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Original Article

Increased HIF-1alpha expression in tumor cells and lymphocytes of tumor microenvironments predicts unfavorable survival in esophageal squamous cell carcinoma patients

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Abstract: The expression of hypoxia-induced factor (HIF)-1α is up-regulated in tumor microenvironments under hypoxia condition. However, the prognostic significance of HIF-1α in esophageal squamous cell carcinoma (ESCC) is still elusive. We measured the HIF-1α expression by immunochemistry in tumor specimens from 136 resected ESCC; in the current study, the HIF-1α expression in tumor cells was significantly associated with tumor stage (P = 0.003) and lymph node metastasis (P = 0.006); whereas the HIF-1α expression in tumor-infiltrating lymphocytes (TILs) had no relationship with patients' clinicopathological parameters. Patients with high HIF-1α expression in tumor cells or in TILs showed worse survival related to those with low HIF-1α expression. Multivariate analysis demonstrated that expression of HIF-1α in TILs was an independent factor for DFS (P = 0.007) and OS (P = 0.013). Additionally, the expression of HIF-1α in tumor cells was an independent factor for DFS (P = 0.037) and OS (P = 0.033) in locoregional ESCC patients, whereas the expression of HIF-1α in TILs was an independent factor for DFS (P = 0.048) and OS (P = 0.039) in metastatic ESCC patients. Correlation analysis revealed that expressions of HIF-1α in tumor cells and in TILs were positively correlated, and patients with combined high HIF-1α in both tumor cells and TILs had the worst survivals (P < 0.05). These findings suggest that the HIF-1α expressions in different cell populations of ESCC microenvironments have different clinical relevance and prognostic impact on patients.

Keywords: Esophageal squamous cell carcinoma, HIF-1α, tumor microenvironments, clinical prognosis, tumor-infiltrating lymphocytes

Introduction

Esophageal squamous cell carcinoma (ESCC), one of the major histopathological subtypes of esophageal cancer, is the fourth most prevalent malignancy in China and a leading cause of cancer-related death. The prognosis of ESCC is generally unfavorable, with a five-year survival rate of less than 30% [1, 2]. Despite general advances in diagnosis and treatment, the prognosis of ESCC patients remains poor because of high rates of recurrence/metastasis and resistance to adjuvant therapy [3, 4]. Therefore, there is an urgent clinical need to explore novel prognostic markers for ESCC patients. In addition to the traditional prognostic factors determined at diagnosis, such as TNM stage and cell differentiation, the molecular markers related to tumor cell apoptosis, epithelial-mesenchymal transition (EMT), the function of infiltrated immune cells and angiogenesis in tumor microenvironments have been evaluated for their contribution to the prognoses of ESCC patients in recent studies [5-10]. However, reliable markers are still lacking in ESCC. Furthermore, to the best of our knowledge, there are no reports on the correlation between the expression level of a given molecular marker in different cell populations within the tumor microenvironment and the clinical prognosis of patients with ESCC, although it has been noted that the biological function of a gene product, as well as its effect on the clinical progression of cancer, varies depending on the cell population in which it is located.

Table 1. Clinical characteristic of 136 patients with ESCC

Characteristics	No. (%)
Total case	136
Age (Years)	
Median	62
Range	35-90
Gender	
Male	111 (81.6%)
Female	25 (18.4%)
WHO degree	
G1	40 (29.4%)
G2	59 (43.4%)
G3	37 (27.2%)
Tumor (T) status	
T1	8 (5.9%)
T2	36 (26.5%)
T3	88 (64.7%)
T4	4 (2.9%)
Lymphoid Nodal (N) status	
N0	69 (50.7%)
N1	67 (49.3%)
Distant metastasis (M) status	
M0	130 (95.6%)
M1	6 (4.4%)
TNM stage	
I	6 (4.4%)
IIa-IIb	68 (50.0%)
III	56 (41.2%)
IV	6 (4.4%)
Death	
No	33 (24.3%)
Yes	103 (75.7%)
Therapy after surgery	
Radiation therapy alone	10 (7.4%)
Chemotherapy alone	12 (8.8%)
Radiation therapy + chemotherapy	7 (5.1%)
No	107 (78.7%)

Hypoxia is one of the defining features and poor prognostic markers of several solid cancers. Under low oxygen conditions (hypoxia), transcription of the a-subunit of hypoxia-induced factor (HIF) is rapidly induced. Hypoxic HIF activity is controlled primarily through post-translational modification and stabilization of the HIF-1α and HIF-2α subunits, leading to the transcription of a diverse set of target genes that are broadly involved in the adaptation to low oxygen and contribute to metabolic changes, angiogenesis and survival [11, 12]. Recent studies demonstrated that HIF-1α was overexpressed in a number of cancers, including gastric cancer, breast cancer, prostate cancer and colon cancer, furthermore, overexpression of HIF-1α had an important effect on the biological action of tumor cells, the recruitment of infiltrated lymphocytes and angiogenesis in tumor microenvironments [13-16]. To date, however, the of the expression status of HIF-1α in ESCCs, particularly in its tumor microenvironment, and their clinico-prognostic significances remain to be elucidated [17-19].

In the present study, we measured the expression pattern of the hypoxia-induced protein HIF-1α in different cell populations, including tumor cells and tumor-infiltrating lymphocytes (TILs) in tumor microenvironment of ESCC and investigated their associations with patients' clinical outcomes.

Methods

Patients and tissue samples

Paraffin-embedded tumor tissue samples were obtained from 136 ESCC patients who underwent surgery at Sun Yat-Sen University Cancer Center in Guangzhou City of China from November of 2000 to December of 2002. None of the patients had received anticancer treatment prior to surgery, and all of the patients had histologically confirmed primary ESCC in this retrospective study. The follow-up data from the 136 patients with ESCC in this study were available and complete. The diagnostic examinations consisted of esophagography, CT, chest X-ray, abdominal ultrasonography and bone scan when necessary to detect recurrence and/or metastasis. The OS was defined as the time interval from the date of surgery to the date of cancer-related death or the end of follow-up (December 2011), and the DFS was defined as the time interval from the date of surgery to the date of tumor recurrence or tumor metastasis. This study was approved by the Research Ethics Committee of the Sun Yat-Sen University Cancer Center.

Immunohistochemistry

The paraffin-embedded tissues were sectioned continuously into 4-μm-thick sections. The tissue sections were dewaxed in xylene, rehydrat-

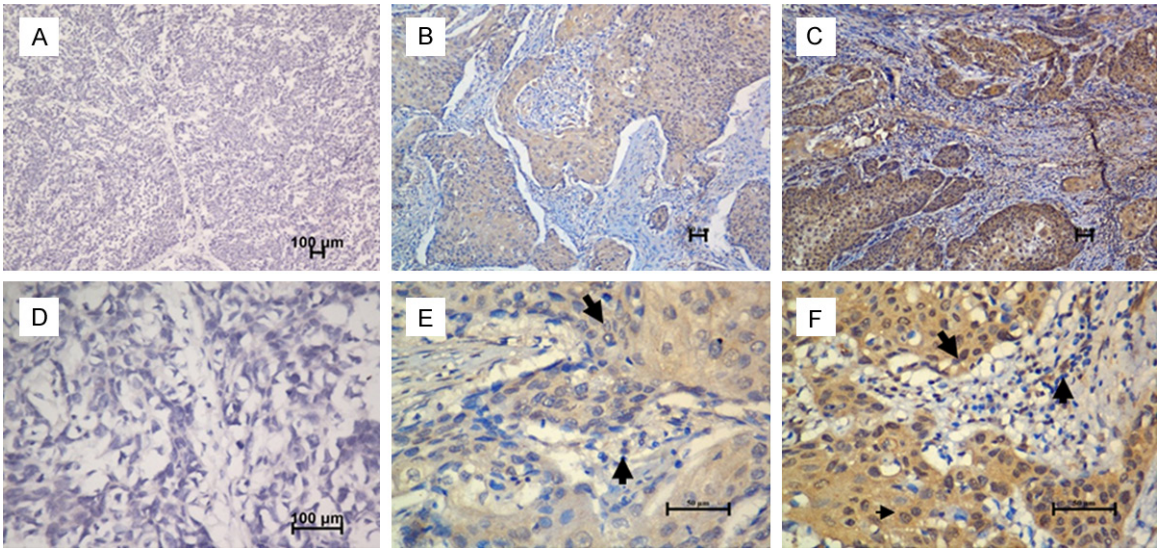

Figure 1. Immunohistochemical staining for HIF-1α in human esophageal carcinoma. Our data showed negative expression level (A, X 100; D, X 400), low expression (B, X 100; E, X 400) and high expression level of HIF-1α (C, X 100; F, X 400) in tumor tissues from patients with ESCC. The arrows point to the positive staining of tumor cells or TILs.

Table 2. Clinicopathological associations of HIF-1α expression levels and the density of tumor-infiltrating lymphocytes in 136 patients with ESCC

Clinicopathologic parameter	Total case (n = 136)	High level of HIF-1α in tumor (%)	P	High level of HIF-1α in TIL (%)	P
Age					
≤ 62 y	71	34 (47.9)	0.292	34 (47.9)	0.574
> 62 y	65	37 (56.9)		28 (43.1)	
Gender					
Female	25	10 (40.0)	0.176	12 (48.0)	0.789
Male	111	61 (55.0)		50 (45.0)	
WHO grade					
G1	40	24 (60.0)	0.213	20 (50.0)	0.221
G2	59	32 (54.2)		22 (37.3)	
G3	37	15 (40.5)		20 (54.1)	
Tumor size					
< 12 cm^3	63	30 (47.6)	0.320	26 (41.3)	0.348
≥ 12 cm^3	73	41 (56.2)		36 (49.3)	
T status					
T1-2	44	15 (34.1)	0.003*	21 (47.7)	0.729
T3-4	92	56 (60.9)		41 (44.6)	
N status					
N0	69	28 (40.6)	0.006*	29 (42.0)	0.398
N1	67	43 (64.2)		33 (49.3)	
Clinical stage					
I-II	74	34 (45.9)	0.110	34 (45.9)	0.927
III-IV	62	37 (33.3)		28 (45.2)	

*$P < 0.05$, as determined by Pearson's X^2 test.

ed and rinsed in graded ethanol solutions. The antigens were retrieved by heating the tissue sections at 100°C for 30 min in citrate (10 mmol/L, pH 6.0) or EDTA (1 mmol/L, pH 9.0) solution when necessary. The sections were then immersed in a 0.3% hydrogen peroxide solution for 30 min to block endogenous peroxidase activity, rinsed in phosphate-buffered saline (PBS) for 5 min, and incubated with the primary antibody mouse anti-human HIF-1α (Clone H1alpha67; Novus Biologicals, Inc., Littleton, CO) at 4°C overnight. A negative control was performed by replacing the primary antibody with a normal murine IgG antibody. The sections were then incubated with a horseradish peroxidase-labeled goat antibody against a mouse/rabbit secondary antibody (Envision; Dako, Glostrup, Denmark) at room

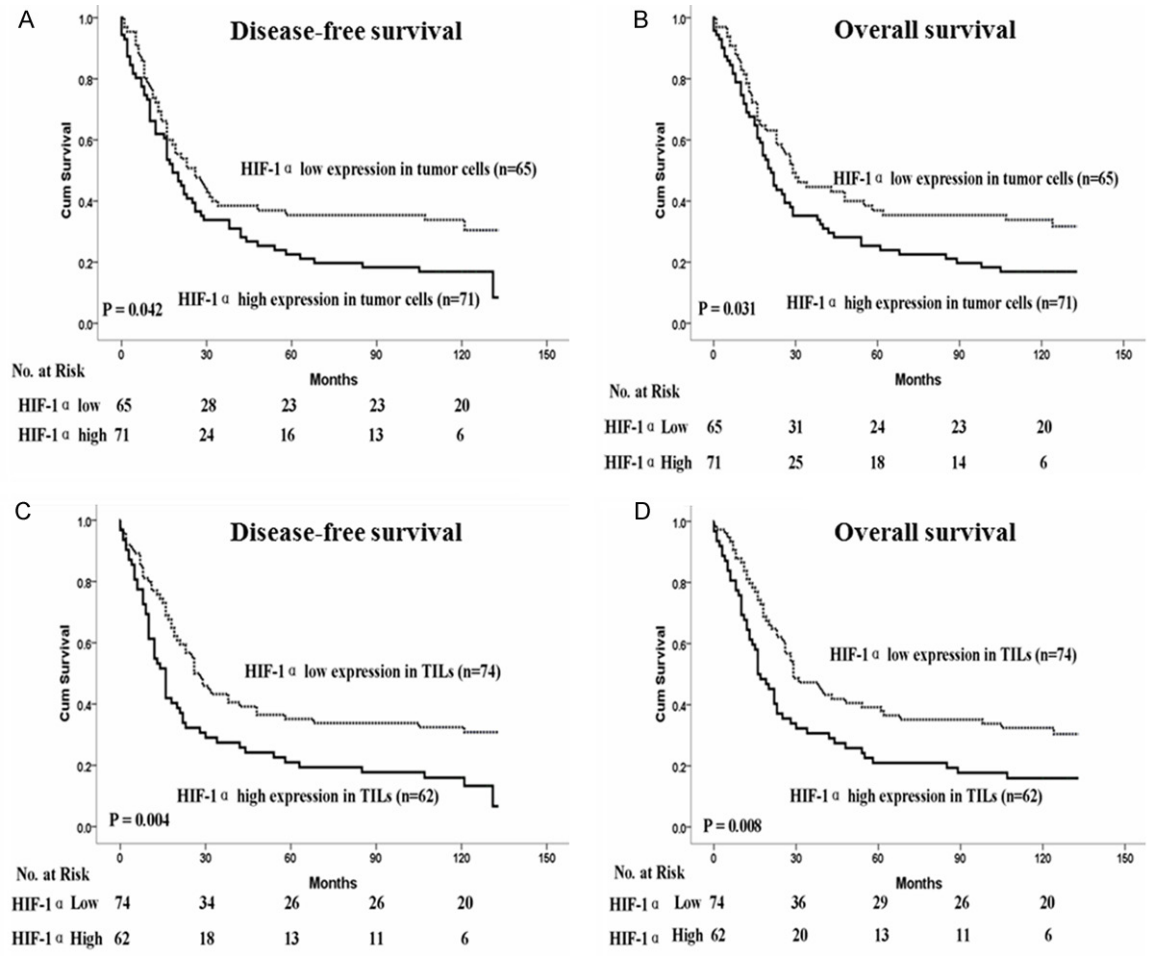

Figure 2. Kaplan-Meier survival analysis in patients with ESCC. A, B. Disease-free survival and overall survival curves for patients according to the low and high expression level of HIF-1α in tumor cells. C, D. Disease-free survival and overall survival curves for patients according to the low and high expression level of HIF-1α in TILs.

temperature for 30 min. Finally, the signal was developed for visualization with 3, 3'-diaminobenzidine tetrahydrochloride (DAB), and all of the slides were counterstained with hematoxylin.

Evaluation of immunohistochemical staining

Two independent observers (Lin Zhang and Xiao-Feng Tang) blinded to the clinicopathological information scored the HIF-1α expression level in tumor cells by assessing (a) the proportion of positively stained cells (0, < 5%; 1, 6 to 25%; 2, 26 to 50%; 3, 51 to 75%; 4, > 75%) and (b) the intensity of staining (0, negative staining; 1, mild staining; 2, moderate staining; 3, strong staining). The score was the product of a × b. The levels of HIF-1α expression in lymphocytes were obtained by counting the positively and negatively stained lymphocytes in five to ten separate 400 × high-power microscopic fields and calculating the mean percentage of positively stained lymphocytes among the total lymphocytes per field. The patients were divided into subgroups: a high-level group (an X b ≥ 7 score in tumor cells; or ≥ 44% of HIF-1α-positive cells in lymphocytes) and a low-level group (an X b < 7 score in tumor cells; or < 44% of HIF-1α-positive cells in lymphocytes) based on the medians of immunohistochemical variable values in diverse cell subsets in our data.

Statistical analysis

All analyses were conducted with SPSS 16.0 (SPSS Inc., Chicago, IL, USA). Pearson's chi-square test and Fisher's chi-square test were used to analyze the correlation between HIF-1α

Table 3. Multivariate Cox regression analysis for DFS and OS of 136 patients with ESCC

Variables[a]	DFS		OS	
	HR (95% CI)	*p*	HR (95% CI)	*p*
HIF-1α expression in tumor cells				
Gender (Male/Female)	0.462 (0.255-0.838)	0.011*	0.422 (0.235-0.760)	0.004*
WHO Grade (1/2/3)	1.321 (1.009-1.730)	0.043*	1.310 (1.000-1.716)	0.050*
Tumor (T) status (1-2/3-4)	0.944 (0.510-1.746)	0.853	/	
Nodal (N) status (0/1)	2.205 (1.015-4.789)	0.046*	2.158 (1.029-4.527)	0.042*
TNM stage (I-II/III-IV)	0.875 (0.567-1.350)	0.547	0.855 (0.594-1.231)	0.400
HIF-1α in tumor cells (low/high)	1.234 (0.794-1.920)	0.350	1.297 (0.856-1.964)	0.220
HIF-1α expression in TILs				
Gender (Male/Female)	0.461 (0.254-0.837)	0.011*	0.412 (0.229-0.741)	0.003*
WHO Grade (1/2/3)	1.289 (0.990-1.677)	0.059	1.263 (0.971-1.644)	0.082
Tumor (T) status (1-2/3-4)	0.977 (0.544-1.752)	0.937	/	
Nodal (N) status (0/1)	2.215 (1.070-4.585)	0.032*	2.156 (1.064-4.367)	0.033*
TNM stage (I-II/III-IV)	0.896 (0.590-1.361)	0.606	0.885 (0.623-1.258)	0.497
HIF-1α in TILs (low/high)	1.697 (1.152-2.500)	0.007*	1.635 (1.108-2.412)	0.013*
HIF-1α expression in locoregional ESCC				
Gender (Male/Female)	0.406 (0.168-0.981)	0.045*	0.341 (0.132-0.886)	0.027*
WHO grade (1/2/3)	1.897 (1.204-2.989)	0.006*	1.881 (1.181-2.994)	0.008*
HIF-1α in tumors cells (low/high)	1.938 (1.040-3.611)	0.037*	1.991 (1.058-3.748)	0.033*
HIF-1α expression in metastatic ESCC				
HIF-1α in TILs (low/high)	1.669 (1.005-2.772)	0.048*	1.711 (1.028-2.847)	0.039*

[a]Variables with *P* values greater than 0.05 in the univariate models were not included in the multivariate analysis. *Significant. DFS, disease-free survival; OS, overall survival; HR, hazard ratio; CI, confidence interval.

expression in different cell subsets and the patients' clinicopathological parameters. HIF-1α expression level was examined in tumor cells and in TILs in relation to the patients' clinical prognosis using the Kaplan-Meier method and the log-rank survival analysis. Prognostic factors were assessed by univariate and multivariate analyses using the Cox proportional hazards model. The correlations among the expression levels of HIF-1α in tumor cells and in TILs were determined using Pearson's correlation coefficient and linear regression analyses. A two-tailed *P*-value < 0.05 was considered statistically significant in this study.

Results

Patient characteristics

A total of 136 eligible patients were enrolled in the current study. The patients had a median age of 62 years (range, 35 to 90 years); 111 (81.6%) were males and 25 (18.4%) were females. There were 74 (54.4%) cases of Stage I and II tumors and 62 (45.6%) cases of Stage III and IV tumors based on the International Union against Cancer 2002 TNM staging system and WHO classification criteria [20]. Of the 136 patients, 103 (75.7%) had died. Of the136 patients one-hundred and seven cases (78.7%) didn't get any anti-tumor therapy until tumor progression, whereas 10 cases (7.4%) received adjuvant radiotherapy, twelve cases received adjuvant chemotherapy (8.8%), and seven cases (5.1%) received both of radiotherapy and chemotherapy. The patients' clinical parameters are detailed in **Table 1**.

Expression patterns of HIF-1α in ESCC and their correlations with clinicopathological parameters

In the present study, the protein expression level of HIF-1α was examined in tumor specimens from 136 patients with ESCC. HIF-1α was expressed mainly in the nucleus and cytoplasm of tumor cells and TILs (**Figure 1**). Based on the criteria described in the Methods section, high expression level of HIF-1α in tumor cells was noted in samples from 71 (52.2%) of the 136

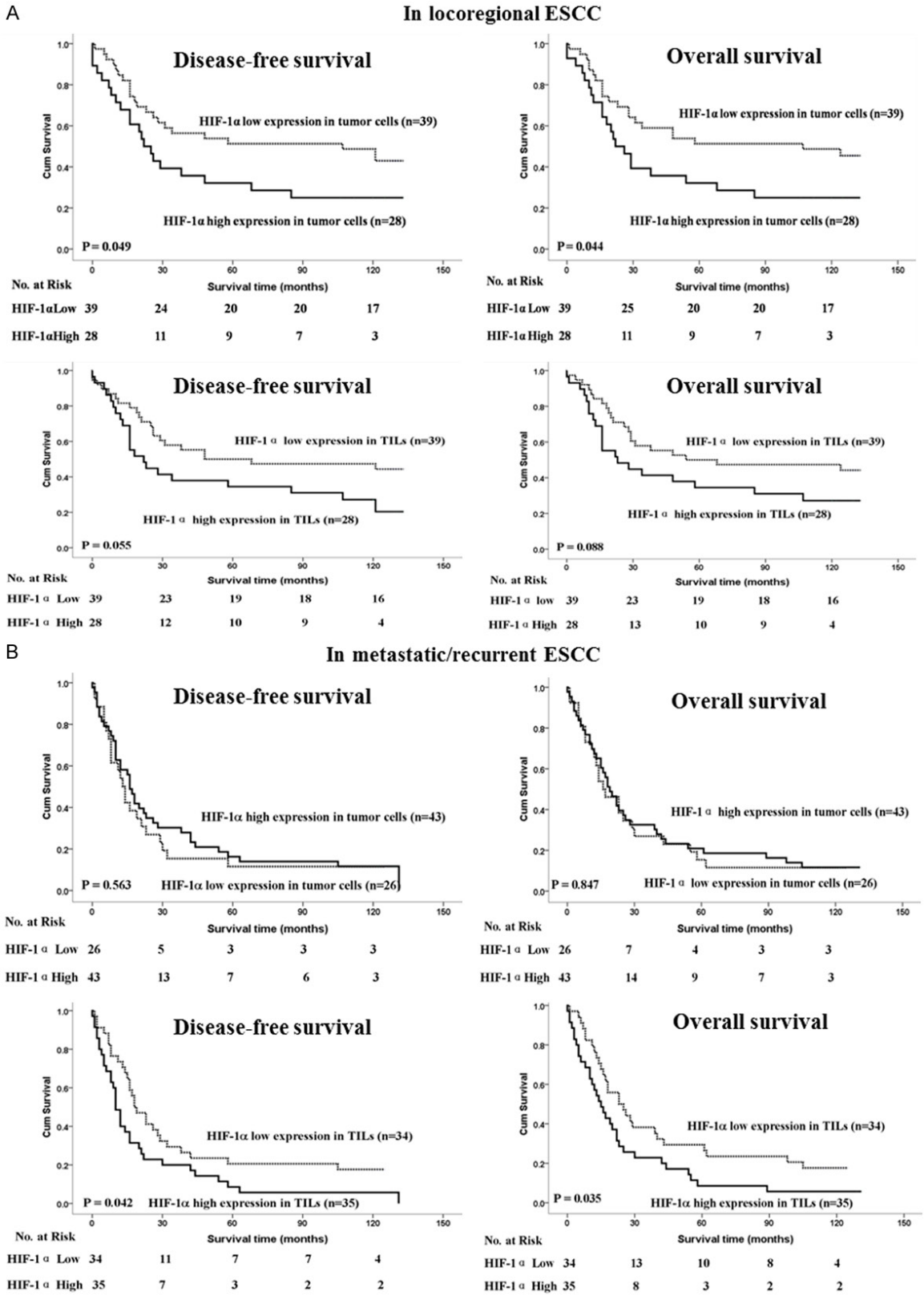

Figure 3. Kaplan-Meier survival analysis in patients with locoregional ESCC and metastatic ESCC. A. Disease-free survival and overall survival curves for patients with low and high expression levels of HIF-1α in tumor cells of locoregional ESCC. B. Disease-free survival and overall survival curves for patients with low and high expression levels of HIF-1α in TIL of metastatic ESCC.

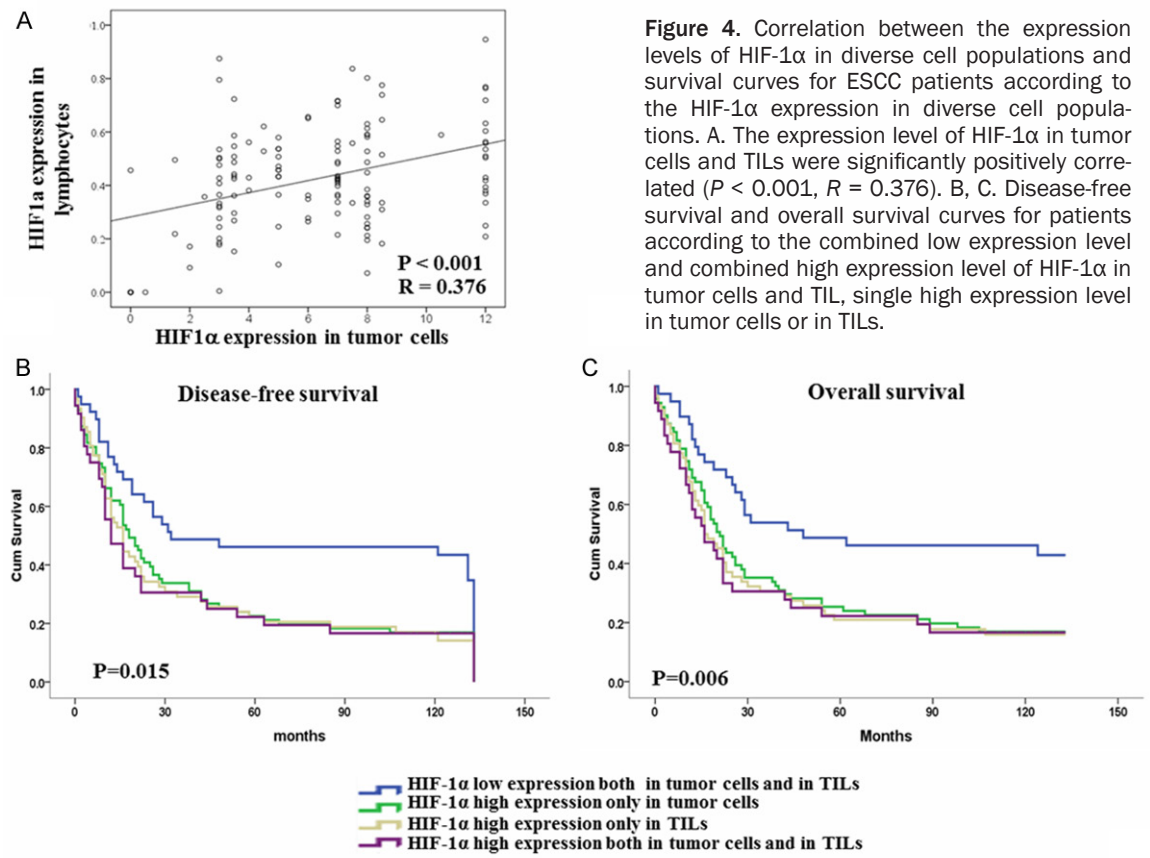

Figure 4. Correlation between the expression levels of HIF-1α in diverse cell populations and survival curves for ESCC patients according to the HIF-1α expression in diverse cell populations. A. The expression level of HIF-1α in tumor cells and TILs were significantly positively correlated ($P < 0.001$, $R = 0.376$). B, C. Disease-free survival and overall survival curves for patients according to the combined low expression level and combined high expression level of HIF-1α in tumor cells and TIL, single high expression level in tumor cells or in TILs.

Table 4. Kaplan-Meier Survival Analysis (Log-Rank Test) analyses of factors associated with OS and DFS

Variables	DFS (n = 136)		OS (n = 136)	
	Median (Months)	*p*	Median (Months)	*p*
Low expression of HIF-1α in tumor cells and in lymphocytes	32	0.015	48	0.06
High expression of HIF-1α only in tumor cells	18		21	
High expression of HIF-1α only in lymphocytes	16		16	
High expression of HIF-1α in tumor cells and in lymphocytes	12		16	

patients. The mean percentage and the range of the percentage of patients with TILs positive for HIF-1α expression per high-power light microscopic field were 43% (range, 0 to 95%) among the 136 patients assessed.

The associations between clinicopathological features and HIF-1α in different cell subsets within the tumor microenvironment in samples from 136 ESCC patients are summarized in **Table 2**. In the present study, high expression level of HIF-1α in tumor cells were closely associated with advanced clinicopathological characteristics, including tumor (T) status ($P = 0.006$), node (N) status ($P = 0.006$) and clinical stage ($P = 0.019$). Furthermore, the expression level of HIF-1α in lymphocyte was not related to any of the clinicopathological parameters, including age, gender, WHO grade, T status, N status and clinical stage.

Expression level of HIF-1α in diverse cell subsets and ESCC patient survival

Among the 136 patients with ESCC, the median survival time was 25 months (range: 0 to 33 months). The cumulative five-year OS rate and DFS rate of the patients in this study were 29

and 31%, respectively. The statistical analysis showed a significant negative correlation between DFS, OS and the HIF-1α expression in tumor cells and TILs ($P < 0.05$, **Figure 2**).

The multivariate analysis demonstrated that except some conventional clinicopathological parameters such as gender, WHO grade, nodal status and TNM stage the HIF-1 in TILs but not in tumor cells was an independent unfavorable factor for DFS ($P = 0.007$) and OS ($P = 0.013$) (**Table 3**).

Among the 136 patients with ESCC, there were 67 (49.3%) patients with locoregional ESCC and 69 cases (50.7%) with metastastic ESCC. The multivariate analysis demonstrated that the high expression of HIF-1α in tumor cells was noticeably associated with reduced DFS ($P = 0.051$) and OS ($P = 0.039$) in patients with locoregional ESCC (**Figure 3A**), whereas high expression level of HIF-1 in TILs was only significantly correlated with poor DFS ($P = 0.042$) and OS ($P = 0.035$) in patients with metastatic ESCC (**Figure 3B**). Furthermore, our multivariate analysis revealed that the expression of HIF-1α in tumor cells was an independent prognostic marker for patients with locoregional ESCC; and the expression of HIF-1α in TILs was an independent prognostic marker for patients with metastatic ESCC (**Table 3**).

The combined expression levels of HIF-1 in both tumor cells and TILs and the survival of ESCC patients

In the current study, Pearson's correlation coefficient and a linear regression analysis were applied to evaluate the correlation between the expression levels of HIF-1α in tumor cells and in TILs. The HIF-1α expression level in tumor cells were significantly positively associated with the HIF-1α expression level in TILs ($P < 0.001$, $R = 0.376$). Therefore, the patients with a combined low expression level of HIF-1α both in tumor cells and TILs had the longest DFS (Median 32 months versus 18, 16 and 12 months) and OS (median 48 months versus 21, 16 and 16 months) related to those with a single high expression level only in tumor cells or in TILs or with a combined high expression level both in tumor cells and in TILs (**Figure 4** and **Table 4**).

Discussion

It has been suggested that the tumor microenvironment contains immune cells in addition to the cancer cells and their surrounding stromal cells, and the interactions of the diverse cell subsets in the tumor microenvironment play an important role in the clinical prognosis of human cancer patients [21]. Hypoxia has been defined as a common feature in tumor microenvironments. It was reported that under low oxygen conditions, a series of genes related to tumor cell proliferation, angiogenesis and immune cell functions and recruitment are overexpressed in tumor microenvironments through the regulation of HIF-1α protein [22, 23]. In this study, we examined the expression pattern of hypoxia-induced protein HIF-1α in different cell populations in tumor tissues from 136 patients with ESCC.

The HIF-1α protein is a good intrinsic marker of tumor hypoxia. Our results suggest that HIF-1α is located in the nucleus and cytoplasm of both tumor cells and TILs (**Figure 1**). The increased level of HIF-1α in tumor cells was significantly associated with high T status and N status in ESCC patients, and increased levels of HIF-1α in both tumor cells and TILs were significantly correlated with poor DFS and OS in patients with ESCC, according to the univariate Kaplan-Meier analysis (**Figure 2**). However, only HIF-1α expression in TILs, but not in tumor cells, was an independent prognostic marker for ESCC in a multivariate Cox model analysis that included gender, T status and N status, which were significantly associated with DFS and OS in ESCC patients in the univariate analysis (**Table 2**). Interestingly, we did observe that high expression of HIF-1α in tumor cells was an independent predictor of DFS and OS in patients with locoregional ESCC (**Figure 3A**), whereas HIF-1α expression in TILs was an independent predictor of DFS and OS in patients with metastatic ESCC, but not in locoregional tumors. These results suggest that increased expression of HIF-1 in tumor cells has an important effect on the progression of malignant ESCC cells at early disease stage, while the expression of HIF-1α in TILs has an important effect on the clinical prognosis of ESCC patients in the whole disease stage. Previous reports have also indicated that HIF-1 expression was upregulated in the malignant cells of cancers, including ESCC, to promote tumor cell proliferation and survival, EMT and angiogenesis [24-26]. Recent studies about the impact of HIF-1α expression on the survivals of ESCC patients were contrast in dif-

ferent research groups, discrepancies from these studies may arise from different populations and not to distinguish the HIF-1α positive cell populations in tumor microenvironments [27-31]. We know that under hypoxic conditions, immune cells can switch to glycolysis by regulating HIF-1α protein expression to alter the balance of Th1 and Th2 helper cells and by regulating the activities of immune cells to alter immune responses [32]. Some studies determined that the overexpression of HIF-1α in lymphocytes could upregulate the expression of Foxp3, increasing the suppressive function of regulatory T cells and impairing the function of cytotoxic T cells [33, 34]. These results also support our finding that high HIF-1α expression in TILs was significantly associated with poor survival in total ESCC patients and in ESCC patients with late disease stage. Furthermore, to the best of our understanding, there is no other report about the correlation between HIF-1 expression level in TILs and cancer patients' survival.

In summary, our results revealed for the first time that the hypoxia-induced protein HIF-1α is overexpressed in both tumor cells and TILs within the tumor microenvironment of ESCCs. The expression of HIF-1α in tumor cells or in TILs was all significantly associated with poor survivals in ESCC patients. Interestingly, the expression of HIF-1α in tumor cells was an independent predictor of survival for locoregional ESCCs, whereas the expression levels of HIF-1 in TILs was an ideal survival predictive factor for total ESCC patients and metastatic ESCC patients. More importantly, patients with the combined high expression of HIF-1α in both tumor cells and in TILs showed the worst survival in all ESCC patients in this retrospective study.

Taken together, these data suggest that the expression levels of HIF-1α in different cell populations of tumor microenvironment have different clinical relevance and prognostic values in patients with ESCC.

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Disclosure of conflict of interest

None.

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