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Endothelin protein expression as a significant prognostic factor in oesophageal squamous cell carcinoma

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Abstract

The aim of this study was to investigate if the expression of endothelin (ET), a vasoactive peptide, in cancerous oesophageal lesions, adjacent dysplastic tissue and normal mucosa might be prognostic. Tissue samples from a total of 101 patients with oesophageal squamous cell carcinoma were obtained and stained with ET antibody in an immunohistochemical analysis. High staining levels of ET within normal mucosa were related to lymph vessel invasion, regional lymph node metastasis and distant metastasis, as well as a reduced relapse-free survival (log-rank test; $P = 0.0066$). After adjustment for several histological prognostic risk factors and each component of the TNM classification system, high ET expression within dysplastic tissue more than doubled the hazard ratio of relapse with significant model improvement. These results suggest that, in addition to known histological risk factors and TNM classification criteria, measurement of ET expression with a simple immunohistochemical analysis might further help in predicting the prognosis of patients with oesophageal squamous cell carcinoma.

Keywords: Keywords; Endothelin; Angiogenesis; Oesophagus; Cancer; Prognosis; Dysplasia

1. Introduction

Oesophageal squamous cell carcinoma is an aggressive cancer and carries a poor prognosis. Five-year survival rates range from 20 to 36%, even with chemoradiotherapy [1,2]. In addition to the TNM classification criteria, further sub-classification of the primary tumour and an intensive examination of lymph node metastasis with monoclonal antibodies have been shown to improve the accuracy of staging and prediction of prognosis [3–5]. Moreover, combined analysis of BAX and $p16^{INK4A}$ expression might be another way to identify tumours which carry a poor prognosis [6]. However, at present, the TNM classification system is the only internationally accepted method for determining prognosis [7].

Angiogenesis is essential for tumour growth, invasion and metastasis. The mechanism by which angiogenesis is triggered remains to be elucidated, but endothelial cells are somehow recruited to form new blood vessels for tumour growth. Endothelin (ET) [8] was discovered as a vasoactive peptide essential for the development of cardiovascular disease, as well as glomerulonephritis in transgenic mice [9,10]. Furthermore, it has been shown to play a multifunctional role in a variety of tissues and cells [11]. Recent studies suggest that ET may be involved in the pathophysiology of a variety of neoplasms [12].

In this study, we retrospectively investigated the significance of ET protein expression by immunohistochemical staining and found that high ET protein expression reduced relapse-free survival in patients with oesophageal squamous cell carcinoma, independent of other histological and clinical prognostic factors.

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2. Patients and methods

2.1. Patients and histological specimens

Between February 1994 and December 2001, 117 patients with oesophageal tumours were listed on file in the Department of Pathology, Clinical Service, Jikei University Hospital. Their clinical information was abstracted from their surgical and clinical charts. Some patients received radiotherapy to improve their quality of life. Tumour stages were classified according to the fifth edition of the TNM classification system of the International Union against Cancer [7]. Patients were periodically (every 1–3 months) examined on an outpatient basis to make sure that they had not relapsed using standard investigations, including endoscopy, and computed tomography (CT) of the chest and abdomen.

Specimens were obtained by endoscopic mucosal resection or surgery. Formalin-fixed, paraffin-embedded specimens of oesophageal tumours were retrieved from the Department of Pathology in the Jikei University Hospital and processed for conventional histological assessment by haematoxylin and eosin (HE) staining. Only patients with squamous cell carcinoma of the oesophagus, confirmed by two or more board pathologists, were included. Patients with all other forms of carcinoma, including partly adenomatous lesions, were excluded. All specimens were free of cancer invasion of the tumour margin and cases of carcinoma *in situ* were excluded. Histological features of the extent of the lesions, invasion into lymphatic or blood vessels, intramural metastasis, and lymph node metastasis, were evaluated. Pathological diagnosis and classification were made according to the Guidelines for the Clinical and Pathologic Studies on Carcinoma of the Esophagus (Japanese Society of Esophageal Disease, 1999). Lymphatic vessel invasion was regarded as definite when cancer cells were detected in thin-walled endothelium-lined spaces containing no red blood cells and occasional lymph fluid [13]. Blood vessel invasion was defined by the presence of cancer cells and red blood cells within round or ovoid endothelium-lined spaces surrounded by a layer of smooth muscle [13–15]. Intramural metastasis was defined as evidence of tumour within the oesophageal wall, not directly related to the primary tumour [16,17]. Histological grades of differentiation were assigned, according to whether a tumour was 'well', 'moderately' or 'poorly' differentiated [18].

2.2. Immunohistochemical staining for endothelin

ET protein was detected using anti-ET rabbit polyclonal antibody (PC266) (Oncogene Research Products, San Diego, CA, USA) that reacts to ET-1, ET-2 and ET-3, using a dextran polymer conjugate two-step visualisation system [19]. Staining levels were defined as follows: level 0, no staining; level 1, partial staining; level 2, strong staining in less than half of the cells examined; level 3, strong staining in more than half of the cells examined; level 4, strong staining in almost all of the cells examined. The examiners were blinded to patients' clinical and histological (HE staining) information when assigning staining levels. Two investigators evaluated the staining levels independently, after which discordant evaluations were adjusted by connected microscopes.

2.3. Statistics

Chi-squared tests were used to evaluate the relationship between ET staining levels and several clinicohistological parameters. Survival curves of the patients were compared by the Kaplan–Meier method and analysis was done by the log-rank test. Cox proportional hazards models were fitted for the multivariate analysis.

3. Results

3.1. Patient characteristics

From the list of 117 patients, paraffin-embedded specimens were unavailable for 4. 6 patients displayed adenocarcinoma and another 6 had carcinoma *in situ*. Thus, 16 patients were excluded from this study. 101 patients, for whom specimens and clinical information could be obtained, were eligible for this study. 3 patients were lost to follow-up and counted as censored events on the final day of the outpatient clinic.

Patients ranged from 43 to 83 years old (mean $\pm$standard deviation (S.D.): 61 ± 9 years) and more males ($n = 69$) than females ($n = 32$) were enrolled in this study. Patients were followed from day 16 onwards for a maximum of 2744 days after surgery (median: 784 days). 44 patients died from oesophageal carcinoma and 6 died of other causes and were counted as censored events. Thus, 51 patients were alive, and 50 had died or been lost to follow-up, by the end of the follow-up period.

3.2. ET protein expression in patients with oesophageal lesions

Typical histological pictures of ET staining of normal, dysplastic and cancerous tissue are shown in Fig. 1a–c, respectively. Cancerous cells around small vessels occasionally expressed ET (Fig. 1d). Since ET protein-staining patterns were not always concordant among the normal mucosa (level $\geqslant 1$), dysplastic tissue (level $\geqslant 2$) and cancerous lesions (level $\geqslant 1$) of each patient (Fig. 2), staining levels of these three kinds of tissues were judged separately.

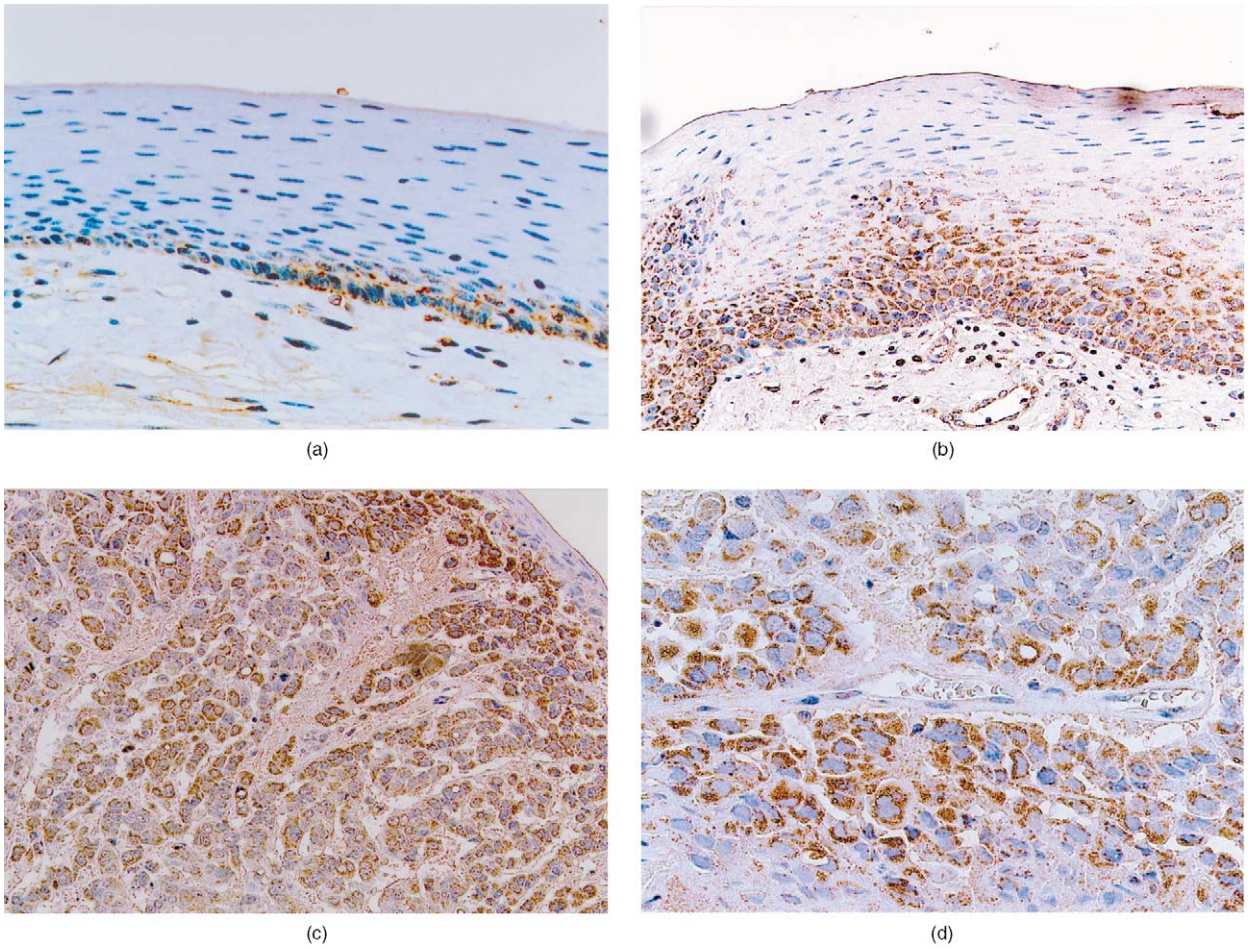

Fig. 1. ET protein expression in oesophageal lesions. Paraffin-embedded sections from patients with oesophageal squamous cell carcinoma were treated with anti-endothelin polyclonal antibody, followed by a dextran polymer conjugated two-step visualisation system. Normal mucosa (original magnification: ×400) (a), dysplastic tissue (original magnification: ×100) (b), cancerous tissue (original magnification: ×100) (c), and cancer cells surrounding a vessel (original magnification: ×400) (d).

3.3. ET expression and clinicopathological variables

ET protein levels within the normal mucosa, dysplastic tissue and cancerous lesions of each patient were compared with several clinicopathological variables (Table 1). A high ET expression level within normal mucosa (level $\geqslant 1$) was significantly related to lymphatic vessel invasion ($P < 0.05$), regional lymph node metastasis ($P < 0.005$), distant metastasis ($P < 0.005$) and TNM stage ($P < 0.005$).

3.4. ET expression and relapse-free survival

According to pattern of ET expression noted among normal, dysplastic and cancerous cells, Kaplan–Meier survival curves were compared using the log-rank test (Fig. 3). A high level of ET staining was taken as level 1 staining within normal mucosa and cancerous tissues, and level 2 staining within dysplastic tissues, in order to maximise differences in relapse-free survival. A high level of ET staining within the normal mucosa (level

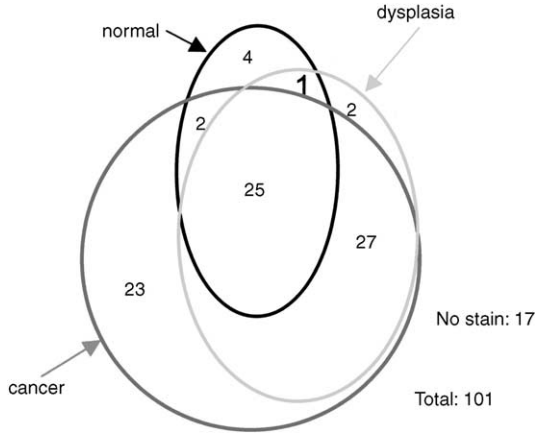

Fig. 2. Overlap of positive staining among the normal mucosa (level $\geqslant 1$), dysplastic tissue (level $\geqslant 2$) and cancerous tissue (level $\geqslant 1$). For example, 25 patients in the center had positive staining in their normal, dysplastic and cancerous tissue.

Table 1
Patients' characteristics ($n = 101$)

Prognostic factor	Endothelin protein (ET) expression levels		
	Normal	Dysplastic	Cancerous
	Low/high (%[a])	Low/high (%)	Low/high (%)
Age (years)			
⩽43–<54	14/7 (67)	7/14 (33)	6/15 (29)
⩽54–<61	17/11 (61)	14/14 (50)	5/23 (18)
⩽61–<68	20/7 (74)	10/17 (37)	6/21 (22)
⩽68–<84	18/7 (72)	15/10 (60)	7/18 (28)
Gender			
Female	8/2 (80)	6/4 (60)	3/7 (30)
Male	61/30 (67)	40/51 (44)	21/70 (23)
Multiple lesions			
(+)	16/5 (76)	8/13 (38)	5/16 (24)
(−)	53/27 (66)	38/42 (48)	19/61 (24)
Location			
Ce	2/0 (100)	0/2 (0)	0/2 (0)
Ut	8/1 (89)	2/7 (22)	1/8 (11)
Mt	25/13 (66)	20/18 (53)	8/30 (21)
Lt	26/14 (65)	19/21 (48)	14/26 (35)
Ae	8/4 (67)	5/7 (42)	1/11 (8)
Size			
Smaller than the median	38/12 (76)	24/26 (48)	9/41 (18)
Larger than the median	31/20 (61)	22/29 (43)	15/36 (29)
Intramural metastasis			
(+)	5/5 (50)	5 /5 (50)	3/7 (30)
(−)	61/25 (71)	40/46 (47)	21/65 (24)
Blood vessel invasion			
(+)	29/19 (60)	22/26 (46)	10/38 (21)
(btl)	40/13 (75)	24/29 (45)	14/39 (26)
Lymphatic vessel invasion*			
(+)	36/23 (61)	27/32 (46)	16/43 (27)
(−)	33/9 (79)	19/23 (45)	8/34 (19)
T, Primary tumour			
T1, submucosa	31/15 (67)	15/31 (33)	5/41 (11)
T2, muscularis propria	12/2 (86)	10/4 (71)	6/8 (43)
T3, adventitia	26/13 (67)	20/19 (51)	13/26 (33)
T4, contiguous structures	0/2 (0)	1/1 (50)	0/2 (0)
N, Regional lymph nodes**			
N0	40/9 (82)	20/29 (41)	11/38 (22)
N1	29/23 (56)	26/26 (50)	13/39 (25)
M, Distant metastasis**			
M0	68/26 (72)	45/49 (48)	23/71 (24)
M1	1/6 (14)	1/6 (14)	1/6 (14)
Stage**			
I	25/8 (76)	11/22 (33)	4/29 (12)
IIA	15/1 (94)	9/7 (56)	7/9 (44)
IIB	12/7 (63)	10/9 (53)	4/15 (21)
III	16/10 (62)	15/11 (58)	8/18 (31)
IV	1/6 (14)	1/6 (14)	1/6 (14)

Ce: cervical esophagus; Ut: upper thoracic; Mt: middle thoracic; Lt: lower thoracic; Ae: abdominal esophagus.

[a] ET low/total (percentage).

* $P < 0.05$; ** $P < 0.005$.

⩾1) was significantly associated with a shorter relapse-free survival ($P = 0.0066$) (Fig. 3a), but a high level of ET staining in dysplastic (level ⩾2) and cancerous tissues (level ⩾1) was not. However, when 33 patients with stage I were excluded from analysis, patients with high ET expression levels within their normal mucosa (level ⩾1) (Fig. 3b) and dysplastic tissue (level ⩾2) (Fig. 3c) had significantly shorter relapse-free survival ($P = 0.0044$ and $P = 0.0209$, respectively). A significant relationship between high levels of ET expression in cancerous lesions and survival was not found. The relationship between high levels of ET expression and survival was more pronounced when patients with T2 or more advanced stage cancers were selected for analysis; normal mucosa ($P = 0.0002$) (level ⩾1) (Fig. 3d), dysplastic tissue ($P = 0.0004$) (level ⩾2) (Fig. 3e), and cancerous lesions (level ⩾1) (Fig. 3f) ($P = 0.044$).

The presence of regional lymph node metastasis and distant metastasis, as defined by the TNM classification system, was significantly related to a reduced relapse-free survival ($P = 0.0002$ and $P = 0.0001$, respectively). Invasion into lymphatic vessels, blood vessels and intramural metastasis among known histological prognostic factors also had a large impact on relapse-free survival ($P = 0.0001$, $P = 0.0009$ and $P = 0.0001$, respectively).

3.5. *Effect of high ET expression on prognosis estimated with the Cox hazard model*

Cox regression analysis was performed to determine the prognostic significance of ET staining independent of the TNM staging results and various other clinical and histological findings. Even after adjustment for several histological prognostic factors, including tumour size, grade of differentiation, depth of primary tumour, intramural metastasis, blood and lymphatic vessel invasion, a high level of ET expression within dysplastic tissue (level ⩾2), but not normal mucosa or cancerous tissue, was associated with a higher risk of tumour relapse (Hazard Ratio (HR) = 2.10; 95% Confidence Interval (CI) 1.02 to 4.33), with significant model improvement ($P = 0.02$) (Table 2). Moreover, a high level of ET expression within dysplastic tissue (level ⩾2) decreased relapse-free survival (HR = 2.21; 95% CI 1.10 to 4.40), with significant model improvement after adjustment for each component of TNM classification system ($P = 0.03$) (Table 3).

4. Discussion

In this study, we demonstrated an association between high levels of ET expression in oesophageal tissue and poor prognosis in patients with oesophageal squamous cell carcinoma. Recently, expression of vascular endothelial cell growth factor (VEGF), another

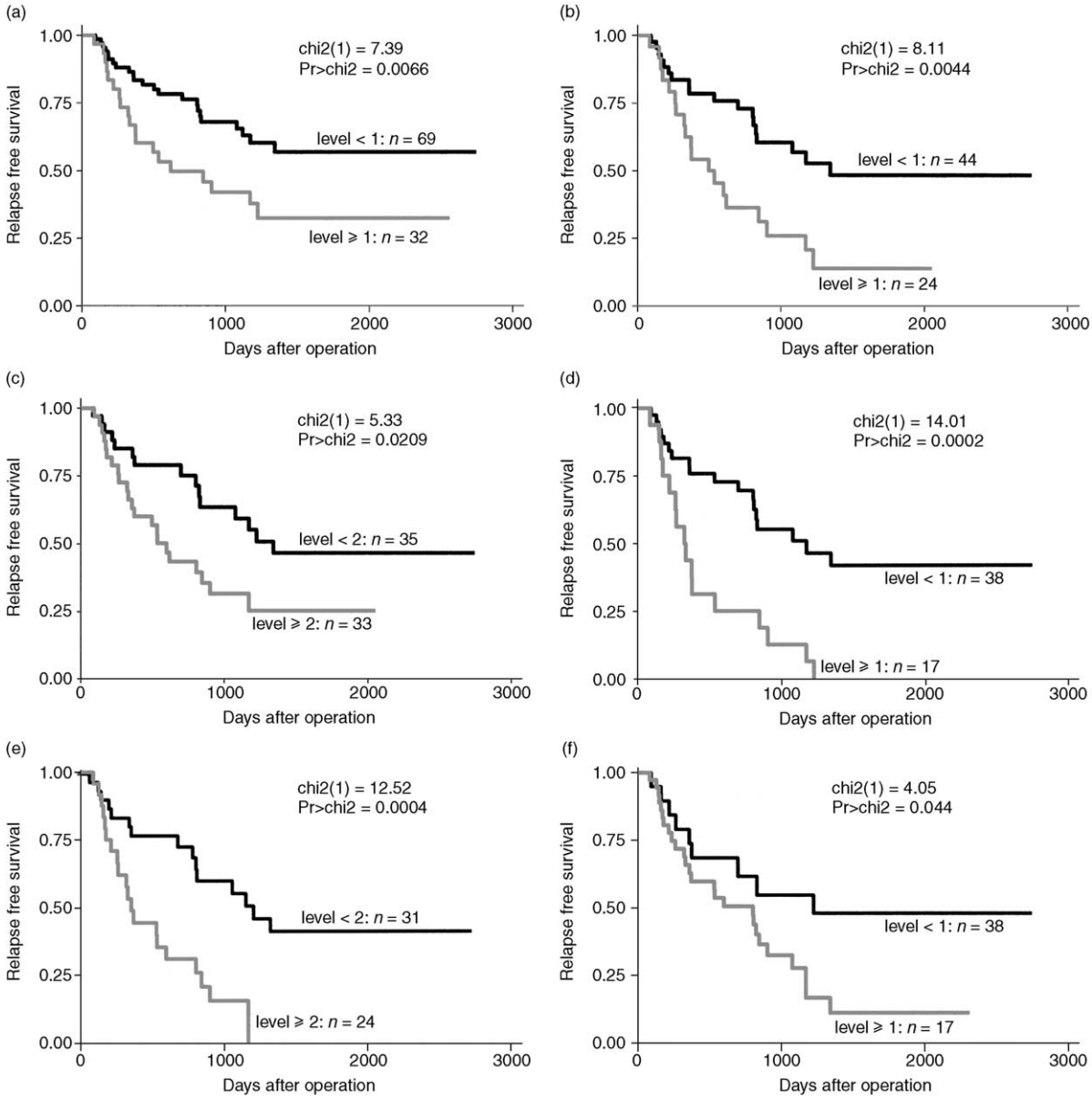

Fig. 3. Kaplan–Meier curves of relapse-free survival among patients with high levels of ET staining of their normal mucosa (a). Curves were also done for patients with stage IIA or more advanced stage cancers and a high level of ET staining within their normal mucosa (b) and dysplastic tissue (c). In addition, curves were done for patients with T2 or more advanced primary tumours and a high level of ET staining of their normal mucosa (d), dysplastic tissue (e), and cancerous lesions (f).

important mediator of tumour angiogenesis, has been demonstrated as an adverse prognostic indicator in oesophageal carcinoma [20]. A significant correlation has been found between VEGF expression and depth of invasion, lymph node metastasis and *p53* mutation [21]. In ovarian carcinoma cells, ET stimulates the secretion of VEGF [22]. In addition, ET induces angiogenic responses in cultured endothelial cells and has been observed to stimulate neovascularisation *in vitro* in concert with VEGF [23]. Thus, it is plausible that ET expression might correlate with prognosis in patients with oesophageal carcinoma.

Growth of a tumour beyond a certain size requires angiogenesis, which may also permit metastasis [24]. In fact, an independent correlation has been established between the number of microvessels found within areas of intense neovascularisation in invasive breast cancer and metastatic disease in axillary lymph nodes and at distant sites [25]. In this study, a significant relationship was observed between a high level of ET staining of the normal mucosa of patients with squamous cell oesophageal carcinoma and pathological evidence of lymphatic vessel invasion, as well as regional lymph node and distant metastasis. This suggests that ET production may

Table 2
Cox regression analysis of high ET expression in dysplastic tissue adjusted for various histological findings

Prognostic factor	Hazard Ratio	(95% CI)	*P* value
Primary tumour			
T1	0.40	(0.11–1.43)	0.160
T2[a]	1.55	(0.55–4.40)	0.405
T3			
T4	2.92	(0.35–24.4)	0.322
Size, less than or equal to the median	0.65	(0.31–1.34)	0.240
Intramural metastasis	2.05	(0.83–5.04)	0.118
blood vessel invasion	1.27	(0.59–2.71)	0.541
lymphatic vessel invasion	2.02	(0.72–5.62)	0.180
Moderately differentiated	1.45	(0.67–3.15)	0.347
Poorly differentiated	1.26	(0.49–3.22)	0.632
High ET staining of dysplastic tissue Level $\leqslant 1$ versus $2\leqslant$	2.10	(1.02–4.33)	0.044

[a] T2 and 'well differentiated' dropped due to collinearity.

Table 3
Cox regression analysis of high ET expression in dysplastic tissue adjusted for each component of TNM classification system

Prognostic factor	Hazard Ratio	(95% CI)	*P* value
T1	0.34	(0.10–1.10)	0.071
T2[a]			
T3	1.66	(0.62–4.46)	0.318
T4	1.79	(0.27–11.99)	0.548
N	1.85	(0.89–3.84)	0.097
M	1.60	(0.96–2.67)	0.070
High ET staining of dysplastic tissue Level $\leqslant 1$ versus $2\leqslant$	2.21	(1.10–4.40)	0.026

[a] T2 data dropped due to collinearity.

facilitate metastasis of tumour cells via vessels and thus, relate to poorer outcomes. When patients with stage I and T1 disease were excluded from analysis, the relationship between a high level of ET staining and reduced relapse-free survival was more evident. By penetrating the muscularis propria, tumour cells are more likely to encounter vessels, resulting in outcomes of regional and distant metastasis and poor prognosis.

A significant correlation between high ET expression and poor prognosis was found mainly in normal mucosa and dysplastic tissue, rather than in the cancerous lesions themselves, suggesting a paracrine growth mechanism for this tumour. This paracrine growth mechanism via ET production has been suggested for other types of carcinoma [26,27], although, autocrine growth mechanisms have also been suggested [28,29]. ET promotes ovarian carcinoma cell invasion by upregulating the secretion and activation of multiple tumour proteinases [30], another suspected mechanism behind ET's possible contribution to a poor prognosis in oesophageal cancer.

ET staining was a significant prognostic factor, independent of other clinical and histological prognostic factors. High ET expression in dysplastic tissue more than doubled the hazard ratio, even after adjustment for certain histological findings and each component of the TNM classification system. These findings suggest that high ET expression may be an important prognostic factor, in addition to and independent of, known histological and clinical prognostic factors. In oesophageal squamous cell carcinoma, chemo-radiotherapy does not significantly alter overall survival. However, being able to accurately predict the prognosis of a patient is of obvious clinical importance and benefit to the patient. ET staining is based on simple immunohistochemical techniques, which can be performed at every institute.

ET receptors have been reported to participate in the pathophysiology of prostate and other cancers [31,32]. Administration of a selective ET receptor B antagonist significantly slows human melanoma tumour growth in nude mice, leading to complete growth arrest in half of the mice examined [33]. The antagonist has been tried in patients with pulmonary hypertension, resulting in improved clinical outcomes in the absence of an increase in adverse events [34–36]. Atrasentan, an ET receptor A antagonist, decreased prostate-specific antigen levels and relieved pain in a subset of patients with prostate cancer [37]. ET antagonists are therefore potential candidates for the treatment of patients with oesophageal squamous cell carcinoma, perhaps with chemotherapy, for which. at present, few alternatives to surgery exist. Molecular targeting therapy, such as $p16^{INK4A}$, may be an alternative.

In conclusion, a high level of ET staining is a significant prognostic factor in oesophageal squamous cell carcinoma, independent of other known histological factors and each component of TNM classification system. These findings further suggest the possibility that an ET receptor antagonist might be used to treat these patients in the future.

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