Clinical Significance of HIF-1α Expression in Patients with Esophageal Cancer Treated with Concurrent Chemoradiotherapy

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Abstract. *Aim: We investigated whether hypoxia-inducible factor-1α (HIF-1 α) expression in pretreatment biopsies of esophageal cancer is predictive of clinical outcome in patients with esophageal cancer undergoing concurrent chemo-radiotherapy (CRT). Patients and Methods: A total of 25 patients were reviewed. Radiotherapy was administered to total doses of 40-66.6 Gy (median: 66.6 Gy) with a single fraction of 1.8-2 Gy. Cisplatin (80 mg/m^2 on day 1) and 5-fluorouracil (800 mg/m^2 on days 2-6) were administered concurrently with radiotherapy, every 3-4 weeks to a total of 1-2 courses. Tissue samples from esophageal cancer were obtained from all 25 patients by biopsy before concurrent CRT, and semiquantitative analyses of HIF-1α expression were performed using immunohistochemical staining. Results: High HIF-1α expression was observed in 11 out of 25 patients (42.7%), and HIF-1α expression was significantly correlated with initial response to CRT (p=0.0027). Patients with high HIF-1α expression had significantly poorer local control (LC) (5-year LC: 42.7%) than those with low expression (5-year LC: 72.5%; p=0.0322). Patients with high HIF-1α expression also had significantly lower recurrence-free survival (RFS) (5-year RFS: 18.2%) compared to those with low HIF-1α expression (5-year RFS: 39.8%; p=0.0009), and on multivariate analysis, HIF-1α (p=0.001) and number of chemotherapy courses (p=0.010) were independent prognostic factors for RFS. Conclusion: HIF-1α expression is significantly correlated with initial response to concurrent CRT, and is predictive of RFS for patients with esophageal cancer receiving concurrent CRT.*

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Esophageal cancer has proven to be one of the most difficult malignancies to cure, and the prognosis for these patients has been extremely poor (1-3). Although surgery has been the mainstay of curative treatment for these tumors, chemoradiotherapy (CRT) has recently been recognized as a viable option for esophageal cancer. The Radiation Therapy Oncology Group (RTOG) 8501 trial demonstrated that CRT is superior to radiotherapy alone as a primary treatment (4). However, several reports indicated that the patterns of failure observed after definitive CRT showed that locoregional failure is frequent, with approximately 50% of patients experiencing local failure (4, 5). Identification of predictive markers of response to CRT would improve patient selection and may allow response modifications for poor responders by introduction of more intensive treatments.

Hypoxia-inducible factor-1α (HIF-1α) is a heterodimetric transcriptional factor that regulates O_2 homeostasis and the physiologic response to O_2 deprivation. HIF-1α has also been recognized 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 (6-8). In various types of cancer, HIF-1α has been found to play important roles in tumor growth, invasion and metastasis (9-12). HIF-1α has also been reported to be a marker for poor prognosis in several types of cancer, such as head and neck, uterine cervical, prostate, gastric and breast cancer (13-17). In esophageal cancer, HIF-1α was found to be significantly related to disease-free survival (DFS) and overall survival in patients with esophageal cancer treated with surgical resection (11, 18-21). However, concerning patients with esophageal cancer treated with concurrent CRT, the prognostic significance of HIF-1α expression has not fully investigated.

In addition, hypoxia has been reported to induce wild-type p53 expression by a pathway different from that of DNA-damaging agents. The hypoxic induction of *p53* selects for tumor cells lacking functional p53, and hence, displaying a diminished apoptotic potential (22). Several reports have

indicated that the combination of HIF-1α overexpression with nonfunctional p53 carries a dismal prognosis for several types of cancer (16, 23). On the other hand, the combined effects of HIF-1α and p53 have not been fully investigated in patients with esophageal cancer.

In the current study, we retrospectively assessed HIF-1 α expression semiquantitatively and investigated whether HIF-1α levels were associated with clinicopathologic parameters and clinical outcomes in patients with esophageal cancer treated with concurrent CRT. We also examined the combined effect of HIF-1α and p53 regarding the prognosis for patients with these tumors.

Patients and Methods

Patients and sample collection. Between 1997 and 2002, 37 patients with esophageal cancer were treated with concurrent CRT at the University of the Ryukyus Hospital. Of these, primary esophageal cancer specimens from pretreatment biopsies were obtained from 25 patients, and these 25 patients were the subject of this study. The disease characteristics of the 25 patients, such as tumor stage and tumor locations, were not significantly different from those of the 12 patients from whom cancer specimens were not obtained. The histopathological diagnosis of all 25 patients was squamous cell carcinoma. No patients received chemotherapy or radiotherapy prior to biopsy. Cancer specimens were obtained from the tumor edge avoiding the necrotic center. All specimens were immediately fixed in 10% buffered formalin.

Patient characteristics of all 25 patients are shown in Table I. Of the 25 patients, 13 were female, and the ages ranged from 45 to 78 years with a median age of 62 years. This study was performed according to the guidelines approved by the Institutional Review Board of our institution, with written informed consent being obtained from all 25 patients.

Concurrent CRT. External beam radiotherapy (EBRT) was administered with megavoltage equipment of photon energies of 4 MeV or more. The total doses of EBRT ranged from 40 to 66.6 Gy with a single fraction of 1.8-2 Gy administered 5 days per week. The median total dose of all 25 patients was 66.6 Gy, and 22 of 25 patients (88.0%) were treated with a total doses of 60 Gy or more. The treatment field of EBRT consisted of localized field in 4 patients (16%), and the primary tumor plus regional lymph nodes in the remaining 21 patients (84%). In most patients, computed tomography (CT)-based treatment planning and conformal radiotherapy were used. Anterior-posterior opposed fields were used up to 32.4-40 Gy, and a booster dose of 14-34.2 Gy was given, using bilateral oblique or multiple fields. The clinical target volume for the primary tumor was defined as the gross tumor volume plus 3 cm craniocaudally. The planning target volumes for the primary tumor and the metastatic lymph nodes were determined with 1- to 1.5-cm margins to compensate for setup variations and internal organ motion. Lung heterogeneity corrections were not used.

Chemotherapy was administered concurrently with radiotherapy. One course of chemotherapy consisted of cisplatin (80 mg/m^2 on day 1) and 5-fluorouracil (800 mg/m^2 on days 2-6), with 3-4 week intervals to a total of 1-2 courses. All patients received antiemetics with granisetron and metoclopramide before chemotherapy administration.

Table I. *HIF-1α expression and clinicopathological characteristics in 25 patients with esophageal cancer treated with concurrent chemoradiotherapy.*

Variable	No. of patients	HIF-1α expression		*p*-Value
		Negative	Positive	
Gender				
Male	24	14	10	0.2496
Female	1	0	1	
Age (years)				
<60	8	4	4	1.0000
≥60	14	7	7	
Clinical T stage (UICC 2002)				
T1-3	15	10	5	0.1882
T4	10	4	6	
Clinical N stage (UICC 2002)				
N0	8	6	2	0.1892
N1	17	8	9	
Clinical M stage (UICC 2002)				
M0	20	13	7	0.0698
M1	5	1	4	
KPS (%)				
100-70	23	13	10	0.8586
≤60	2	1	1	
Tumor site				
Ce or Ut	12	5	7	0.1564
Mt or Lt	13	9	4	
Total radiation dose				
<60 Gy	3	2	1	0.6915
≥60 Gy	22	12	10	
No. of chemotherapy courses				
1	7	2	5	0.0849
2	18	12	6	

UICC: International Union Against Cancer; KPS: Karnofsky performance status; Ce: cervical esophagus; Ut: upper thoracic; Mt: middle thoracic; Lt: lower thoracic.

Immunohistochemical staining for HIF-1α and p53, and evaluation of staining. Formalin-fixed, paraffin-embedded tumor sections were dewaxed in xylene and dehydrated using a series of ethanol solutions of increasing dilution. Staining for HIF-1α and p53 was then carried out using the EnVision Dual Link system-HRP kit (DAKO Co. Ltd., Tokyo, Japan). This protocol uses a 3,3'-diaminobenzidine substrate system that enables visualization of HIF-1α and p53 protein as a brown stain. For HIF-1α staining, a 1/200 (0.1 mg/ml protein) concentration of monoclonal anti-human/mouse/rat HIF-1α antibody (R&D Systems Inc., Minneapolis, MN, USA) was used. For p53 staining, a 1/50 concentration of monoclonal mouse antihuman p53 protein (DAKO Co. Ltd., Tokyo, Japan) was used. Microwave pretreatment in 10 mM citrate buffer, pH 6.0, was performed for 15 min at 500 W. For HIF-1α, an incubation overnight at 4˚C was used for the primary antibody step, whereas for p53, an incubation time of 30 min at room temperature was used for the primary antibody steps; an incubation time of 30

min at room temperature was chosen for each secondary antibody. Negative controls were prepared by omitting the primary antibodies. After staining, sections were rinsed with water, counter-stained with Gill's hemotoxylin, and coverslipped using an aqueous mountant.

Two independent pathologists blinded to the clinicopathological information performed the scoring of immunohistochemical staining. The percentage of positive tumor cells was semiquantitatively determined by assessing the whole biopsy specimen, and the mean percentage of positive tumor cells by the two pathologists was calclulated. For HIF-1α and p53, each sample was assigned to one of the following categories: (I) low (0–10% positivity); (II) high (11-100% positivity).

Statistical analysis. The median follow-up of 9 surviving patients was 57.8 months (range, 2.8-107.7 months). In the current study, the initial response of the primary tumor was evaluated according the criteria of the Japanese Society for Esophageal disease, which were based on findings from esophagograms and esophagoscopy (24). In brief, complete response (CR) was defined as the complete disappearance of tumor and no appearance of any new lesion at least 4 weeks after treatment. Partial response (PR) was defined as a >50% reduction in the product of the perpendicular diameters of tumor and no appearance of any new lesion at least 4 weeks after treatment. Progressive disease (PD) was defined as a >25% increase in the product of the perpendicular diameters of tumor or any new tumor. All other situations were defined as no change (NC). Disease recurrence was defined as recurrence/progression at the site of initial disease or the occurrence of new disease after CRT detected by computed tomography scans and/or esophagoscopy, which were taken every 3-4 months for 2 years and then twice a year. Overall survival (OS), recurrence-free survival (RFS) and local control (LC) rates were calculated actuarially according to the Kaplan–Meier method (25), and were measured from the first day of CRT. Differences between groups were estimated using the chi-square test, and the generalized Wilcoxon test (26). Multivariate analysis was performed using the Cox regression model (27). A probability level of 0.05 was chosen for statistical significance. Statistical analysis was performed with SPSS software package (version 6.1; SPSS, Inc., Chicago, IL, USA).

Results

Figure 1 shows representative examples of high (Figure 1a) and low (Figure 1b) HIF-1α expression by immunohistochemical analysis. In the current study, high HIF-1α expression was observed in 11 out of 25 (42.7%) patients, and patients were divided into low and high HIF-1α expression groups. Table I shows the clinical data and HIF-1α expression in tumor biopsies from 25 patients. None of the factors included correlated with the HIF-1α expression. Figure 2 shows representative examples of high (Figure 2a) or low (Figure 2b) p53 expression by immunohistochemical analysis. In the current study, high p53 expression was observed in 13 out of 25 (52.0%) patients, and patients were divided into low and high p53 expression groups.

Table II indicates the HIF-1α expression and the initial response in 25 patients. Eleven out of 13 patients (84.6%) in the low HIF-1α expression group had a CR, while 3 out of 12 patients (25.0%) in the high HIF-1α expression had a CR. There were significant differences between the low and high HIF-1α expression groups concerning the initial response to concurrent CRT (p=0.0027).

At the time of this analysis, 16 patients (91.0%) experienced disease recurrence (local only in 6 patients; regional lymph nodes only in 2 patients; distant metastasis, such as to bone or lung, to single sites in 4 patients, and multiple sites in 4 patients). For 16 patients with multiple recurrence, 2 patients had simultaneous local recurrences. Therefore, local recurrence occurred in 8 patients (32.0%) in total. The 5-year actuarial LC rate in all 25 patients was 61.9%. Figure 3 shows the LC curves according to the HIF-1α expression. Patients with high HIF-1α expression had a significantly poorer LC (5-year LC: 42.7%) than those with low HIF-1α expression (5-year LC: 72.5%; p=0.0322). On univariate analysis, HIF-1α expression, number of chemotherapy courses, total radiation dose and clinical M stage had significant impact on LC (Table III), and on multivariate analysis, total radiation dose and number of chemotherapy courses were independent prognostic factors for LC (Table IV). On the other hand, HIF-1α expression was not an independent prognostic factor for LC.

Sixteen out of the 25 patients (64.0%) died during the period of this analysis. Of these 16 patients, 13 patients died of esophageal carcinoma and the remaining 3 patients died without any sign of clinical recurrence (1 died of radiation pneumonitis, 1 died of penumonia and 1 died of unknown causes). The 5-year actuarial RFS rate for all 25 patients was 31.3%. Figure 4 indicates the RFS curves according to the HIF-1α expression. Patients with high HIF-1α expression had a significantly lower RFS (5-year RFS: 18.2%) compared to those with low HIF-1α expression (5-year RFS: 39.8%; p=0.0009). On univariate analysis, HIF-1α expression, number of chemotherapy courses, clinical N stage and clinical M stage had a significant impact on RFS(Table V), and on multivariate analysis, HIF-1α expression (p=0.001) and number of chemotherapy courses (p=0.010) were significant prognostic factors for RFS (Table VI).

Figure 5 indicates the RFS curves according to HIF-1α and p53 expression. The 5-year RFS in patients with high HIF-1α/high p53 expression, patients with high HIF-1α/low p53 or low HIF-1α/high p53 and patients with low HIF-1α/low p53 expression were 16.7%, 28.6% and 47.6%, respectively (Figure 5). There were significant differences regarding RFS between patients with high HIF-1 α/high p53 expression and patients with lowHIF-1α/low p53 expression (p=0.0181).

The 5-year actuarial OS rate for all 25 patients was 27.3%. There were no significant differences regarding OS for potential prognostic factors, including clinical N stage, clinical M stage, number of chemotherapy courses, HIF-1α expression and p53 expression.

Late complications of NCI-CTC Grade 4-5 were observed in two patients (4.0%). One patient suffered grade 4 pericardial

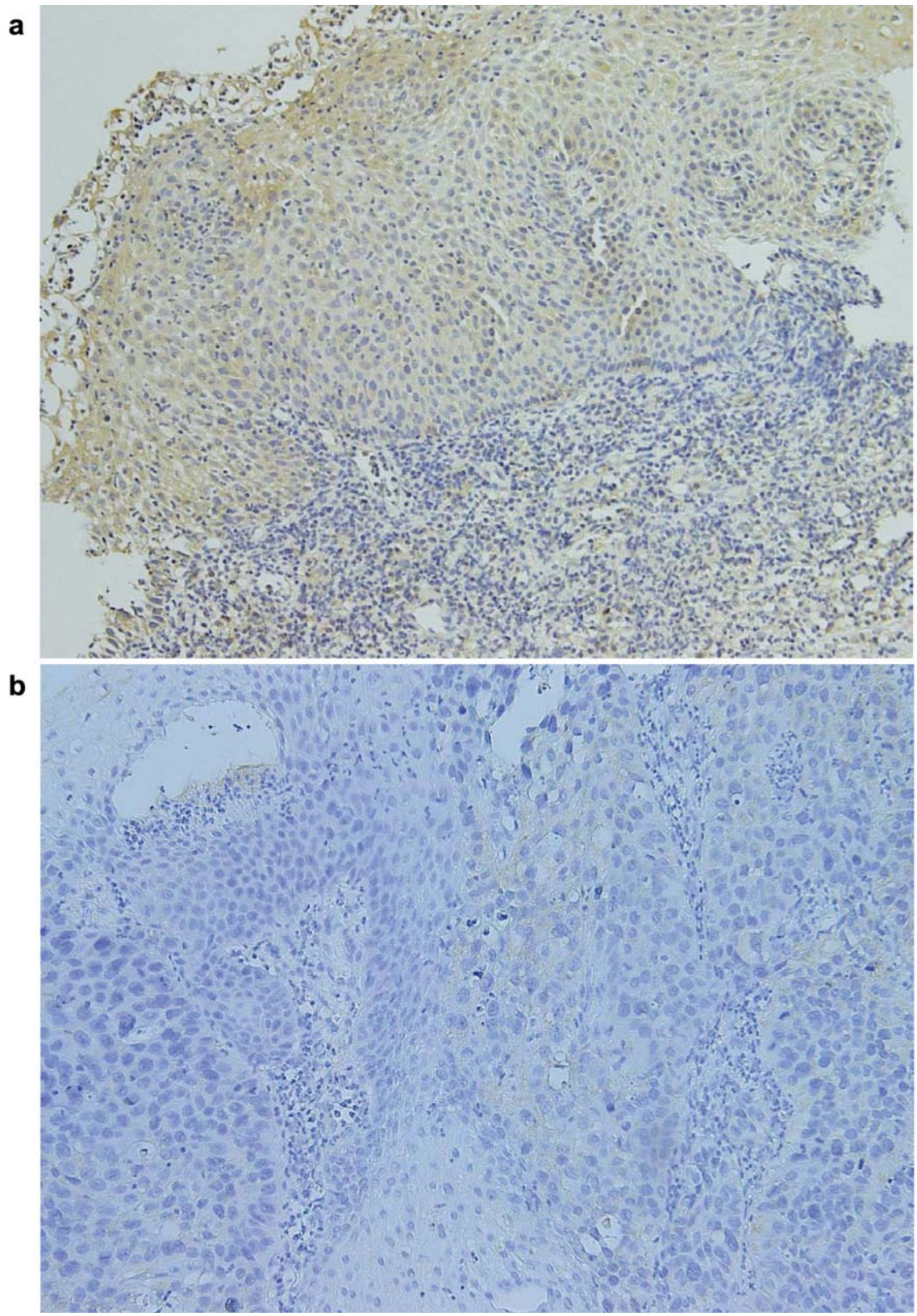

Figure 1. *Representative example of (a) high and (b) low HIF-1α expression in esophageal carcinoma. Immunohistochemical staining with anti-HIF-1α antibody in esophageal carcinoma cells.*

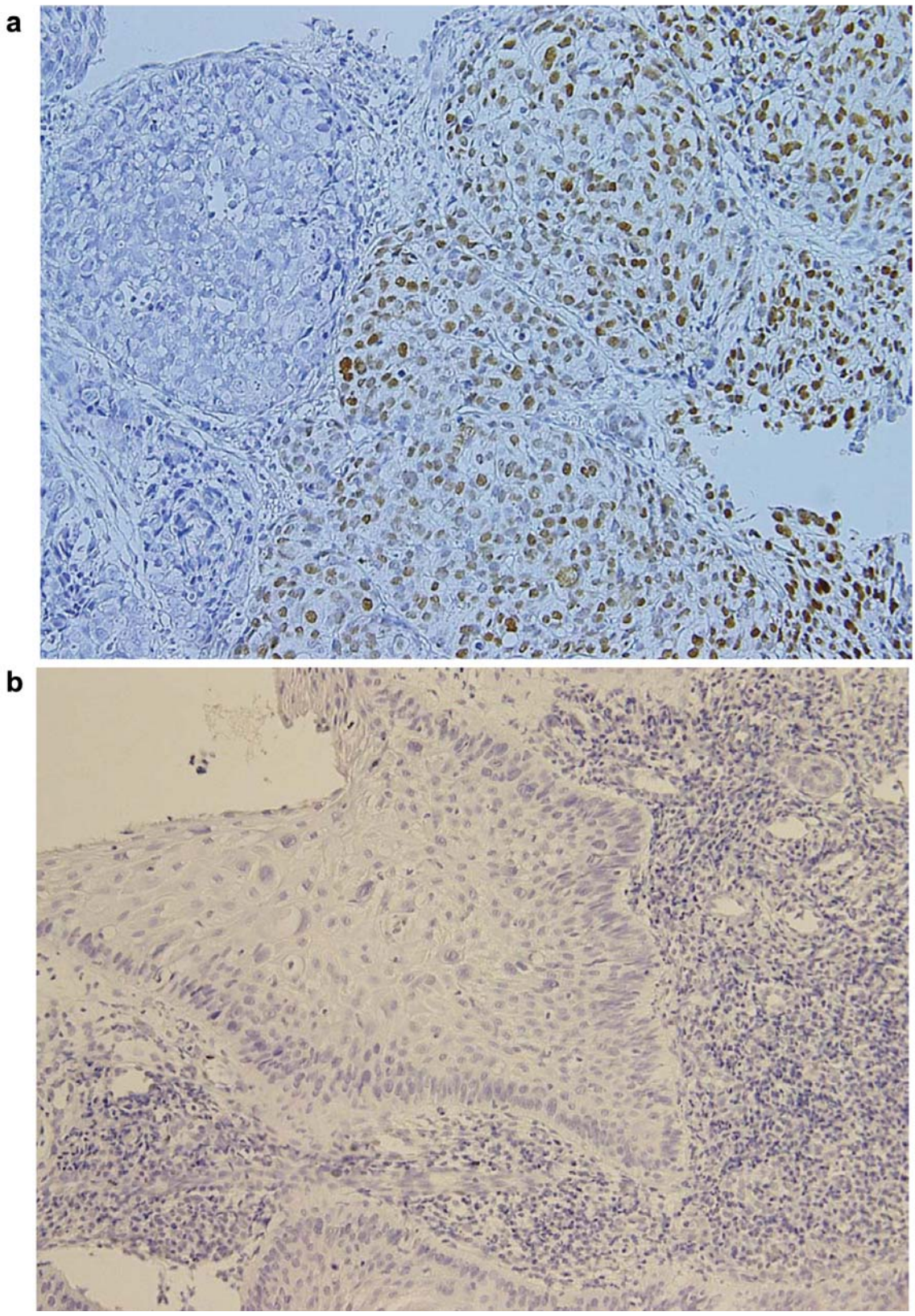

Figure 2. *Representative example of (a) high and (b) low p53 expression in esophageal carcinoma. Immunohistochemical staining with anti-p53 antibody in esophageal carcinoma cells.*

Table II. *HIF-α expression and initial response in 25 patients with esophageal cancer treated with concurrent chemoradiotherapy.*

Initial response	No. of patients	HIF-1α expression		*p*-Value
		Low	High	
CR	13	11 (84.6%)	2 (15.4%)	0.0027
PR or NC	12	3 (25.0.0%)	9 (75.0%)	

CR: Complete response; PR: partial response; NC: no change.

effusion requiring pericardial puncture 58 months after the completion of CRT. The other patient suffered grade 5 radiation pneumonitis and died of radiation pneumonitis 4 months after CRT. Both patients had been treated with a total dose of 66.6 Gy radiotherapy and two courses of chemotherapies.

Discussion

The current study indicated that HIF-1α expression was significantly correlated with initial response to concurrent CRT of patients with esophageal cancer. Eleven out of 13 patients (84.6%) in the low HIF-1α expression group had a CR, while 3 of 12 patients (25.0%) in the high HIF-1α expression group had a CR. The relationship between HIF-1α expression and reduction in response to radiotherapy and chemotherapy is explained by the fact that HIF-1α expression is a marker of cellular adaptive responses to hypoxia (6, 8). Concerning radiotherapy, the biological effect of radiotherapy has been reported to be increased approximately 3-fold when irradiation is performed under well-oxygenated conditions compared to under anoxic conditions (28). Therefore, HIF-1α expression might be a suitable marker of hypoxia, which could be measured simply and inexpensively as part of the routine histological assessment of tumors. Sohda *et al*. indicated that concerning patients with esophageal cancer treated with CRT, the initial response of patients with HIF-1α-negative tumors was significantly higher (p=0.009) than that of patients with HIF-1α-positive tumors (29). These results, together with our results, indicate that HIF-1α expression appears to be a surrogate marker for initial response to CRT in patients with esophageal cancer.

Concerning LC, patients with high HIF-1α expression tumors had a significantly poorer LC (5-year LC: 42.7%) than those with low HIF-1α expression (5-year LC: 72.5%; p=0.0322), however, HIF-1α expression was not an independent prognostic factor for LC. Regarding head and neck cancer, several reports have indicated that HIF-1α expression was a significant prognostic factor for LC (13, 30). Further studies are required to investigate the significance of HIF-1α on LC in patients with esophageal cancer treated with CRT.

Table III. *Univariate analysis of various potential prognostic factors for local control (LC) in patients with esophageal cancer treated with concurrent chemoradiotherapy.*

	Univariate analysis		
	No. of patients	LC 5-year rate	*p*-Value
Gender			
Male	24	59.7%	0.2087
Female	1	100.0%	
Age (years)			
<60	8	62.5%	0.3847
≥60	14	59.6%	
Clinical T stage (UICC 2002)			
T1-3	15	63.3%	0.2851
T4	10	60.0%	
Clinical N stage (UICC 2002)			
N0	8	68.6%	0.2048
N1	17	62.3%	
Clinical M stage (UICC 2002)			
M0	20	68.1%	0.0067
M1	5	40.0%	
KPS (%)			
100-70	23	62.3%	0.1776
≤60	2	50.0%	
Tumor site			
Ce or Ut	12	71.4%	0.8294
Mt or Lt	13	59.4%	
Total radiation dose			
<60 Gy	3	33.3%	0.0058
≥60 Gy	22	66.1%	
No. of chemotherapy courses			
1	7	19.5%	0.0012
2	18	78.7%	
HIF-1α expression			
Low	14	72.5%	0.0322
High	11	42.7%	
p53 expression			
Low	12	91.7%	0.0624
High	13	39.1%	

UICC: International Union Against Cancer; KPS: Karnofsky performance status; Ce: cervical; Ut: upper thoracic; Mt: middle thoracic; Lt: lower thoracic.

The current study also indicated that patients with high HIF-1α expression had significantly lower RFS (5-year RFS: 18.2%) compared to those with low HIF-1α expression (5-year RFS: 39.8%; p=0.0009), and on multivariate analysis, HIF-1α and number of chemotherapy courses were independent prognostic factors for RFS. To our knowledge, this is the first report to indicate the clinical significance of HIF-1α expression on survival in patients with esophageal cancer undergoing concurrent CRT. Regarding patients with esophageal cancer undergoing surgical resection, previous reports have indicated that HIF-1α overexpression is a significant risk factor for death (11, 18-21). Matsuyama *et al*.

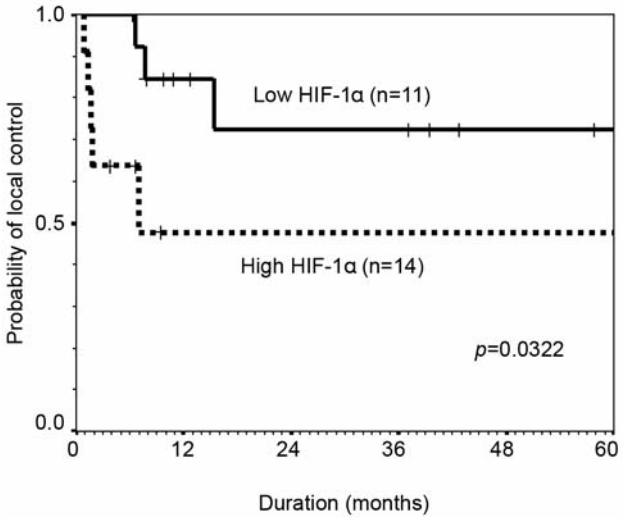

Figure 3. *Local control (LC) curves according to the HIF-1α expression in esophageal carcinoma patients treated with concurrent chemoradiotherapy.*

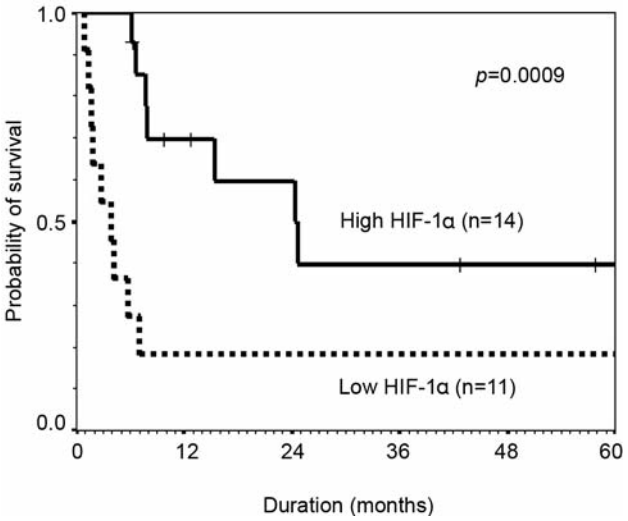

Figure 4. *Recurrence-free survival curves according to the HIF-1α expression in esophageal carcinoma patients treated with concurrent chemoradiotherapy.*

Table IV. *Multivariate analysis of various potential prognostic factors for local control (LC) in patients with esophageal cancer treated with concurrent chemoradiotherapy.*

	Multivariate analysis	
	RR (95% CI)	*p*-Value
HIF-1α expression (Low *vs*. high)	0.320 (0.061-1.678)	0.178
No. of chemotherapy courses (1 *vs*. 2)	12.816 (2.136-76.888)	0.005
Clinical M stage (M0 *vs*. M1)	8.507 (1.098-65.940)	0.919
Total radiation dose (<60 Gy *vs*. ≥60 Gy)	0.683 (0.000-3409.927)	0.040

RR: Relative ratio; CI: confidence intervals.

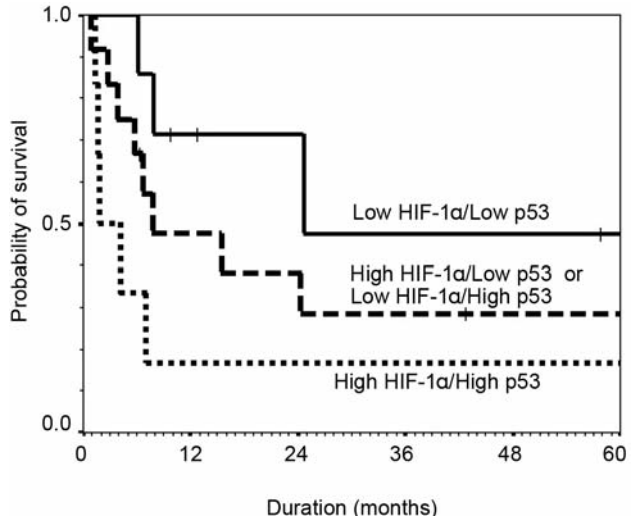

Figure 5. *Recurrence-free survival curves according to the HIF-1α and p53 expression in esophageal carcinoma patients treated with concurrent chemoradiotherapy.*

investigated 215 patients with esophageal cancer treated with surgical resection and found that HIF-1α had a significant effect on DFS (20). Kimura *et al*. indicated that the survival rate of patients with high HIF-1α expression was significantly worse than that of patients with low HIF-1α expression (11). Ogane *et al*. analyzed 96 surgically resected patients with esophageal cancer and found that HIF-1α was significantly related to DFS and OS (18). These results indicate that HIF-1α expression is predictive of clinical outcome for patients with esophageal cancer undergoing concurrent CRT, as well as for patients undergoing surgical resection.

HIF-1α has been shown to interact with the tumor suppressor protein p53, and the overexpression of HIF-1α and p53 has been shown in a variety of human cancer types using immunohistochemistry (16, 31, 32). Sumiyoshi *et al*. indicated that when patients with gastric cancer were stratified for HIF-1α and p53 positivity, the patients who were p53-positive and HIF-1α-positive had the worst prognosis (16). Theodoropoulos *et al*. found that the combination of HIF-1α and p53 overexpression was a significant factor for progression-free survival in superficial urothelial cancer (32). However, the interaction of HIF-1α and p53 has not been fully investigated in esophageal cancer. In the current study, we analyzed the combined

Table V. *Univariate analysis of various potential prognostic factors for recurrence-free survival (RFS) in patients with esophageal cancer treated with concurrent chemoradiotherapy.*

	Univariate analysis		
	No. of patients	RFS, 5-year rate	*p*-Value
Gender			
Male	24	27.9%	0.3125
Female	1	100.0%	
Age (years)			
<60	8	25.0%	0.3782
≥60	14	34.2%	
Clinical T stage (UICC 2002)			
T1-3	15	25.9%	0.5743
T4	10	40.0%	
Clinical N stage (UICC 2002)			
N0	8	68.6%	0.0084
N1	17	14.7%	
Clinical M stage (UICC 2002)			
M0	20	39.1%	< 0.0001
M1	5	0.0%	
KPS (%)			
100-70	23	31.6%	0.4884
≤60	2	50.0%	
Tumor site			
Ce or Ut	12	35.9%	0.5099
Mt or Lt	13	25.9%	
Total radiation dose			
<60 Gy	3	33.3%	0.0539
≥60 Gy	22	32.4%	
No. of chemotherapy courses			
1	7	0.0%	0.0004
2	18	43.8%	
HIF-1α expression			
Low	14	39.8%	0.0009
High	11	18.2%	
p53 expression			
Low	12	37.5%	0.7328
High	13	26.0%	

UICC: International Union Against Cancer; KPS: Karnofsky performance status; Ce: cervical; Ut: upper thoracic; Mt: middle thoracic; Lt: lower thoracic.

Table VI. *Multivariate analysis of various potential prognostic factors for recurrence-free survival in patients with esophageal cancer treated with concurrent chemoradiotherapy.*

	Multivariate analysis	
	RR (95% CI)	*p*-Value
HIF-1α (Low *vs.* high)	0.071 (0.015-0.335)	0.001
No. of chemotherapy courses (1 *vs.* 2)	5.472 (1.496-20.014)	0.010
Clinical M stage (M0 *vs.* M1)	0.382 (0.057-2.555)	0.321
Total radiation dose (< 60 Gy *vs.* > 60 Gy)	5.165 (0.768-34.759)	0.091
Clinical N stage (N0 *vs.* N1)	3.331 (0.9788-11.352)	0.054

RR: Relative ratio; CI: confidence intervals.

effect of HIF-1α and p53 expression for prognosis in patients with esophageal cancer. Our results indicated that there were significant differences regarding RFS between patients with high HIF-1α/high p53 expression and patients with low HIF-1α/low p53 expression (p=0.0181). Moreover, stratification by combined HIF-1α and p53 expression (Figure 5) appears to provide a more detailed prediction of prognosis compared to stratification by HIF-1α alone (Figure 4). These results suggest that the use of combination of HIF-1α and p53 expression may be useful in predicting the survival of these patients. However, in the current study, the number of patients analyzed was small, and p53 alone was not a significant prognostic factor for survival. Therefore, further studies are required to confirm the combined effect of HIF-1α and p53 with a larger number of patients.

In conclusion, our results indicated that HIF-1α expression is significantly correlated with initial response to concurrent CRT in patients with esophageal cancer. Our results also indicated that HIF-1α is predictive of RFS. These findings suggest a possible role for HIF-1α as a new prognostic biomarker for patients with esophageal cancer undergoing CRT, and would allow selection of patients most likely to benefit from more intensive treatments. Furthermore, understanding the biological function of HIF-1α may allow response modification by targeting of specific pathways. In combination with p53 expression, more detailed prediction of prognosis may be possible for these patients.

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