SPANDIDOS PUBLICATIONS

Reduced expression of p63 has prognostic implications for patients with esophageal squamous cell carcinoma

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Received August 17, 2005; Accepted October 24, 2005

Abstract. The p63 gene is a member of the p53 family that plays a role in cell differentiation, development and carcinogenesis. The relationship between p63 expression and the prognosis of esophageal squamous cell carcinoma (ESCC) remains unknown. The present study examines the clinical impact of p63 in patients with ESCC. Resected specimens from 180 patients with ESCC were immunostained for p63 and p53. After establishing a cut-off value for p63 expression, we statistically examined its clinical impact and relationship to p53 expression. At a 50% cut-off value for p63 expression, the 5-year overall survival was significantly longer in p63-positive (46.4%) than -negative patients (11.1%, p=0.05). Among the 180 ESCC patients, 171 (95.0%) were p63 immunoreactive and only 9 (5.0%) were negative. The correlation between p63 status and clinicopathological parameters was not significant, although p63-negativity tended to correlate with distant metastasis (p=0.06) and clinical stage (p=0.08). Univariate analysis demonstrated significant correlations between patient survival and tumor diameter, depth of invasion, lymphatic invasion, vascular invasion, lymph node metastasis and distant metastasis. The survival of patients who did not express p63 and p53 was obviously unfavorable (p=0.03). Multivariate analysis revealed that only lymph node metastasis was a critical independent prognostic marker for overall survival (p=0.0015). Expression of p63 was not an independent prognostic factor for overall survival in this study (p=0.69). These data suggest that, although a reduced expression of p63 is infrequent, it has a prognostic impact upon patients with ESCC.

Introduction

Esophageal carcinoma is a widespread malignancy with a high mortality rate, especially among males and in less developed areas of the world (1). However, the underlying molecular mechanisms that determine the biological behavior of esophageal carcinoma remain obscure despite considerable efforts at clarification.

The p63 gene maps to 3q27-29 and it is a p53 tumor suppressor (2). The gene contains 15 exons, and encodes six isotypes (TAp63, ß, γ, ΔNp63α, ß, γ) that share significant structural homology with p53 at the protein level, so they are thought to induce G1 cell cycle arrest or apoptosis (3-5). In addition to the nasopharynges (6), oral cavity (7), cervix (8), bladder (9), lung (10,11) and skin (12), p63 plays an important role in carcinogenesis.

Several investigators have reported that increased expression of p63 is an early event in esophageal squamous cell carcinomas (ESCC) that might play an important role in the development of the disease (13,14). On the other hand, the clinical impact of p63 expression has not been reported. The present study investigates p63 expression in ESCC using immunohistochemistry and examines the prognostic impact. We also examined the relationship between p63 and p53 expression in ESCC using p53 immunohistochemistry.

Patients and methods

Patients. We obtained specimens from 180 patients with ESCC (154 males and 26 females, mean 64.9 years, age range 36-84) who had undergone resection of the esophagus with lymph node dissection at the Department of Oncological Science (Surgery II) of the Oita University, Faculty of Medicine between January 1990 and December 2000. None of the patients received preoperative adjuvant therapy, and none had macroscopic residual cancer except distant metastasis.

Resected specimens were classified based on positive or negative vessel invasion according to the TNM system of the UICC.

Immunohistochemical staining. Resected specimens fixed in 10% buffered formalin for 24 h and embedded in paraffin were sectioned (4 μm thick) and placed on silane-coated slides. The sections were deparaffinized and rehydrated, then endogenous peroxidase activity was blocked by incubation in 3% hydrogen peroxide for 20 min. The sections were autoclaved at 121˚C in citrate buffer (10 mM, pH 6.0) for 10 min to retrieve antigen, cooled at room temperature for 30 min, and then incubated with normal goat serum for 15 min at room

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Key words: p63, p53, esophageal squamous cell carcinoma

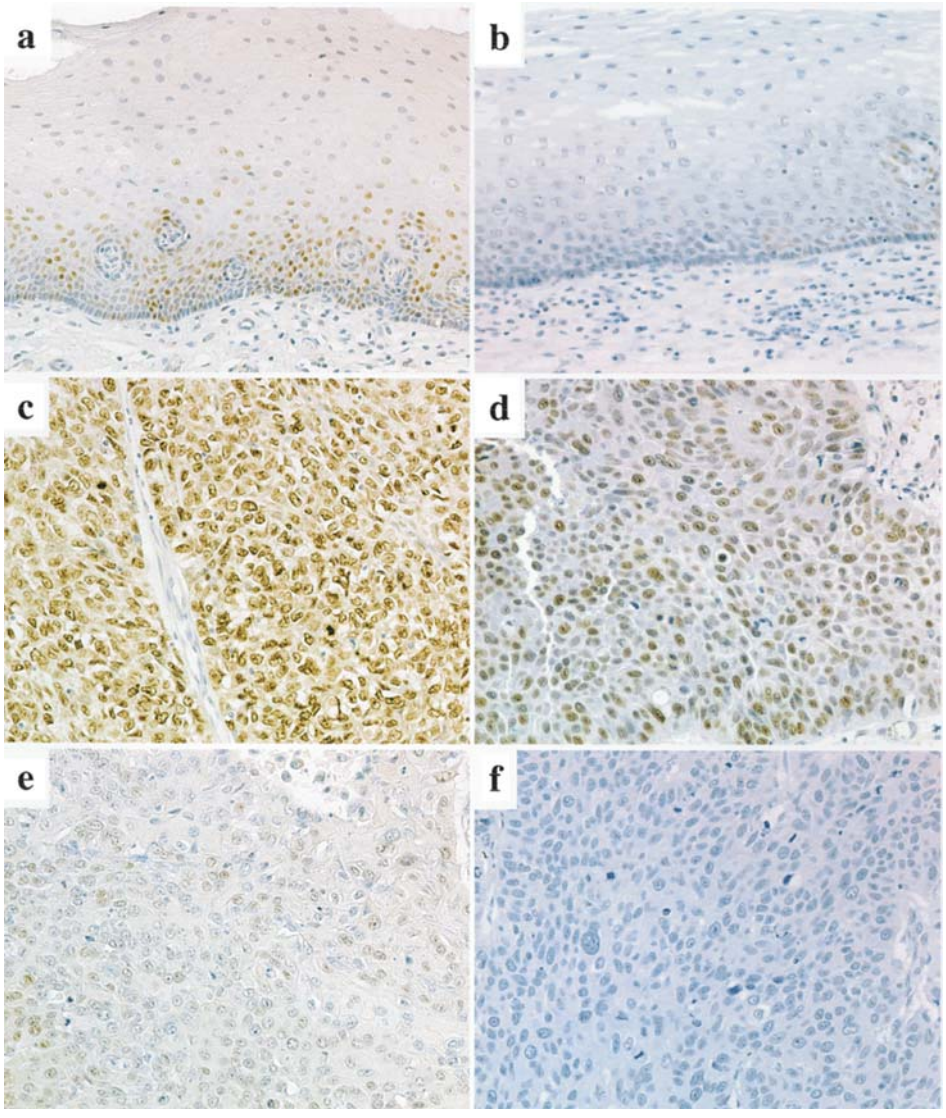

Figure 1. Immunohistochemistry to detect p63 (a, c, e) and p53 (b, d, f) (original magnification, x200; counterstained with hematoxylin). In normal squamous epithelium, p63 (a) and p53 (b) were localized in basal and suprabasal layers. Nuclei of ESCC tumor cells were intensely immuno-reactive for p63 (c) and p53 (d) in samples that were immunopositive for both proteins. On the contrary, tumor cells from samples that were immuno-negative for p63 (e) and p53 (f) were rarely immunoreactive.

temperature followed by anti-p63 or anti-p53 monoclonal antibody (p63; 1:50, p53; 1:80, Dako, Glostrup, Denmark) for 12 h at 4˚C. Standard immunohistochemical staining proceeded using the avidin-biotin-peroxidase complex with a Histofine SAB-PO (M) kit (Nichirei, Tokyo, Japan). The chromogen was 3, 3'-diaminobenzidine and nuclei were counterstained with hematoxylin.

At least 500 neoplastic cells per specimen were immunohistochemically evaluated at x400 magnification, and the ratio (%) of p63 immunoreactive cells was recorded. To establish an adequate cut-off value, we considered 50% to 80% immunoreactive cells as evidence of p63 positivity, and evaluated the survival after surgery for each value using the Kaplan-Meier method. We also evaluated the p53 immunoreactivity in each specimen, and a ratio of immunoreactive neoplastic cells >10% was defined as p53 positivity as described (15,16). Two independent observers who were blinded to the clinical information evaluated the immunohistochemical staining.

Clinicopathological parameters. We examined patient age and gender, tumor diameter, histological grade (differentiation) and TNM classification, degree of lymphatic or blood vessel invasion present and p53 expression. Patients were divided according to a median age of 65 years (older or younger) and a median tumor diameter of 47 mm (larger or smaller).

Statistical methods. After establishing the cut-off value for p63 immunohistochemistry, we analyzed the correlation between p63 expression and clinicopathological factors using the Mann-Whitney U test, Fisher's exact probability test or the χ^2 test. Overall survival was calculated according to the Kaplan-Meier method from the time of surgery until either death or the date of the last follow-up. In addition to the evaluation of survival using clinicopathological parameters, we compared survival with the immunohistochemical expression of both p63 and p53.

The differences between individual study variables and outcome were examined using the log-rank test. Variables

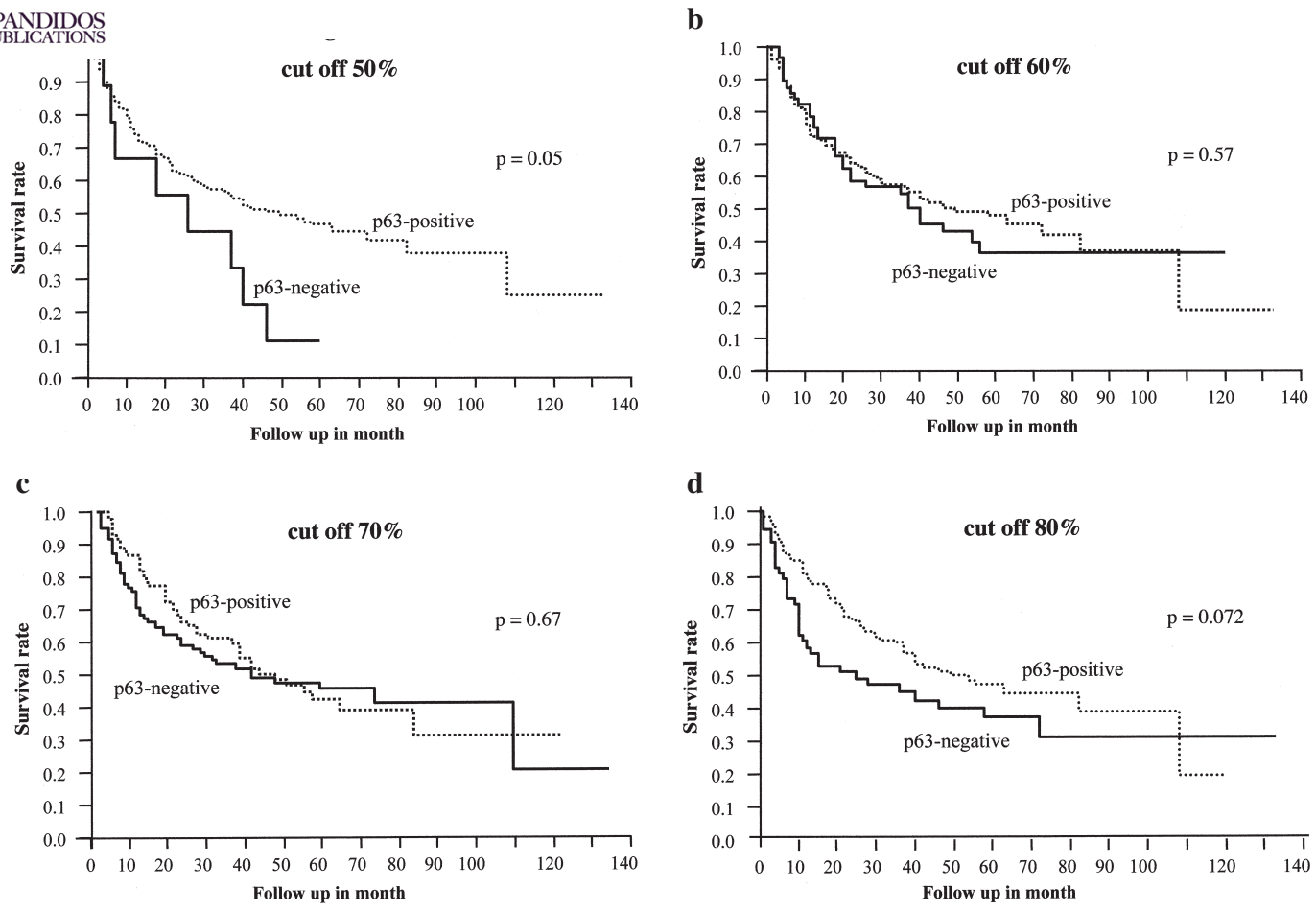

Figure 2. Kaplan-Meier survival curves of patients with ESCC according to p63 cut-off values of 50% (a), 60% (b), 70% (c) and 80% (d). Only 50% (a) exhibited prognostic implication (p=0.05).

that achieved statistical significance at p≤0.20 in univariate analyses were placed into multivariate models. The Cox proportional hazards model then analyzed interrelationships between variables and their relative impact on outcome. A p-value of <0.05 was considered statistically significant.

Results

Immunohistochemical staining. Immunoreactivity for p63 was intense in the nuclei of esophageal squamous mucosa and positive in all basal and suprabasal layer cells of the normal esophageal squamous epithelium (Fig. 1a). In positive samples of squamous cell carcinoma, p63 immunoreactivity extended to the superficial area of the esophageal mucosa. Similarly, p63 was obviously diffused throughout the tumor cells of invasive squamous cell carcinoma (Fig. 1c). On the contrary, negative specimens contained few p63-immunoreaction cells among the tumor cells (Fig. 1e). The nucleus or cytoplasm of esophageal squamous mucosa was immunoreactive for p53. In normal squamous epithelium, p53 expression was focal to patchy and confined to basal and/or parabasal layer cells (Fig. 1b). The nuclei of cancer cells at the periphery of positive tumors expressed high levels of p53 (Fig. 1d). On the other hand, negative tumors expressed very low levels of p53 that was limited to the tumor cells (Fig. 1f).

Relationship between survival and p63 expression. Univariate analyses of p63 expression with several cut-off values revealed a significant correlation only at a cut-off value of 50% (Fig. 2). At this value, the overall survival was significantly longer in p63-positive than in p63-negative patients (46.4% vs. 11.1%, p=0.05; Fig. 2a). We therefore adopted 50% as the cut-off value for p63 positivity.

Of the 180 ESCC patients, 171 (95.0%) were intensely p63-positive and 9 (5.0%) were -negative. On the other hand, 111 (61.7%) and 69 (38.3%) patients were immunopositive and negative, respectively for p53. Furthermore, 4 of the 9 patients (2.2%) who were p63-negative were also p53-negative (Table I).

Correlation with clinicopathological parameters. Table I shows the clinicopathological impact of p63 expression. None of the tested clinicopathological parameters significantly correlated with p63 status although p63-negativity tended to correlate with distant metastasis (p=0.06) and clinical stage (p=0.08).

The correlation between the expression of p63 and p53 was also not significant (p=0.76).

Survival analysis. Univariate analysis demonstrated significant correlations between patient survival and tumor diameter

Table I. Immunohistochemical status of p63 in patients with esophageal squamous cell carcinoma.

Factor investigated	p63		
	Positive (%)	Negative (%)	P-value
Patient age (years)			
<Median (65)	77 (42.8)	4 (2.2)	0.97
>Median (65)	94 (52.2)	5 (2.8)	
Diameter (mm)			
<Median (47)	109 (61.6)	4 (2.3)	0.22
>Median (47)	59 (33.3)	5 (2.8)	
Histological grade of squamous cell carcinoma			
Well differentiated	42 (23.3)	1 (0.6)	0.60
Moderately differentiated	77 (42.8)	5 (2.8)	
Poorly differentiated	52 (28.9)	3 (1.7)	
Depth of invasion			
Tis	3 (1.7)	0 (0.0)	0.76
Tl	78 (43.8)	3 (1.7)	
T2	21 (11.8)	2 (1.1)	
T3	68 (38.2)	3 (1.7)	
Lymphatic invasion			
Positive	76 (49.7)	7 (4.6)	0.13
Negative	68 (44.4)	2 (1.3)	
Vascular invasion			
Positive	38 (23.8)	1 (0.62)	0.30
Negative	113 (70.6)	8 (5.0)	
Lymph node metastasis			
Positive	85 (47.2)	5 (2.8)	0.73
Negative	86 (47.8)	4 (2.2)	
Distant metastasis			
Positive	1 (0.6)	1 (0.6)	0.06
Negative	170 (94.4)	8 (4.4)	
Stage			
0	26 (14.5)	0 (0.0)	0.08
1	39 (21.8)	1 (0.6)	
2	36 (20.1)	5 (2.8)	
3	48 (26.8)	1 (0.6)	
4	21 (11.7)	2 (1.1)	
p53 expression			
Positive	106 (58.9)	5 (2.8)	0.76
Negative	65 (36.1)	4 (2.2)	

Table II. Univariate analysis of prognostic factors.

Factor investigated	No. of patients	5-year survival (%)	Univariate analysis P-value (log-rank test)
Patient age (years)			
<Median (65)	81	46.1	0.257
>Median (65)	99	42.7	
Diameter (mm)			
<Median (47)	114	50.2	0.002
>Median (47)	64	31.7	
Histological grade of squamous cell carcinoma			
Well differentiated	43	50.9	0.272
Moderately or poorly differentiated	137	41.8	
Depth of invasion			
Tl, T2	107	57.7	<0.001
T3, T4	73	24.3	
Lymphatic invasion			
Positive	83	31.5	0.006
Negative	70	62.7	
Vascular invasion			
Positive	40	22.3	0.0132
Negative	122	51.0	
Lymph node metastasis			
Positive	90	23.0	<0.001
Negative	90	65.2	
Distant metastasis			
Positive	2	0	0.040
Negative	178	44.7	
IHC for p63			
Positive	171	46.4	0.050
Negative	9	11.1	
IHC for p53			
Positive	112	46.4	0.936
Negative	68	11.1	

IHC, immunohistochemistry.

(p=0.002), depth of invasion (p<0.001), lymphatic invasion (p=0.006), vascular invasion (p=0.0132), lymph node metastasis (p<0.001) and distant metastasis (p=0.04) (Table II).

The survival of patients who expressed neither p63 nor p53 was poor (Fig. 3a, log-rank test, p=0.03). Among p53-positive patients, p63-negativity was not significant according to the survival analysis (Fig. 3b, log-rank test, p=0.43). On the other hand, the survival of patients who were negative for both p63 and p53 was worse (0%) than that of patients who were p63-positive and p53-negative (log-rank test: 45.2%, p=0.03; Fig. 3c).

Multivariate analysis. Factors with statistical significance in the univariate analysis (p<0.2) were entered into a multivariate analysis (Table III). This procedure revealed that only lymph node metastasis was an important independent prognostic marker for overall survival (odds ratio, 0.63; 95% CI, 0.472-0.841; p=0.0015). The expression of p63 (odds ratio, 1.13; 95% CI, 0.555-1.891; p=0.69) and the absence of both p63 and p53 expression (odds ratio, 1.57; 95% CI; 0.720-3.606;

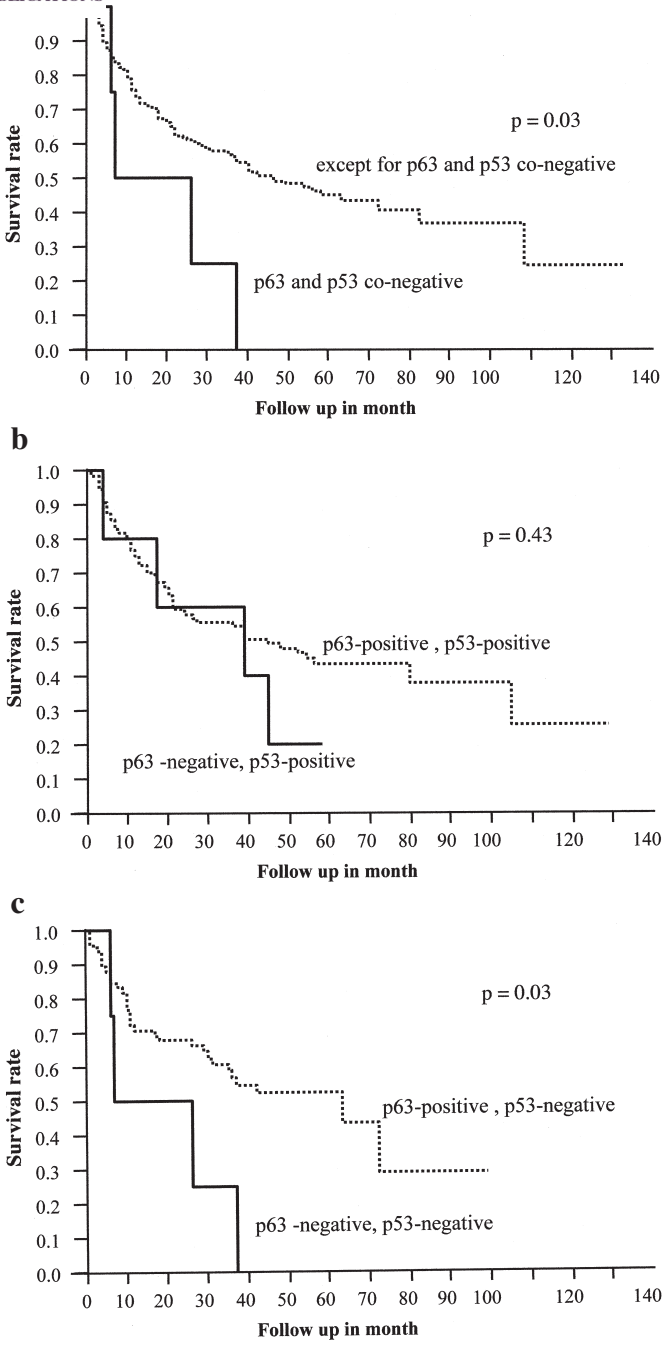

Figure 3. Kaplan-Meier survival curves of patients with ESCC according to immunohistochemical expression of p63 and p53. (a) Prognosis of patients who were negative for both p63 and p53 was poorer than that of other patients (p=0.03). (b) Among p53-positive patients, p63-negativity was not significant in survival analyses (p=0.43). (c) Among p53-negative patients, p63-negativity was associated with a significantly poor prognosis (p=0.03).

p=0.25) were not independent prognostic factors for overall survival.

Discussion

All basal and suprabasal layer cells of the normal esophageal squamous epithelium and almost all ESCC cells are p63 immunoreactive (13,14,17). At a 50% cut-off value of p63 immunoreactivity determined using survival analysis, 171 of 180 patients (95%) were positive for p63. This cut-off value is easy to count and should support further p63 studies. Our data showed that almost all squamous epithelial and cancer cells express p63. Studies with p63 knockout mice revealed that p63 plays a role in the differentiation or development of squamous epithelium (18,19). Park *et al* (20) reported that reduced TAp63 expression in invasive bladder cancer is associated with tumor stage and grade. Urist *et al* (21) concluded that a loss of p63 expression in an invasive bladder cancer cell line contributes to a loss of differentiation or development of cancer cells. In this study, only 9 of 180 patients (5%) were p63-negative. Furthermore, their prognoses were poor and tended to correlate with distant metastasis (p=0.06) and clinical stage (p=0.08). These data suggested that p63 is involved in the differentiation or development of ESCC.

The function of p63, which is a member of the p53 gene family in carcinogenesis, is similar to that of p53 and affects cell cycle arrest and apoptosis (6,7,12,22-26). Pruneri *et al* (27) reported that p63 immunoreactivity is not associated with p53 gene mutations and p53 immunoreactivity. Shimada *et al* (28) revealed that a cellular signal on p63 cross-talks, although not completely, with that of the p53 pathway. We found no correlation between the expression of p63 and of p53, and the prognosis of patients who were immunonegative for both factors was obviously poor. These results suggest that patients with ESCC accompanied by reduced expression of both p63 and p53 have biologically malignant potential and a poor prognosis. Furthermore, p63 might function in cell cycle arrest and apoptosis using a pathway different to that of p53. Further study is required to examine these notions.

In conclusion, we demonstrated that reduced expression of p63 is infrequent but it has a prognostic impact for patients with ESCC. The absence of p63 expression might represent a poor prognosis and reflect biologically malignant potential.

Table III. Multivariate analysis of prognostic factors.

Variable	P-value	Ratio of risk	95% CI
Diameter	0.26	0.86	0.660-1.125
Depth of invasion	0.28	0.84	0.611-1.153
Lymphatic invasion	0.90	0.98	0.725-1.320
Vascular invasion	0.46	0.89	0.641-1.227
Lymph node metastasis	0.0015	0.63	0.472-0.841
Distant metastasis	0.21	0.42	0.140-1.939
p63-negative	0.69	1.13	0.555-1.891
p63 and p53 co-negative	0.25	1.57	0.720-3.606

CI, confidence interval.

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