Endothelin B receptor expression correlates with tumour angiogenesis and prognosis in oesophageal squamous cell carcinoma

Running title: Endothelin B Receptor Expression in OSCC

T Tanaka[1], M Sho*[,1], T Takayama[1], K Wakatsuki[1], S Matsumoto[1], K Migita[1], M Ito[1], K Hamada[2], Y Nakajima[1]

[1]Department of Surgery, Nara Medical University, Nara, Japan and [2]Division of Clinical and Investigative Medicine, Nara Medical University, 840 Shijo-cho, Kashihara, Nara, 634-8522, Japan

*Correspondence:

Masayuki Sho, M.D., Ph.D.,

Department of Surgery, Nara Medical University, 840 Shijo-cho, Kashihara, Nara, 634-8522, Japan.

Phone: 81-744-29-8863; Fax: 81-744-24-6866;

E-mail: m-sho@naramed-u.ac.jp

Abstract

Background: The endothelin-axis has been shown to play a pivotal role in several human malignancies. The aim of this study was to clarify the clinical importance of endothelin B receptor (ETBR) in human oesophageal squamous cell carcinoma (OSCC).

Methods: We evaluated ETBR expression in 107 patients with OSCC by immunohistochemistry. Microvessel density (MVD) and Lymphatic vessel density (LVD) were assessed by CD31 and D2-40 immunostaining, respectively. Furthermore, CD4, CD8, and CD45RO+ tumour-infiltrating lymphocytes (TILs) were immunohistochemically analysed.

Results: Sixty-one (57%) cases showed high expression of ETBR. ETBR expression was correlated with several clinicopathological factors including tumour differentiation, tumour depth, and lymph node metastasis. The overall and disease-specific survival rates were significantly lower in patients with high ETBR expression than patients with low expression. Furthermore, multivariate analysis revealed that ETBR status was an independent prognostic factor for patient survival. Mechanistic analysis indicated that MVD was significantly higher in tumour tissues with high ETBR expression compared to those with low expression, suggesting that angiogenesis may be a key mechanism in tumour

progression and metastasis of OSCC mediated by ETBR expression. By contrast, there were no significant correlations between TILs and ETBR expression.

Conclusion: ETBR plays a pivotal role in oesophageal cancer and may be therapeutic target for this intractable malignancy.

Keywords: Endothelin B receptor, oesophageal cancer, angiogenesis

Oesophageal cancer is highly aggressive and is the sixth leading cause of cancer deaths in the world (Parkin *et al*, 2005). Although several advancements in the treatment including chemotherapy and radiotherapy have been achieved, the prognosis of patients remains poor. Even in curatively resected patients, the 5-year survival rate is below 50% after surgery (Courrech Staal *et al*, 2009). In addition, most cases are diagnosed at advanced stage with lymphatic and hematogenous dissemination (Okines *et al*, 2010). Therefore, to elucidate the underlying mechanisms of tumour progression, and to identify new biomarkers and therapeutic targets for oesophageal cancer are critically important to improve patients' prognosis.

The endothelial cell-derived peptide endothelin (ET) was discovered as a potent vasoconstrictor in 1988 (Yanagisawa *et al*, 1988). ET family includes three 21-amino acid peptides, ET-1, ET-2 and ET-3, which bind to two G-protein-coupled receptors, endothelin receptor type A (ETAR) and endothelin receptor type B (ETBR) (Levin, 1995). The system of these three endothelin peptides and two receptors is referred to as the endothelin axis (ET-axis). Extensive studies have revealed the various roles of endothelin system in cardiovascular and renal disorders (Feldstein & Romero, 2007; Iglarz & Clozel, 2007; Lehrke *et al*, 2001; Tomobe *et al*, 1988). Furthermore, the ET-axis has also been shown to play significant roles in a variety of human malignancies including ovarian, prostate, cervical, breast, colorectal, lung cancers, and

melanoma (Nelson *et al*, 2003). ET-axis has been revealed to regulate tumour growth and metastasis via various mechanisms including cell proliferation, angiogenesis, antiapoptotic activity, and immune modulation (Bagnato *et al*, 2008; Buckanovich *et al*, 2008; Herrmann *et al*, 2006; Nelson *et al*, 2003; Spinella *et al*, 2002; Wulfing *et al*, 2004). Whilst upregulation of ET-1 expression has been consistently reported in various malignancies, the different expression and function of its receptors ETAR and ETBR have been shown in distinct tumours. Thus, each receptor has unique roles and its function may be dependent on cancer cell type. Furthermore, selective antagonists for each receptor as well as dual ETAR/ETBR antagonist have been widely investigated, and some of them were evaluated in clinical trials (Bagnato *et al*, 2008).

A few previous studies have addressed the role of ET-axis in oesophageal cancer (Ishibashi *et al*, 2003; Jiao *et al*, 2008; Jiao *et al*, 2007). These studies have shown that tissue or circulating serum expression of endothelin has a significant prognostic value in oesophageal squamous cell carcinoma (OSCC). A study has also shown that a dual receptor antagonist inhibited migration of human oesophageal cancer cell in vitro (Jiao *et al*, 2007). Furthermore, a recent study has identified aberrant promoter methylation of EDNRB gene, which encodes ETBR in humans, in OSCC (Zhao *et al*, 2009). Although these studies have suggested a potential role of ETBR in OSCC, little is known about its clinical importance. In this study, we evaluated tumour ETBR expression to investigate

its clinical significance in OSCC.

MATERIALS AND METHODS

Patients

We examined 107 patients with oesophageal squamous cell carcinoma. The patients underwent surgery including subtotal oesophagectomy with dissection of regional lymph nodes at Nara Medical University Hospital between November 1995 and May 2007. None of them have received pre-operative treatment, such as radiation or chemotherapy. The patient's median age was 61 years (range, 42-75). Postoperative follow-up data were obtained from all patients. The pathologic features of the specimens were classified based on the 7th edition of the pathological tumour-node-metastasis (TNM) classification of the International Union Against Cancer (UICC). Documented informed consent was obtained from individual patients for use of their tissue samples and clinical record.

Immunohistochemical staining

Formalin-fixed, paraffin-embedded tissues were cut into 5-μm sections, deparaffinised and rehydrated in a graded series of ethanol. Immunohistochemical staining for ETBR

was performed with a Dako Envision™ kit (DAKO Cytomation, Tokyo, Japan). Antigen retrieval was performed by heating tissue sections using a target retrieval solution, pH 9·0 (DAKO). Then, the samples were incubated for 5 min in peroxidase blocking solution (DAKO) to inhibit endogenous peroxidase and washed thrice in fresh PBS, each of 5 min duration. The anti-human ETBR antibody (LS-A54, MBL, USA) diluted 1:50 with Antibody Diluent (DAKO) was added and incubated at 37°C for 30 min. After the sections were washed thrice in PBS, each of 5 min duration, a subsequent reaction was carried out using second antibodies (DAKO) at 37°C for 30 min. The sections were then washed thrice in PBS and the colour was displayed subsequently with diaminobenzidine (DAB) (DAKO) for approximately 5 min and rinsed in distilled water. Sections were counterstained with haematoxylin, dehydrated in ethanol, cleared in xylene and coverslipped. Furthermore, for the evaluation of tumour angiogenesis and tumour infiltrating lymphocytes (TILs), immunohistochemical stainings for CD31, D2-40, CD4, CD8, and CD45RO were carried out by the same method as above. Monoclonal antibodies were purchased from Abcam (Tokyo, Japan); anti-CD31 (JC/70A), anti-D240 (D2-40), anti-CD4 (mAb51312), anti-CD8 (144B), and anti-CD45RO (UCH-L1).

Extraction of total RNAs and real-time reverse transcriptase PCR analysis

Total RNA was isolated using RNAspin Mini (GE Healthcare, Tokyo, Japan) and the first-strand cDNA was synthesised from 1 μg RNA using a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA), according to the instructions of the manufacturer. Real-time quantitative PCR analysis was carried out using an ABI Prism 7700 sequence detector system (Applied Biosystems). All primer/probe sets were purchased from Applied Biosystems. PCR was carried out using the TaqMan Universal PCR Master Mix (Applied Biosystems) using 1 μl of cDNA in a 20 μl final reaction volume. The PCR thermal cycle conditions were as follows: initial step at 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. The expression level of the housekeeping gene β2-microglobulin was measured as an internal reference with a standard curve to determine the integrity of template RNA for all specimens. The ratio of the mRNA level of each gene was calculated as follows: (absolute copy number of each gene) / (absolute copy number of β2-microglobulin).

Evaluation of ETBR expression

All cases were classified into two groups according to the percent of positively stained cells of ETBR. The evaluation was performed by authorised pathologists who had no knowledge of the patients' clinical status and outcome. Low ETBR immunoreactivity was defined as staining of < 50% of tumour cells, whereas high ETBR

immunoreactivity was defined as staining of $\geq$ 50% of tumour cells.

Evaluation of microvessel density (MVD) and lymphatic vessel density (LVD)

Vessel count was assessed by light microscopy in areas of the tumour containing the highest numbers of capillaries. The highly vascular areas were identified at low power (×40 magnification). After six areas of most abundant positively stained vessels were identified, a vessel count was performed on a ×200 field, and the average count of six fields was determined as the MVD or LVD respectively as previously described (Ikeda *et al*, 1999; Matsumoto *et al*, 2007; Weidner *et al*, 1991).

Evaluation of tumour-infiltrating lymphocytes (TILs)

We selected five areas with the most abundant positively stained cells in each tissue under ×40 magnification. We then counted these cells and calculated the mean number of each samples stained by anti-CD4, CD8, and CD45RO antibody, respectively.

Statistical analysis

Comparisons among the clinical and pathological features were evaluated using χ^2 and Fisher's exact tests. Statistical significance between two groups of parametric date was evaluated using an unpaired Student's t-test. Survival curves were estimated using the

Kaplan-Meier method, and the significances of differences between survival curves were determined using log-rank test. Multivariate comparisons of survival distributions were made using Cox proportional hazard models. A P value < 0.05 was considered to indicate statistically significance.

RESULTS

ETBR expression in human oesophageal squamous cell carcinoma. We first examined the expression of ETBR on 107 oesophageal squamous cell carcinoma tissues by immunohistochemistry. Sixty-one (57.0%) cases showed high expression of ETBR. ETBR expression was identified in the cytoplasm of carcinoma cells (Figure 1). We then compared the relative expression of ETBR between oesophageal cancer and non-cancer tissues using available frozen tissues by quantitative real-time PCR analysis. The ETBR expression in oesophageal carcinoma tissues was significantly higher than in non-carcinoma tissues ($P = 0.036$; Figure 2A). Furthermore, the ETBR expression of cancer tissue was consistently highly than that of non-cancer tissue in each individual patient with OSCC (Figure 2B). These data suggested that ETBR might have some role in OSCC and may be a potential therapeutic target.

Correlation between ETBR expression and clinicopathological findings. To clarify the clinical significance of ETBR in OSCC, we compared ETBR expression with clinicopathological features. As a result, there was no significant correlation between ETBR expression and several clinicopathological variables including gender, age, and distant metastatic status (Table 1). In contrast, ETBR expression was significantly correlated with tumour differentiation, tumour depth, lymph node metastasis, lymphatic invasion, venous invasion, and tumour stage (P = 0.013, 0.041, 0.037, 0.002, 0.019, and 0.036, respectively). Moreover, larger tumour and metastatic lymph nodes were found more often in patients with high ETBR expression (P = 0.012 and 0.016, respectively). Thus, ETBR expression in OSCC may play a critical role in tumour growth and metastasis.

Prognostic value of ETBR expression in OSCC. Next we examined the prognostic importance of ETBR expression in patients with OSCC. The overall and disease-specific survival rates were significantly lower in patients with high ETBR expression than in those with low expression (P = 0.003 and 0.002; Figure 3). The 5-year overall survival rate for ETBR high expression patients was 56.2 % and for low expression patients was 20.1%. In this study, gender, tumour status, lymph node metastasis and venous invasion were also found to have a prognostic value for patient

survival of OSCC. To determine independent variables among these prognostic factors, we performed a multivariate analysis using Cox proportional hazard models. The analysis revealed that, in addition to gender and lymph node metastasis, ETBR expression was one of the independent prognostic factors for the patients with OSCC (Table 2). Taken together, ETBR is functional and plays a significant role in OSCC.

Association of ETBR expression with tumour angiogenesis and lymphangiogenesis. To investigate the underlying mechanisms of ETBR expression in OSCC, we examined tumour angiogenesis and lymphangiogenesis. To this end, we performed immunohistochemical staining of CD31 and D2-40 in the same tissues of OSCC in which ETBR expression was evaluated (Figure 4A and B). We found that MVD was significantly higher in tissues with high ETBR expression compared to those with low expression (8.04±4.31 and 4.03±3.99; $P < 0.001$; Figure 4C). On the other hand, there was no significant correlation between tumour ETBR expression and LVD (high ETBR group and low group; 2.96±2.54 and 4.02±2.70, respectively). We further analyzed the postoperative recurrence pattern. In this series, a total of 61 patients had recurrence during follow-up period. Thirty-eight patients had hematogenous metastasis including 12 in the liver, 21 in the lung, and 8 in the bone. Furthermore, 40 patients had lymphatic metastasis. There were significant correlations of ETBR expression with hematogenous

and lymphatic metastasis ($P = 0.042$ and 0.025, respectively). On the other hand, neither MVD nor LVD correlated with each metastatic pattern. Taken together, angiogenesis might play some roles in tumour progression rather than metastasis in relation to ETBR.

Association between ETBR expression and tumour infiltrating lymphocytes. Finally, we investigated the immunomodulatory function of ETBR in OSCC. We performed immunohistochemical analysis of TILs including CD4, CD8, and CD45RO. As a result, there were no significant correlations of tumour ETBR expression with TILs in any T cell subsets (Figure 5).

DISCUSSION

Accumulating evidence demonstrates that ET-axis plays significant roles in tumours by a number of complex mechanisms including cell survival, proliferation, migration, invasion, epithelial-mesenchymal transition, methylation, angiogenesis, and immune modulation (Bagnato *et al*, 2008; Carducci *et al*, 2003; Eltze *et al*, 2007; Nelson *et al*, 2003; Nelson, 2003). Furthermore, several clinical studies have shown that ET-1 and its two receptors, ETAR and ETBR, are overexpressed in various actual human cancer tissues (Bagnato *et al*, 2008). Although a few studies have also reported a potential role of ET-1 in human oesophageal cancer, the data are very limited. Since a basic research

has suggested a potential involvement of ETBR in OSCC, this study focused on the clinical significance of ETBR expression in OSCC tissues (Zhao *et al*, 2009). As a result, we found several important findings. First, ETBR was highly expressed in cancer tissues compared to non-cancer tissues. Similar results were also reported in several other tumours (Eltze *et al*, 2007; Wulfing *et al*, 2004). Second, there were significant associations of ETBR expression with some clinicopathological features including tumour differentiation, tumour size, lymph node metastasis, and venous invasion. Thus, data suggest that ETBR expression in OSCC may play an important role in both tumour progression and metastasis. Third, most importantly, ETBR expression has a significant prognostic value in OSCC. The patients with high ETBR expression had a worse prognosis in overall and disease-specific survival compared to patients with low expression. To our knowledge, this is the first report to demonstrate that ETBR is an independent prognostic marker for human oesophageal cancer. Previous studies have also demonstrated that tumour ETBR expression was a negative prognostic marker in other cancer (Shen *et al*, 2011). On the other hand, ETBR gene expression was reported as a positive prognostic marker in renal cell carcinoma (Wuttig *et al*, 2012). Therefore, the function of ETBR may be dependent on tumour type.

Next, we investigated the underlying mechanisms in ETBR expression of OSCC. We first focused on angiogenesis that plays a key role in tumour growth and metastasis.

ET-1 has been shown to promote angiogenesis both directly and indirectly by inducing endothelial cell survival, proliferation, invasion, and upregulating VEGF production in the vasculature through ETBR (Kandalaft *et al*, 2009; Salani *et al*, 2000). In this study, we found a significant positive correlation of ETBR expression with angiogenesis evaluated by counting intratumour microvessel density. This is consistent with previous data in different tumour (Wulfing *et al*, 2004). Although several studies have demonstrated that angiogenesis has a significant role and prognostic value in OSCC, the mechanisms to control angiogenesis are not fully elucidated (Igarashi *et al*, 1998; Kitadai *et al*, 1998; Tanigawa *et al*, 1997). Furthermore, our data also indicated that there was a significant association between ETBR expression and venous invasion. Taken together, tumour ETBR expression may promote neovascularisation and enhance venous invasion. In addition, ETBR has been shown to play a critical role in tumour lymphangiogenesis (Spinella *et al*, 2009). However, we had no significant correlation between tumour ETBR expression and lymphangiogenesis determined by D2-40 immunostaining. Therefore, lymphangiogenesis may not be a key mechanism in tumour progression mediated by ETBR in OSCC.

Then, we evaluated the association of ETBR with tumour immunity. Previous studies have shown that ET-1 axis also has a unique role to regulate immune response in tumour environment (Buckanovich *et al*, 2008; Grimshaw *et al*, 2004; Guruli *et al*,

2004). ET-1 axis modulate the activation, differentiation and trafficking of tumour-infiltrating immune cells. Furthermore, ETBR plays a crucial role in lymphocyte homing and overexpression of endothelial ETBR in tumours prevents T cell homing. We have recently reported that TILs, especially CD45RO-expressing memory T cells, have significant prognostic value in OSCC (Enomoto *et al*, 2012). Therefore, we hypothesised that ETBR may regulate TILs in OSCC. To clarify this possibility, we evaluated association of tumour expressing ETBR with TILs including CD4, CD8, and CD45RO by immunohistochemistry. As a result, we found no significant correlations between them. Thus, our data suggest that ETBR may have little role in modulating immune response in the progression and metastasis in OSCC. However, further studies are required to completely rule out the immunological role of ETBR in OSCC.

Based on diverse functions and involvement of ET-axis in a variety of tumours, several agents targeting ET-axis have been extensively investigated (Bagnato *et al*, 2008; Kandalaft *et al*, 2009). Furthermore, some endothelin antagonists have been clinically evaluated (Bagnato *et al*, 2011). Although most of them are selective ETAR antagonists, ETBR antagonist is also available for clinical use and expected of clinical benefit. Compared to other cancers, there were only a few therapeutic agents clinically available in OSCC. In addition, OSCC is generally difficult to treat and cure by nonsurgical antitumour treatments. Therefore, combination therapy of ETBR antagonist

with other antitumour agents may be desirable for this intractable tumour. Our data indicates a significant relationship between ETBR and angiogenesis. Therefore, combination of ETBR with anti-angiogeneic treatment may exert a synergistic effect. Since anti-angiogenic treatment including anti-VEGF monoclonal antibody is widely used in clinical cancer therapy, this therapeutic strategy can be relatively easily tested. However, there are limitations in this study and further studies are essential before clinical application. The number of samples evaluated for this study is relatively small. In fact, we have treated more patients with oesophageal cancer in the study period. Due to some reasons including sample limitations, we randomly chose samples of 107 patients and investigated. Therefore, a large-scale and careful evaluation for ETBR is critically important to confirm reproducibility of our data.

In conclusion, we have shown that ETBR is expressed in human oesophageal cancer and has a significant prognostic value. This study may provide a rationale for developing a novel cancer therapy targeting ETBR for this fatal malignant disease. However, more experimental and clinical evidence is needed to prove the significance of ETBR as a new therapeutic target.

ACKNOWLEDGEMENTS

This work was supported by the following grants: Grants-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology of Japan (M. Sho).

REFERENCES

Bagnato A, Loizidou M, Pflug BR, Curwen J, Growcott J (2011) Role of the endothelin axis and its antagonists in the treatment of cancer. *Br J Pharmacol* **163**(2): 220-233

Bagnato A, Spinella F, Rosano L (2008) The endothelin axis in cancer: the promise and the challenges of molecularly targeted therapy. *Can J Physiol Pharmacol* **86**(8): 473-484

Buckanovich RJ, Facciabene A, Kim S, Benencia F, Sasaroli D, Balint K, Katsaros D, O'Brien-Jenkins A, Gimotty PA, Coukos G (2008) Endothelin B receptor mediates the endothelial barrier to T cell homing to tumors and disables immune therapy. *Nat Med* **14**(1): 28-36

Carducci MA, Padley RJ, Breul J, Vogelzang NJ, Zonnenberg BA, Daliani DD, Schulman CC, Nabulsi AA, Humerickhouse RA, Weinberg MA, Schmitt JL, Nelson JB (2003) Effect of endothelin-A receptor blockade with atrasentan on tumor progression in men with hormone-refractory prostate cancer: a randomized, phase II, placebo-controlled trial. *J Clin Oncol* **21**(4): 679-689

Courrech Staal EF, van Coevorden F, Cats A, Aleman BM, van Velthuysen ML, Boot H, Peeters MJ, van Sandick JW (2009) Outcome of low-volume surgery for esophageal cancer in a high-volume referral center. *Ann Surg Oncol* **16**(12): 3219-3226

Eltze E, Bertolin M, Korsching E, Wulfing P, Maggino T, Lelle R (2007) Expression and prognostic relevance of endothelin-B receptor in vulvar cancer. *Oncol Rep* **18**(2): 305-311

Enomoto K, Sho M, Wakatsuki K, Takayama T, Matsumoto S, Nakamura S, Akahori T, Tanaka T, Migita K, Ito M, Nakajima Y (2012) Prognostic importance of tumour-infiltrating memory T cells in oesophageal squamous cell carcinoma. *Clin Exp Immunol* **168**(2): 186-191

Feldstein C, Romero C (2007) Role of endothelins in hypertension. *Am J Ther* **14**(2): 147-153

Goseki N, Koike M, Yoshida M (1992) Histopathologic characteristics of early stage esophageal carcinoma. A comparative study with gastric carcinoma. *Cancer* **69**(5): 1088-1093

Grimshaw MJ, Hagemann T, Ayhan A, Gillett CE, Binder C, Balkwill FR (2004) A role for endothelin-2 and its receptors in breast tumor cell invasion. *Cancer Res* **64**(7): 2461-2468

Guruli G, Pflug BR, Pecher S, Makarenkova V, Shurin MR, Nelson JB (2004) Function and survival of dendritic cells depend on endothelin-1 and endothelin receptor autocrine loops. *Blood* **104**(7): 2107-2115

Herrmann E, Bogemann M, Bierer S, Eltze E, Hertle L, Wulfing C (2006) The endothelin axis in urologic tumors: mechanisms of tumor biology and therapeutic implications. *Expert Rev Anticancer Ther* **6**(1): 73-81

Igarashi M, Dhar DK, Kubota H, Yamamoto A, El-Assal O, Nagasue N (1998) The prognostic significance of microvessel density and thymidine phosphorylase expression in squamous cell carcinoma of the esophagus. *Cancer* **82**(7): 1225-1232

Iglarz M, Clozel M (2007) Mechanisms of ET-1-induced endothelial dysfunction. *J Cardiovasc Pharmacol* **50**(6): 621-628

Ikeda N, Adachi M, Taki T, Huang C, Hashida H, Takabayashi A, Sho M, Nakajima Y, Kanehiro H, Hisanaga M, Nakano H, Miyake M (1999) Prognostic significance of angiogenesis in human pancreatic cancer. *Br J Cancer* **79**(9-10): 1553-1563

Ishibashi Y, Hanyu N, Nakada K, Suzuki Y, Yamamoto T, Takahashi T, Kawasaki N, Kawakami M, Matsushima M, Urashima M (2003) Endothelin protein expression as a significant prognostic factor in oesophageal squamous cell carcinoma. *Eur J Cancer* **39**(10): 1409-1415

Jiao W, Xu J, Zheng J, Shen Y, Lin L, Li J (2008) Elevation of circulating big endothelin-1: an independent prognostic factor for tumor recurrence and survival in patients with esophageal squamous cell carcinoma. *BMC Cancer* **8**: 334

Jiao WJ, Xu J, Pan H, Wang TY, Shen Y (2007) Effect of endothelin-1 in esophageal squamous cell carcinoma invasion and its correlation with cathepsin B. *World J Gastroenterol* **13**(29): 4002-4005

Kandalaft LE, Facciabene A, Buckanovich RJ, Coukos G (2009) Endothelin B receptor, a new target in cancer immune therapy. *Clin Cancer Res* **15**(14): 4521-4528

Kitadai Y, Haruma K, Tokutomi T, Tanaka S, Sumii K, Carvalho M, Kuwabara M, Yoshida K, Hirai T, Kajiyama G, Tahara E (1998) Significance of vessel count and vascular endothelial growth factor in human esophageal carcinomas. *Clin Cancer Res* **4**(9): 2195-2200

Lehrke I, Waldherr R, Ritz E, Wagner J (2001) Renal endothelin-1 and endothelin receptor type B expression in glomerular diseases with proteinuria. *J Am Soc Nephrol* **12**(11): 2321-2329

Levin ER (1995) Endothelins. *N Engl J Med* **333**(6): 356-363

Matsumoto S, Yamada Y, Narikiyo M, Ueno M, Tamaki H, Miki K, Wakatsuki K, Enomoto K, Yokotani T, Nakajima Y (2007) Prognostic significance of platelet-derived growth factor-BB expression in human esophageal squamous cell carcinomas. *Anticancer Res* **27**(4B): 2409-2414

Nelson J, Bagnato A, Battistini B, Nisen P (2003) The endothelin axis: emerging role in cancer. *Nat Rev Cancer* **3**(2): 110-116

Nelson JB (2003) Endothelin inhibition: novel therapy for prostate cancer. *J Urol* **170**(6 Pt 2): S65-67; discussion S67-68

Okines A, Sharma B, Cunningham D (2010) Perioperative management of esophageal cancer. *Nat Rev Clin Oncol* **7**(4): 231-238

Parkin DM, Bray F, Ferlay J, Pisani P (2005) Global cancer statistics, 2002. *CA Cancer J Clin* **55**(2): 74-108

Salani D, Taraboletti G, Rosano L, Di Castro V, Borsotti P, Giavazzi R, Bagnato A (2000) Endothelin-1 induces an angiogenic phenotype in cultured endothelial cells and stimulates neovascularization in vivo. *Am J Pathol* **157**(5): 1703-1711

Shen C, Yang L, Yuan X (2011) Endothelin B receptor expression in human astrocytoma: association with clinicopathological variables and survival outcomes. *Int J Neurosci* **121**(11): 626-631

Spinella F, Garrafa E, Di Castro V, Rosano L, Nicotra MR, Caruso A, Natali PG, Bagnato A (2009) Endothelin-1 stimulates lymphatic endothelial cells and lymphatic vessels to grow and invade. *Cancer Res* **69**(6): 2669-2676

Spinella F, Rosano L, Di Castro V, Natali PG, Bagnato A (2002) Endothelin-1 induces vascular endothelial growth factor by increasing hypoxia-inducible factor-1alpha in ovarian

carcinoma cells. *J Biol Chem* 277(31): 27850-27855

Tanigawa N, Matsumura M, Amaya H, Kitaoka A, Shimomatsuya T, Lu C, Muraoka R, Tanaka T (1997) Tumor vascularity correlates with the prognosis of patients with esophageal squamous cell carcinoma. *Cancer* 79(2): 220-225

Tomobe Y, Miyauchi T, Saito A, Yanagisawa M, Kimura S, Goto K, Masaki T (1988) Effects of endothelin on the renal artery from spontaneously hypertensive and Wistar Kyoto rats. *Eur J Pharmacol* 152(3): 373-374

Weidner N, Semple JP, Welch WR, Folkman J (1991) Tumor angiogenesis and metastasis--correlation in invasive breast carcinoma. *N Engl J Med* 324(1): 1-8

Wulfing P, Kersting C, Tio J, Fischer RJ, Wulfing C, Poremba C, Diallo R, Bocker W, Kiesel L (2004) Endothelin-1-, endothelin-A-, and endothelin-B-receptor expression is correlated with vascular endothelial growth factor expression and angiogenesis in breast cancer. *Clin Cancer Res* 10(7): 2393-2400

Wuttig D, Zastrow S, Fussel S, Toma MI, Meinhardt M, Kalman K, Junker K, Sanjmyatav J, Boll K, Hackermuller J, Rolle A, Grimm MO, Wirth MP (2012) CD31, EDNRB and TSPAN7 are promising prognostic markers in clear-cell renal cell carcinoma revealed by genome-wide expression analyses of primary tumors and metastases. *Int J Cancer* 131(5): E693-704

Yanagisawa M, Kurihara H, Kimura S, Tomobe Y, Kobayashi M, Mitsui Y, Yazaki Y, Goto K, Masaki T (1988) A novel potent vasoconstrictor peptide produced by vascular endothelial cells. *Nature* 332(6163): 411-415

Zhao BJ, Sun DG, Zhang M, Tan SN, Ma X (2009) Identification of aberrant promoter methylation of EDNRB gene in esophageal squamous cell carcinoma. *Dis Esophagus* 22(1): 55-61

FIGURE LEGEND

Figure 1. Expression of ETBR in oesophageal squamous cell carcinoma. Representative case of positive expression of ETBR. (**A**) Original magnification, x20. (**B**) Original magnification, x100. (**C**) Representative case of negative expression of ETBR. Original magnification, x100.

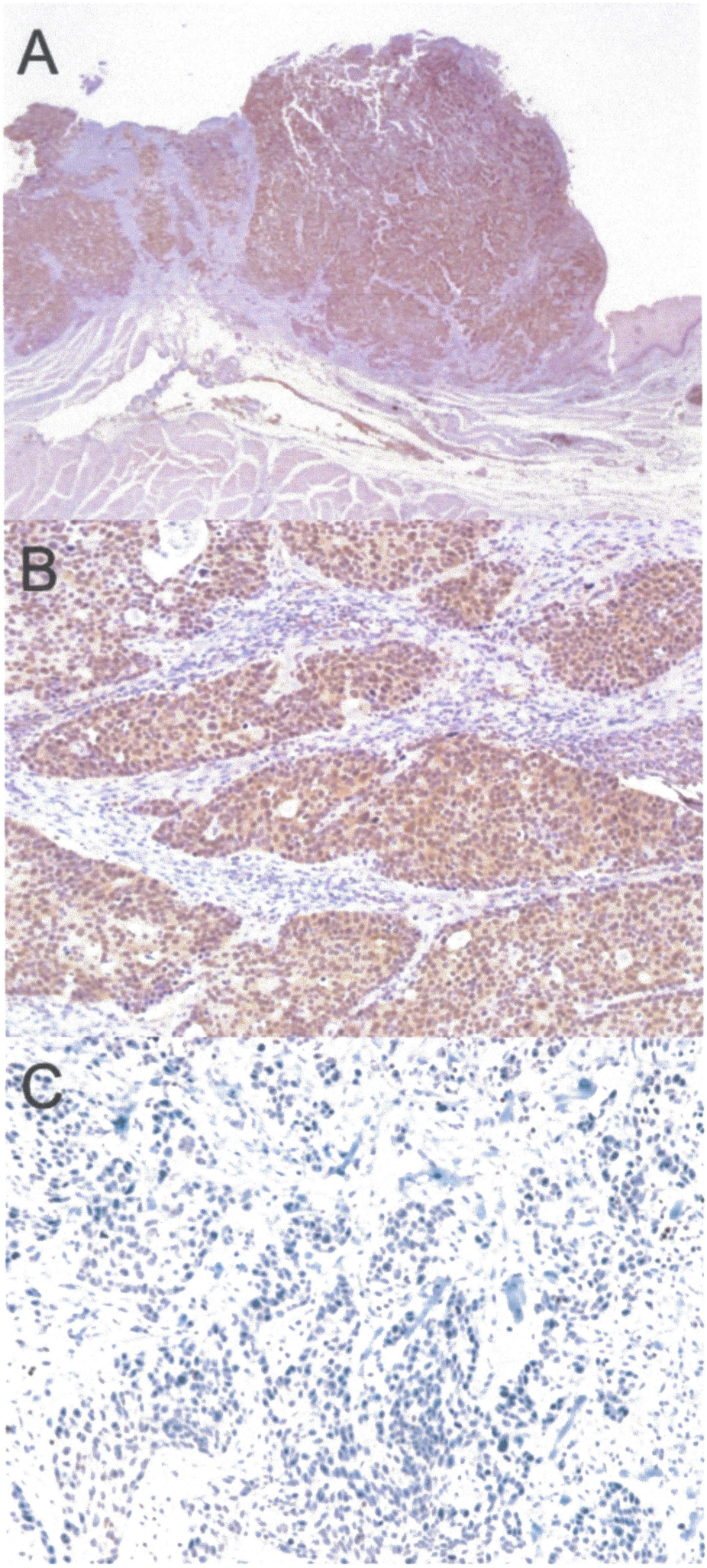
Figure 2. Comparison of ETBR expression between cancer and non-cancer tissue of the oesophagus. (**A**) The cumulative ETBR expression in cancer tissue was significantly higher compared with that in non-cancer tissue as determined by real-time PCR (n = 10 of each, P = 0.036). (**B**) The expression in cancer tissue is consistently higher than that in non-cancer tissue of individual oesophageal cancer patients.

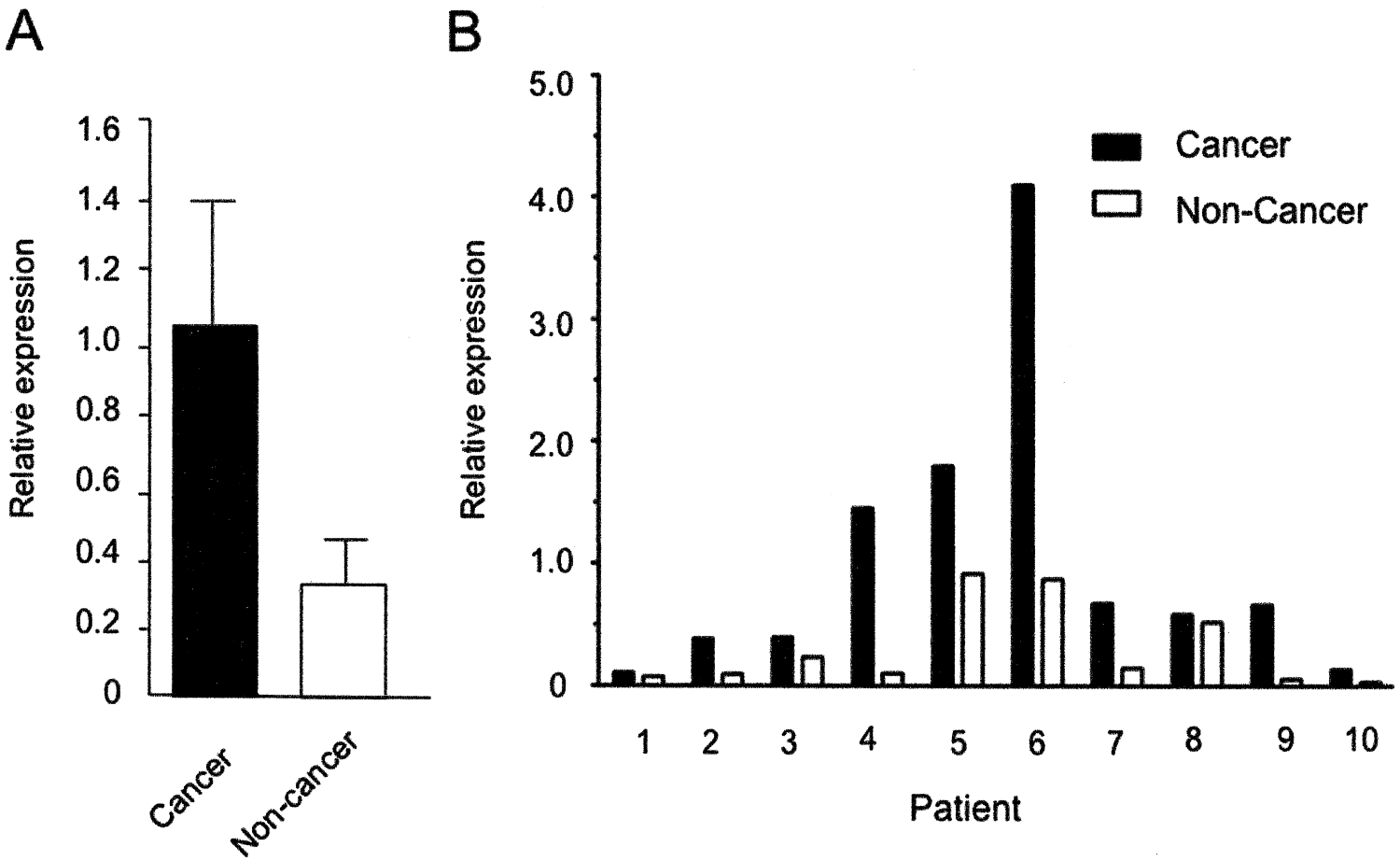
Figure 3. Prognosis of oesophageal cancer patients according to tumour ETBR expression status. (**A**) Overall survival rate (P = 0.003). (**B**) Disease-specific survival rate (P = 0.002).

Figure 4. Association of tumour angiogenesis with ETBR expression. A: CD31 expression was detected in endothelial cells of tumour vasculature (Original

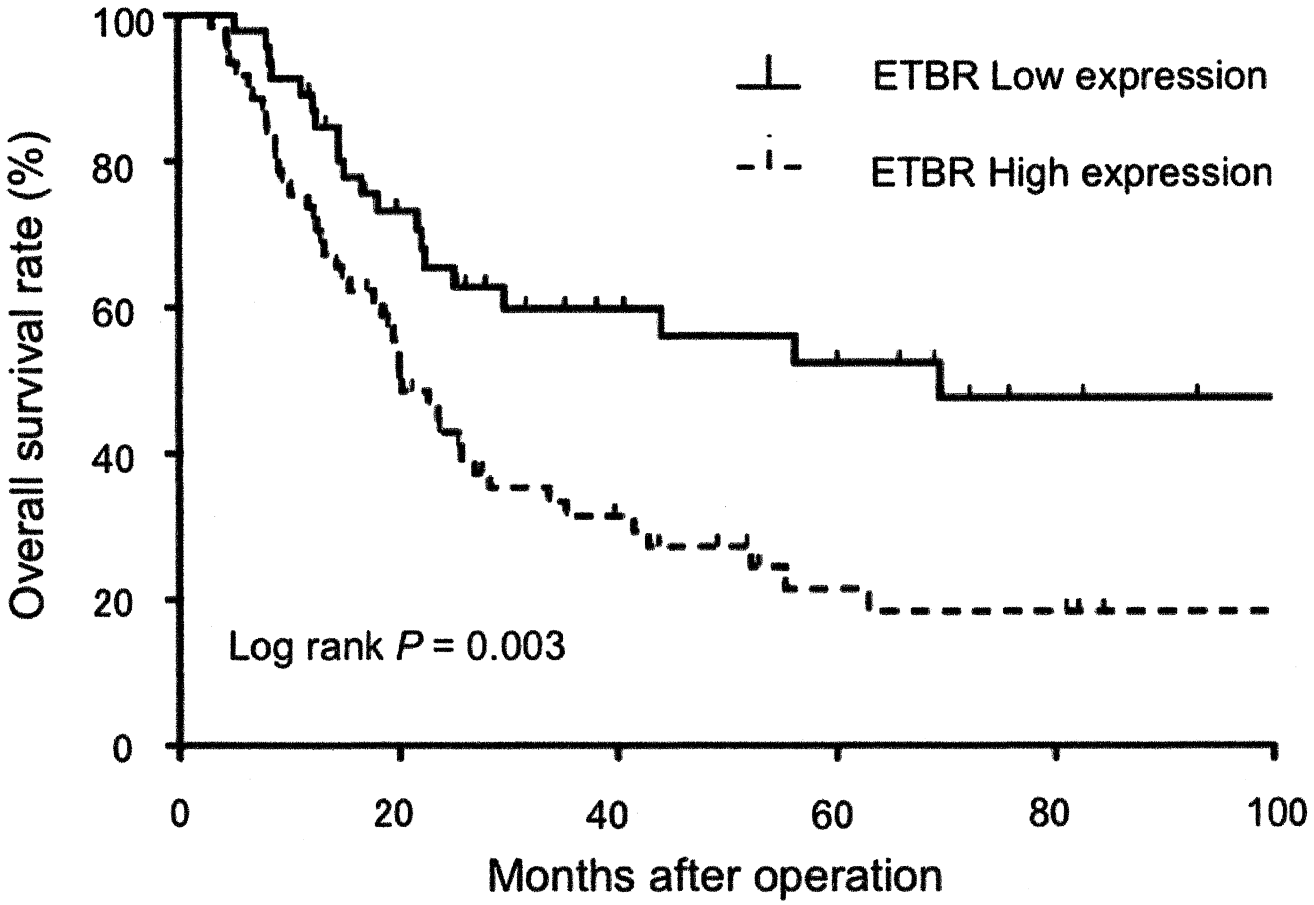
magnification, x200). B: Immunohistochemistry for D2-40 showing lymphatic channels with staining of endothelium (Original magnification, x200). C: Microvessel density (MVD) was significantly higher in tissues with high ETBR expression compared to those with low expression.

Figure 5. Association of tumour infiltrating lymphocytes with ETBR expression. There were no significant correlations of tumour ETBR expression with the number of tumour infiltrating lymphocytes in CD4, CD8, and CD45RO, respectively.

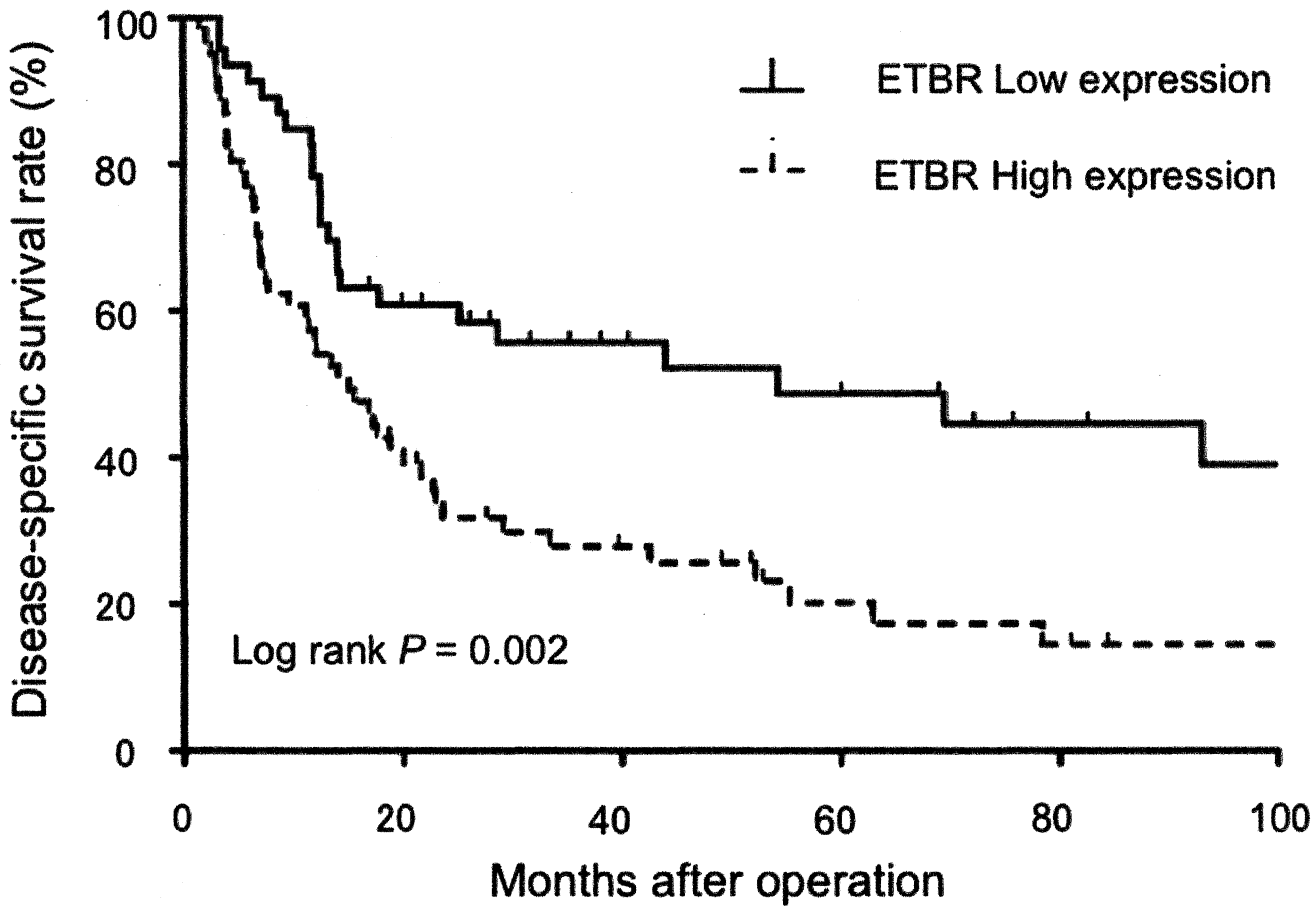

A
B
C

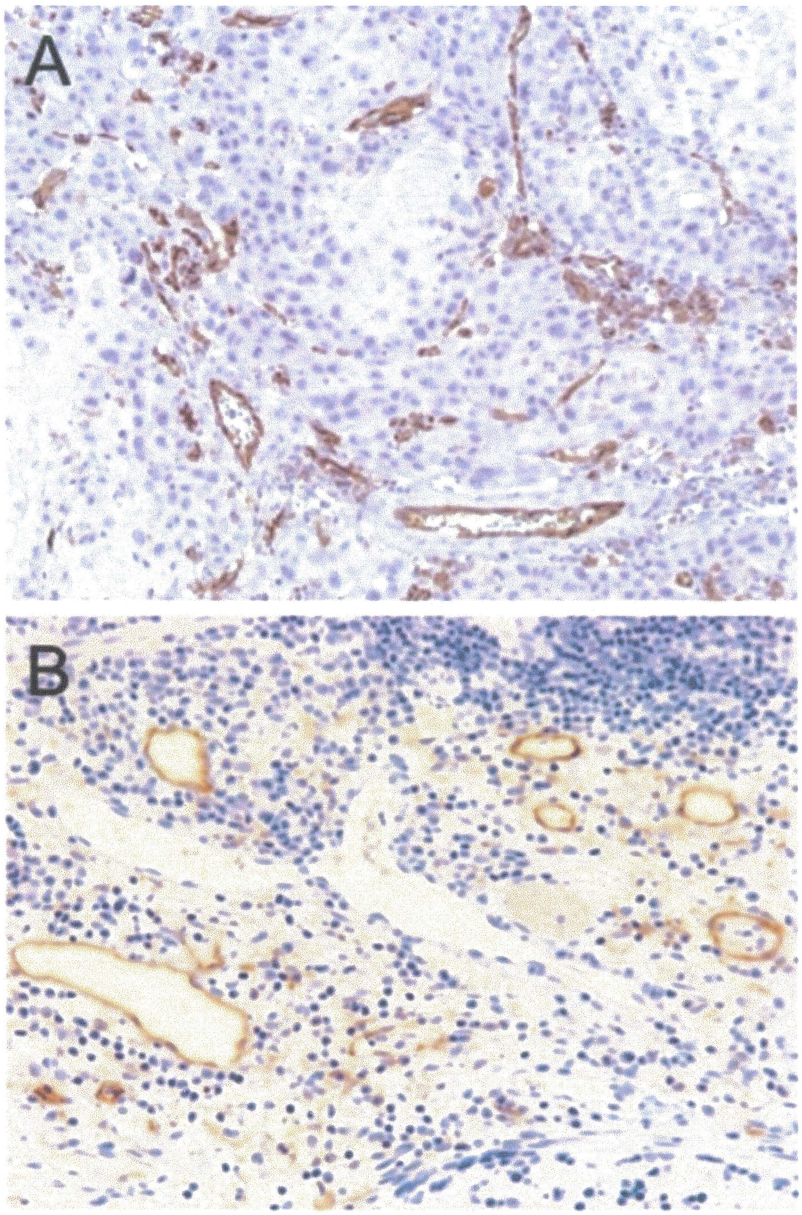

A
Relative expression
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
Cancer
Non-cancer
B
Relative expression
5.0
4.0
3.0
2.0
1.0
0
Cancer
Non-Cancer
1
2
3
4
5
6
7
8
9
10
Patient

A


100
80
60
40
20
0
Overall survival rate (%)
ETBR Low expression
ETBR High expression
Log rank P = 0.003
0
20
40
60
80
100
Months after operation

B


100
80
60
40
20
0
Disease-specific survival rate (%)
ETBR Low expression
ETBR High expression
Log rank P = 0.002
0
20
40
60
80
100
Months after operation


A
B
C
P < 0.001
Microvessel Density
0
1
2
3
4
5
6
7
8
9
High
Low
ETBR expression

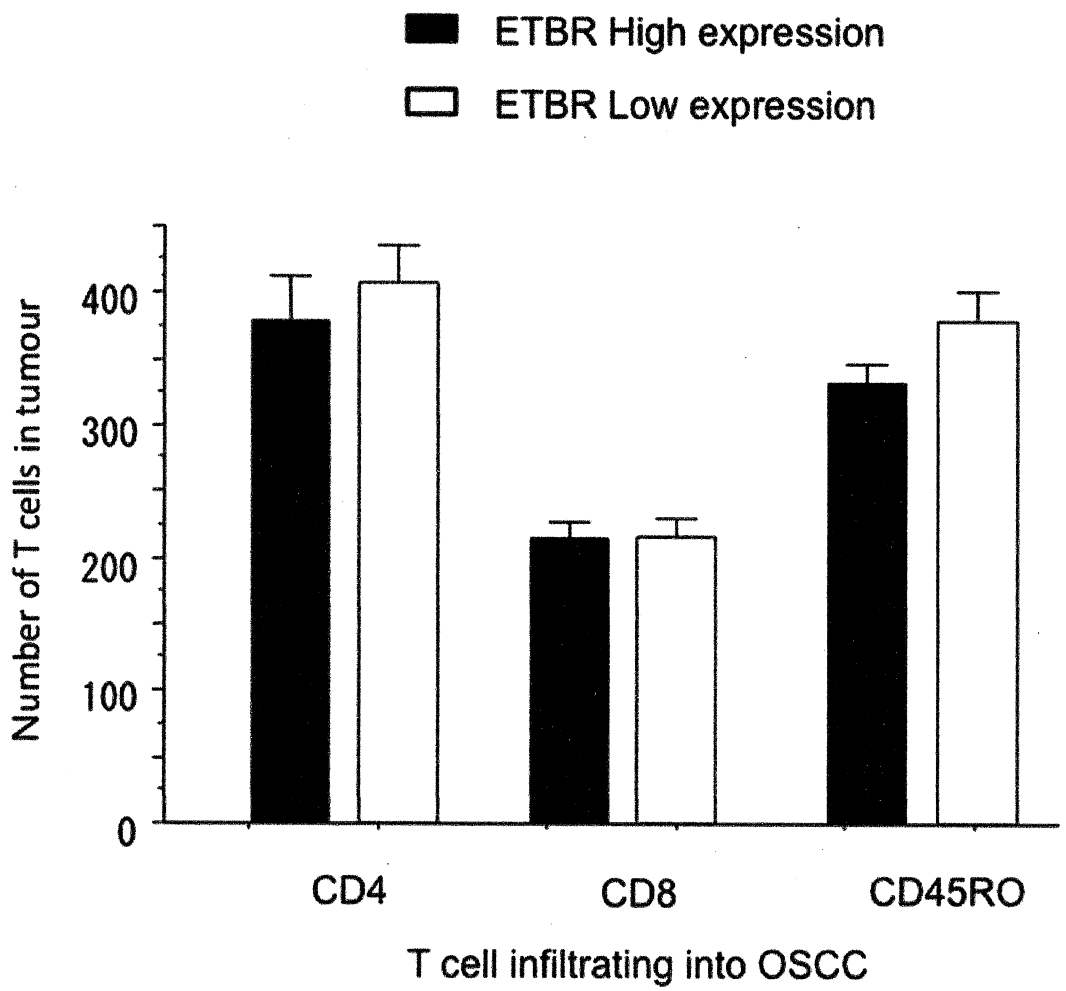

ETBR High expression
ETBR Low expression
Number of T cells in tumour
400
300
200
100
0
CD4
CD8
CD45RO
T cell infiltrating into OSCC

Table 1. Relationship between ETBR expression and clinicopathological characteristics in oesophageal sequamous cell carcinoma

Characteristics	Total (n = 107)	ETBR expression High (n = 61)	ETBR expression Low (n = 46)	*P*-value
Gender				
Male	88	48	40	0.315
Female	19	13	6	
Age (years) (mean ± s.d.)		61.1±7.01	61.0±7.66	0.948
Diffrentiation				
Well	39	16	23	0.013
Moderate	52	37	15	
Poor	16	8	8	
Tumour depth				
*p*T1	23	9	14	0.041
*p*T2	21	9	12	
*p*T3	58	39	19	
*p*T4	5	4	1	
Lymph node metastasis				
*p*N0	37	16	21	0.037
*p*N1	70	45	25	
Distant metastasis				
M0	106	61	45	0.431
M1	1	0	1	
Lymphatic invasion				
Negative	20	5	15	0.002
Positive	87	56	31	
Venous invasion				
Negative	60	28	32	0.019
Positive	47	33	14	
Tumour stage				
I	16	6	10	0.036
II	32	14	18	
III	42	29	13	
IV	17	12	5	
Tumour size(mm) (mean ± SD)		43.7±18.9	33.5±17.6	0.012
Number of lymph node metastasis (mean ± SD)		4.94±7.15	1.90±3.40	0.016

Table 2. Univariate and multivariate analysis for overall survival in 107 patients with oesophageal sequamous cell carcinoma

Characteristics	Univariate analysis	Multivariate analysis		
	P-value	HR	95% CI	*P*-value
Gender (Femare vs. Male)	0.017	2.967	1.274 – 6.944	0.012
Tumour depth (T1 vs. T2-4)	0.003	1.693	1.112 – 2.577	0.014
Lymph node metastasis (No vs. N1)	<0.001	2.165	1.119 – 4.186	0.022
Venous invasion (Negative vs. Positive)	0.015	0.725	0.400 – 1.312	0.287
Tumour size	0.014	0.999	0.981– 1.018	0.948
ETBR expression (Low vs. High)	0.003	2.104	1.108 – 3.996	0.023

Abbreviations: HR=hazard ratio; 95% CI=95% confidence interval