasing apoptosis and increasing cellular proliferation [27-30]. Cigarette smoking is a

Table 5. Multivariate Cox proportional hazard model of variables associate with prognosis

Variables	RR	95% CI	*P*
Overall survival			
COPD	2.948	1.290-6.737	$P < 0.05$
Clinicopathological stage	3.967	1.741-9.038	$P < 0.001$
Progression free survival			
COPD	3.144	1.401-7.058	$P < 0.05$
Expression of HIF-1α	2.045	1.342-3.118	$P < 0.001$
Disease specific survival			
COPD	2.653	1.087-7.132	$P < 0.05$
Clinicopathological stage	4.732	1.631-13.727	$P < 0.05$

COPD: chronic obstructive pulmonary disease; HIF-1α: hypoxia inducible factor-1α.

definitive risk factor for bladder cancer. About 30-50% of bladder cancers are caused by cigarette smoking. The current risk of bladder cancer to smokers is double to quadruple the risk for those who have never smoked, and there is a positive correlation with the intensity and length of smoking [6]. Furthermore, chronic exposure to cigarette smoke plays an important role in the development of COPD, and a previous study suggests that cigarette smoke activates epidermal growth factor receptor-mediated signaling pathways, leading to HIF-1α production and activation [31], which may lead to a negative impact on the prognosis of patients with BUC. However, previous clinical studies about the impact of smoking on the prognosis of lower tract urothelial cancer have not yet drawn an absolute conclusion. Aveyard P et al [32] undertook a systematic review of the effect of stopping smoking on prognosis. Their study suggested that stopping smoking might favorably alter the course of bladder cancer. Similarly, Chen CH et al [33] studied 297 men with primary nonmuscle-invasive bladder cancer who were treated by transurethral resection. They found smoking cessation might be associated with a lower recurrence rate for patients with nonmuscle-invasive bladder cancer. Lammers RJ et al [34] evaluated the role of smoking status on the clinical outcome of patients with non-muscle-invasive bladder cancer. In univariate analyses, recurrence-free survival (RFS) was significantly shorter in ex-smokers and current smokers (P=0.005). Similarly, in multivariate analyses, smoking status remained a significant factor for predicting RFS. Their study concluded that ex-smokers and current smokers had a significantly shorter RFS compared with nonsmokers. In our study, smoking status was not an independent prognostic factor for BUC. One possible reason is the different behavior of patients after the diagnosis of BUC. Few patients smoked continually, and the majority stopped smoking as soon as they were diagnosed. Meanwhile, all tissue samples were obtained from consecutive patients who underwent operation, and all patients underwent operation as soon as they were diagnosed. Therefore, the smoking status of patients upon BUC diagnosis did not have any short-term effect on immunohistochemical staining patterns. The mechanism of tumor hypoxia caused by smoking included both acute and chronic effects. The acute effect correlated with the carboxyhemoglobin formation and constriction of peripheral vessels, and the chronic effect correlated with the chronic damage of airway and alveolus. Both of them were related to tumor hypoxia.

Bladder cancer is a common tumor in older populations, and morbidity has a positive correlation with age [35]. Thus, patients with bladder cancer usually have other comorbidities. However, our results showed that other comorbidities had no statistical correlations with PFS and DSS. Patients with cardiovascular and cerebrovascular disease had shorter OS because of higher mortality caused by cardiovascular incidence and stroke.

Because of the retrospective design, this study has some inevitable limitations, including selection bias. Furthermore, the long-term effect of changing smoking habit after diagnosis on immunohistochemical staining pattern was not studied, and the severity of COPD could not be graded. The prognostic effect of chronic tumor hypoxia resulting from COPD in patients with BUC needs to be investigated by further prospective studies and *in vivo* animal models.

Conclusion

COPD may contribute to poor prognosis in patients with BUC, and a possible explanation may be tumor hypoxia caused by COPD. High expression levels of HIF-1α in BUC patients with COPD were associated with higher clinicopathological stage and histological grade. The expression level of HIF-1α may be a predictive

factor for the prognosis of BUC patients with COPD. Furthermore, the invasion, recurrence and metastasis of tumors may be inhibited through blocking the transcription and expression of HIF-1α.

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Disclosure of conflict of interest

None.

Address correspondence to: Dr. Xiaokun Zhao, Department of Urology, The Second Xiangya Hospital, Central South University, Changsha 410011, Hunan province, China. Tel: 0086-731-85295133; E-mail: hzc0305@163.com

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