Prognostic impact of the combination of HIF-1α and GLUT1 in patients with oesophageal squamous cell carcinoma

HANJIE YI[1,2*], YONGQIN HAN[3*], QIN LI[1], RUNDUAN LIN[2], JIA ZHANG[4], YUN YANG[5], XUEPING WANG[2] and LIN ZHANG[2,6]

[1]Department of Oncology, The Second Affiliated Hospital of Nanchang University, Nanchang, Jiangxi 330000; [2]Department of Oncology, State Key Laboratory of Oncology in South China, Sun Yat-Sen University Cancer Center, Guangzhou, Guangdong 510060; [3]Department of Oncology, Shangrao People's Hospital, Shangrao, Jiangxi 334000; [4]Department of Laboratory Medicine, The Third Hospital of Changsha, Changsha, Hunan 410015; [5]Department of Laboratory Medicine, The 921st Hospital of The Joint Logistics Support Force of The Chinese People's Liberation Army, Changsha, Hunan 410003; [6]Department of Laboratory Medicine, Yunfu People's Hospital, Yunfu, Guangdong 527300, P.R. China

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Abstract. Oesophageal squamous cell carcinoma (ESCC) is a common type of carcinoma. Hypoxia is associated with chemo- and radio-resistance, which may lead to a poor prognosis. Hypoxia-inducible factor-1α (HIF-1α) is the main transcriptional regulator of the cellular response to low oxygen levels. Moreover, it can trigger the expression of critical genes, including glucose transporter protein type 1 (GLUT1). The aim of the present study was to evaluate the roles of HIF-1α and GLUT1 in ESCC and their usefulness as prognostic markers. HIF-1α and GLUT1 were measured in four ESCC cell lines, namely Eca109, KYSE150, TE-1 and TE-10, by western blotting following culture under normoxic and hypoxic conditions. In addition, xenograft tumors were established in mice using normoxic and hypoxic Eca109 cells and the chemosensitivity of the xenografts to 5-fluorouracil (5-FU) was evaluated. Furthermore, HIF-1α and GLUT1 were analysed by immunochemistry in the tumor tissues of patients with ESCC and the associations of their expression levels with clinicopathological parameters were investigated. The results revealed that HIF-1α and GLUT1 protein expression was weak in all four cell lines under a normoxic atmosphere but increased following culture in a hypoxic environment. *In vivo*, 5-FU inhibited tumor growth more strongly in normoxic Eca109 xenografts than hypoxic Eca109 xenografts. Higher levels of apoptosis were also detected in the normoxic Eca109 xenografts via western blotting and TUNEL analysis. In patients with ESCC, HIF-1α expression was associated with advanced ESCC while GLUT1 expression was associated with the sex of the patients. Multivariate analysis demonstrated that HIF-1α and GLUT1 were negatively associated with progression-free survival (PFS) and overall survival (OS). Additionally, a combination of HIF-1α and GLUT1 expression was a predictor of RFS and OS in patients with ESCC without lymph node metastasis but not those with lymph node metastasis. The study demonstrated that HIF-1α and GLUT1 were strongly expressed *in vitro* and in xenograft models when cells were exposed to hypoxia. The simultaneous high expression of HIF-1α and GLUT1 was associated with poorer survival, and may play an important role in ESCC chemoresistance and the prognosis of ESCC.

Correspondence to: Dr Lin Zhang or Dr Xueping Wang, Department of Oncology, State Key Laboratory of Oncology in South China, Sun Yat-Sen University Cancer Center, 651 Dongfeng Road East, Guangzhou, Guangdong 510060, P.R. China
E-mail: zhanglin@sysucc.org.cn
E-mail: wangxp@sysucc.org.cn

*Contributed equally

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Introduction

Oesophageal cancer (EC) was the fourth most common type of cancer in China in 2015 and the sixth most frequent cause of cancer-associated death worldwide (1). Squamous cell carcinoma is the main histopathological type of EC in Asia, particularly in China, and its five-year overall survival (OS) rate is <10% (2).

Hypoxia-inducible factor-1α (HIF-1α) plays a key role in the maintenance of human oxygen homeostasis. Its expression increases in a hypoxic atmosphere and is maintained at a normal level in a normoxic atmosphere. The overexpression of HIF-1α has been shown to cause the transcription of certain genes associated with angiogenesis, cell proliferation and glucose metabolism (3,4). Upregulated expression of HIF-1α has been detected in various cancers, including brain, breast and uterine cancers (5). Hypoxic conditions are known to be common in cancers. HIF-1α is critical for glucose uptake and

glycolysis; Glucose transporter 1 (GLUT1) is upregulated during glycolysis and regulated by HIF-1α (6). The upregulation of GLUT1 may be an important mechanism by which cancer cells increase glucose intake and compensate for the lack of energy triggered by hypoxia (7,8). Hypoxia plays a major role in radio- and chemoresistance, which may lead to a poor prognosis for patients (9). The association between the expression of GLUT1 and the prognosis of patients with various cancers has been investigated previously. Specifically, several studies have shown that the high expression of GLUT1 protein in tumors is associated with poor survival in patients with various tumors, including, lung, breast and liver cancer (10-13). However, there have been few reports on HIF-1α and GLUT1 in oesophageal squamous cell carcinoma (ESCC) and their association with the prognosis of patients with ESCC (14).

In the present study, the *in vitro* and *in vivo* expression levels of HIF-1α and GLUT1 under hypoxic or normoxic conditions were investigated and compared. In addition, the associations between the expression levels of HIF-1α and GLUT1 and chemoresistance were evaluated *in vivo*. Furthermore, the relationships between HIF-1α and GLUT1 and the prognosis of ESCC were also analysed.

Materials and methods

Cell lines. The Eca109, Kyse150, TE-1 and TE-10 human ESCC cell lines were confirmed by cell morphology and genomic short tandem repeats. All cell lines were incubated in RPMI-1640 with 10% foetal bovine serum (Yeasen Biotech Co., Ltd.) and 1% penicillin-streptomycin (Invitrogen, USA) at 37˚C in a humidified atmosphere with 5% CO_2. In the hypoxic experiments, the cells were treated with 150 μM $CoCl_2$ for 24 h at 37˚C and then cultured in a humidified atmosphere with 5% CO_2 at 37˚C.

Western blot analysis. All cell lines were separately cultured under normoxic and hypoxic conditions. Cell lysates were collected. Proteins extracted from mouse tumor tissues (80 μg) were analyzed using western blotting. Samples of tissue and cells were homogenized in radioimmunoprecipitation buffer containing a protease inhibitor cocktail (Roche Applied Science). The protein concentration was determined using a bicinchoninic acid kit (Beyotime Institute of Biotechnology). A total of 50 μg of protein per lane were resolved on 10% SDS-PAGE gels and transferred to PVDF membranes (Roche Diagnostics). Membranes were blocked using 5% skimmed milk for 1 h at room temperature. The membranes were incubated at 4˚C with primary antibodies targeting HIF-1α (1:1,000; cat. no. sc-13515; Santa Cruz Biotechnology), GLUT1 (1:1,000; cat. no. sc-377228; Santa Cruz Biotechnology), cleaved caspase 3 (1:1,000; cat. no. ab2302; Abcam), H2A histone family member X (H2AX) (1:1,000; cat. no. sc-517336; Santa Cruz Biotechnology), phosphorylated H2AX (γH2AX) (1:100; cat. no. A0264; ABclonal Biotech Co., Ltd.) and GAPDH (1:1,000; cat. no. sc-47724 Santa Cruz Biotechnology). Next day, secondary antibody (goat anti-rabbit; cat. no. SC2004; Santa Cruz Biotechnology) was applied for 2 h at room temperature. The Clarity™ Western ECL substrate (Bio-Rad Laboratories, Inc.) was used to detect the antigen-antibody complexes.

Table I. Clinical characteristic of 157 patients with oesophageal squamous cell carcinoma.

Characteristics	N (%)
Total cases	157
Age (years)	
Median	61.75
Range	35-90
Sex	
Male	125 (79.6)
Female	32 (20.4)
Degree of differentiation	
G1	48 (30.6)
G2	69 (43.9)
G3	40 (25.5)
Tumor status	
T1	12 (7.6)
T2	42 (26.8)
T3	98 (62.4)
T4	5 (3.2)
Lymph node status	
N0	84 (53.5)
N1	73 (46.5)
Distant metastasis status	
M0	149 (94.9)
M1	8 (5.1)
TNM stage	
I	10 (6.4)
II	68 (43.3)
III	71 (45.2)
IV	8 (5.1)
Death	
No	42 (26.8)
Yes	115 (73.2)

Patient characteristics. A total of 157 tissue specimens from patients with ESCC were collected from the Cancer Center of Sun Yat-Sen University between January 2012 and December 2014. All patients were histologically confirmed to have ESCC before surgery and received surgery without radiation or chemotherapy. The clinical information of the patients is presented in Table I. The median age of the patients was 61.75 years (range, 35-90 years). There were 125 males and 32 females; 78 cases had TNM stage I and II tumors, and 79 cases had TNM stage III and IV tumors according to the TNM staging system of the World Health Organization published in 2002 (15).

Xenograft tumor models. A total of 16 male 6-8-week-old BALB/c-nude mice (20-25 g) were provided by Beijing Vital River Laboratory Technology Co., Ltd. The animals were housed in the Laboratory Animal Center of Sun Yat-Sen University at 21˚C with 50% relative humidity and a 12 h

light/dark cycle. The animal experimentation ethics committee of Sun Yat-Sen University approved the animal experimentation protocol (L201501054). The animals were assigned to two groups: Normoxia Eca109 and hypoxia Eca109 (n=8/group). Hypoxic and normoxic Eca109 cells ($2x10^6$) were each combined with Matrigel in a 1:5 ratio and subcutaneously inoculated into the right infra-axillary area of the BALB/c nude mice in the respective group. When the volumes of the tumours reached 200-300 mm^3, treatment with 5-fluorouracil (5-FU) by intraperitoneal injection was initiated, using a dosage of 20 mg/kg twice a week for 2 weeks. The mice were anesthetized using 1% pentobarbital sodium (50 mg/kg of body weight) during the intraperitoneal injection. The mice were sacrificed by CO_2 inhalation using a 30% vol/min air displacement rate when they met any of the humane endpoint criteria, namely severe tumor burden (tumor size >1,500 mm^3), prostration, significant body weight loss, difficulty breathing, rotational motion and body temperature drop. The volume of the xenograft tumor and the body weight of each mouse were recorded twice a week. The tumor volumes were calculated using the following formula: Volume (mm^3)=1/2 x (length x width^2). The maximum tumor diameter measured in this experiment did not exceed 17 mm.

Immunohistochemical (IHC) staining. The IHC analysis of HIF-1α and GLUT1 was performed using 4-μm formalin-fixed paraffin-embedded sections of the patient tumor specimens. The sections underwent deparaffinization using xylene, followed by hydration with a decreasing ethanol series. To quench endogenous peroxidases, the sections were immersed in Dako REAL peroxidase blocking solution (Agilent Technologies) for 5 min at room temperature and then rinsed in PBS for 1 min using a magnetic stirrer. Staining was performed overnight at 4˚C using GLUT-1 and HIF-1α mouse/rabbit polyclonal antibodies (1:100; cat. nos. ab8366 and ab252403; Abcam). Subsequently, the slides were washed three times for 5 min each with PBS containing 0.2% Triton. The sections were then incubated with a horseradish peroxidase-conjugated rabbit anti-mouse Ig antibody or goat anti-rabbit IgG antibody (1:100; cat. nos. ab6728 and ab288151; Abcam, USA) at room temperature for 1 h, followed by DAB staining at room temperature for 15 min. Finally, hematoxylin was applied as a counterstain at room temperature for 10 min. The sections were imaged using a Leica microscope (Leica Microsystems GmbH). When evaluating HIF-1α expression, homogenously and darkly stained nuclei and >1% positive nuclei were considered positive. GLUT1 was considered as positive when membrane staining was observed in >1% of the cells. The immunohistochemically stained slides were scanned, imaged and digitized using a Panoramic Midi digital slide scanner (3DHISTECH Ltd.). Panoramic Viewer software (version 1.15.2; 3DHISTECH Ltd.) was used to analyse the data. The IHC scores of HIF-1α and GLUT1 expression were determined by a semi-quantitative method according to the percentage and intensity of positively stained cells (15). The positive staining was scored as follows: 0, <5% positively stained cells; 1, 5-24% positively stained cells; 2, 25-49% positively stained cells; 3, 50-74% positively stained cells; and 4, 75-100% positively stained cells. The intensity was scored as follows: 0, negative staining; 1, weak staining; 2, moderate staining; and 3, strong staining. The final score was generated by multiplying the percentage score by the staining intensity score. Two independent observers blindly evaluated the IHC scores of HIF-1α and GLUT1 expression in all specimens, and the mean values were calculated. The cut-off value for high HIF-1α and GLUT1 expression was determined based on the median IHC score, and high HIF-1α and GLUT1 expression was defined as an IHC score greater than the cut-off value.

Table II. Association between HIF-1α and GLUT1 expression determined by immunohistochemical analysis in patients with oesophageal squamous cell carcinoma.

GLUT1 expression	HIF-1α expression			P-value
	High	Low	Total	
High	46	32	78	0.008
Low	34	45	79	
Total	80	77	157	

Analyzed using χ^2 test. HIF-1α, hypoxia-inducible factor 1α; GLUT1, glucose transporter protein type 1.

In situ TUNEL staining. An *In Situ* Cell Death Detection Kit (Roche Diagnostics GmbH) was used to perform TUNEL staining of the mouse xenograft tissues. The deparaffinized sections were treated with Proteinase K solution without DNase I (Sigma-Aldrich; Merck KGaA) at 37˚C for 30 min. The slices were then exposed to terminal deoxynucleotidyl transferase (TdT) equilibration buffer, recombinant TdT enzyme and fluorescein isothiocyanate (FITC)-dUTP Labeling Mix. This reaction processed for 60 min at 37˚C in the dark. The slices were washed twice with 1x PBS and then incubated with DAPI (Beyotime Institute of Biotechnology) for 5-10 min at room temperature after the reaction was stopped using 50 ml of 1x TdT Stop Buffer at room temperature for 5 min. The labelling solution alone was used to incubate sections as negative controls. Fluorescent images were captured using an Olympus BX51 microscope. Twenty-six microscopic fields were examined for each sample.

Statistical analysis. Each experiment was performed in triplicate, at least three times. Analyses were performed using SPSS (version 19.0; IBM Corp.). Differences in tumour volume and body weight between mice in the two treatment groups were assessed using unpaired Student's t-tests. The TUNEL results were also evaluated using an unpaired Student's t-test. The associations between clinicopathological features and the expression levels of HIF-1α and GLUT1 were analysed using the Kruskal-Wallis test. Kaplan-Meier curves were assessed using the log-rank test to analyse the relationship of HIF-1α and GLUT1 expression with the clinical prognosis of the patients. Prognostic factors for progression-free survival (PFS) and OS were evaluated by multivariate Cox regression analyses. The relationship between HIF-1α and GLUT1 expression was analysed by Spearman's correlation analysis and a χ^2 test. A receiver operating curve analysis was also performed to investigate the

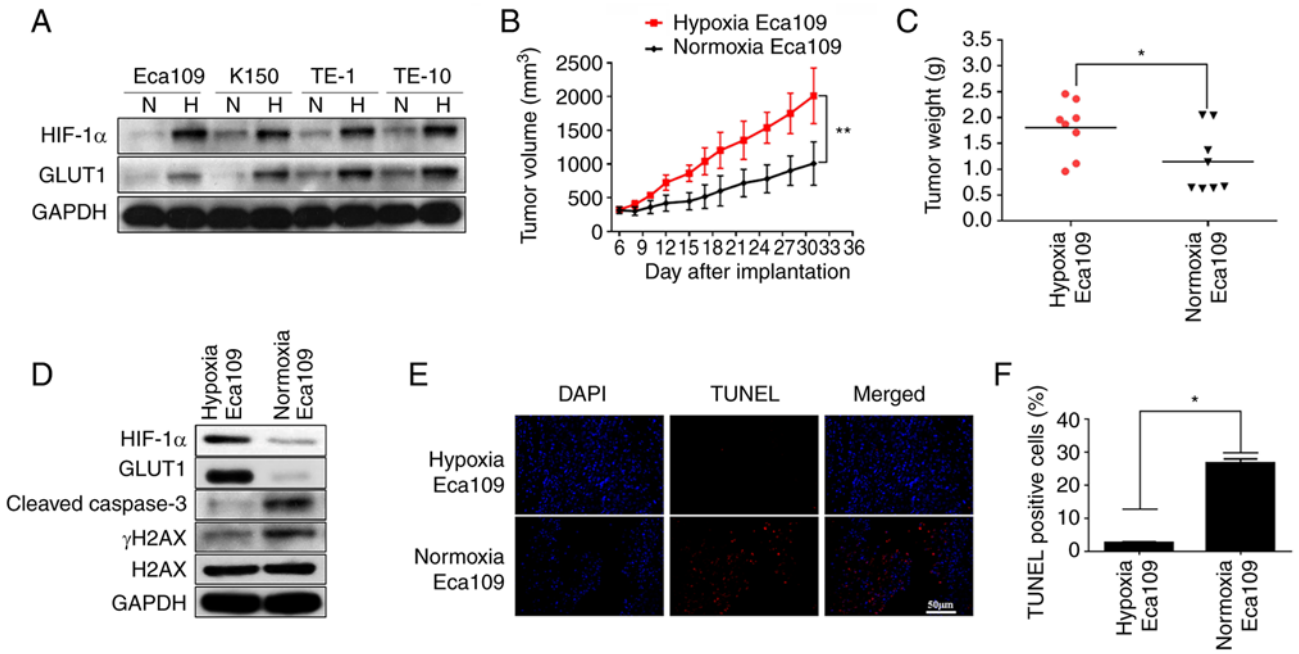

Figure 1. HIF-1α and GLUT1 expression in oesophageal squamous cell carcinoma. (A) Expression of HIF-1α and GLUT1 in four oesophageal squamous cell carcinoma cell lines cultured in normoxic and hypoxic conditions detected by western blotting. Changes in the (B) volume and (C) weight of subcutaneous Eca109 xenografts in mice treated with 5-fluorouracil. The tumor volumes and weights in the normoxia Eca109 group were lower than those in the hypoxia Eca109 group. (D) Western blot analysis of proteins associated with DNA damage. (E) Representative TUNEL images and (F) quantified TUNEL results. HIF-1α, hypoxia-inducible factor 1α; GLUT1, glucose transporter protein type 1; N, normoxia; H, hypoxia; H2AX, H2A histone family member X; γH2AX, phosphorylated H2AX. *P<0.05 and **P<0.01.

sensitivity and specificity of HIF-1α and GLUT1 expression in the prediction of death. A two-tailed P<0.05 was considered to indicate a statistically significant result.

Results

HIF-1α and GLUT1 expression in ESCC cell lines and xenografts derived from cells cultured under a normoxic or hypoxic atmosphere. Western blotting revealed that the expression of HIF-1α and GLUT1 in all four ESCC cell lines cultured with hypoxic stress was increased compared with that of the respective cells cultured under normoxic conditions (Fig. 1A). Subsequently, Eca109 cells cultured under normoxic or hypoxic conditions were used to establish xenografts in nude mice and investigate their chemosensitivity to 5-FU. When compared with the hypoxic Eca109 xenografts, the normoxic Eca109 xenografts were more sensitive to 5-FU; the tumor volume in the normoxia Eca109 group was smaller than that in the hypoxia Eca109 group (Fig. 1B). After treatment with 5-FU for 2 weeks, the mean tumor volume in the hypoxia Eca109 group reached ~1,800 mm^3 at the time of last measurement, while the tumor volume in the normoxia group was ~750 mm^3 at the same time point. A comparable result was observed for tumor weights (Fig. 1C). The levels of HIF-1α and GLUT1 in the two xenograft groups were consistent with those obtained *in vitro* as revealed by western blotting (Fig. 1D). In addition, the protein levels of cleaved caspase 3 and γH2AX were higher in the normoxia Eca109 xenograft group compared with the hypoxia Eca109 xenograft group (Fig. 1D). The percentage of TUNEL positive cells in the normoxia Eca109 xenograft group was ~25%, which was significantly higher compared with that in the hypoxia Eca109 xenograft group (5%; Fig. 1E and F). These results indicate that the chemoresistance of the hypoxia Eca109 xenograft group to 5-FU was increased compared with that of the normoxia Eca109 xenograft group.

Expression of HIF-1α and GLUT1 in normal and ESCC tissues. To investigate the expression of HIF-1α and GLUT1 protein in ESCC tissues, the expression of HIF-1α and GLUT1 in tumor tissues and matched adjacent tissues was detected using IHC staining. As shown in Fig. 2, the expression of HIF-1α in the tumor tissue was higher than that in the matched adjacent tissue. Similarly, higher expression of GLUT1 was detected in the tumor tissue compared with the adjacent normal tissue.

Relationship between HIF-1α and GLUT1. To determine the relationship between HIF-1α and GLUT1, IHC scores for HIF-1α were compared with those for GLUT1 (Table II; Fig. 3). HIF-1α expression was significantly associated with GLUT1 (Chi-square test, P=0.008; Spearman's r=0.204, P=0.01). The optimal cut-off values for HIF-1α and GLUT1 expression were investigated for sensitivity and specificity in the prediction of death by receiver operating curve analysis (Table III; Fig. 4). Both HIF-1α and GLUT1 had statistically significant areas under the curve (0.689 and 0.648, respectively; P<0.001 and P=0.005, respectively). A high expression level of HIF-1α protein was detected in 51.0% of patients (80/157, cut-off score 4) and a high expression level of GLUT1 was observed in 49.7% of patients (78/157, cut-off score 7).

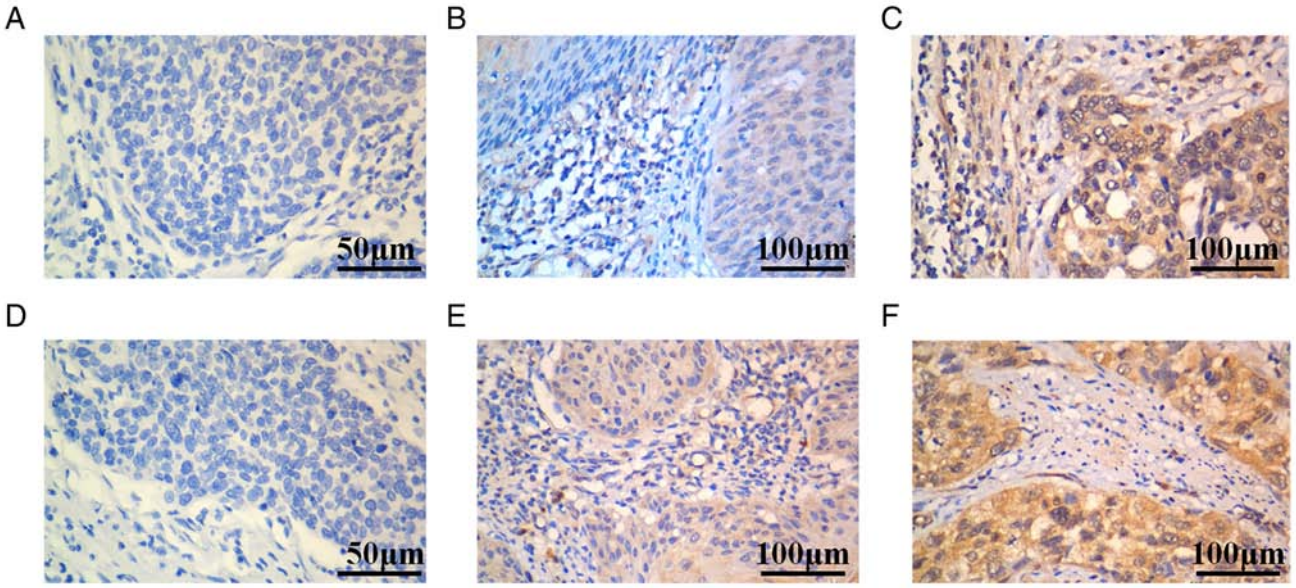

Figure 2. Immunohistochemical staining of GLUT1 and HIF-1α in oesophageal squamous cell carcinoma specimens from patients. (A) Negative, adjacent normal specimens, (B) low expression and (C) high expression of GLUT1. (D) Negative, adjacent normal specimens, (E) low expression and (F) high expression of HIF-1α. GLUT1, glucose transporter protein type 1; HIF-1α, hypoxia-inducible factor 1α.

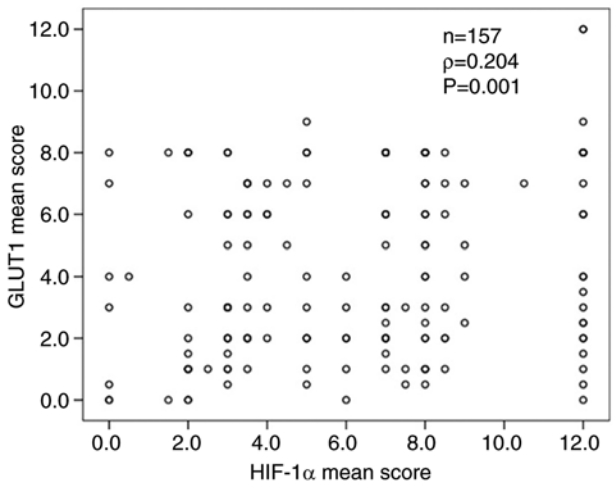

Figure 3. Scatter plot showing the correlation between mean HIF-1α and GLUT1 immunohistochemistry scores in oesophageal squamous cell carcinoma specimens. HIF-1α, hypoxia-inducible factor 1α; GLUT1, glucose transporter protein type 1. Bolder circles represent more than one data point.

Clinicopathological characteristics and their association with HIF-1α and GLUT1 expression. The associations between the expression levels of HIF-1α and GLUT1 and clinicopathological characteristic were analysed, based on the protein levels of HIF-1α and GLUT1 determined by IHC in the 157 formalin-fixed paraffin-embedded ESCC tissues. The associations between clinicopathological features and the protein expression levels of HIF-1α and GLUT1 are listed in Table IV. High expression levels of HIF-1α protein were found to be significantly associated with advanced ESCC, including tumor status (P=0.007), lymph node status (P=0.011) and clinical TNM stage (P=0.04), but not with age, sex, degree of tumour differentiation and distant metastasis. However, GLUT1 expression levels were only associated with sex (P=0.047), and not with the other clinical pathological features, namely age, degree of differentiation, tumour status, lymph node status, metastasis status and TNM stage.

Relationship between the levels of HIF-1α and GLUT1 protein and the survival of patients with ESCC. The median OS of the 157 patients with ESCC was 25 months (range, 0-133 months). The cumulative 5- and 10-year PFS rates were 28.8 and 22%, respectively, whereas the cumulative 5- and 10-year OS rates were 32.8 and 22.3%, respectively. Fig. 5A and B demonstrate a negative association of HIF-1α expression with PFS and OS (both P<0.001). In addition, a statistically significant negative association was also detected for the expression of GLUT1 with PFS and OS (both P<0.001; Fig. 5C and D). In addition to sex and nodal status, the multivariate Cox analysis indicates that HIF-1α and GLUT1 expression levels are independent unfavourable factors for PFS and OS in patients with ESCC (P<0.05; Table V).

Combined expression levels of HIF-1α and GLUT1 and the survival of patients with ESCC. The patients were assigned to four groups, according to whether the HIF-1α and GLUT1 expression levels were low or high. As shown in Fig. 6, the patients with combined low expression levels of HIF-1α and GLUT1 had the longest PFS and OS times compared with those with high expression of HIF-1α and/or GLUT1. Additionally, the patients with high expression levels of HIF-1α and GLUT1 had the shortest PFS and OS times among the four groups. The results presented in Fig. 6 indicate that the combined expression of HIF-1α and GLUT1 is likely to be a marker for prognosis in patients with ESCC. The impact of GLUT1 on PFS and OS may be greater than the effect of HIF-1α. The results also indicate that HIF-1α and GLUT1 are negatively associated with PFS and OS; however, GLUT1 was not compared with HIF-1α in this analysis.

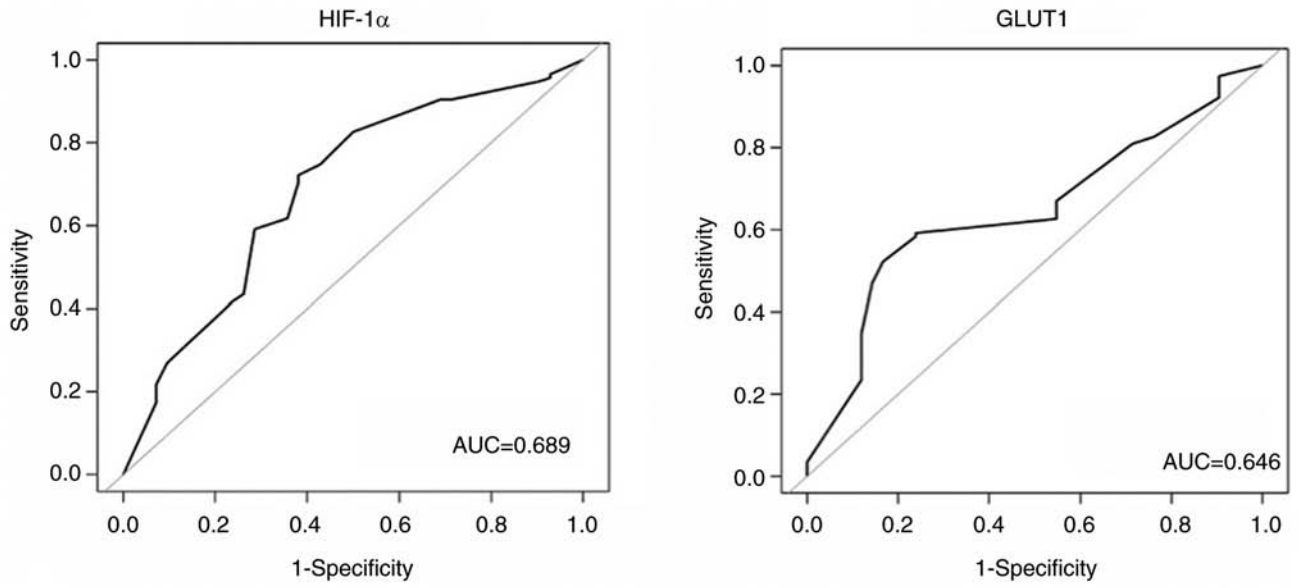

Figure 4. Receiver operating characteristic curves for HIF-1α and GLUT1 in the prediction of death for patients with oesophageal squamous cell carcinoma. HIF-1α, hypoxia-inducible factor 1α; GLUT1, glucose transporter protein type 1.

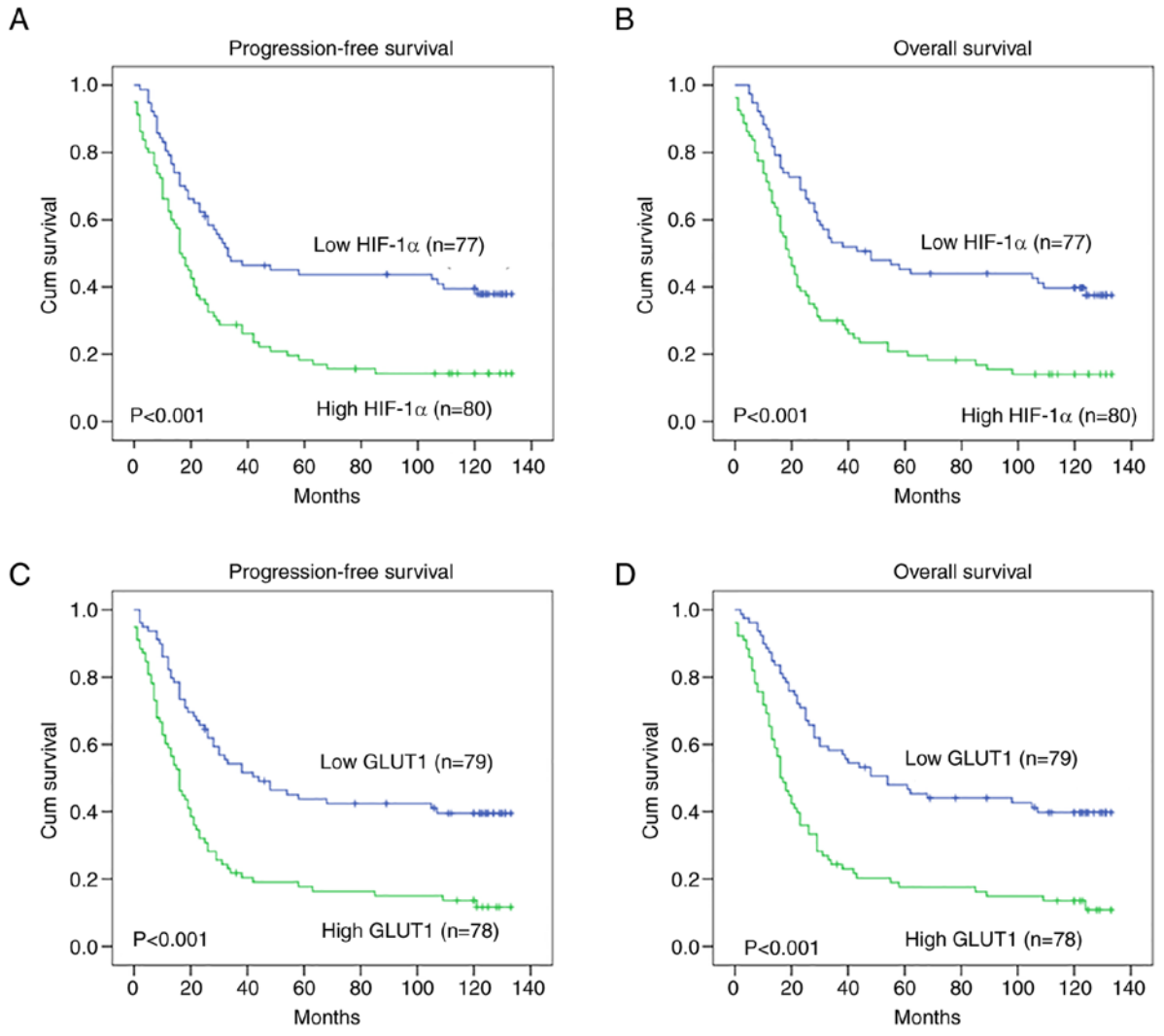

Figure 5. Progression-free survival and overall survival analysis of patients with oesophageal squamous cell carcinoma according to the expression levels of HIF-1α and GLUT1. HIF-1α, hypoxia-inducible factor 1α; GLUT1, glucose transporter protein type 1. (A) Progression-free survival and (B) overall survival according to HIF-1α expression. (C) Progression-free survival and (D) overall survival according to GLUT1 expression. HIF-1α, hypoxia-inducible factor 1α; GLUT1, glucose transporter protein type 1; Cum, cumulative.

Table III. Optimal cut-off values for high expression of markers in the prediction of death.

				Prediction of death	
Marker	AUROC (95%CI)	P-value	Cut-off score	Sensitivity (%)	Specificity (%)
HIF-1α	0.689 (0.593-0.785)	<0.001	4	0.722	0.619
GLUT1	0.646 (0.554-0.739)	0.005	7	0.552	0.833

HIF-1α, hypoxia-inducible factor 1α; GLUT1, glucose transporter protein type 1; AUROC, area under the receiver operating characteristic curve.

Table IV. Association of HIF-1α and GLUT1 expression with the clinicopathological characteristics of patients with oesophageal squamous cell carcinoma.

		HIF-1α score		GLUT1 score	
Characteristics	N	Median (Q1-Q3)	P-value	Median (Q1-Q3)	P-value
Sex			0.096		0.047
Male	125	7.0 (3.5-8.0)		4.0 (2.0-7.0)	
Female	32	4.2 (2.9-8.0)		3.0 (1.0-4.2)	
Age (years)			0.705		0.169
≥61	84	7.0 (3.4-8.0)		3.0 (1.9-6.0)	
<61	73	6.0 (3.0-8.0)		4.0 (2.0-7.0)	
Degree of differentiation			0.139		0.606
G1	48	7.0 (3.5-8.0)		4.0 (2.4-7.0)	
G2	69	7.0 (3.5-8.5)		3.0 (1.5-7.0)	
G3	40	4.5 (3.0-7.6)		3.5 (2.0-7.2)	
Tumor status			0.007		0.218
T1-2	54	4.0 (3.0-7.4)		3.0 (2.0-6.0)	
T3-4	103	7.0 (3.5-8.5)		4.0 (2.0-7.0)	
Lymph node status			0.011		0.576
N0	84	5.0 (3.0-8.0)		3.5 (2.0-6.2)	
N1	73	7.0 (4.0-8.5)		3.0 (2.0-8.0)	
Distant metastasis status			0.776		0.347
M0	149	7.0 (3.0-8.0)		3.0 (2.0-7.0)	
M1	8	6.5 (2.8-9.8)		7.5 (1.8-8.0)	
TNM stage			0.040		0.396
I-II	89	5.0 (3.0-8.0)		3.0 (2.0-6.0)	
III-IV	68	7.0 (3.5-8.5)		3.8 (2.0-8.0)	

Among the 157 patients with ESCC, there were 84 (53.5) patients without lymph node metastasis and 73 (46.5) patients with lymph node metastasis. Kaplan-Meier survival analysis showed that the combined high expression of HIF-1α and GLUT1 was significantly associated with poor PFS (P<0.001) and OS (P<0.001) in patients with ESCC without lymph node metastasis (Fig. 7A and B), but not with either poor OS (P=0.133) or PFS (P=0.24) in patients with ESCC with lymph node metastasis (Fig. 7C and D). The results indicate that the combined expression of HIF-1α and GLUT1 may be a prognostic marker for patients without lymph node metastasis, but not those with lymph node metastasis.

Discussion

Locally advanced ESCC may be treated using radiotherapy; however, ESCC frequently becomes resistant to radiation (16). The resistance of tumors to radiotherapy and chemotherapy is associated with hypoxia, and HIF-1 serves a major role in the regulation of the adaptive responses of tumors to hypoxic conditions (17). Tumor cells adapt to hypoxia via the activation of various signaling pathways (18,19), such as the Wnt/β-catenin signaling pathway (18) and the p-JNK signaling pathway (20). In addition, HIF-1α contributes to tumor growth and metastasis. Tumor-associated vasculature

Table V. Multivariate Cox regression analysis of OS and PFS for 157 patients with oesophageal squamous cell carcinoma.

	OS		PFS	
Variables	Hazard ratio (95% CI)	P-value	Hazard ratio (95% CI)	P-value
Sex (male/female)	0.440 (0.250-0.775)	0.003	0.499 (0.287-0.870)	0.014
Age (≥61/<61 years)	0.849 (0.580-1.243)	0.401	0.850 (0.581-1.245)	0.405
Degree of differentiation (G1/2/3)	1.226 (0.952-1.579)	0.115	1.250 (0.971-1.609)	0.083
Tumor status (T1-2/T3-4)	0.735 (0.431-1.254)	0.259	0.717 (0.423-1.218)	0.219
Lymph node status (N0/N1)	2.778 (1.440-5.359)	0.002	2.260 (1.180-4.329)	0.014
Distant metastasis status (M0/M1)	1.113 (0.435-2.843)	0.823	1.034 (0.407-5.634)	0.943
TNM stage (I-II/III-IV)	0.800 (0.556-1.151)	0.228	0.937 (0.653-1.345)	0.725
HIF-1α	1.745 (1.177-2.588)	0.006	1.629 (1.090-2.435)	0.017
GLUT1	2.341 (1.595-3.435)	0.001	2.114 (1.439-3.105)	0.001

OS, overall survival; PFS, progression-free survival; HIF-1α, hypoxia-inducible factor-1α; GLUT1, glucose transporter protein type 1.

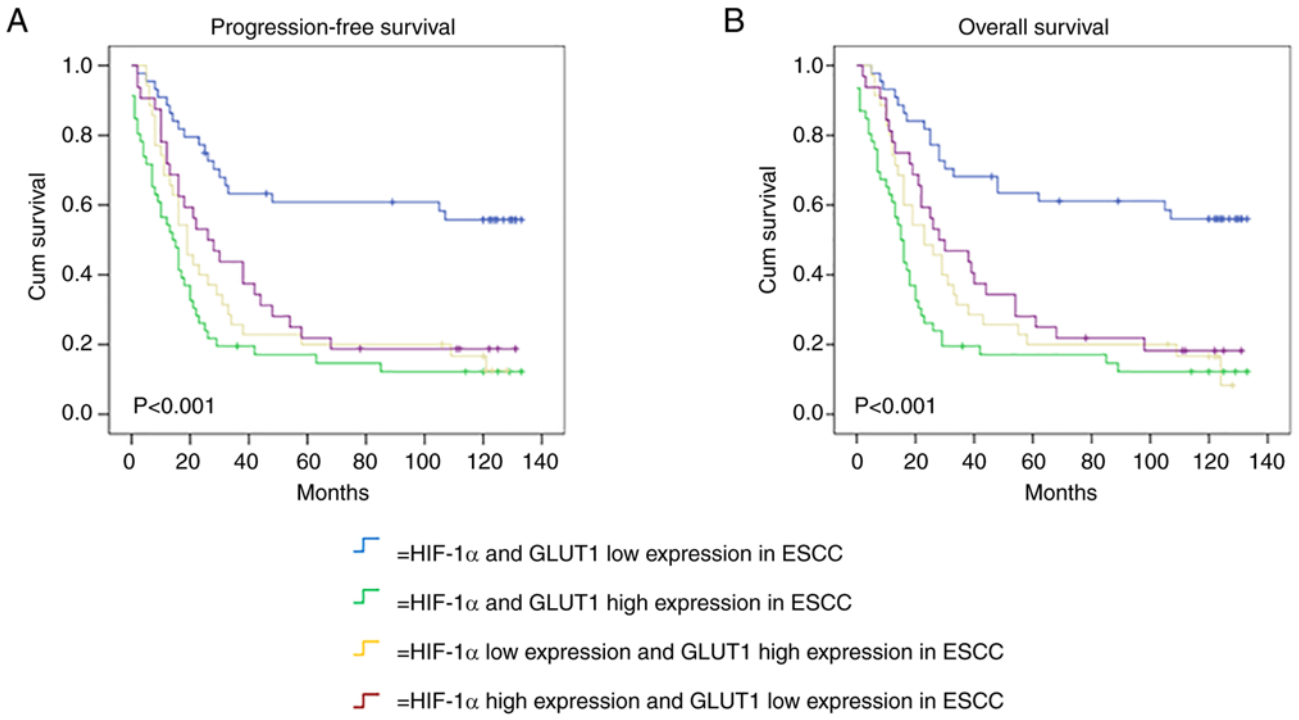

Figure 6. Survival analysis of patients with ESCC according to the combined expression levels of HIF-1α and GLUT1. (A) Progression-free survival and (B) overall survival. ESCC, oesophageal squamous cell carcinoma; HIF-1α, hypoxia-inducible factor 1α; GLUT1, glucose transporter protein type 1; Cum, cumulative.

is poorly organized and hyperpermeable compared with normal blood vessels, which makes effective drug delivery challenging and creates an abnormal microenvironment that causes radio- and chemotherapy to be less effective. The upregulation of HIF-1α and GLUT1 has been shown to be associated with reduced sensitivity to radiotherapy and chemotherapy in numerous solid tumors (21,22). Consistently, the *in vivo* experiment in the present study demonstrated that the sensitivity of xenografts to 5-FU generated from hypoxic cells was reduced compared with those generated from normoxic cells. However, researchers have demonstrated that anti-angiogenic drugs can normalize the blood vessels of tumors, causing them to be more sensitive to chemotherapy and radiotherapy (23).

HIF-1α activates the glucose transporter GLUT1. The protein expression level of GLUT1 has been reported to be an important biomarker in a number of different cancers, including ESCC, breast cancer and gastric cancer (24-26). Furthermore, a review confirmed that GLUT1 is a valid biomarker in various types of solid cancers (27); specifically, it firmly established that the upregulation of GLUT1 is associated with a poor prognosis in patients with solid tumors. GLUT1 is regulated by numerous transcription factors, including HIF-1α, which has been shown to elevate

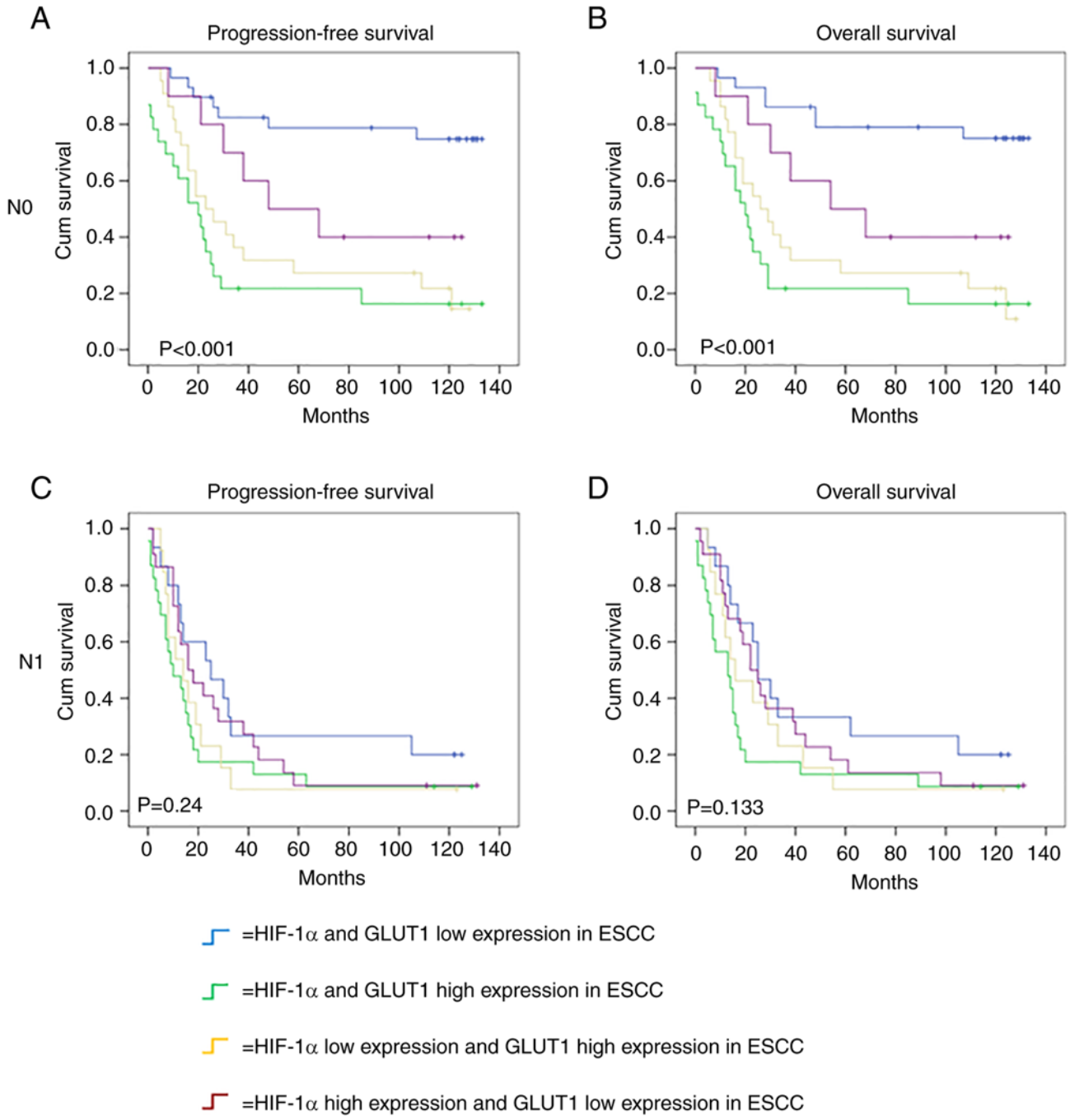

Figure 7. Survival analysis of patients with ESCC according to the combined expression levels of HIF-1α and GLUT1 in patients with different lymph node metastasis status. (A) Progression-free survival and (B) overall survival in patients with lymph node status N0. (C) Progression-free survival and (D) overall survival in patients with lymph node status N1. ESCC, oesophageal squamous cell carcinoma; HIF-1α, hypoxia-inducible factor 1α; GLUT1, glucose transporter protein type 1; N0, no lymph node metastasis; N1, lymph node metastasis; Cum, cumulative.

the expression of GLUT1 under a hypoxic atmosphere (28). In the present study, GLUT1 was only found to be associated with sex among the various clinicopathological features that were analyzed. The reason may be that most of the patients were male (125 patients, ~80%), and the expression of GLUT1 may differ between the sexes. The differential expression of GLUT1 between males and females has also been observed in colorectal adenocarcinomas (29).

Previous data also showed that the upregulation of HIF-1α was closely associated with a poor prognosis and chemo-radiation effectiveness in patients with ESCC (30). High levels of HIF-1α have previously been suggested to be a predictive marker of poor prognosis in patients ESCC and to be significantly associated with invasion and metastasis (31). In a hypoxic environment, HIF-1α has been shown to reduce tissue integrity via the loss of E-cadherin, which is considered as a suppressor of invasion and metastasis in numerous cancers (32). The cell basement membrane and extracellular matrix are also undermined by HIF-1α (33). As aforementioned, hypoxia is a common pathological feature in solid tumors, which results from insufficient blood supply and rapid tumor growth (34). Under anoxic and hypoxic

conditions, tumor cells produce several different proteins that stimulate cell invasiveness, promote angiogenesis, and result in chemotherapy or radiotherapy resistance (35). The prognostic value of HIF-1α in EC remains unclear. Although a number of studies have shown that the expression level of HIF-1α in tumor cells is closely associated with clinical tumor stage (TNM stage) (32), another study found that HIF-1α was not a significant independent prognostic factor for PFS and OS (33). Although HIF-1α may regulate p53 and VEGF downstream signalling pathway (36,37), the relationships between these factors remain unclear in patients with EC.

Since ESCC is a common pathological type of EC, it is important to identify the clinical significance of HIF-1α and GLUT1 in patients with ESCC as this may improve upon the current prognostic system based on TNM staging. Notably, the present study examined the roles of HIF-1α and GLUT1 in the hypoxic signalling of ESCC by IHC analysis combined with *in vivo* and *in vitro* experiments. The correlation between HIF-1α and GLUT1 was confirmed, and both proteins were shown to be associated with the outcomes of patients with ESCC. In addition, only HIF-1α were found to be associated with lymph node metastasis. Furthermore, the results of the multivariate analysis demonstrated that high expression levels of HIF-1α and GLUT1 are prognostic factors that indicate poorer OS and PFS in patients with ESCC.

Further studies of HIF-1α and GLUT1 may focus on their use as targets for therapeutic intervention. In addition, their use as molecular biomarkers to identify the cancer patients who would respond best to radiation therapy and chemotherapy merits further investigation, as it may improve the clinical treatment outcomes of patients with ESCC.

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Availability of data and materials

All data generated or analysed during this study are included in this published article.

Authors' contributions

HY and YH performed the main experiments and drafted the manuscript. QL, RL, JZ and YY collected the data and analyzed the statistical analysis. XW and LZ conceived and designed the experiments. HY, YH and LZ confirm the authenticity of all the raw data. All authors read and approved the final version of the manuscript.

Ethics approval and consent to participate

The animal studies were performed under the guidance of Sun Yat-Sen University Committee for Use and Care of Laboratory Animals and approved by the animal experimentation ethics committee of Sun Yat-Sen University (L201501054). The use of clinical materials was performed with the written informed consent of all patients and approved by the Institutional Research Ethics Committee of Sun Yat-Sen University Cancer Center (GZR2015-093).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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