대흉외지 2004;37:691-701 □ 임상연구 □

식도 편평세포암에서 Hypoxia-inducible Factor-1 α의 발현: 예후와 종양표지자와의 상관성

양일종* · 조성래* · 김종인* · 이해영* · 천봉권**

Expression of Hypoxia-inducible Factor-1 α in Esophageal Squamous Cell Carcinoma: Relationship to Prognosis and Tumor Biomarkers

Il Jong Yang, M.D.*, Sung Rae Cho, M.D.*, Jong In Kim, M.D.*
Hae Young Lee, M.D.*, Bong Kwon Chun, M.D.**

Background: Tissue hypoxia is a characteristic of many human malignant neoplasms, and hypoxia inducible factor-1 (HIF-1) plays a pivotal role in essential adaptive response to hypoxia, and activates a signal pathway for the expression of the hypoxia-regulated genes, resulting in increased oxygen delivery or facilitating metabolic adaptation to hypoxia. Increased level of HIF-1 α has been reported in many human malignancies, but in esophageal squamous cell carcinoma, the influence of HIF-1 α on tumor biology, including neovascularization, is not still defined. ***Material and Method:*** The influence of HIF-1 α expression on angiogenic factors, correlation between the tumor proliferation and HIF-1 α expression, interaction of HIF-1 α expression and p53, and correlation between HIF-1 α expression and clinicopathological prognostic parameters were investigated, using immunohistochemical stains for HIF-1 α, VEGF, CD34, p53, and Ki-67 on 77 cases of resected esophageal squamous cell carcinoma. ***Result:*** HIF-1 α expression in cancer cells was found in 33 of 77 esophageal squamous cell carcinoma cases. The 33 cases (42.9%) showed positive stain for HIF-1 α. High HIF-1 α expression was significantly associated with several pathological parameters, such as histologic grade (p=0.032), pathological TMN stage (p=0.002), the depth of tumor invasion (p=0.022), regional lymph node metastasis (p=0.002), distant metastasis (p=0.049), and lymphatic invasion (p=0.004). High HIF-1 α expression had significant VEGF immunoreactivity (p=0.008) and Ki-67 labeling index ($p<0.001$), but was not correlated with microvascular density within tumors (p=0.088). The high HIF-1 α expression was correlated with aberrant p53 accumulation with a marginal significance (p=0.056). The overall 5-year survival rate was 34.9%. The survival rate of patients with a high HIF-1 α expression was worse than that of patients with low-expression tumors (log-rank test, p=0.0001). High HIF-1 α expression was independent unfavorable factors although statistical significance is marginal in multivariate analysis. ***Conclusion:*** It is suggested that (1) high HIF-1 α expression in esophageal squamous cell carcinoma is associated with tumor hypoxia, or with genetic alteration in early carcinogenesis and progressive stages, (2) high HIF-1 α expression may be associated with intratumoral neovascularization through HIF-VEGF pathway, and (3) high HIF-1 α expression is associated with poor prognosis in patients with esophageal squamous cell carcinoma and may play a role as biomarker for regional lymph node metastasis.

(Korean J Thorac Cardiovasc Surg 2004;37:691-701)

*고신대학교 의과대학 흉부외과학교실
Department of Thoracic & Cardiovascular Surgery, Kosin University College of Medicine
**고신대학교 의과대학 병리학교실
Department of Pathology, Kosin University College of Medicine
†2003년 11월 제35회 대한흉부외과학회 추계학술대회에서 발표되었음.
논문접수일 : 2004년 5월 12일, 심사통과일 : 2004년 7월 2일
책임저자 : 조성래 (602-702) 부산광역시 서구 암남동 34번지, 고신대학교 의과대학 흉부외과학교실
(Tel) 051-990-6237, (Fax) 051-254-5446, E-mail: srcho@kosinmed.or.kr

대흉외지
2004;37:691-701

Key words: 1. Esophageal neoplasms
2. Neoplasm marker
3. Neoplasm outcomes

INTRODUCTION

Tissue hypoxia is characteristic of many human malignant neoplasm, and cellular response results in neovascularization and increased glycolysis. Markedly proliferative cancer cells fall into hypoxic condition in the absence of neovasculization because the diffusion of oxygen, glucose, and other nutrients from blood vessels are limited[1]. Thus tumor cells cannot grow beyond several cubic millimeters. But in hypoxic condition cells sense low oxygen partial pressure condition and cellular adaptive response includes activation of a signaling pathway for the expression of the hypoxia-regulated genes[2].

Hypoxia inducible factor-1 (HIF-1) plays a pivotal role in essential adaptive response to hypoxia, its expression and transcriptional activity increasing exponentially with decreases in levels of cellular oxygen[3]. This key protein HIF-1 activates a signal pathway for the expression of the hypoxia-regulated genes, including those encoding vascular endothelial growth factor (VEGF), erythropoietin, transferrin, heme oxygenase-1, inducible nitric oxide synthetase, glucose transporters, and glycolytic pathway enzymes aldolase A, enolase 1, lactate dehydrogenase A, phosphofructokinase I, and phophoglycerate kinase I[4,5]. These protein products increase oxygen delivery or facilitate metabolic adaptation to hypoxia.

HIF-1 is a heterodimeric basic-helix-loop-helix-PER-ARNT-SIM transcription factor that is composed of two subunits, HIF-1 α and HIF-1 β[6]. Whereas HIF-1 β is identical to aryl hydrocarbon nuclear receptor translocator (ARNT) which is constitutively present, the level of HIF-1 α expression is primary determinant of HIF-1 DNA binding and transcription activity[4,7]. Thus, induction of HIF-1 α appears to be a critical step in the hypoxic response and occurs via increased mRNA expression, protein stabilization, and augmented activity of its transcriptional activation domains[7]. Increased level of HIF-1 α has been reported in many human malignancies, including squamous cell carcinoma of head-and-neck[8], lung[9], and uterine cervix[10]. Koukourakis reported the association of HIF-1 α expression with response to photodynamic therapy in esophageal squamous cell carcinoma[11]. Several reports revealed HIF-1 α expression was associated with angiogenic factors, such as VEGF, platelet derived endothelial cell growth factor (PD-ECGF)[9], and microvessel density, and proliferative index[12]. Also a few workers found the interaction of HIF-1 and p53 in carcinogenesis and tumor progression in experimental models and in other human malignancies[13]. But in esophageal squamous cell carcinoma the influence of HIF-1 α on tumor biology, including neovascularization, is not still defined.

In present study the influence of HIF-1 α expression on angiogenic factors, such as VEGF and microvessel density was studied, using imunohistochemical (IHC) analysis. In addition, correlation between the tumor proliferation and HIF-1 α expression and interaction of HIF-1 α expression and p53 were investigated. Also correlation between HIF-1 α expression and clinicopathological prognostic parameters is investigated.

MATERIAL AND METHOD

1) Materials

Archival surgical material recruited in this study were retrieved from seventy-seven patients with primary esophageal squamous cell carcinomas, who underwent radical esophagectomy and lymph node dissection from 1995 to 2000 at the Department of Cardiothoracic Surgery at Kosin University, College of Medicine. All patients have not suffered from preoperative chemotherapy or radiotherapy.

2) Methods

(1) Clinico-pathological parameters: Patient's age and sex, duration of survival, gross feature of surgical samples were obtained from patient's charts and archival pathological reports. For histopathological review, one of these sections was stained with hematoxylin-eosin. Pathological parameters, such as histological differentiation, depth of tumor invasion, nodal metastasis, and lymphatic and/or vascular invasion, were also analyzed. Degree of histological differentiation was divided into three degrees, such as well differentiated, intermediate differentiated, and poorly differentiated. The clinicopathologic stage was determined according to the TMN classification system of the International Union Against Cancer (UICC).

(2) Immunohistochemical staining: Immunohistochemical staining was performed using anti-hypoxia-inducible factor 1 α (HIF-1 α) monoclonal IgG1 (Clone ESSE122, Novus, Littletone, USA), Vascular endothelial growth factor (VEGF) (Clone A20, Santa Cruz, California, USA), p53 mouse monoclonal antibody (Clone DO-7, NeoMarkers, Fremont, USA), Ki-67 (Clone 7B11, Zymed, South San Francisco, USA). Dilutions were 1:500 for anti-HIF-1, and 1 : 50 for VEGF, p53, and Ki-67: 4- m sections of routine formalin-fixed, parraffin- embedded tissues were mounted on 2% 3-aminopropyletoxysilane-treated glass slides (Probe On Plus Microscope Slides, Fisher Scientific, Pittsburgh, USA), air dried overnight, in a 60°C dry-oven, deparaffinized twice in xylene for 3 min each, rehydrated I a descending ethanol series (in 100% ethanol twice for 3 min each, and in 95% ethanol twice for 3 min each), and rinsed thoroughly in distilled water for 10 min. The slides were placed in a plastic jar filled with 10mM sodium citrate buffer at pH 6.0 and then treated by being boiled in a microwave oven three times for a total 10 min. After gradual cooling to room temperature and being rinsed thoroughly in distilled water for 10 min, the slides were treated with 3% hydrogen peroxide in distilled water for 6 min to block endogenous peroxidase activity and washed with distilled water and placed in Tris buffer (×10 Immunoassay bufferR, Biomeda, Foster, USA) for 10 min. Tissue sections were incubated with HIF-1 α 3 hr at room temperature in moisture chamber, thereafter, Catalysed Signal Amplication System (DAKO Co., Carpinteria, CA, USA) was used according to the manufacture's instructions, which is based on streptavidin-biotin-horseradish peroxidase complex formation. For VEGF, p53, and Ki-67, tissue sections were incubated with these antibodies overnight at 4°C and then immunohistochemical procedure was performed with LSAB KitR (DAKO, Carpinteria, USA), which is based on streptavidin-biotin complex formation. The reaction products were visualized by exposing sections to 3,3-diaminobenzidine for HIF-1 α, and 3-amino-9-ethylcarbazole for others. Nuclei were lightly counterstained for about 20 sec with placed in 3-amino-9-ethylcarbazole (AEC, DAKO company, Carpinteria, USA), counterstained with Mayer's haematoxylin. Sections were then mounted in diluted malinol after the application of

Table 1. Clinicopathological characteristics in esophageal squamous cell carcinoma

Clinicopathological characteristics	Number of patients (%)
Age (Yrs)	
<60	35 (45.5)
≥60	42 (54.5)
Gender	
Male	74 (96.1)
Female	3 (3.9)
Histologic type	
Well diferentiated	36 (46.8)
Moderately differentiated	34 (44.2)
Poorly differentiated	7 (9.1)
Pathological stage (UICC)	
I	16 (20.8)
II	32 (41.6)
III	23 (29.9)
IV	6 (7.8)
pT status	
T1	18 (23.4)
T2	19 (24.7)
T3	40 (51.9)
T4	0 (0)
pN status	
N0	37 (48.1)
N1	40 (51.9)
pM status	
M0	71 (92.2)
M1	6 (7.8)

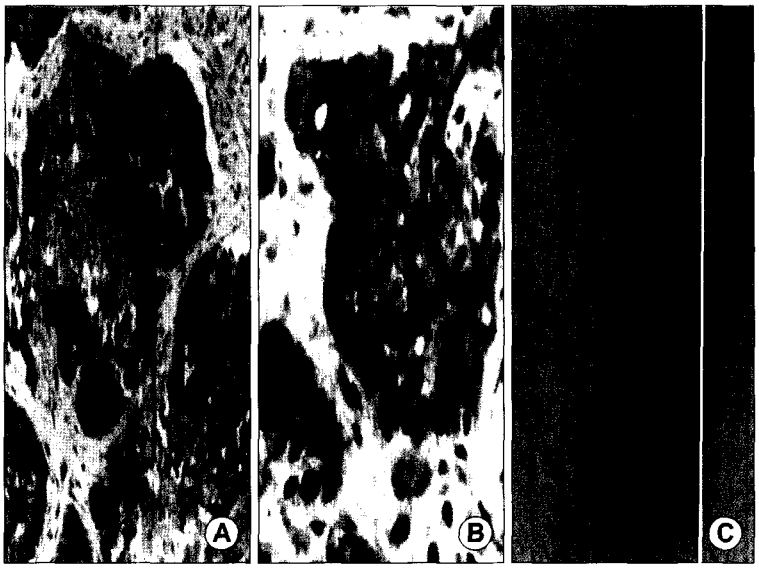

Fig. 1. Immunostaining for HIF-1 α: The patterns of HIF-1 α expression in cancer cells were diffuse throughout nuclear and cytoplasms. Clusters of cancer cells showing high HIF-1 α expression were most dense at the periphery of necrotic regions (A, ×200) and the invading edge of tumor margins (B, ×400). Negative control (C, ×100).

Universal Mount (Biomeda, Foster, USA).

For each batch of IHC, positive and negative control specimens were also incubated and reviewed along with the test slides. Sections from one lung squamous cell carcinoma showing positive immunostaining were used as positive control for HIF-1 α, VEGF, p53, and Ki-67. Negative controls were run without the primary antibody to monitor background staining.

3) Interpretation of Immunohistochemical Staining

All immunohistochemical evaluations were performed by one pathologist. The immunohistochemical results for HIF-1 α protein were classified as follows: -, no staining; 1+, nuclear staining in less than 1% of tumor cells; 2+, nuclear staining in 1~10% of tumor cells and/or with weak cytoplasmic staining; 3+, nuclear staining in 10~50% of tumor cells and/or with distinct cytoplasmic staining; 4+, nuclear staining in more than 50% of tumor cells and/or with strong cytoplasmic staining. HIF-1 α 3+ to 4+ were regarded as high expression patterns and the 1+ and 2+ cases were considered to be low expression[12,13]. For evaluation of VEGF expression a score corresponding to the sum of both (a) staining intensity (0, negative; 1, weak; 2, intermediate; 3, strong) and (b) percentage of positive cells (0, 0% positive cells; 1, less than 25% positive cells; 2, 26~50% positive cells; 3, more than 50% positive cells) was determined. Their sum reached a maximum score of 6. A score greater than 3 was regraded as positive. Aberrant p53 accumulation was scored positive if nuclear staining was present in >10% of tumor cells. For Ki-67 analysis, nuclei from approximately 1000 tumor cells from randomly selected fields were counted at high power (×200), and the labeling index (LI) was determined as the percentage of positive nuclei.

4) Statistical Analysis

Statistical analysis was performed with the SPSS software (SPSS for Windows Standard version 10.1, SPSS Inc., Chicago, USA). To analyze whether the elevated level of HIF-1 α correlated with VEGF expression, abberrant p53 accumulation, histologic differentiation, depth of tumor invasion, lymph nodal invasion, vascular invasion, pathological stage, Pearson chi-square test and Fisher exact probability test were performed. Independent T-test was also applied to evaluate the correlation between HIF-1 α expression and

micro vascular density. Kaplan-Meier method, and the significance of differences in survival was analysed by the log-rank test. The univariate and multivariate analyses were performed using the Cox proportional hazard regression model. p value less than 0.05 was regarded as significant in all analyses. All statistical tests were two-sided.

RESULTS

1) Cliniopathological characteristics of esophageal squamous cell carcinoma

The age of seventy-seven patients ranged between 44 to 77 years (median: 60 years), and males and females were 74 and 3, respectively. Well differentiated tumors were seen in 36 patients, moderately differentiated tumors in 34 patients, and poorly differentiated tumors in 7 patients. Adventitial involvement by tumor cells were seen in 40 patients, other 37 patients show tumor invasion within the level of proper muscle layer. Forty patients (51.9%) had regional lymph node metastases. Distant metastases at the time of operation were seen in six patients (7.8%)(Table 1).

2) Association of HIF-1 α expression with clinicopathological characteristics in esophageal squamous cell carcinoma

The staining patterns of HIF-1 α expression in cancer cells were diffuse throughout nuclear and cytoplasms (Fig. 1). Adjacent non-neoplastic squamous epithelium showed a varying intensity of cytoplasmic staining. Staining of tissue sections from normal esophagus showed no immunostaining for HIF-1 α in the normal epithelium. Within tumors, clusters of cancer cells showing high HIF-1 α expression were most dense at the periphery of necrotic regions and the invading edge of tumor margins. High HIF-1 α expression in cancer cells was found in 33 of 77 cases of esophageal squamous cell carcinoma (42.9%). High HIF-1 α expression was significantly more frequent in tumors of high histologic grade (p=0.032). High HIF-1 α expression correlated with pathological TMN stage (p=0.002), the depth of tumor invasion (p=0.022), regional lymph node metastasis (p=0.002), distant metastasis (p=0.049), lymphatic invasion (p=0.004)(Table 2).

Table 2. Correlation between HIF-1 α expression and clinicopathologic characteristics in esophageal squamous cell carcinoma

Clinicopathological characteristics	No.of cases	HIF-1 α		p value
		Positive (n=33)	Negative (n=44)	
Age (Yrs)				*0.244*
<60	35	17	18	
≥60	42	16	26	
Sex				*0.392*
Male	74	31	43	
Female	3	2	1	
Histological type				*0.032*
Well differentiated	36	10	26	
Moderately differentiated	34	20	14	
Poorly differentiated	7	3	4	
Pathological stage				*0.002*
I-II	48	14	34	
III-IV	29	19	10	
pT status				*0.022*
T1-T2	37	11	26	
T3-T4	40	22	18	
3		12	8	
pN status				*0.002*
N0	37	9	28	
N1	40	24	16	
pM status				*0.049*
M0	71	28	43	
M1	6	5	1	
Lymphatic invasion				*0.004*
Positive	42	23	16	
Negative	35	10	28	
Vascular invasion				*0.593*
Positive	12	5	7	
Negative	65	28	37	

3) Association of HIF-1 α expression with VEGF, p53 and Ki-67 labeling index in esophageal squamous cell carcinoma

Clusters of VEGF immunoreactive cells were most dense at the invading edge of tumor margins (Fig. 2). VEGF immunoreactivity was seen in 53 of 77 cases (68.8%). High HIF-1 α expression was significantly more frequent in tumors showing VEGF immunoreactivity (p=0.008)(Table 3). But

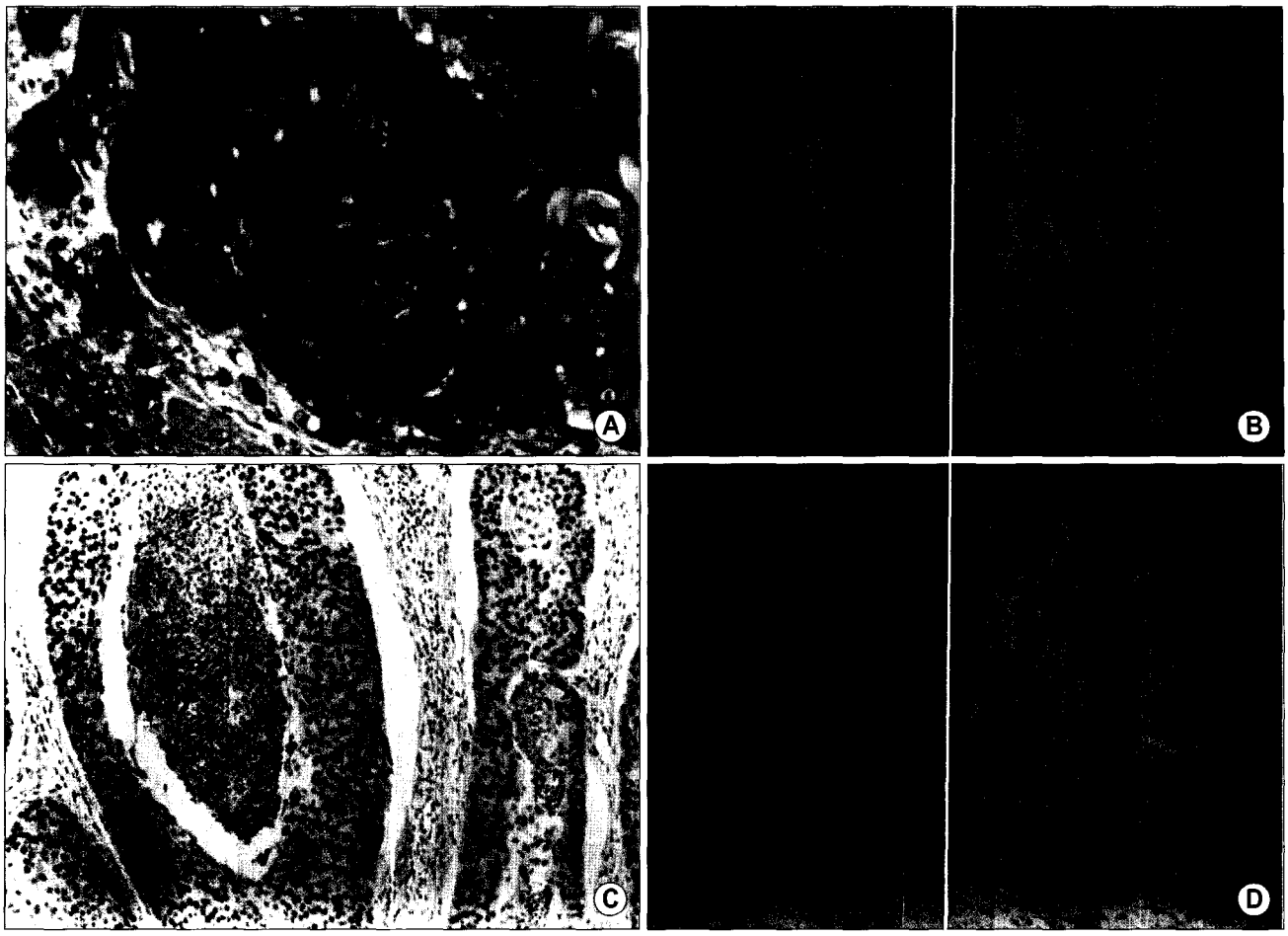

Fig. 2. Immunostaining for VEGF, CD34, p53, and Ki-67: Cancer cells show reddish brown cytoplasmic staining for VEGF in a diffuse fashion (A, ×400), and increased CD34-positive microvessels in the interfaces between carcinoma nests and stroma are seen (B, ×200). Most of tumor cells show positive nuclear staining for p53 (C, ×40), and for Ki-67 (D, ×100).

high HIF-1 α expression is not correlated with microvascular density within tumors (p=0.088). Aberrant p53 accumulation was seen throughout the tumors, including the cancer cells in mucosal surface (Fig. 3). The correlation of high HIF-1 α expression with aberrant p53 accumulation was marginal statistical significance (p=0.056)(Table 3). Statistical analysis demonstrated a highly significant correlation of high HIF-1 α with Ki67 labeling index as a marker of cellular proliferation ($p<0.001$)(Table 3).

4) Kaplan-Meier survival analysis according to HIF-1 α expression in esophageal squamous cell carcinoma

During the course of this study (median follow-up 34 months), 3 cases were lost to follow-up. Data on postoperative treatment were not available. The overall 5-year survival rate was 34.9%. The survival curve of patients with a high HIF-1 α expression tumors was worse than that of patients with low expression tumors (log-rank test, p=0.0001)(Fig. 3).

5) Univariate survival analysis in esophageal squamous cell carcinoma

Cox regression univariate analysis identified pT status (p=0.028), pN status ($p<0.001$), pM status (p=0.004), lymphatic invasion ($p<0.001$), high HIF-1 α expression ($p<0.001$) as correlating with survival (Table 5).

Table 3. Association of HIF-1 α expression with VEGF, p53, and Ki-67 labeling index in esophageal squamous cell carcinoma

Parameters	No. of cases	HIF-1 α		p value
		Positive (n=33)	Negative (n=44)	
VEGF				*0.008*
Positive	53	28	25	
Negative	24	5	19	
MVD (microvessels/mm^2)		22.0±6.6	19.5±6.1	*0.088*
p53				*0.056*
Positive	47	24	23	
Negative	30	9	21	
Ki-67 labeling index (%)				
≥21	38	26	12	*0.001*
<21	39	7	32	

VEGF=Vascular endothelial growth factor; MVD=Microvessel density.

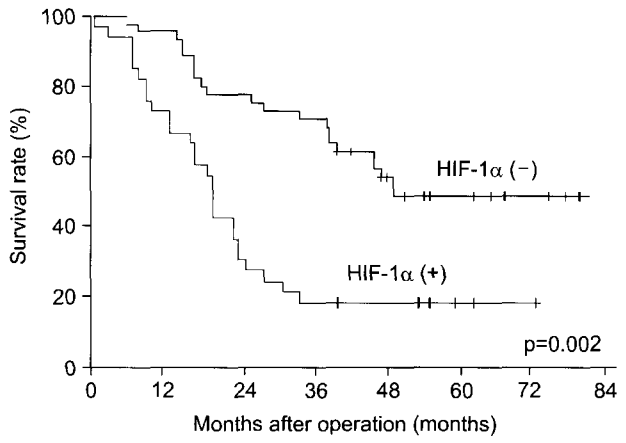

Fig. 3. Kaplan-Meier overall survival curves according to HIF-1 α expression in esophageal squamous cell carcinoma.

6) Multivariate survival analysis in esophageal squamous cell carcinoma

Multivariate analysis performed by Cox regression identified no independent unfavorable factors, except for high HIF-1 α expression which had a marginal significance (Table 5).

Table 4. Microvascular density according to clinicopathological and molecular parameters in esophageal squamous cell carcinoma

Parameters	No. of cases	MVD (microvessels/mm^2)	p value
Age			*0.183*
<60	35	19.5±6.2	
≥60	42	21.5±6.5	
Patholgical stage			*<0.001*
I-II	48	18.5±5.6	
III-IV	29	23.9±6.4	
pT status			*0.003*
T1-T2	37	18.4±6.1	
T3-T4	40	22.6±6.0	
pN status			*0.14*
N0	37	18.7±5.5	
N1	40	22.3±6.8	
pM status			*0.050*
M0	71	20.2±6.4	
M1	6	25.3±4.8	
Lymphatic invasion			*0.020*
Positive	42	22.2±6.8	
Negative	35	18.8±5.6	
Vascular invasion			*0.054*
Positive	12	23.8±6.3	
Negative	65	20.0±6.3	
VEGF			*<0.001*
Positive	53	22.9±5.2	
Negative	24	15.5±5.9	
p53			*0.62*
Positive	74	20.9±6.8	
Negative	3	20.1±5.9	
Ki-67 LI			*0.12*
<21	38	18.8±6.0	
≥21	39	22.4±6.4	

VEGF=Vascular endothelial growth factor.

DISCUSSION

In the present study, the patterns of HIF-1 α expression in cancer cells were mixed nuclear/cytoplasmic, and adjacent normal mucosa showed a varying intensity of cytoplasmic staining (weak or even strong). Staining of tissue sections from normal esophagus showed that the normal epithelium did not express HIF-1 α protein. Although some studies

Table 5. Univariate and multivariate analysis of HIF-1 α and clinicopathological parameters in esophageal squamous cell carcinoma

Factors	Odds ratio	95% confidence interval (CI)	p value
Univariate			
HIF-1 α (high vs. Low)	2.949	1.656~5.252	*<0.001*
Age (<60 yrs, ≥60 yrs)	1.048	0.596~1.841	*0.872*
Gender (male vs. female)	1.448	0.449~4.675	*0.535*
Pathological grade (G1, G2, G3)	1.424	0.932~2.177	*0.102*
pT status (T1-2 vs. T3)	1.898	1.072~3.363	*0.028*
pN status (N0 vs. N1)	4.893	2.590~9.245	*<0.001*
pM status (M0 vs. M1)	3.729	1.538~9.038	*0.004*
Lymphatic invasion (positive vs. negative)	4.763	2.554~8.879	*<0.001*
Vascular invasion (positive vs. negative)	1.196	0.559~2.555	*0.645*
VEGF (positive vs. negative)	1.703	0.886~3.272	*0.110*
p53 (positive vs. negative)	.851	0.483~1.501	*0.578*
Multivariate			
HIF-1 α (high vs. low)	1.829	0.979~3.416	*0.058*
Pathological grade (G1, G2, G3)	0.889	0.509~1.552	*0.680*
pT status (T1-2 vs. T3)	1.246	0.645~2.407	*0.512*
pN status (N0 vs. N1)	2.444	0.560~10.672	*0.235*
pM status (M0 vs. M1)	2.204	0.826~5.883	*0.115*
Lymphatic invasion (positive vs. negative)	1.562	0.324~7.530	*0.578*

VEGF=Vascular endothelial growth factor.

reported only nuclear pattern of HIF-1 α in oropharyngeal cancer and lung cancers, this mixed nuclear/cytoplasmic patterns of HIF-1 α in cencer cells have been reported by Talks et al. and Zhong et al., but normal mucosa was persisitently negative in both studies[12,13]. Hypoxia directly increases the half-life of HIF-1 α by decreasing the rate of ubiquitination and proteasomal degradation of the protein[14].

In this study, clusters of cancer cells showing high HIF-1 α expression were most dense at the periphery of necrotic regions within tumors, and this finding may support that the hypoxic tumor microenvironment may directly contribute to increased HIF-1 α activity in esophageal squamous cell carcinomas. Of interest, tumor cells in the invading edge of tumor margins away from the tumor necrosis also showed increased level of HIF-1 α protein. Although intratumoral oxygen partial pressure of tumor cells away from the tumor necrosis was not measured by Eppendorf microelectrode, this finding may reflect that in addition to hypoxia alternative mechanism regulates the induction of HIF-1 α activity in esophageal squamous cell carcinoma.

A growing number of evidence indicates that genetic alterations is also associated with increased HIF-1 α expression in cancer cells[15]. Stimulation of growth factors, including insulin-like growth factors (IGFs) 1 and 2, basic fibroblast growth factor, epidermal growth factor induces HIF-1 α expression[15]. Activation of a signal transduction pathway involving phosphatidylinositol-3-kinase (PI3K) and the serine-threonine kinases AKT (protein kinase B) and FKBP-rapamycin-associated protein (FRAP) has been shown to result in a dramatic increase in HIF-1 α protein synthesis[16]. Thus, gain-of-function mutation in oncogenes which activate growth factor-PI3K signaling pathway lead to increased HIF-1 α expression via this mechanism, as to lose-of function mutations in the tumor suppressor gene encoding PTEN[16]. Cells expressing the v-Src oncogene have increased expression of HIF-1 and downstream genes including VEGF under both hypoxic and non hypoxic conditions[17]. Thus the high HIF-1 α expression in esophageal squamous cell carcinoma demonstrated by immunohistochemistry results both as a response to the physiologic stimulus of hypoxia and as a result of tumor specific genetic alterations. But which genetic alteration is associated with high HIF-1 α expression in these tumor cells away from the tumor necrosis requires further studies.

In the study of Zhong et al[12]. HIF-1 α protein was overexpressed in preneoplastic and premalignant lesons such as colonic adenoma, breast ductal carcinoma in situ, and prostate intra-epithelial neoplasia, whereas every benign tumor was negative for HIF-1 α expression. They suggested that overexpression of HIF-1 α can occur very early in carcinogenesis, before histological evidence of angiogenesis or invasion. In present study, one case of three carcinoma in situ stage and three intra-epithelial neoplasias adjacent to the invasive tumors were positive for HIF-1 α immunoreactivity. Additional studies is required to assess whether HIF-1 α may

represent a novel biomarker for precancerous lesions that warrant clinical surveillance or therapeutic intervention.

As concern with Ki-67 proliferation index, high HIF-1 α expression had a significant association with high proliferation index as both studies of Zhong et al.[12] and Bos et al.[18], which showed a significant association of HIF-1 α expression with Ki-67 proliferation index. High proliferation of tumor cells are associated with increased need of oxygen supply and energy metabolism, resulting in induction of HIF pathway, with increased oxygen delivery via new blood vessel formation. Activation of HIF-1-regulated genes, such as glucose transporters and glycolytic enzymes allow the tumor cells to adapt their metabolism to an oxygen-deprived environment.

In present study microvascular density was strongly correlated with VEGF expression, 22.9±5.2 microvessels/mm^2 in VEGF-positive group and 15.5±5.9 microvessels/mm^2 in VEGF-negative group, as the study of Ogata et al.[19]. Microvascular density was also strongly associated with pathological stage (UICC), 18.5±5.6 microvessels/mm^2 in pathological stage I and II group and 23.9±6.4 microvessels/mm^2 in pathological stage III and IV group. These results suggest that capacity of new blood vessel formation evaulated by measurement of MVD comes under the influence of intratumoral VEGF level, and is associated with tumor progression. The association of high HIF-1 α expression with MVD in present study is not found. Although high HIF-1 α expression was strongly correlated with VEGF expression as other studies, microvascular density had an association of berderline significance with high HIF-1 α expression. This result is in contrast with those of Birner et al.[10] and Bos et al.[18], who reported the significant association between high HIF-1 α expression and MVD, but is consistent with those of Beasley et al.[8] and Aebersold et al.[20], who reported that no significant correlation existed between immunostaining for HIF-1 α and MVD. Also this result suggests that the process of tumor angiogenesis in esophageal squamous cell carcinoma is also subject to other modulators such as PD-ECGF and TGF-alpha expression, although tumor necrosis and peritumoral inflammation affect the angiogenesis process in invasive squamous cell carcinoma. Also in present study MVD was associated with lymphatic invasion, but was not correlated with regional lymph node metastasis, accumualtion of aberrant p53, and Ki-67 proliferation index. These results differed from those of both studies of Li et al.[21] and Koide et al.[22], who reported the significant association between MVD and lymph node metastasis.

In present study high HIF-1 α expression was marginally associated with accumulation of aberrant p53 in esophageal squamous cell carcinomas. This result is consistent with those of both studies of Zhong et al.[12] and Talks et al.[13], but is in contrast with those of the study of Bos et al.[18]. Under hypoxic conditions HIF-1 α has been shown to interact with the tumor suppressor protein p53, which is itself a DNA-binding transcription factor. This interaction appears to increase the half-life of p53 and decrease the half-life of HIF-1 α as a result of increased ubiquitination by MDM2, a ubiquitin protein ligase that binds to p53. Loss of p53 in tumor cells enhances HIF-1 α levels and augments HIF-1-dependent transcriptional activation of the expression of vascular endothelial growth factor gene in response to hypoxia.

In present study high HIF-1 α expression may strongly influence both tumor proliferation and lymph node metastasis in esophageal squamous cell carcinoma. However, it has been reported that HIF-1 α overexpression was not significantly correlated with pathological parameter in other cancers, including head and neck and oropharyngeal cancer[20]. Thus, HIF-1 α expression seems to behave in a tissue-dependent manner. The strong association between high HIF-1 α expression and regional nodal metastasis in esophageal squamous cell carcinoma suggest high HIF-1 α expression may be biomarker for nodal metastasis in preoperative biopsy material.

Solid tumor contain regions of hypoxia, and tumor hypoxia is an important determinant of clinical prognosis. It was observed that a significant association between the overall survival of the patients and HIF-1 α expression ($p=0.002$).

This result is in concordance with a very recent study by Koukourakis et al.[11], who reported that survival in patients with a high HIF-1 α expression was significantly worse than in those with low expression in patients treated with adjuvant therapy. Also other studies of early esophageal carcinomas, operable non-small cell lung cancer, oropharyngeal cancer, and head and neck cancer revealed a significant association

of HIF-1 α expression with poor overall survival[8,9,11, 20,23]. In addition, it was found that a significant correlation between the overall survival and pathological parameters, including depth of tumor invasion, regional lymph node metastasis, distant metastasis, and lymphatic invasion. However, no association between the overall survival and pathological grade and molecular parameters, including VEGF expression and accumulation of aberrant p53.

As concerned with VEGF expression and MVD, some researchers suggested that the two parameters were not correlated with the overall survival in esophageal carcinomas as this study[24]. However, other workers reported that both VEGF expression and MVD were linked to poor survival in esophageal squamous cell carcinomas[25]. This discripancy may be due to the different methodologies applied, the approaches to the estimated results, and the cut-off levels applied.

In multivariate models, of all parameters with a statistically significant prognostic role in univariate analysis, high HIF-1 α expression was one predictive factors of overall survival with a marginal significance (p=0.058).

CONCLUSION

In conclusion, it is suggested that (1) high HIF-1 α expression in esophageal squamous cell carcinoma is associated with tumor hypoxia or with genetic alteration in early carcinogenesis or progressive stages, (2) high HIF-1 α expression may be associated with intratumoral neovascularization through HIF-VEGF pathway, and (3) high HIF-1 α expression is associated with poor prognosis in patients with esophageal carcinoma and may play a role as biomarker for regional lymph node metastasis.

REFERENCE

1. Dang CV, Semenza GL. *Oncogenic alteration of metabolism.* Trends Biochem Sci 1999;24:68-72.
2. Chandel NS, McClintock DS, Feliciano CE, et al. *Reactive oxygen species generated at mitochondrial complex III stabilize hypoxia-inducible factor-1 alpha during hypoxia: a mechanism of O2 sensing.* J Biol Chem 2000;275:25130-8.
3. Yu AY, Frid MG, Shimoda LA, Wiener CM, Stenmark K, Semenza GL. *Temporal, spatial, and oxygen-regulated expression of hypoxia-inducible factor-1 in the lung.* Am J Physiol 1998;275(4 Pt 1):L818-26.
4. Forsythe JA, Jiang BH, Iyer NV, et al. *Activation of vascular endothelial growth factor gene transcription by hypoxia-inducible factor 1.* Mol Cell Biol 1996;16:4604-13.
5. Jung F, Palmer LA, Zhou N, Johns RA. *Hypoxic regulation of inducible nitric oxide synthase via hypoxia inducible factor-1 in cardiac myocytes.* Circ Res 2000;86:319-25.
6. Wang GL, Semenza GL. *Purification and characterization of hypoxia-inducible factor 1.* J Biol Chem 1995;270:1230-7.
7. Wiesener MS, Turley H, Allen WE, et al. *Induction of endothelial PAS domain protein-1 by hypoxia: characterization and comparison with hypoxia-inducible factor-1 alpha.* Blood 1998;92:2260-8.
8. Beasley NJ, Leek R, Alam M, Turley H, Cox GJ, Gatter K. *Hypoxia-inducible factors HIF-1 alpha and HIF-2 alpha in head and neck cancer: relationship to tumor biology and treatment outcome in surgically resected patients.* Cancer Res 2002;62:2493-7.
9. Giatromanolaki A, Koukourakis MI, Sivridis E, et al. *Relation of hypoxia inducible factor 1 alpha and 2 alpha in operable non-small cell lung cancer to angiogenic/molecular profile of tumours and survival.* Br J Cancer 2001;85:881-90.
10. Birner P, Schindl M, Obermair A, Plank C, Breitenecker G, Oberhuber G. *Overexpression of hypoxia-inducible factor 1 alpha is a marker for an unfavorable prognosis in early-stage invasive cervical cancer.* Cancer Res 2000;60:4693-6.
11. Koukourakis MI, Giatromanolaki A, Skarlatos J, et al. *Hypoxia inducible factor (HIF-1a and HIF-2a) expression in early esophageal cancer and response to photodynamic therapy and radiotherapy.* Cancer Res 2001;61:1830-2.
12. Zhong H, De Marzo AM, Laughner E, Lim M, Hilton DA, Zagzag D. *Overexpression of hypoxia-inducible factor 1 alpha in common human cancers and their metastases.* Cancer Res 1999;59:5830-5.
13. Talks KL, Turley H, Gatter KC, et al. *The expression and distribution of the hypoxia-inducible factors HIF-1 alpha and HIF-2 alpha in normal human tissues, cancers, and tumor-associated macrophages.* Am J Pathol 2000;157:411-21.
14. Kallio PJ, Wilson WJ, O'Brien S, Makino Y, Poellinger L. *Regulation of the hypoxia-inducible transcription factor 1 alpha by the ubiquitin-proteasome pathway.* J Biol Chem 1999;274:6519-25.
15. Feldser D, Agani F, Iyer NV, Pak B, Ferreira G, Semenza GL. *Reciprocal positive regulation of hypoxia-inducible factor 1 alpha and insulin-like growth factor 2.* Cancer Res 1999;59:3915-8.
16. Zhong H, Chiles K, Feldser D, et al. *Modulation of hypoxia-inducible factor 1 alpha expression by the epidermal growth factor /phosphatidylinositol 3-kinase/PTEN/AKT/FRAP pathway in human prostate cancer cells: implications for*

tumor angiogenesis and therapeutics. Cancer Res 2000; 60:1541-5.
17. Jiang BH, Agani F, Passaniti A, Semenza GL. *V-SRC induces expression of hypoxia-inducible factor 1 (HIF-1) and transcription of genes encoding vascular endothelial growth factor and enolase 1: involvement of HIF-1 in tumor progression.* Cancer Res 1997;57:5328-35.
18. Bos R, Zhong H, Hanrahan CF, et al. *Levels of hypoxia-inducible factor-1 alpha during breast carcinogenesis.* J Natl Cancer Inst 2001;93:309-14.
19. Ogata Y, Fujita H, Yamana H, Sueyoshi S, Shirouzu K. *Expression of vascular endothelial growth factor as a prognostic factor in node-positive squamous cell carcinoma in the thoracic esophagus: long-term follow-up study.* World J Surg 2003;27:584-9.
20. Aebersold DM, Burri P, Beer KT, et al. *Expression of hypoxia-inducible factor-1 alpha: a novel predictive and prognostic parameter in the radiotherapy of oropharyngeal cancer.* Cancer Res 2001;61:2911-6.
21. Li Z, Shimada Y, Uchida S, et al. *TGF-alpha as well as VEGF, PD-ECGF and bFGF contribute to angiogenesis of esophageal squamous cell carcinoma.* Int J Oncol 2000;17: 453-60.
22. Koide N, Nishio A, Hiraguri M, Hanazaki K, Adachi W, Amano J. *Coexpression of vascular endothelial growth factor and p53 protein in squamous cell carcinoma of the esophagus.* Am J Gastroenterol 2001;96:1733-40.
23. Koukourakis MI, Giatromanolaki A, Sivridis E, et al. *Hypoxia-inducible factor (HIF1A and HIF2A), angiogenesis, and chemoradiotherapy outcome of squamous cell head-and-neck cancer.* Int J Radiat Oncol Biol Phys 2002;53: 1192-202.
24. Ahn MJ, Jang SJ, Park YW, et al. *Clinical prognostic values of vascular endothelial growth factor, microvessel density, and p53 expression in esophageal carcinomas.* J Korean Med Sci 2002;17:201-7.
25. Shimada H, Hoshino T, Okazumi S, Matsubara H, Funami Y. *Expression of angiogenic factors predicts response to chemoradiotherapy and prognosis of oesophageal squamous cell carcinoma.* Br J Cancer 2002;86:552-7.

=국문 초록=

배경: 악성종양에서 신생혈관 생성 및 당분해의 증가는 저산소 상태의 미세환경을 나타내며, 이는 종양의 침습성, 전이 등으로 환자의 예후와 관련이 있는 것으로 알려져 있다. Hypoxia-inducible factor 1 (HIF-1)는 당원 수송체, 당분해 효소, 혈관내피세포 성장인자 등의 유전자의 전사를 활성화한다고 알려져 있다. 그리고 HIF-1의 전사 활성도는 HIF-1 α subunit의 표현이 조절되는 정도에 의존한다. 그러나 식도암에서 HIF-1의 발현과 혈관 생성능 및 종양세포 증식능과의 관계 및 예후에 관한 연구는 전무하다. **대상 및 방법**: 고신대학교 의과대학 흉부외과학교실에서 1995년부터 2000년까지 수술치험한 77예의 식도 편평세포암 환자의 조직에서 채취한 정상 편평상피와 암조직에서 면역조직화학검사를 이용하여 HIF-1 α의 발현을 조사하고 혈관생성인자, 증식지수, p53 단백과의 상관관계, 임상-병리학적인 인자 및 생존율과의 상관관계를 분석하였다. **결과**: HIF-1 α의 고발현율은 42.9% (33예/77예)였다. HIF-1 α의 고발현은 조직학적 등급(p=0.032), 병리학적 병기(p=0.002), 종양 침윤의 깊이(p=0.022), 주위 림프절 전이(p=0.002), 원격전이(p=0.049), 림프관 침윤(p=0.004)과 관련이 있었다. HIF-1 α의 고발현은 혈관내피세포 성장인자의 발현, Ki-67 증식지수와 관련이 있었으나, 미세혈관수와는 관련이 없었고, p53의 발현과는 관련이 있는 경향을 보였다. 단변량분석과 다변량분석에서 HIF-1 α의 고발현은 불량한 예후를 나타내는 인자로 보였다. **결론**: 식도 편평세포암 조직에서 HIF-1 α의 발현은 종양조직내 신생혈관의 생성과 관련이 있는 것으로 나타났고, 고발현된 경우는 림프절 전이와 수술 후 불량한 예후를 나타내었으므로 보다 강화된 치료전략이 필요할 것으로 사료된다.

중심 단어 : 1. 식도 종양
2. 종양지표
3. 종양예후