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Expression of hypoxia-inducible factor (HIF)-1α is associated with vascular endothelial growth factor expression and tumour angiogenesis in human oesophageal squamous cell carcinoma

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Abstract

The purpose of this study was to examine the relationship between hypoxia inducible factor (HIF)-1α expression, vascular endothelial growth factor (VEGF) expression, and tumour vascularity in squamous cell carcinoma of the oesophagus. Expression of HIF-1α and VEGF was examined in two oesophageal squamous cell carcinoma cell lines (TE2, TE3) and 82 archival surgical specimens of human oesophageal squamous cell carcinoma tissue. In both cell lines, the levels of HIF-1α protein and VEGF mRNA were increased under hypoxic conditions. Thirty-two of the 82 (39%) tumour specimens showed high levels of HIF-1α immunoreactivity in the nuclei and/or cytoplasm of cancer cells. HIF-1α expression correlated significantly with venous invasion, VEGF expression, and microvessel density. Among the 47 patients who did not receive pre-operative chemotherapy, the outcome of those with high HIF-1α-expressing tumours was significantly poorer than that of those with low HIF-1α-expressing tumours. These results suggest that HIF-1α and VEGF expression are important determinants of survival in squamous cell carcinoma of the oesophagus.

Keywords: Hypoxia-inducible factor-1α; Angiogenesis; Vascular endothelial growth factor; Oesophageal squamous cell carcinoma

1. Introduction

Angiogenesis is essential for tumour growth and metastasis and is dependent on the production of angiogenic factors by tumour and host cells [1,2]. Tumours cannot grow larger than 1–2 mm^3 in the absence of angiogenesis because the lack of oxygen in the centre of the tumour results in apoptosis and necrosis. Hypoxia is a key signal for the induction of angiogenesis, and a key angiogenic factor whose expression is regulated by hypoxia is the vascular endothelial growth factor (VEGF) [3]. There is a strong correlation between VEGF expression and cancer progression and metastasis [4]. The prognostic significance of VEGF expression has been reported in gastric, colon, breast, bladder and oesophageal carcinomas, as well as in other malignancies [5,6]. Transfection and inhibitory experiments have confirmed the importance of VEGF in angiogenesis and tumour growth [7,8].

Hypoxia inducible factor (HIF)-1 activates transcription and plays a critical role in oxygen homeostasis [9]. HIF-1 is a heterodimer of HIF-1α and HIF-1β subunits and HIF-1β has been previously identified as an aryl hydrocarbon nuclear translocator (ARNT) [10]. Both transcription factors contain basic helix–loop–helix (bHLH) and per-ARNT-sim (PAS) domains that are required for dimerisation and DNA binding, and these domains control various critical embryogenic and

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Table 1
Clinicopathological features and HIF-1α expression in oesophageal squamous cell carcinoma

Clinicopathological findings	No. of cases	High HIF-1α expression (%)		*P* value[a]
		Low	High	
All	82	50 (61)	32 (39)	
Gender				
Male	72	41 (57)	31 (43)	0.045*
Female	10	9 (90)	1 (10)	
Age (62.2 ± 9.9 years)				
⩽60	29	19 (66)	10 (35)	0.533
⩾61	53	31 (59)	22 (42)	
Location				
Cervical	1	0 (0)	1 (100)	0.778
Upper thoracic	8	6 (75)	2 (25)	
Middle thoracic	36	22 (61)	14 (39)	
Lower thoracic	37	22 (60)	15 (41)	
Histological grade				
Well	29	16 (55)	13 (45)	0.134
Moderately	36	23 (64)	13 (36)	
Poorly	17	11 (65)	6 (35)	
T stage[b]				
T1	19	15 (79)	4 (21)	0.061
T2	9	6 (67)	3 (33)	
T3	47	25 (53)	22 (47)	
T4	7	4 (57)	3 (43)	
N stage[c]				
N0	32	20 (63)	12 (36)	0.821
N1	50	30 (60)	20 (40)	
Lymphatic invasion				
(+)	71	41 (58)	30 (42)	0.128
(−)	11	9 (82)	2 (18)	
Venous invasion				
(+)	48	24 (50)	24 (50)	0.016*
(−)	34	26 (82)	8 (18)	
Clinical stage				
I, II	30	21 (70)	9 (30)	0.203
III, IV	52	29 (56)	23 (44)	

[a] χ^2 test.
[b] T1, tumour invades submucosa; T2, tumour invades muscularis propria; T3, tumour invades adventitia; T4, tumour invades adjacent structures.
[c] N0, no regional lymph node metastasis; N1, regional lymph node metastasis.
* $P < 0.05$.

pathogenic events [9,11–13]. Under non-hypoxic conditions, HIF-1α is subject to rapid ubiquitination and proteasomal degradation [10]. This degradation is affected by the von Hippel–Lindau (VHL) protein [14].

HIF-1α has been recognised as an important regulatory protein in the transcription of a large number of genes related to glucose transport, glycolysis, erythropoiesis, cell proliferation/survival and angiogenesis [3,15,16]. In human tumours, overexpression of HIF-1α may activate metabolic and pathogenic pathways that are related to tumour angiogenesis, growth, invasion and metastasis [17]. Tumours derived from cells lacking HIF-1α or HIF-1β show significantly reduced vascularisation and, in most cases, reduced growth rates compared with parental cells [4,7,18,19].

The purpose of this study was to examine the relationship between HIF-1α expression, VEGF expression and tumour vascularity in human squamous cell carcinoma of the oesophagus.

2. Materials and methods

2.1. Cell cultures

Two cell lines established from human oesophageal carcinomas (TE2, poorly differentiated squamous cell carcinoma; TE3, well differentiated squamous cell carcinoma) were kindly provided by Dr. T. Nishihira (Tohoku University School of Medicine, Sendai, Japan).

Cells were maintained in Roswell Park Memorial Institute (RPMI) 1640 media (Nissui Co., Ltd., Tokyo, Japan) with 10% fetal bovine serum (FBS, M.A. Bioproducts, Inc.,Walkersville, MD). Cells were grown to confluency and then subjected to 4 h of hypoxia (0% O_2, 5% CO_2, 95% N_2) with Anaero Packs (Mitsubishi, Tokyo, Japan).

2.2. Patients and tumour specimens

Paraffin-embedded tumour specimens from 82 patients with oesophageal squamous cell carcinoma who had undergone surgery at the Hiroshima University Hospital were studied by immunohistochemistry (IHC). Thirty-five patients with invasive carcinoma received neo-adjuvant/pre-operative chemotherapy (cisplatin, 5-doxorubicin, bleomycin, uracil plus tegafur). Fifty-two patients received adjuvant chemotherapy and/or radiation therapy.

Pathology reports and clinical histories at the time of surgery were reviewed to determine an accurate staging. Stage and histological classifications were according to the International Union Against Cancer (UICC) TNM classification system [20]. Patients ranged in age from 39 to 81 years (mean, 62.2 years) and included 72 men and 10 women. Histological grades, tumour stages and the depth of invasion are reported in Table 1.

2.3. Reverse transcriptase-polymerase chain reaction analysis

Total RNA was extracted from human oesophageal carcinoma cell lines with the RNeasy kit (Qiagen, Tokyo, Japan). Two oligomers of primers were synthesised on the basis of the reported sequences of HIF-1α (5′-CCT GCA CTC AAT CAA GAA GTT GC-3′ and 5′-TTC CTG CTC TGT TTG GTG AGG CT-3′), VEGF (5′-CAC ATA GGA GAG ATG AGC-3′ and 5′-CCG CCT CGG CTT GTC ACA-3′), and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (5′-ATC ATC CCT GCC TCT ACT GG-3′ and 5′-CCC TCC GAC GCC TGC TTC AC-3′) [16,21]. The primer pair amplified a 620 base pair (bp) fragment as HIF-1α, a 100 bp fragment as $VEGF_{121}$, a 230 bp fragment as $VEGF_{165}$, and a 188 bp fragment as GAPDH. Reverse transcriptase-polymerase chain reaction (RT-PCR) was performed with the isolated RNA and the oligomers as templates and primers, respectively [22]. For HIF-1α, the cDNA was amplified with 27 cycles of denaturation for 1 min at 94 °C, annealing for 1 min at 60 °C, and extension for 1 min at 72 °C. For VEGF, the cDNA was amplified with 28 cycles of denaturation for 1 min at 94 °C, annealing for 1 min at 57 °C, and extension for 1 min at 72 °C. For GAPDH, the cDNA was amplified with 25 cycles of denaturation for 1 min at 94 °C, annealing for 1 min at 51 °C, and extension for 1 min at 72 °C. After amplification, products were loaded onto a 5% non-denaturing polyacrylamide gel in Tris–borate–ethylene diamine tetraacetic acid (EDTA) buffer, and the specific bands were visualised with ethidium bromide and photographed under ultraviolet light. RT-PCR reactions without reverse transcriptase yielded no specific bands. VEGF and GAPDH mRNA levels were quantified with the aid of computer software (Photoshop 6.0, Adobe, USA and NIH Image 1.63, Wayne Resband, NIH, USA). For semi-quantitation, VEGF PCR products were normalised to GAPDH.

2.4. Western blotting analysis

Cells were homogenised in lysis buffer containing 8 M urea, 10% sodium dodecyl sulphate (SDS), 1 M dithiothreitol (DTT), and protease inhibitors. Protein (30 μg) was dissolved in sample buffer with 2-mercaptoethanol, boiled at 3 min, separated by electrophoresis on a 7.5% SDS–polyacrylamide gel electrophoresis (PAGE) gel, and transferred to Immobilon membranes (Millipore, Bedford, MA). Membranes were blocked with phosphate buffered solution (PBS) containing 5% non-fat dried milk and 0.1% Tween 20. HIF-1α protein was detected with a mouse anti-HIF-1α monoclonal antibody (1:250; Transduction Laboratories, Lexington, KY). Peroxidase-conjugated anti-mouse IgG goat antibody (diluted 1:1000; Medical and Biological Laboratories, Nagoya, Japan) was used as the secondary antibody. Protein–antibody complexes were detected by enhanced chemiluminescence (ECL, Amersham Pharmacia Biotech, Buckinghamshire, UK) and exposed to Kodak X-Omat AR Film (Rochester, NY).

2.5. Immunohistochemistry

Immunohistochemistry (IHC) was performed on 4-μm thick sections of formalin-fixed, paraffin-embedded tissues. IHC for HIF-1α was performed with the Catalyzed Signal Amplification (CSA) system (DAKO, Carpinteria, CA). HIF-1α was detected with a mouse monoclonal antibody Mab H1α67 (1:1000; Novus Biologicals, Littleton, CO). Sections were deparaffinised and microwaved with target retrieval solution (DAKO) for 5 min, three times. The primary antibody reaction was carried out at 4 °C overnight. Subsequent steps were performed according to the manufacturer's instructions.

IHC studies for VEGF and CD34 were performed with Labelled Streptavidin Biotin 2 (LSAB2) System (DAKO). VEGF was detected with a rabbit polyclonal antibody (A-20, 1:300; Santa Cruz Biotechnology, Santa Cruz, CA), and CD34 was detected with a mouse monoclonal antibody (NU-4A1, Nichirei, Tokyo, Japan). After deparaffinisation and rehydration, tissue sections were pretreated with 0.1% trypsin at 37 °C for 30 min and then treated with 3% H_2O_2 for 30 min to

block endogeneous peroxidase activity. Sections were then washed in PBS and blocked with PBS containing 5% non-fat dried milk for 30 min. The primary antibody reaction was carried out at 4 °C overnight. Subsequent steps were performed according to the manufacturer's instructions. 3,3,-diaminobenzidine (DAB)/hydrogen peroxide was used to detect antigen-antibody binding, and slides were counterstained with Mayer's haematoxylin.

HIF-1α immunoreactivity was present in both nuclei and cytoplasm. The percentages of cancer cells with nuclear localisation of HIF-1α and with strong cytoplasmic localisation were assessed separately in all optical fields (Fig. 1(a) and (b)). The mean value of the percentages obtained from all optical fields examined in each case was used to calculate the nuclear and cytoplasmic scores for each case. Using these scores, we calculated the overall mean value and range of the percentages of cells with nuclear and strong cytoplasmic HIF-1α localisation in this series of 82 tissues. The mean values for nuclear and/or strong cytoplasmic expression of HIF-1α were used as the cut-off points to define two groups: high and low HIF-1α expression.

Expression of VEGF was defined as positive if distinct staining of the cytoplasm was observed in at least 10% of tumour cells (Fig. 1(c)). CD34 staining was used to assess microvessel density (MVD) by light microscopy at the site of the highest number of capillaries and small venules (Fig. 1(d)). Highly vascular areas were identified by scanning tumour sections at low power (40× and 100×). After six areas with the highest neovascularisation (termed hot spots) were identified, a vessel count was performed on a ×200 field (×20 objective and ×10 ocular, 0.723 mm^2 per field), and the average count of the six fields was calculated. As Weidner and colleagues [23] described, a visible lumen was not necessary for a structure to be defined as a vessel. Microvessels were counted independently by two investigators who had no knowledge of the HIF-1α expression status of the tumours.

2.6. Statistical analysis

Statistical significance of differences was evaluated with the χ^2 test and Mann–Whitney U tests, as appropriate curve for overall survival were drawn according

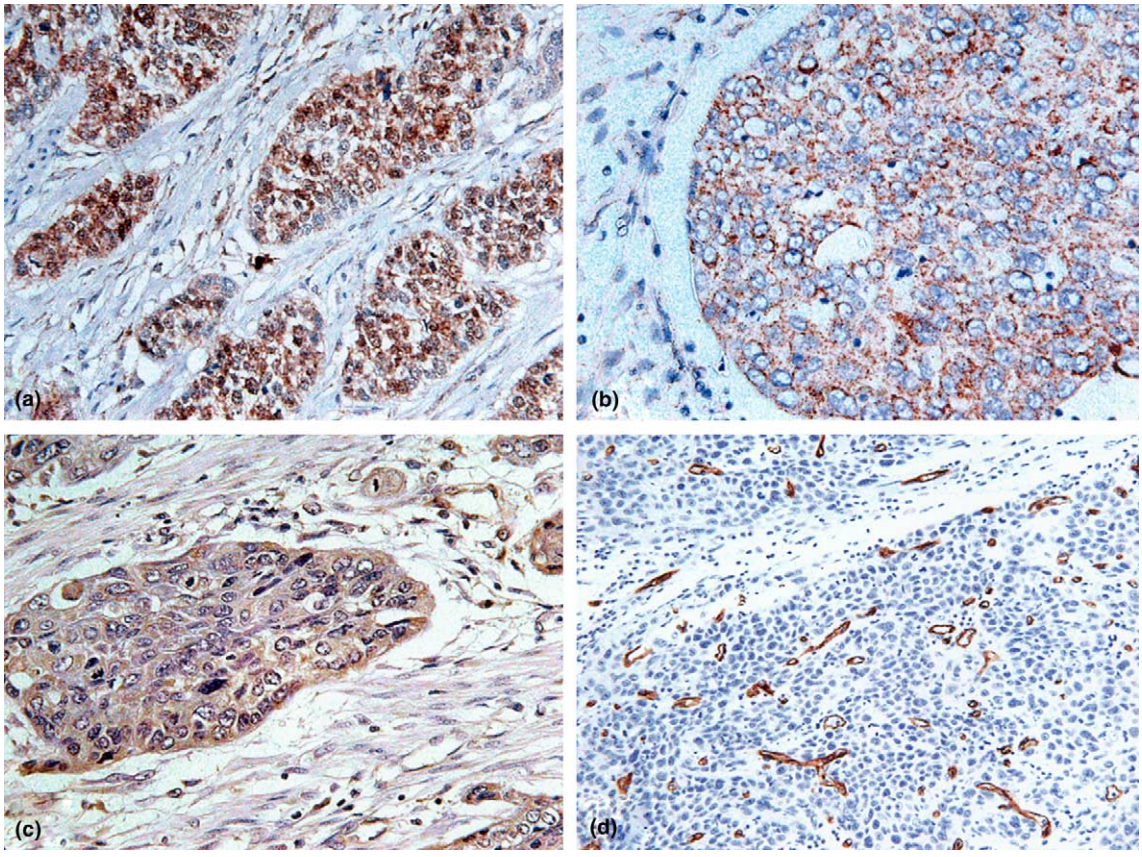

Fig. 1. IHC staining for HIF-1α and VEGF in oesophageal squamous cell carcinoma. (a) HIF-1α immunoreactivity in nuclei of tumour cells. (b) Strong HIF-1α immunoreactivity in cytoplasm of tumour cells. (c) Positive IHC staining of VEGF. VEGF immunoreactivity is present in the cytoplasm of tumour cells. (d) CD34 staining of the same HIF-1α-high carcinoma tissue.

to the Kaplan–Meier method, and differences between the curves were analysed with the log-rank test. The significance level was set at 5% for each analysis.

3. Results

3.1. Expression of HIF-1α and VEGF in oesophageal squamous cell carcinoma cell lines

We initially examined the influence of hypoxia on the expression of HIF-1α mRNA and protein in two oesophageal squamous cell carcinoma cell lines. Expression of HIF-1α protein was increased by hypoxia in both cell lines (Fig. 2(a)). In contrast, HIF-1α mRNA levels were similar in both cell lines, regardless of the level of HIF-1α protein expression (Fig. 2(b)), indicating that hypoxia influences HIF-1α at the protein level in oesophageal squamous carcinoma cell lines. We also examined expression of VEGF mRNA in the same samples. In both cell lines, hypoxia slightly increased the expression of VEGF mRNA (Fig. 2(b)).

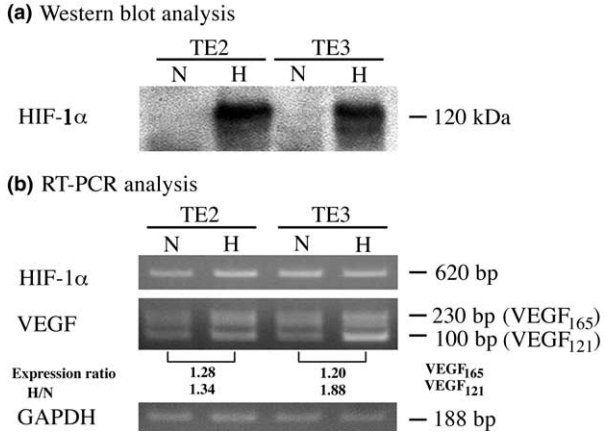

Fig. 2. Expression of HIF-1α and VEGF in oesophageal squamous cell carcinoma cell lines TE2 and TE3 under normoxic and after hypoxic conditions. (N) normoxia; (H) 4 h treatment under hypoxic conditions. (a) Western blotting analysis of HIF-1α protein levels after hypoxia. (b) Reverse transcriptase-polymerase chain reaction (RT-PCR) amplification of HIF-1α and VEGF mRNAs by oesophageal squamous cell carcinoma cell lines after hypoxia. The ethidium bromide staining values were calculated as described in Section 2. The expression ratio between hypoxia and normoxia (H/N ratio) is included as an internal control.

3.2. Relationship between HIF-1α expression and clinicopathological features

We used IHC to investigate the association between HIF-1α expression and clinicopathological features in 82 oesophageal squamous cell carcinomas. The mean percentage of cells with nuclear localisation of HIF-1α was 4.2% (range 0–70%), and the mean percentage of cells with strong cytoplasmic HIF-1α immunoreactivity was 20.7% (range 0–80%). On the basis of the criteria described in Section 2, 32 (39%) of the 82 oesophageal squamous cell carcinomas were judged to have high HIF-1α expression (i.e. nuclear and/or strong cytoplasmic staining above the mean) (Table 1). HIF-1α expression in male was significantly higher than that in female. HIF-1α immunoreactivity was correlated significantly with venous invasion ($P < 0.05$) (Table 1).

3.3. Relationship between VEGF expression, MVD, and HIF-1α expression

Of the 82 oesophageal squamous cell carcinomas, 51 (62.2%) showed intense VEGF immunoreactivity in the cytoplasm of cancer cells (Fig. 1(c)). In the 32 oesophageal squamous cell carcinomas classified as high-HIF-1α tumours, the VEGF-positive and VEGF-negative tumour frequencies were 27/32 (84%) and 5/32 (16%), respectively (Table 2). In contrast, in the 50 oesophageal squamous cell carcinomas classified as low-HIF-1α tu-

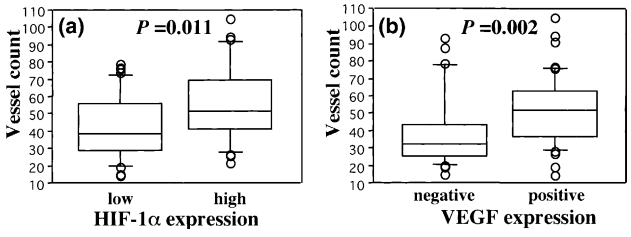

Fig. 3. Relationship between the expression of HIF-1α: (a) VEGF, and; (b) microvessel count obtained from CD34 staining. *P* values were determined by the Mann–Whitney *U* test.

Table 2
VEGF expression and MVD in relation to HIF-1α expression

HIF-1α expression	No. of cases	VEGF[a]		MVD (Mean ± SD)	
		Positive	Negative		
High	32	27 (84%)	5 (16%)	56.1 ± 22.4	$P < 0.05$[b]
Low	50	24 (48%)	26 (52%)	42.0 ± 18.1	

SD, standard deviation.
[a] χ^2 test, $P < 0.001$.
[b] Mann–Whitney *U* test.

mours, 24/50 (48%) were VEGF-positive, and 26/50 (52%) were VEGF-negative. This difference was statistically significant ($P < 0.001$) (Table 2).

Seventy-three of the 82 cases were evaluated for MVD. Nine cases were excluded from the analysis because of damage to the sections or because there was a marked

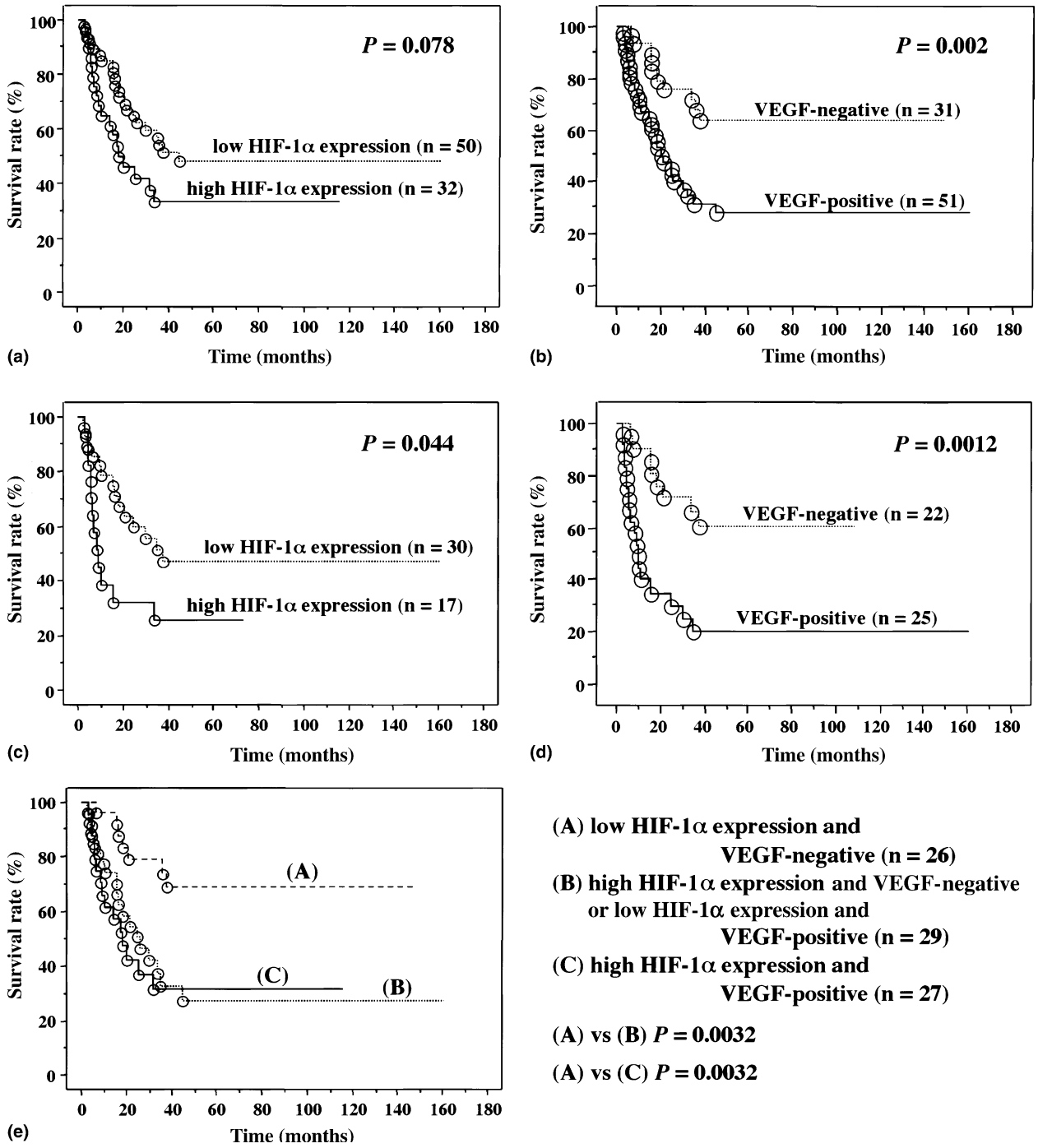

Fig. 4. Kaplan–Meier survival curves for all patients with oesophageal squamous cell carcinoma with regard to HIF-1α protein expression (a) and VEGF protein expression (b). (a) There was a trend for shorter survival in patients with HIF-1α-high tumours; however, this trend was not statistically significant. (b) The survival rate of patients with VEGF-positive tumours was significantly worse than that of patients with VEGF-negative tumours. Kaplan–Meier survival curves for patients without pre-operative chemotherapy with regard to HIF-1α protein expression (c) and VEGF protein expression (d). (c) and (d) The survival rate of patients with HIF-1α-high tumours and VEGF-positive tumours was significantly worse than that of patients with HIF-1α-low tumours and VEGF-negative tumours. Kaplan–Meier survival curves with regard to a combination of HIF-1α and VEGF expression analysis (e). We classified 3 groups: (A) HIF-1α-low tumours and VEGF-negative tumours ($n = 26$); (B) HIF-1α-high tumours, VEGF-negative tumours or HIF-1α-low tumours, VEGF-positive tumours ($n = 29$); (C) HIF-1α high tumours and VEGF-positive tumours ($n = 27$). Survival rates of (B) and (C) were significantly worse than that those of patients in group (A). *P* values were determined by the log-rank test.

discrepancy in the microvessel counts of the two investigators. MVD in the tissue adjacent to the tumour ranged from 14 to 105 (median, 46). The median of MVD in the HIF-1α-high and HIF-1α-low groups was 52 and 39, respectively, (Table 2; Fig. 3(a)). This difference was statistically significant ($P < 0.05$), indicating that there was greater neovascularisation in the HIF-1α-expressing cancers. The median of MVD in the VEGF-positive and VEGF-negative groups was 52 and 33, respectively. MVD in the VEGF-positive group was significantly higher than that in the VEGF-negative group (Fig. 3(b)).

3.4. Relationship between HIF-1α expression and patients' survival

To investigate whether HIF-1α and VEGF expression is associated with outcome of patients with oesophageal squamous cell carcinoma, a Kaplan–Meier analysis was performed. All 82 patients were followed-up in our hospital. The median follow-up time of the 42 surviving patients was 60.2 months (range 1.0–160.3 months) and that of remaining 40 patients who died was 15.7 months (range 2.5–44.9 months). Kaplan–Meier analysis showed that there was a tendency toward a poorer outcome in patients with HIF-1α-high tumours compared with that in patients with HIF-1α-low tumours. However, this difference was not statistically significant (Fig. 4(a)).

Subsequently, we selected 47 patients who had not received neo-adjuvant (preoperative) therapy in order to exclude any influence from the therapy received. The median follow-up time of the 21 surviving patients was 63.4 months (range 1.0–160.3 months) and that of the remaining 26 patients who died was 9.7 months (range 2.5–37.8 months). The survival rate of patients with HIF-1α-high tumours was also lower in this subgroup analysis than that of patients with HIF-1α-low tumours – a statistically significant difference ($P < 0.05$) (Fig. 4(c)). The survival rate of patients with VEGF-positive tumours was significantly lower than that of patients with VEGF-negative tumours ($P < 0.01$) (Fig. 4(b) and (d)).

Bivariate analysis was also assessed in all 82 patients. The survival rate of patients with HIF-1α-low and VEGF-negative tumours was significantly higher than that of the other groups (Fig. 4(e)).

4. Discussion

VEGF is a potent stimulator of angiogenesis, both *in vitro* and *in vivo* [24], and it is now widely accepted that VEGF expression is mediated by HIF-1α during hypoxia [25]. HIF-1α activates the VEGF promoter directly through the HIF-1α binding site [26]. In rat glioma cells, overexpression of HIF-1α increases the half-life of VEGF mRNA [27]. It was recently reported that HIF-1α expression correlates with VEGF expression and MVD in several tumour types [17,25].

IHC studies of HIF-1α in human tissues have been reported recently [28]. Mixed nuclear and cytoplasmic staining patterns were observed in these studies. HIF-1α expression was absent in most normal tissues, and the staining patterns were variable in each organ [28]. In our study, HIF-1α immunoreactivity was localised in the nuclei and/or cytoplasm of carcinoma cells. The reason for these differences in staining patterns is unknown. We observed two distinct patterns of HIF-1α immunostaining. One pattern was heterogeneous and was detected only in viable tumour cells surrounding the area of necrosis, whereas the other pattern was diffuse and homogeneous. The heterogeneous staining may have been the result of hypoxia, whereas the homogeneous pattern may have been due to regulatory modes other than hypoxia. Expression of HIF-1α is enhanced by genetic alterations in tumour suppressor genes (*VHL*, *p53*, *PTEN*) and oncogenes (*v-src*, *HER2neu*, *H-ras*) and by the induction of several growth factors (insulin-like growth factor (IGF)-1 and IGF-2, basic fibroblast growth factor (bFGF), and epidermal growth factor (EGF)). It is possible that the homogeneous HIF-1α immunostaining pattern in oesophageal carcinoma was associated with these factors, at least in part. Additional studies are needed to clarify these associations.

The prognosis and choice of therapy for most oesophageal carcinoma patients are based on both the histological type and tumour stage. Previous studies have shown that angiogenesis is associated with prognosis for several malignancies [5,6,23]. In the present study, we followed 82 patients to determine whether the expression of HIF-1α and VEGF can predict which patients are likely to recur. As expected, the outcome of patients was significantly associated with VEGF expression. This result is consistent with results from previous studies [29]. Although there was a tendency for shorter survival in patients with high HIF-1α expression, this correlation did not reach statistical significance. In several previous studies, high expression of HIF-1α has predicted a poor outcome [30]; however, the opposite scenario has also been reported [31]. HIF-1 activation regulates many processes that are advantageous to tumour growth [16], but Sowter and colleagues [32] suggested that activation of HIF-1 during the evolution of cancer also activates other pathways, such as Bcl-2/adenovirus E1B 19 kDa-interacting protein 3 (BNIP3) and Nip3-like protein X (NIX), that may have antitumour effects. Piret and colleagues [33] suggested that HIF-1α has both pro- and anti-apoptotic effects. Mild hypoxia causes the expression of various anti-apoptotic proteins, which are protective, whereas severe hypoxia leads to cell death, at least in part, through stabilisation of *p53* by HIF-1α [33]. The overall balance of the activation effects of HIF-1α may depend on the type of cancer and treatment modality used [31].

Chemotherapy and radiotherapy have variable effects on angiogenesis. Previous studies have been shown that the production of angiogenic factors by tumour cells is upregulated during chemotherapy and radiotherapy [34,35]. In head and neck cancers, expression of HIF-1α is upregulated in post-radiotherapy biopsy specimens in approximately 35% of patients [17]. In contrast, several investigators have reported reduced expression of angiogenic factors during chemotherapy [36]. Therefore, we selected 47 patients who had not received preoperative chemotherapy and analysed their outcomes separately.

In conclusion, we showed that HIF-1α expression is associated with VEGF expression and angiogenesis in human oesophageal squamous cell carcinoma. Overexpression of HIF-1α is also a useful marker for predicting patient outcome, at least in a subset of patients who had not received preoperative chemotherapy and/or in conjuction with VEGF expression. HIF-1α may be a potential target for angiogenic therapies to treat oesophageal squamous cell carcinoma.

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