Expression of HIF-1α is a predictive marker of the efficacy of neoadjuvant chemotherapy for locally advanced cervical cancer

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Abstract. Platinum-based, arterial infusion chemotherapy as a neoadjuvant chemotherapy (NACT) followed by hysterectomy may be efficient for the treatment of locally advanced cervical cancer and improve prognosis. It is important to predict whether the NACT would be effective before it is launched. Hypoxia inducible factor-1α (HIF-1α) is the master transcriptional regulator of the cellular response to altered oxygen concentration. HIF-1α protein expression is elevated in numerous human malignancies, contributes to poor disease outcome, and has been reported to induce tumorigenesis and chemoresistance. In the present study, patients with International Federation of Gynecology and Obstetrics stage IIB-IIIB cervical cancer (n=59) between 2008 and 2014 were assessed for HIF-1α expression by immunohistochemistry. Tumor samples were obtained by biopsy before any treatment. A double-path chemotherapy regimen, paclitaxel (intravenous) plus cisplatin (intra-arterial injection into the uterine region), was used as NACT. The patients were then separated into two groups according to NACT response: One group comprised patients with NACT, for whom the response to treatment was efficient resulting in complete/partial remission of the tumor (CR + PR group; n=52), the other group contained patients with NACT, for whom the result of the treatment was a stable/progressive disease (SD + PD group; n=7). HIF-1α expression was tested in paraffin-embedded sections using immunohistochemistry. HIF-1α expression was significantly higher in the SD + PD group compared with the CR + PR group (P=0.029). The overall survival time was significantly longer in the CR + PR group compared with the SD + PD group (P<0.001). When the patients were divided into two groups based on HIF-1α expression levels. Low (weighted score ≤4, n=39) and high (weighted score ≥6, n=20) expression level groups; the low HIF-1α expression group was significantly more susceptible to NACT treatment (P=0.025). Cox hazard analysis revealed that a high level of HIF-1α expression and lymph node metastases were significant independent predictors of poor overall survival (P=0.025, HR=6.354; P=0.020, HR=6.909, respectively). These results indicated that the expression of HIF-1α may be able to predict the efficiency of NACT and may be considered an independent prognostic factor for stage IIB-IIIB cervical cancer.

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Introduction

According to cervical cancer clinical guidelines, since 1999 the standard treatment for patients with locally advanced cervical cancer (LACC), defined as International Federation of Gynecology and Obstetrics (FIGO) stage ≥IIB, is platinum-based concurrent chemoradiotherapy (CCRT) (1); however, the prognosis is still unsatisfactory. Several studies have demonstrated a 40-60% reduction in the relative risk of recurrence and a 30-50% reduction of the risk of death with CCRT (2-4).

Platinum-based neoadjuvant chemotherapy (NACT), followed by radical hysterectomy, has been reported to be effective in patients with LACC (5), with a prognosis equal to that of CCRT (6). Furthermore, a previous study reported that patients who had a good response to NACT had longer tumor-free survival and a lower recurrence rate than patients who had no response to NACT (P<0.001; P=0.013) (7). However, chemoresistance to NACT is still a major challenge. For patients that do not respond to NACT, hysterectomy cannot be performed; consequently, the treatment strategy must be changed from surgery to radiation therapy, which results in a long period treatment delay, affecting prognosis (8-10). Therefore, it is important to identify prognostic factors in patients with LACC that predict whether NACT will be efficient before treatment (11-15).

The development of hypoxia in solid tumors is associated with tumor progression, metastasis and recurrence following treatment (16). Hypoxia-inducible factor-1 (HIF-1) is the master transcriptional factor that regulates oxygen homeostasis (17). It comprises a constitutive β-subunit and an α-subunit whose protein level depends on surrounding oxygen concentration. When oxygen is available, HIF-1α is rapidly degraded (18). Under hypoxic conditions, HIF-1α escapes degradation and rapidly dimerizes with HIF-1β. The dimers subsequently translocate to the nucleus and regulate transcription of a series of hypoxia-dependent genes (19).

At the genetic level, HIF-1α gene polymorphisms cause substantially higher transcriptional activity than the wild-type, and the C1772T polymorphism has been reported to be significantly related to response in patients undergoing NACT for LACC (20). At the protein level, HIF-1α has been demonstrated to be upregulated in a wide range of solid tumors due to hypoxic conditions or aberrant activation of some oncogenes (21). Elevated HIF-1α levels makes tumor cells more resistant to chemotherapy and increases the likelihood of metastasis and poor outcome (22). HIF-1α expression has previously been shown to be associated with tumor stage and histology of cervical cancer (23). In addition, high expression of HIF-1α resulted in worse 5-year survival rates than those patients with low HIF-1α expression (24,25). However, to date, to the best of our knowledge, there is no study available on the association between HIF-1α protein expression and the chemoresistance of cervical cancer. To the best of our knowledge, the present study is the first to identify HIF-1α protein expression as a biomarker of chemoresistance in patients with LACC.

The present study was designed to investigate whether the expression levels of HIF-1α were associated with the chemoresistance of NACT for patients with FIGO stage IIB-IIIB LACC.

Patients and methods

Patients and samples. Between January 2008 and December 2014, >600 patients with cervical cancer were referred to the Gynecologic Oncology Department, Maternal and Child Health Hospital of Hubei Province (Wuhan, China). Patients received a standard evaluation, including physical and gynecological examination, colposcopy, biopsy, laboratory examinations and image examinations, including chest X-ray, intravenous pyelography, and hepatic and pelvic ultrasonography. Exclusion criteria included the lack of informed consent, the lack of tumor samples, existing complicating disease or prior malignant disease, and patients who did not undergo NACT. Finally, 59 patients aged <70 years with complete data on age, clinical stage, grade, histology, size of tumor and main therapy, who had primary and previously untreated LACC (FIGO stages IIB-IIIB) were enrolled and analyzed retrospectively. The tumor samples were obtained by biopsy prior to any treatment. The tumor size was measured by the combination of pelvic examination and ultrasonography. Two senior oncological gynecologists participated in the evaluation. Written informed consent was obtained from all patients prior to the tumor biopsy. The Ethics Committee of Maternal and Child Healthcare Hospital of the Hubei province approved the current study protocol.

NACT with transcatheter arterial chemoembolization (TACE) technology. NACT was administered using gelatin sponge particles (GSPs) combined with TACE (26) using the Seldinger technique (27), and a paclitaxel/cisplatin treatment regimen was applied. Briefly, a catheter (5-French diameter) was inserted into the left uterine artery region under the guidance of digital subtraction angiography to locate the tumor feeding vessels. Cisplatin (75 mg/m^2) was divided into six doses, one dose was injected into the left uterine artery, one dose with 700-1,000 μm GSPs was injected into the peripheral uterine artery, then 2-3 mm GSPs were injected into the main uterine artery, and one dose was injected when the catheter came back to the anterior trunk of the iliac artery (not the superior gluteal artery). The same operation was done using the other three doses in the right uterine artery region, with adequate hydration prior to and following TACE to preserve renal function. After TACE, but on the same day, paclitaxel (175 mg/m^2) was administered intravenously for 3 h.

Treatment after NACT. NACT was administered for 1-3 cycles at 21-day intervals (between the start day of two cycles). One cycle of NACT was initially given. Only responders received the next cycle. A total of 2 weeks after each cycle, the clinical response to NACT and the operability was evaluated by magnetic resonance imaging and pelvic examination according to the World Health Organization (WHO) criteria, and defined as: Complete remission (CR), partial remission (PR), stable disease (SD) and progressive disease (PD) (28). NACT responders included patients with CR or PR, while non-NACT responders were patients with SD or PD. The cases were divided into two groups according to the efficiency of NACT: A complete/partial remission (CR + PR) group and a stable/progressive disease (SD + PD) group (Table I).

NACT responders could have surgery followed by radiation therapy; some patients chose to have radiation therapy directly due to age or financial reasons. Non-responders received radiation therapy after one cycle of NACT. According to their treatment after NACT, patients were divided into two groups: One group consisted of patients who had surgery and radiation therapy following NACT (NACT + S + R group; n=40); the other group consisted of patients where only radiation therapy was performed (NACT + R group; n=19) (Table I).

Blood counts, and liver and renal function exams were performed weekly, or more frequently if there was evidence of toxicity. Treatment was delayed if the white blood cell (WBC) count was <3,000/mm^3 or the platelet (PLT) count was <100,000/mm^3. The drug doses would be reduced by 20% if WBC count was <1,000//mm^3 or PLT count was <50,000/mm^3 over a period of >5 days. Recombinant human granulocyte colony-stimulating factor was administered with persistent grade 3-4 myelotoxicity.

A total of 2 weeks after the last cycle, patients in the NACT + S + R group underwent type III radical hysterectomy with pelvic lymphadenectomy. For patients with squamous cancer and those <40 years old, one ovary was preserved and suspended outside the pelvis. The NACT + S + R and

Table I. Chemotherapeutic response according to clinicopathological parameters.

Variables	Total no. of patients	Response to NACT		Response rate (%)	P-value
		CR + PR	SD + PD		
Number of patients	59	52	7	88.14	
Age, years (range)		47 (28-62)	50 (40-60)		0.284[a]
Clinical stage					
Stage IIB	56	49	7	87.50	0.514[b]
Stage IIIA	0	0	0		
Stage IIIB	3	3	0	100	
Grade					
G1	12	11	1	91.67	0.672[b]
G2/G3	47	41	6	87.23	
Histology					
SCC	53	49	4	92.45	0.017[b]
A	6	3	3	50	
AS	0	0	0		
Others	0	0	0		
Size of tumor					
<4 cm	10	9	1	90	0.841[b]
≥4 cm	49	43	6	87.76	
Main therapy					
NACT + S + R	40	38	2	95	0.053[b]
NACT + R	19	14	5	73.68	

[a]Independent two-sample t-test; [b]χ^2 test. CR + PR, complete remission + partial remission; SD + PD, stable disease + progressive disease; NACT + S + R, neoadjuvant chemotherapy + surgery + radiotherapy; NACT + R, neoadjuvant chemotherapy + radiotherapy; FIGO, International Federation of Gynecology and Obstetrics; SCC, squamous cell carcinoma; A, adenocarcinoma; AS, adenosquamous carcinoma.

NACT + R groups had radiotherapy after NACT + surgery or NACT directly.

Follow-up study. All patients were followed up periodically until May 2019. Overall survival was defined as the period of time from initial treatment until cervical cancer-related death. Surviving patients were censored on the date of the last follow-up.

Immunohistochemical analysis. The expression of HIF-1α was detected in 4% formalin-fixed for 24 h at room temperature, paraffin-embedded sections (size, 4 μm) by immunohistochemical staining, as previously described (29).

Briefly, 4-μm paraffin-embedded sections were deparaffinized and immersed in 3% hydrogen peroxidase in methanol for 10 min at room temperature to block endogenous peroxidase activity. The antigen was retrieved by immersing the slides in 10 mM citrate buffer (pH 6.0) and heating at 110˚C for 5 min, followed by washing in PBS. The sections were then incubated with a monoclonal rabbit anti-human HIF-1α antibody (clone EP1215Y; 1:200; cat. no. ab51608; Abcam) overnight at 4˚C. The samples were washed with PBS for 15 min and incubated with a HRP-conjugated anti-rabbit secondary antibody (1:200; cat. no. Sb 129; Servicebio) for 30 min at room temperature. 3,3'-diaminobenzidine was used as the chromogen for 5 min at room temperature. Finally, the sections were counterstained with Mayer's hematoxylin for 10 sec at room temperature.

Two independent pathologists blinded to the clinical parameters used a light Olympus-IX71 microscope (magnification, x400; Olympus Corporation) to observe the images. HIF-1α expression was semi-quantitatively analyzed based on the scoring method of Sinicrope *et al* (30). Briefly, the staining results were scored based on the following criteria: i) The percentage of positive staining was determined in five separate areas (magnification, x400); 0 (<5%), 1 (5-25%), 2 (25-50%), 3 (50-75%) and 4 (>75%); ii) staining intensity was scored as 0 (none), 1 (weak), 2 (moderate) and 3 (strong). The weighted score was calculated by multiplying the staining intensity score by the percentage of positive staining for each tissue specimen. The mean value of the weighted score was 5, so a weighted score of 0, 1, 2, 3, 4 was defined as low HIF-1α expression, and a weighted score of 6, 8, 9, 12 was defined as high HIF-1α expression.

Statistical analysis. Data are presented as the mean ± standard deviation. The Kaplan-Meier and log-rank tests were used for survival analysis and to determine the significance of differences in survival distribution. The weighted scores were compared using the Mann-Whitney U test. The independent two-sample t-test and a χ^2 test were performed for intergroup

Table II. Pathological findings from surgical specimens.

	Response to NACT		
Positive rate	CR + PR	SD + PD	P-value[a]
Lymph node metastasis	26.3% (10/38)	100% (2/2)	0.024
Surgical margin	10.5% (4/38)	0% (0/2)	0.629
Vascular invasion	2.6% (1/38)	0% (0/2)	0.816
Depth of cervical invasion	50% (19/38)	0% (0/2)	0.168

[a]χ^2 test. CR + PR, complete remission + partial remission; SD + PD, stable disease + progressive disease.

comparisons. Univariate and multivariate Cox proportional hazard regression model was used to identify the potential independence predictors. SPSS software, version 21.0 (IBM, Corp.), was used for all the statistical analyses. P<0.05 was considered to indicate a statistically significant difference.

Results

Patient characteristics and clinical response to NACT. As summarized in Table I, clinical chemotherapeutic response evaluation identified 52 patients as NACT responders (CR + PR group; 52/59; 88.14%) and seven patients were identified as non-NACT responders (SD + PD group; 7/59; 11.86%). The association between response rate and clinicopathological parameters has been detailed in Table I. Squamous cell carcinoma exhibited a more favorable response than adenocarcinoma (P=0.017). Age, FIGO stage, grade, size of the tumor and main therapy did not exhibit significant differences in NACT response (P>0.05; Table I).

Pathological findings. The pathological findings were analyzed within the NACT + S + R group using the specimens obtained after surgery. Significantly reduced pelvic lymph node metastasis was detected in the CR + PR group compared with SD + PD group (26.3 vs. 100%; P=0.024; Table II). There were no significant differences in surgical margin rates, depth of cervical invasion rates and vascular invasion rates between the two groups (P>0.05; Table II).

Expression of HIF-1α. HIF-1α was expressed in the nuclei and cytoplasm of tumor cells (Fig. 1). The brown staining represents HIF-1α expression, while blue staining represents the nuclei. The staining results were scored based on the following criteria: i) The percentage of positive staining was determined in five separate areas (magnification, x400); 0 (<5%), 1 (5-25%), 2 (25-50%), 3 (50-75%) and 4 (>75%); ii) staining intensity was scored as 0 (none), 1 (weak), 2 (moderate) and 3 (strong). The weighted score was calculated by multiplying the staining intensity score by the percentage of positive staining for each tissue specimen. The mean value of the weighted score was 5, so a weighted score of 0, 1, 2, 3, 4 was defined as low HIF-1α expression, and a weighted score of 6, 8, 9, 12 was defined as high HIF-1α expression. The mean weighted score for HIF-1α expression was significantly lower in the CR + PR group compared with the SD + PD group (3.75 vs. 6.29; P=0.029; Fig. 2). In total, 39 of the 59 patients exhibited low expression levels of HIF-1α (weighted scores, 0-4), and 20 had high HIF-1α expression (weighted score, 6-12). There were no significant differences in clinical characteristics observed between the two groups (Table III).

Association between the expression of HIF-1α and the efficiency of NACT. Of the 39 patients with low HIF-1α expression, 37 patients (94.87%) were in the CR + PR group and two patients (5.13%) were in the SD + PD group, whereas with regards to high HIF-1α expression, 15/20 patients (75%) were in the CR + PR group, and 5/20 (25%) patients were in the SD + PD group. This indicated that patients with low HIF-1α expression were significantly more responsive to NACT compared with patients with high HIF-1α expression (P=0.025; Table IV).

Survival. The overall survival time was significantly longer in the CR + PR group, compared with the SD + PD group (P<0.001; Fig. 3). The low HIF-1α expression group exhibited significantly longer overall survival time compared with the high HIF-1α expression group (P=0.017; Fig. 4).

Multivariate analysis in NACT + S + R group. A multivariate Cox proportional-hazard regression model was used to evaluate the relative strength and potential independence of HIF-1α expression, NACT response and lymph node metastases using post-surgery specimens. Age, FIGO stage, size, histology, surgical margin, depth of cervical invasion and vascular invasion had no significant impact on recurrent free survival (RFS) in univariate analysis (data not shown) and consequently were not included in multivariate analysis. High HIF-1α expression levels and lymph node metastases were significant independent predictors of poor RFS, whereas response to NACT was not significant (Table V).

Discussion

Cervical cancer is a clinical and pathological heterogeneous malignancy, which requires different treatment strategies and has a variety of patient outcomes. For early-stage cervical cancer, surgery is accepted as the standard treatment. For the treatment of patients with LACC, CCRT is recommended as the standard treatment (31). However, limited access to radiation equipment, especially in developing countries,

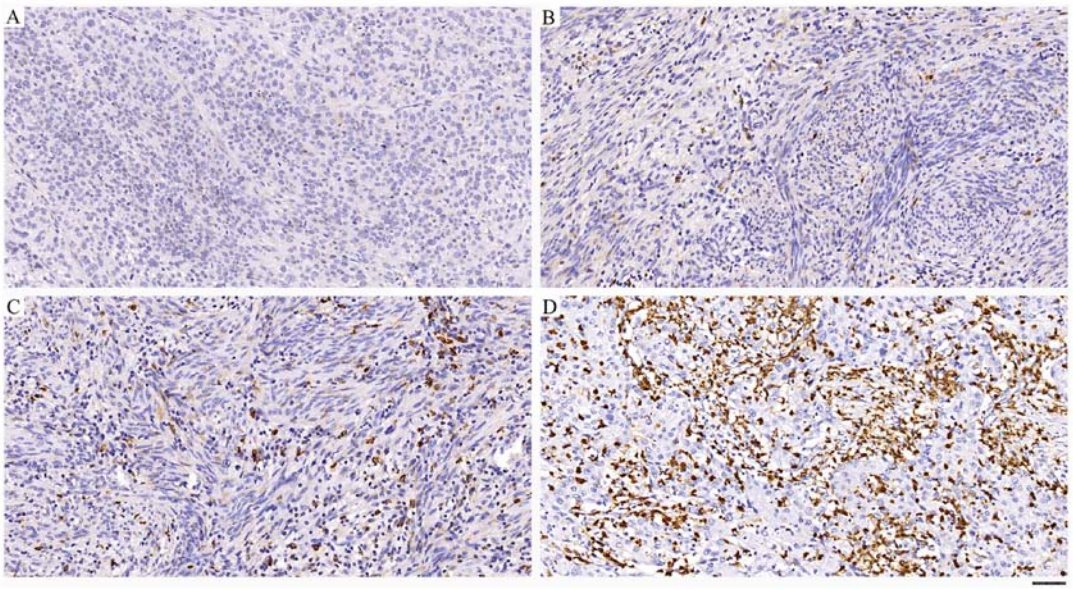

Figure 1. Immunohistochemical staining of HIF-1α in locally advanced cervical cancer. Brown staining represents HIF-1α expression, while blue staining represents the nuclei. (A) Negative control; (B) weighted score 1; (C) weighted score 4; (D) weighted score 9 (scale bar, 50 μm; magnification, x400). HIF-1α was expressed in the nuclei and cytoplasm of tumor cells. HIF-1α, hypoxia-inducible factor-1α.

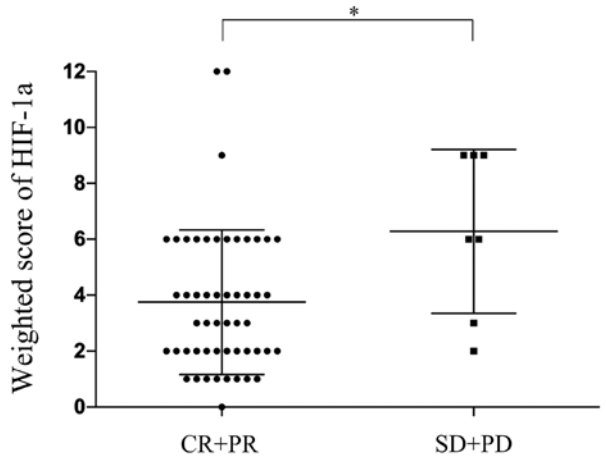

Figure 2. Weighted score for HIF-1α expression in tumor samples from patients with locally advanced cervical cancer. HIF-1α expression was significantly higher in the SD + PD compared group with the CR + PR group. *P=0.029 (Mann-Whitney U test). CR + PR, complete remission + partial remission; SD + PD, stable disease + progressive disease; HIF-1α, hypoxia-inducible factor-1α.

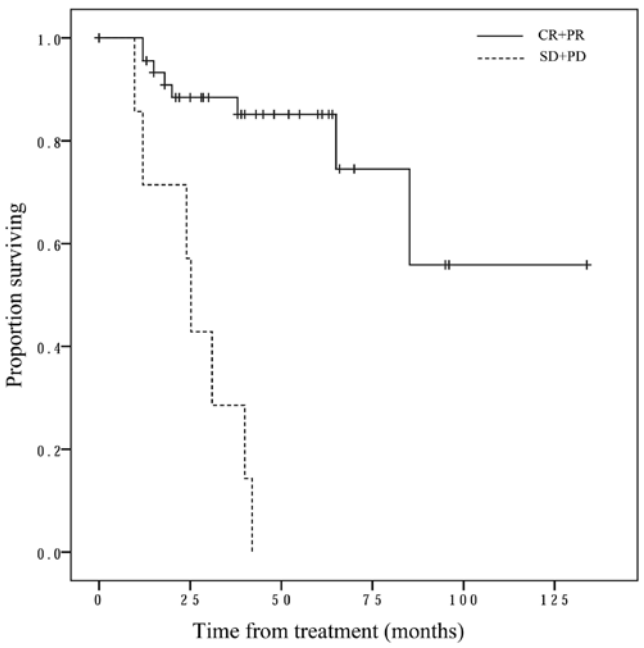

Figure 3. Overall survival rate in the CR + PR (n=52) and SD + PD (n=7) groups. Solid line, CR + PR; dashed line, SD + PD. CR + PR group exhibited significantly longer overall survival time compared with the SD + PD group (P<0.001, Kaplan-Meier and log-rank tests). CR + PR, complete remission + partial remission; SD + PD, stable disease + progressive disease.

poor control of micrometastasis, and the high incidence of permanent local toxicity due to radiation, mainly in young and sexually active women, have brought about the development of different therapeutic approaches such as NACT followed by radical surgery (32).

In clinical practice, only some patients with LACC benefit from chemotherapy treatment followed by radical surgery. Identifying patients who will be responsive to chemotherapy could provide them with proper treatment, which has important implications in personalized treatment and outcomes, while identifying non-responders may reduce the possibility of these patients receiving unsuccessful treatment and thereby enable them to receive more effective treatments as soon as the disease is diagnosed. Therefore, prognostic factors identifying the efficiency of NACT will play a critical role in trials of NACT in these patients.

The main objective of NACT is to reduce tumor volume, reduce the clinical stages of the patients, decrease lymph node metastasis, increase the chance to achieve radical hysterectomy, preserve ovarian function and reduce the dose of postoperative radiation therapy, so as to improve the quality of life of patients, especially in sexually active woman (33-36). However, there are limitations to this strategy; if the NACT

Table III. Characteristics of patients in the low and high HIF-1α expression groups.

Characteristics	Low HIF-1α expression (≤4[a])	High HIF-1α expression (≥6[a])	P-value
Number of patients	39	20	
Age, years (range)	47 (28-62)	48 (34-62)	0.923[b]
Clinical stage			
Stage IIB	36	20	0.544[c]
Stage IIIA	0	0	
Stage IIIB	3	0	
Grade			
G1	11	1	0.079[c]
G2/G3	28	19	
Histology			
SCC	35	18	0.975[c]
A	4	2	
AS	0	0	
Others	0	0	
Size of tumor			
≤4 cm	5	5	0.416[c]
>4 cm	34	15	
Main therapy			
NACT + S + R	29	11	0.132[c]
NACT + R	10	9	

[a]Weighted score; [b]independent two-sample t-test; [c]χ^2 test. HIF-1α, hypoxia-inducible factor-1α; NACT + S + R, neoadjuvant chemotherapy + surgery + radiotherapy; NACT + R, neoadjuvant chemotherapy + radiotherapy; FIGO, International Federation of Gynecology and Obstetrics; SCC, squamous cell carcinoma; A, adenocarcinoma; AS, adenosquamous carcinoma.

Table IV. Number of patients with low and high HIF-1α expression in the CR + PR and SD + PD groups.

Expression	CR + PR, n (%)	SD + PD, n (%)	P-value
Low, ≤4	37 (94.87)	2 (5.13)	0.025[a]
High, ≥6	15 (75)	5 (25)	

[a]χ^2 test. HIF-1α, hypoxia-inducible factor-1α; CR + PR, complete remission + partial remission; SD + PD, stable disease + progressive disease.

Table V. Cox regression multivariate analysis with overall survival as end point (n=40).

Risk factor	HR	95% CI for HR	P-value
Lymph node metastasis	6.909	1.356-35.216	0.020
High HIF-1α expression	6.354	1.262-31.995	0.025
Response to NACT	0.246	0.026-2.541	0.258

HIF-1α, hypoxia-inducible factor-1α; NACT, neoadjuvant chemotherapy; HR, hazard ratio; CI, confidence interval.

is not administered efficiently, there will be a time delay and chemotherapy-induced resistance to radiotherapy, which would result in a worse prognosis (8,9,37,38). Hence, it is crucial to identify factors that could predict the efficacy of NACT in patients with LACC.

Hypoxia, a decrease in oxygen concentration in the tissue microenvironment, affects physiological development and tumorigenesis (39). A key mediator of the response to hypoxia is HIF-1α (40). HIF-1α is inactive and remains at a low concentration in normoxia. In hypoxia, however, HIF-1α is stabilized and activated. In gynecological cancers, HIF-1α is an important factor in carcinogenesis, and high levels of HIF-1α expression seem associated with shorter progression-free survival and overall survival (41). Significantly higher levels of HIF-1α transcript and protein were detected in tumor tissue compared with normal tissue in cervical cancer (42). However, to the best of our knowledge, there is no study on the effect of HIF-1α protein expression on chemoresistance of cervical cancer.

This retrospective study firstly reported that HIF-1α protein expression may be able to distinguish patients with LACC at FIGO stage IIB and IIIB who are relatively chemoresistant. Also, it may be a good prognostic indicator over the current standards with clinical stage, since patients with the same clinical stage often have different prognosis (some patients are cured, while others suffer recurrence). The present results demonstrated that high levels of HIF-1α expression were

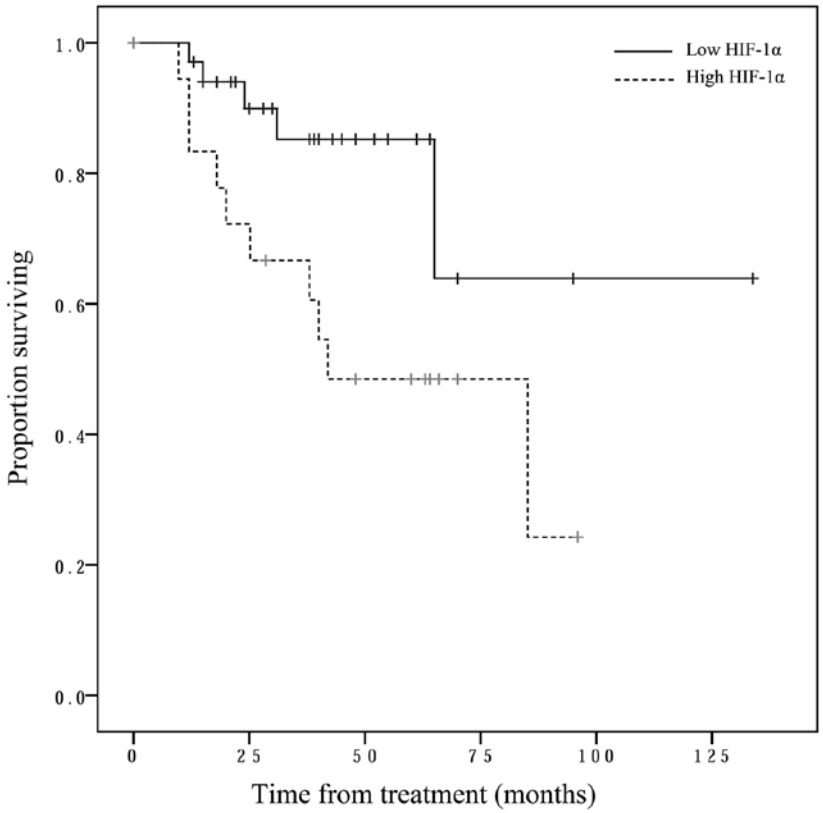

Figure 4. Overall survival rate in the low (n=39) and high HIF-1α expression (n=20) groups. Solid line, low level of HIF-1α expression; dashed line, high level of HIF-1α expression. The low HIF-1α group exhibited significantly longer overall survival time compared with the high HIF-1α group (P=0.017, Kaplan-Meier and log-rank tests). HIF-1α, hypoxia-inducible factor-1α.

associated with resistance to cisplatin-based chemotherapy and may be a prognostic predictor of the efficiency of NACT in patients with LACC at FIGO stage IIB and IIIB. In addition, survival analysis revealed that prognosis was worse when NACT was inefficient, which is in accordance with previous findings (7). Cox hazard analysis using post-surgery specimens indicated that lymph node metastasis and high levels of HIF-1α expression were independent prognostic factors. However, NACT response was not an independent prognostic factor (P=0.258), which may due to the insufficient number of specimens and SD + PD patients. Further study is required to conduct experiments to detect HIF-1α with multiple approaches such as quantitative PCR and western blotting to confirm these findings. The downstream molecules of HIF-1α, such as vascular endothelial growth factor (VEGF) and erythropoietin (EPO), should also be detected, as under a hypoxic environment, with increased levels of HIF-1α, VEGF and EPO promote angiogenesis and erythropoiesis, which will alter the hypoxic condition (43).

In conclusion, these results indicated that inefficient NACT for LACC leads to a worse prognosis. Therefore, factors that predict the efficiency of NACT will play an important role in trials of NACT for cancer. To the best of our knowledge, the present study is the first to show that the level of HIF-1α expression may be a strong predictor of the efficiency of NACT and a prognostic factor in patients with stage IIB and IIIB cervical cancer. Future studies with larger patient numbers and different FIGO stage are required to validate and further elucidate this finding.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author upon reasonable request.

Authors' contributions

BY, XFW and JBH conceived and designed the study. BY, XFW, QFM, WFT, HNC, YLL, ZGZ, FXZ, YJX and HG performed the experiments and collected the data. BY, XFW, JBH, XD, MX and YLG analyzed and interpreted the data. BY and XFW drafted the initial manuscript and revised the important intellectual content. All authors read and approved the final version of the manuscript.

Ethics approval and consent to participate

The research involving human samples was approved by the Ethics Committee of Maternal and Child Health Hospital of Hubei Province. All experiments were conducted according to relevant national and international guidelines. Informed consent was obtained from all participants included in the study.

Patient consent for publication

Informed consent was obtained from all participants included in the study.

Competing interests

The authors declare that they have no competing interests.

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