Quantification of tumour and circulating vascular endothelial growth factor (VEGF) in patients with oesophagogastric cancer: a long-term follow-up study

R. T. GRAY[1], M. E. O'DONNELL[2,3], J. A. McGUIGAN[1] and G. M. SPENCE[2]

[1]Department of Upper Gastrointestinal Surgery, Ulster Hospital, Dundonald; [2]Faculty of Life and Health Sciences, University of Ulster, Belfast; and [3]Department of Thoracic Surgery, Royal Victoria Hospital, Belfast, Northern Ireland, UK

Accepted: 1 May 2012

ABSTRACT

Vascular endothelial growth factor (VEGF) is an angiogenic cytokine that regulates tumour angiogenesis. The prognostic significance of VEGF expression remains incompletely investigated for patients with oesophagogastric cancer. This study assesses the significance of tumour VEGF (T-VEGF) and circulating VEGF (C-VEGF) expression in a 10-year follow-up of patients with oesophagogastric cancer. Patients undergoing surgical resection were prospectively recruited between February 1999 and August 2000. Circulating VEGF, derived both from plasma (P-VEGF) and serum (S-VEGF), and T-VEGF were assessed using a commercial enzyme-linked immunosorbent assay (ELISA). As platelet count may contribute to C-VEGF, pre-operative platelet levels were also recorded to exclude a confounding effect. Patients were followed up over a 10-year period using the Northern Ireland Cancer Registry. Sixty-one patients were recruited (men=45) with a mean age of 65.7 years. The 10-year survival was 19.7% (n=12) with a median follow-up of 808 days (inter-quartile range [IQR]: 349.5–2358.5). Union for International Cancer Control (UICC) tumour staging was Stage I=9 (14.8%), Stage II=15 (24.6%), Stage III=33 (54.1%) and Stage IV=4 (6.6%). The only significant relationship between clinicopathological features and the study variables was for S-VEGF, which was elevated in patients with advanced T-stage (P=0.05). Circulating VEGF did not correlate with platelet count. Although a trend towards decreased survival was observed for patients who had positive lymph nodes (P=0.08) and advanced UICC stage (P=0.09) on univariate analysis, only lymphovascular invasion significantly predicted poor prognosis in this cohort (P=0.05). Therefore, ELISA quantification of circulatory or tumour VEGF does not appear to be a significant predictor of mortality in patients with oesophagogastric cancer.

KEY WORDS: Esophagus.
Neoplasms.
Stomach.
Vascular endothelial growth factors.

Introduction

There is a compelling body of evidence linking tumour growth, metastasis and poor outcome with tumour angiogenesis in many solid malignancies.[1] Vascular endothelial growth factor (VEGF) is a pro-angiogenic cytokine that is important in tumour angiogenesis.[2] Tumour VEGF (T-VEGF) is over-expressed in 30–60% of patients with oesophageal carcinoma. In patients with squamous cell carcinoma it correlates with lymph node metastases and outcome.[3,4] However, T-VEGF expression does not appear to correlate with aggressive tumour characteristics or patient outcome in oesophageal adenocarcinoma, although it does correlate with the transition from Barrett's oesophagus to high-grade dysplasia, and with the transition from carcinoma *in situ* to locally advanced disease.[3,4]

Measurement of VEGF levels within the systemic circulation (C-VEGF) has been identified as a useful predictor of survival in other solid organ malignancies, such as colorectal and small cell lung carcinoma.[5-7] The prognostic significance of C-VEGF in oesophagogastric cancer remains unclear.[3] Circulating VEGF has been assayed predominantly in serum, but the authors have previously implicated *ex vivo* platelet activation in the formation of serum as a potential artificial source of elevated VEGF.[8] Consequently, plasma VEGF (P-VEGF) may be a more accurate marker of circulating VEGF *in vivo*.

In an earlier study, the authors investigated the relationship between T-VEGF and C-VEGF as potential markers of angiogenesis, and the presence of bone marrow micrometastases, a surrogate marker of tumour cell dissemination, in patients with oesophagogastric cancer.[9] Plasma VEGF correlated with the detection of micrometastatic cells in bone marrow, but T-VEGF did not. The significance of bone marrow micrometastases as a marker of tumour cell dissemination and adverse prognosis in oesophagogastric cancer remains unproven.[10]

Consequently, the objective of this study is to assess the direct prognostic significance of P-VEGF, S-VEGF and T-VEGF in patients with oesophagogastric cancer. The prognostic significance of pre-operative platelet count is also assessed as platelets are a potential source of VEGF in the circulation.

Correspondence to: Dr Ronan Gray
42 Brooke Hall Heights, Belfast BT8 6WN, Northern Ireland, UK
Email: rgray05@qub.ac.uk

Table 1. Relationship between clinicopathological factors and T-VEGF (pg VEGF/mg total protein), P-VEGF (pg/mL), S-VEGF (pg/mL) and pre-operative platelet count (x10⁹/L).

	T-VEGF			P-VEGF		
Variable	***n***	**Median (IQR)**	***P* value***	***n***	**Median (IQR)**	***P* value***
Age						
<65	17	242.1 (129.1–435.7)		24	15.36 (15.4–25.4)	
≥65	32	272.3 (152.8–454.2)	0.67	37	19.6 (4.7–44.5)	0.40
Gender						
Male	40	236.3 (137.3–451.8)		45	17.7 (0.0–25.3)	
Female	9	353.4 (216.4–511.6)	0.31	16	19.7 (13.1–54.6)	0.21
Cell type						
Adeno	40	283.9 (155.0–494.9)		47	17.7 (0.0–32.1)	
SCC	9	170.1 (83.3–310.1)	0.07	14	19.1 (12.6–44.4)	0.41
T stage						
1–2	15	168.7 (109.7–620.9)		20	18.6 (9.6–25.2)	
3–4	34	283.9 (161.0–413.9)	0.41	41	18.6 (0.0–41.9)	0.87
N stage						
Positive	33	290.0 (158.1–486.8)		41	18.6 (0–41.9)	
Negative	16	205.3 (113.8–392.4)	0.34	20	18.6 (2.4–25.5)	0.73
UICC stage						
1–2	19	242.1 (103.3–620.9)		24	18.6 (9.6–25.5)	
3–4	30	278.4 (160.1–413.9)	0.58	37	18.6 (0.0–41.9)	0.96
Grade						
Well–moderate	27	242.1 (151.2–368.1)		33	19.6 (11.4–40.7)	
Poor	22	279.9 (131.6–504.9)	0.67	28	13.0 (0.0–30.0)	0.14
LVI						
Yes	39	277.8 (154.1–470.8)		50	17.6 (0.0–36.9)	
No	10	205.3 (100.4–471.2)	0.44	11	22.7 (13.0–27.5)	0.37

Adeno: adenocarcinoma, SCC: squamous cell carcinoma, UICC: Union for International Cancer Control, LVI: lymphovascular invasion.
*Mann-Whitney U.

Materials and methods

Patients with oesophagogastric malignancies scheduled to undergo surgical resection were prospectively recruited between February 1999 and August 2000. As was the standard treatment at time of recruitment, no patient received neoadjuvant chemotherapy. The authors have previously described the methodology,[9] which is summarised briefly below.

Pathological stage was classified according to the Union for International Cancer Control (UICC) tumour/node/metastasis (TMN) classification (5th edn).[11] Tissue blocks were obtained randomly from each tumour specimen and then bisected, with one half embedded in paraffin wax for histological assessment and the other snap-frozen and stored in liquid nitrogen for enzyme-linked immunosorbent assay (ELISA) assessment of VEGF.

Samples (20 mL) of peripheral venous blood were obtained from each patient the day before surgery for measurement of platelet count, P-VEGF and S-VEGF.[8,9]

Vascular endothelial growth factor protein expression in tumour blocks was analysed by ELISA methodology, using the technique of Crew *et al.*[12] The snap-frozen sample was homogenised and underwent ultracentrifugation. The cell cytosolic fraction was assayed quantatively for T-VEGF using a commercial ELISA kit for human VEGF (Quantikine, R&D Systems, Minneapolis MN). Tumour VEGF levels were standardised for total protein concentration to allow for variation in size of the tumour blocks. Results were expressed as pg VEGF/mg total protein.[9] Plasma VEGF and S-VEGF were measured using the same commercial ELISA kit, and were expressed as pg/mL.[8]

Patients were followed up until May 2010 through analysis of the Northern Ireland Cancer Register, supplemented with additional data from the patient's general practitioner, as required. Cause and date of death were recorded for each patient.

Data were expressed as mean (standard error of the mean [SE]) or median (inter-quartile range [IQR]) where appropriate. Mann-Whitney U test was used to analyse the association between the study variables and standard clinicopathological features. Spearman's rank correlation coefficient was used to assess the relationship between C-VEGF and platelet count. Univariate survival analysis was calculated using Cox's proportional hazard model. Survival analysis was calculated using the Kaplan-Meier estimation method with log rank test to assess the equality of survivorship function according to patient characteristics.

S-VEGF			Pre-op platelet count		
n	Median (IQR)	*P* value*	*n*	Median (IQR)	*P* value*
24	229.2 (124.2–431.8)		24	264.0 (201.3–351.3)	
37	305.0 (174.9–493.2)	0.10	37	269.0 (220.0–330.0)	0.85
45	269.3 (136.0–442.8)		45	260.0 (203.5–340.0)	
16	291.2 (140.3–470.2)	0.99	16	292.5 (244.0–338.0)	0.23
47	277.5 (138.4–471.0)		47	265.0 (220.0–337.0)	
14	219.7 (111.8–389.1)	0.43	14	305.5 (217.0–343.8)	0.53
20	255.2 (126.1–304.5)		20	275.0 (224.8–320.8)	
41	314.1 (170.3–536.5)	0.05	41	265.0 (212.5–344.5)	0.98
41	260.3 (136.0–513.4)		41	265.0 (220.0–343.0)	
20	290.3 (140.8–339.8)	0.52	20	272.0 (208.8–320.8)	0.84
24	289.3 (140.8–339.8)		24	272.0 (216.5–320.8)	
37	260.3 (136.0–536.5)	0.49	37	265.0 (220.0–344.5)	0.84
33	313.5 (180.9–438.4)		33	259.0 (195.5–318.5)	
28	229.1 (117.3–475.1)	0.11	28	278.5 (231.5–353.5)	0.17
50	264.8 (137.2–479.5)		50	268.0 (220.0–338.5)	
11	305.0 (125.0–343.3)	0.71	11	259.0 (194.0–354.0)	0.89

All statistical tests were two-sided and differences were considered significant at $P<0.05$. Statistical analysis was performed using Stata/IC 10.1 for Windows, while the software package R 2.12.2 was used to produce the survival graphs. Informed consent was obtained from all patients and ethical approval was obtained from the Research Ethics Committee of the Queen's University of Belfast (Application Number 1/99).

Results

Patient demographics

Sixty-one patients were recruited, of which 45 (73.8%) were men. Mean age was 65.7 years (range: 39–83).

Pathological features

Tumour was located in the middle third of the oesophagus in three patients (4.9%), the lower third in 20 (32.8%) and at the oesophagogastric junction/cardia in 38 (62.3%). Forty-seven adenocarcinomas (77.0%) and 14 squamous cell carcinomas (23.0%) were resected. Histopathological assessment included: T-stage: T1=6 (9.8%), T2=14 (23.0%) and T3=41 (67.2%). Tumour staging (UICC) was Stage I=9 (14.8%), Stage II=15 (24.6%), Stage III=33 (54.1%) and Stage IV=4 (6.6%). Three tumours (4.9%) were well differentiated, 30 (49.2%) were moderately differentiated and 28 (45.9%) were poorly differentiated. Forty-one (67.2%) cases were lymph node-positive, 50 (82.0%) had lymphovascular invasion, and 24 (39.3%) had perineural invasion.

T-VEGF, C-VEGF and platelet count

The median (range) value for P-VEGF was 18.57 (0–363.05) pg/mL, S-VEGF was 277.4 (55.24–926.74) pg/mL, T-VEGF was 260.99 (34.87–3761.1) pg VEGF/mg total protein, and platelet count was 268x10^9/L (102–487). The relationship between clinicopathological features and the variables P-VEGF, S-VEGF, T-VEGF and pre-operative platelet count is summarised in Table 1. Comparing adenocarcinomas with squamous cell carcinomas, there was a trend towards elevated T-VEGF ($P=0.07$). However, the only difference identified was that increased S-VEGF was observed in patients with advanced T-stage ($P=0.05$). Correlation between platelet count and C-VEGF level was low (S-VEGF $r=0.22$, P-VEGF $r=0.27$).

Survival

Overall, five- and 10-year survival rates were 29.5% ($n=18$) and 19.7% ($n=12$), respectively, with a median follow-up of

808 days (IQR: 349.5–2358.5). Kaplan-Meier survival analysis demonstrated no difference in long-term outcome for patients with T-VEGF (P=0.75), P-VEGF (P=0.87), S-VEGF (P=0.92) and pre-operative platelet count (P=0.93) greater than the median value.

Univariate survival analysis of clinicopathological variables and the described biochemical markers revealed that only lymphovascular invasion significantly predicted poor prognosis in this cohort (P=0.05). A trend towards decreased survival was observed in patients with positive lymph nodes (P=0.08) and advanced UICC stage (P=0.09), but all other variables were not significant as predictors of poor prognosis.

Discussion

Although widely reported in squamous cell carcinoma, the role of T-VEGF in oesophageal adenocarcinoma is not as clear. A previous systematic review concluded that tumour VEGF expression failed to give prognostic information in invasive adenocarcinoma of the oesophagus.[3] The data reported in the present study have identified a non-significant trend towards elevation of T-VEGF level in patients with adenocarcinoma of the oesophagus. However, ELISA assessment of T-VEGF provided no obvious prognostic benefit. The findings of this study would concur with previous research on adenocarcinomas of the oesophagus and oesophagogastric junction; thus, the role of T-VEGF as a prognostic indicator is limited.

It has been postulated that C-VEGF may be a reliable and easily accessible surrogate marker of angiogenic activity and tumour progression in cancer patients.[5] Although some studies indicate that C-VEGF may be a useful predictor of survival in patients with cancer, not all studies agree.[13,14] In particular, the prognostic value of C-VEGF analysis in oesophagogastric cancer remains uncertain.[3] Such disparities have been attributed to the possibility that sources other than the tumour may contribute to VEGF level in the circulation.[5]

Vascular endothelial growth factor is released from activated platelets *ex vivo*. Consequently, S-VEGF levels may be artificially higher than *in vivo* levels. Such variance may depend on the total platelet level and may also reflect platelet-derived VEGF concentration, rather than being indicative of tumour derivation and actual tumour burden.[13,15] It has been postulated that the apparent prognostic importance of S-VEGF may simply be a function of the platelet count, rather than the angiogenic capability of the tumour *per se*.

Analysis of plasma levels, rather than that found in serum, avoids the coagulation cascade, thus restricting platelet activation. Consequently, P-VEGF rather than S-VEGF may more accurately represent tumour-derived circulating VEGF in patients with cancer.[13,15,16] Analysis of both of these VEGF derivatives in conjunction with platelet count was undertaken here in an attempt to discover which, if any, had prognostic significance. This study did not demonstrate any prognostic significance for P-VEGF, S-VEGF or platelet count over a 10-year follow-up period. However, the authors acknowledge potential study limitations relating to low patient numbers, recruitment of patients with both histological tumour subtypes, and to biological variation, which may have limited the ability of the study to detect prognostic significance of VEGF in the circulation, should it exist.

Recent studies continue to confirm that circulating and tumour VEGF, in particular the vascular endothelial growth factor C subtype (a known lymphangiogenic factor), correlates with lymph node metastasis and poor prognosis in patients with squamous cell carcinoma of the oesophagus.[17–21]

Future treatment with anti-VEGF therapy remains a focus of current research studies in solid tumour malignancies. Bevacizumab, a monoclonal antibody directed against VEGF, has been approved for the treatment of colorectal and non-small cell lung cancer. Anti-angiogenic properties combined with normalisation of the 'leaky' abnormal vasculature are postulated to be factors associated with its antitumour effects.[4] Bevacizumab is currently being evaluated in oesophagogastric cancer, both for locally advanced and metastatic disease, and long-term results are awaited.[4]

In conclusion, in a cohort of patients undergoing surgical resection for oesophagogastric cancer, where the adenocarcinoma cell subtype predominated, quantification of T-VEGF, C-VEGF and pre-operative platelet count did not demonstrate prognostic significance over a 10-year follow-up period. □

The authors wish to thank Rejina Verghis at the Clinical Research Support Centre, Belfast, for her assistance with the statistical analysis of this paper. The work was supported by grants from the Royal Victoria Hospital and Ulster Hospital Research Funds, the Mason Medical Foundation and the Ulster Cancer Foundation.

References

1 Folkman J. Angiogenesis and angiogenesis inhibition: an overview. *EXS* 1997; **79**: 1–8.
2 Ferrara N, Davis-Smyth T. The biology of vascular endothelial growth factor. *Endocr Rev* 1997; **18** (1): 4–25.
3 Kleespies A, Guba M, Jauch KW, Bruns CJ. Vascular endothelial growth factor in esophageal cancer. *J Surg Oncol* 2004; **87** (2): 95–104.
4 Ku GY, Ilson DH. Esophagogastric cancer: targeted agents. *Cancer Treat Rev* 2010; **36** (3): 235–48.
5 Poon RT, Fan ST, Wong J. Clinical implications of circulating angiogenic factors in cancer patients. *J Clin Oncol* 2001; **19** (4): 1207–25.
6 Hyodo I, Doi T, Endo H *et al.* Clinical significance of plasma vascular endothelial growth factor in gastrointestinal cancer. *Eur J Cancer* 1998; **34** (13): 2041–5.
7 Salven P, Ruotsalainen T, Mattson K, Joensuu H. High pre-treatment serum level of vascular endothelial growth factor (VEGF) is associated with poor outcome in small-cell lung cancer. *Int J Cancer* 1998; **79** (2): 144–6.
8 Spence GM, Graham AN, Mulholland K *et al.* Vascular endothelial growth factor levels in serum and plasma following esophageal cancer resection–relationship to platelet count. *Int J Biol Markers* 2002; **17** (2): 119–24.
9 Spence GM, Graham AN, Mulholland K *et al.* Bone marrow micrometastases and markers of angiogenesis in esophageal cancer. *Ann Thorac Surg* 2004; **78** (6): 1944–9.

10 Hermanek P, Hutter RV, Sobin LH, Wittekind C. International Union Against Cancer. Classification of isolated tumor cells and micrometastasis. *Cancer* 1999; **86** (12): 2668–73.
11 Sobin LH, Wittekind eds. *UICC TNM Classification of Malignant Tumours* 5th edn. New York: John Wiley & Sons, 1997.
12 Crew JP, O'Brien T, Bradburn M *et al.* Vascular endothelial growth factor is a predictor of relapse and stage progression in superficial bladder cancer. *Cancer Res* 1997; **57** (23): 5281–5.
13 Adams J, Carder PJ, Downey S *et al.* Vascular endothelial growth factor (VEGF) in breast cancer: comparison of plasma, serum, and tissue VEGF and microvessel density and effects of tamoxifen. *Cancer Res* 2000; **60** (11): 2898–905.
14 Takigawa N, Segawa Y, Fujimoto N, Hotta K, Eguchi K. Elevated vascular endothelial growth factor levels in sera of patients with lung cancer. *Anticancer Res* 1998; **18** (2B): 1251–4.
15 Banks RE, Forbes MA, Kinsey SE *et al.* Release of the angiogenic cytokine vascular endothelial growth factor (VEGF) from platelets: significance for VEGF measurements and cancer biology. *Br J Cancer* 1998; **77** (6): 956–64.
16 Wynendaele W, Derua R, Hoylaerts MF *et al.* Vascular endothelial growth factor measured in platelet poor plasma allows optimal separation between cancer patients and volunteers: a key to study an angiogenic marker *in vivo*? *Ann Oncol* 1999; **10** (8): 965–71.
17 Ogata Y, Fujita H, Yamana H, Sueyoshi S, Shirouzu K. Expression of vascular endothelial growth factor as a prognostic factor in node-positive squamous cell carcinoma in the thoracic esophagus: long-term follow-up study. *World J Surg* 2003; **27** (5): 584–9.
18 Tanaka T, Ishiguro H, Kuwabara Y *et al.* Vascular endothelial growth factor C (VEGF-C) in esophageal cancer correlates with lymph node metastasis and poor patient prognosis. *J Exp Clin Cancer Res* 2010; **29**: 83.
19 Mobius C, Freire J, Becker I *et al.* VEGF-C expression in squamous cell carcinoma and adenocarcinoma of the esophagus. *World J Surg* 2007; **31** (9): 1768–72.
20 Kozlowski M, Kowalczuk O, Milewski R, Chyczewski L, Niklinski J, Laudanski J. Serum vascular endothelial growth factors C and D in patients with oesophageal cancer. *Eur J Cardiothorac Surg* 2010; **38** (3): 260–7.
21 Kimura H, Kato H, Tanaka N *et al.* Preoperative serum vascular endothelial growth factor-C (VEGF-C) levels predict recurrence in patients with esophageal cancer. *Anticancer Res* 2008; **28** (1A): 165–9.