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Vascular Endothelial Growth Factor Polymorphisms and Esophageal Cancer Prognosis

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Abstract

Statement of translational relevance: application to future practice of medicine—The incidence of esophageal carcinoma is increasing at a rate exceeding that of most other solid malignancies. The disease has a poor prognosis with a <15% 5 year survival. Treatment strategies frequently involve multimodality therapy; however, the benefit from this approach is controversial. Identifying prognostic biomarkers may improve the selection of patients that will benefit from additional treatment. We report on the prognostic significance of vascular endothelial growth factor (*VEGF*) single nucleotide polymorphisms in esophageal cancer. We demonstrate a statistically significant association between the variant allele of a putatively functional *VEGF* polymorphism and overall survival in multivariate analysis. This demonstrates *VEGF* polymorphisms have potential prognostic capabilities in esophageal cancer, which may ultimately enhance the selection of patients with esophageal cancer that require additional treatment.

Purpose—Vascular endothelial growth factor (VEGF) promotes angiogenesis and vascular permeability. The *VEGF* gene is polymorphic. We investigated the prognostic significance of three *VEGF* single nucleotide polymorphisms (SNPs) in esophageal cancer.

Experimental Design—361 patients were genotyped for three *VEGF* SNPs (*−460T/C, +405G/C* and *+936C/T*), using DNA extracted from prospectively collected blood samples. The association of each individual SNP, and haplotypes of the three SNPs, on overall survival (OS) was investigated.

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Results—The variant allele of *+936C/T* was associated with improved OS compared with the wildtype genotype (log rank p<0.001). This association remained significant for OS after adjustments for age, gender, performance status and disease stage (OS AHR *VEGF 936 C/T* 0.70 (95% CI 0.49–0.99), p=0.04; *VEGF 936 T/T* 0.11 (95% CI 0.02–0.82), p=0.03). No independent associations were found for *VEGF −460T/C* and *VEGF 405G/C*. The *CGC* haplotype of the three *VEGF* SNPs *−460T/C,+405G/C* and *+936C/T* combined was associated with reduced OS compared with all other patients *(CGC/CGC* OS AHR 1.51, 95%CI 1.00–2.30, p=0.05).

Conclusions—*VEGF 936 C/T*, and a haplotype of *−460T/C,+405G/C* and *+936C/T* combined, has potential prognostic significance in esophageal cancer.

Keywords

Polymorphism; vascular endothelial growth factor; esophageal cancer; prognosis

INTRODUCTION

The incidence of esophageal cancer, particularly the adenocarcinoma subtype, is rising at an alarming rate (1). In 2008, the number of new cases of esophageal cancer in the United States is estimated to reach 16,470, but the number of deaths is estimated to approach 14,280, highlighting the poor prognosis of this disease (2). Multimodality treatment regimens, with chemoradiation and surgery, are frequently recommended, although the additional benefit from this approach is controversial (3). In addition, trimodality therapy is associated with significant side effects, and treatment delivery is challenging in this patient population in which co-morbidities are common. Prognostic factors have the potential to improve the selection of patients for whom additional treatments are most beneficial.

Angiogenesis is a regulated process of new vessel formation that has a role in a discrete number of normal physiological events, but is activated inappropriately in a range of diseases, and is an early event in carcinogenesis (4). Vascular endothelial growth factor A (VEGF) is the most potent angiogenic factor. VEGF induces endothelial cell proliferation and migration, enhances vascular permeability, reduces endothelial cell apoptosis and promotes stromal proteolysis. As in other malignancies, VEGF is of interest in esophageal cancer as a potential prognostic and predictive marker (5).

Multiple single nucleotide polymorphisms (SNP) have been identified within the *VEGF* gene (6). *VEGF −460T/C*, *+405G/C* and *+936C/T* are in linkage disequilibrium and are of interest due to their potential functional implications. The variant alleles of *VEGF 936C/T* and *VEGF 405G/C* are associated with reduced basal and stimulated levels of VEGF respectively, and haplotyes containing the *VEGF−460C* and *VEGF +405G* alleles are associated with increased promoter activity in luciferase reporter assays (7–9) Several association studies have demonstrated *VEGF* polymorphisms, that are associated with reduced VEGF expression confer prognostic significance (10–12). For example, in a cohort of patients with early stage non-small cell lung cancer, carriage of the *VEGF 405C* allele was associated with an improved overall survival (OS), with a trend for improved outcome associated with the *VEGF 936T* allele (10). Similarly, *VEGF −460C or 405G* genotypes was associated with a decreased OS in a large cohort of patients with breast cancer (11). However, results have not been consistent across all studies (13–17).

The prognostic significance of *VEGF* polymorphisms has not been evaluated in esophageal cancer. We postulated the variant alleles of *VEGF−460T/C*, *+405G/C* and *+936C/T* (associated with reduced VEGF expression) confers an improved prognosis in patients with esophageal cancer. This was investigated, prospectively, in a large cohort of patients with esophageal cancer by evaluating the association of each SNP independently, and in addition exploring the

prognostic significance of haplotypes of the three SNPs (*VEGF−460T/C*, *+405G/C* and *+936C/T*) combined.

METHODS

Patients

Patients diagnosed with esophageal cancer from 1997 onwards were recruited as part of a prospective series at Massachusetts General Hospital (MGH) and Dana Farber Cancer Institute (DFCI) Boston, MA. To ensure adequate follow up time, patients recruited up to October 2004 (n=404), were included in this investigation. The institutional review boards of MGH, DFCI and Princess Margaret Hospital, Toronto, ON, approved the study. Patient demographics and blood samples for genotyping were obtained at the time of entry to the study. Follow up variables including treatment, survival, and treatment toxicity data were collected prospectively (18). The participation rate for this study was 93%. Of the 404 patients recruited prior to October 2004, 361 were included in the analysis. The reasons for excluding 43 cases were as follows: 18 cases had no blood available for genotyping, six cases had no follow up data, 16 cases were missing at least some clinical data and in three cases the genotyping was unsuccessful.

Endpoint

The primary endpoint for the study was OS, defined as the time between date of histological diagnosis to date of death or last follow up.

SNP Selection and Genotyping

All patients were genotyped for three *VEGF* SNPs: *VEGF −460T/C* (rs833061), *VEGF +405G/C* (rs2010963), and *VEGF 936C/T* (rs3025039). These SNPs were selected using the following criteria: (1) associated with altered VEGF expression (7–9,19); (2) prognostic significance in other malignancies (10–12); (3) adequate frequency in Caucasians to enable evaluation.

DNA for genotyping was extracted from whole blood using the Puregene Isolation Kit (Gentra System, Minneapolis MN). Genotyping was performed using the TaqMan assay and the 384 Well ABI 7900HT Sequence Detection System (Applied BioSystems Foster City Ca). Conditions, pimers and probes are summarized in appendix 1.

Statistics

Univariate demographic and genotyping data were tabulated. The association between genotype and OS was assessed using the methods of Kaplan and Meier and Log Rank. Cox proportional Hazard ratios were adjusted for gender, age, performance status (PS), and disease stage. Statistical analyses were conducted at the 0.05 significance level using SAS software version 8.2 (SAS Institute, Cary NC). The association between individual *VEGF* SNPs and outcome was assessed using the additive model whereby the presence of each variant allele was assumed to impart an equal effect on outcome. The Bonferroni correction was applied to adjust the primary analysis for three comparisons.

In exploratory analysis, we evaluated if the *VEGF* SNPs had a different impact on OS in different histological subtypes of disease, or different stage of disease, by performing subgroup analyses in cohorts of patients with adenocarcinoma, squamous carcinoma, node negative disease, and lymph node positive/metastatic disease.

VEGF−460T/C, *+405G/C* and *+936C/T* are in linkage disequilibrium. To investigate the combined effect of the SNPs together haplotype analysis was performed. Haplotype

frequencies were estimated using PHASE (Bayesian approach). The association between haplotype and OS was investigated by applying Cox Proportional Hazard models to haplotype trend regression analyses using the estimated haplotype frequencies (20). The omnibus association test and haplotype specific association tests were performed (20–22). Adjustments were made for gender, age, PFS and disease stage.

Quality Control

The methodology utilized was generally in line with that described in the ReMARK recommendations for evaluation of prognostic markers (23). Details of the prospective data collection procedures have been previously reported (18). In summary, at each patient visit data was collected using a standardized outcomes collection form completed by physicians, which included the end point variables and data required to undertake multivariate analyses. Data abstraction and electronic recording procedures incorporate quality control audits to ensure accuracy and consistency. Blood for genotyping was collected at time of entry into the study. All sample collection and storage procedures were standardized. Genotyping results were double checked and a 5% sample was repeated for additional quality control.

RESULTS

Patient demographics are summarized in Table 1. The median follow up times for both OS and PFS was 34 months. During this time there were 213 deaths, and 237 patients had disease progression. The median OS for the entire cohort of patients was 30 months (53 node negative, 27 node positive and 15 for metastatic disease), with a progression free survival time of 16 months (42 node negative, 16 node positive and 8 for metastatic disease).

Genotype frequency VEGF−460T/C, +405G/C and +936C/T

All three SNPs were in Hardy Weinberg Equilibrium. The genotype frequency for the homozygous wildtype, heterozygous and homozygous variant, for each SNP was as follows: *VEGF −460T/C*- 25%, 50%, and 26%; *VEGF +405G/C*- 49%, 41% and 10%; *VEGF 936C/T*- 76%, 22%, and 2% respectively. The three SNPs were in linkage disequilibrium (Lewontin D' s were 0.88 and 0.81 for VEGF *−460T/C: 405G/C* and *405G/C: 936C/T* respectively).

Individual polymorphisms and overall survival

Of the three VEGF SNPs evaluated, no independent association was found for either *VEGF −460T/C* or *VEGF 405G/C* and OS (Table 2). However, for *VEGF 936C/T* patients carrying the heterozygous (*VEGF 936C/T*) or homozygous variant (*VEGF 936T/T*) genotype had improved OS in univariate analysis, compared to patients carrying the wildtype genotype (Figure 1, log rank $p<0.001$ and $p=0.002$ respectively). This association remained significant after adjustments for age, gender, PS and stage (Table 2).

We also evaluated the association between *VEGF 936C/T* and OS using a dominant model of inheritance, whereby the presence of at least one variant allele is sufficient to affect OS compared to the wildtype genotype. In multivariate analyses, the variant genotype (*VEGF 936 T*/-) was associated with improved OS compared with homozygous wildtype genotype. (AHR OS 0.63, 95%CI 0.44–0.89, $p=0.01$). This association remained significant after applying Bonferroni adjustment for the three SNPs evaluated in this analysis.

Exploratory Subgroup Analyses (Table 3)

We explored whether there was a different association between *VEGF* SNPs and histological subtype of esophageal cancer, by evaluating the association between *VEGF* genotype in patients with adenocarcinoma and squamous carcinoma separately. Adenocarcioma was the

predominant histological subtype (n=293, 81%). The association remained the same as in the primary analyses. Squamous carcinoma accounted for 56 patients. No specific associations were found for any of the *VEGF* SNPs including *VEGF 936C/T* across additive and dominant models of inheritance; however, patient subgroups were small (for example *VEGF 936C/T* homozygous wildtype 50 patients, heterozygous 12 patients, homozygous variant 0 patients). We did not identify any different associations in cohorts of patients defined by disease stage compared with those identified for the entire patient cohort (Table 3). Additional subset analysis by cisplatin treatment, trimodality treatment, and restriction to Caucasians resulted in similar magnitude and direction of results as the entire cohort (data not presented).

Haplotype Analysis

As the three *VEGF* SNPs are in linkage disequilibrium, we performed haplotype analyses to assess the prognostic significance of all three SNPs combined (*VEGF −460T/C*, *405 G/C*, *936C/T*). Six haplotypes were inferred. The frequencies of these haplotypes were as follows *CGC−* 41%, *TCC* −27%, *TGC−* 18%, *CGT−* 10%, *TCT−* 3% and *TGT−* 1%. The global test for all estimated haplotypes on OS was p=0.0001 and p=0.02 after adjustment for age, gender, PS and disease stage. Of these haplotypes, *CGC* was found to confer a poorer prognosis compared with the other haplotypes: *CGC/CGC* (AHR 1.51, 95%CI 1.00–2.30, p=0.05) and *CGC*/---- OS AHR 1.37, 95%CI 1.00–1.88), p=0.05.

DISCUSSION

We evaluated the prognostic significance of three *VEGF* SNPs in a large cohort of patients with esophageal cancer. In multivariate analysis, we demonstrated the heterozygous and homozygous variant genotype of *VEGF +936C/T* conferred an improved OS compared with the homozygous wildtype genotype. Although we did not find an independent association for two of the *VEGF* SNPs evaluated and OS (*VEGF −460T/C* and *+405G/C*), we identified the *CGC* haplotype was associated with a poorer outcome compared with the other haplotypes combined. This association may be driven by *VEGF 936C/T* which we found to be independently associated with OS. Alternatively, this may reflect an accumulative effect of the three SNPs together, or additional SNPs that have not been evaluated in this study but are in the same haplotype block.

This association may arise from reduced VEGF expression reported with the variant allele of *VEGF 936C/T*. (9,19). The premise that increased VEGF protein expression confers a poor prognosis in esophageal cancer has been reported in a number of, but not all, studies (24–26). VEGF protein expression has been correlated with microvascularisation in Barrett's and adenocarcinoma of the esophagous (26–27). Furthermore, a stepwise increase in VEGF expression has been identified from metaplasia to dysplasia and adenocarcinoma, indicating angiogenesis may be an early event in transformation and potential explanation for early micrometastatic spread (28). In squamous carcinoma an association between intra-tumoural VEGF expression and microvessel density (24), disease stage (24), and as a prognostic factor has been reported (24–25).

The prognostic significance of these *VEGF* SNPs has been evaluated in a range of malignancies (10–17). While this is the first study to evaluate *VEGF* SNPs in esophageal cancer, two prior gastric cancer studies reported conflicting results (12,16). In the study of 503 gastric cancer patients by Kim et al. patients carrying the homozygous variant allele of *VEGF 936C/T* had a poorer OS relative to the wildtype genotype. In contradistinction, we identified this as the favorable genotype; however, this genotype is rare (<2%), and therefore caution is required when interpreting these results in small cohorts of patients. In our study, we also found patients carrying the heterozygous genotype (frequency 22%, n=79), had an improved prognosis in both additive and dominant models, in keeping with that of a non-small cell lung cancer study

(n=462) which demonstrated a trend for significance favoring the variant allele of *VEGF 936C/T* (10). Prior studies have reported an association between *VEGF −405T/C* and *VEGF 460G/C* and overall OS predominantly in patients with early stage cancers (10,12). We did not find specific associations in esophageal cancer patients with early stage (lymph node negative) disease; however, our early stage cohort is underpowered, given the propensity to diagnose esophageal cancer at more advanced stages.

Identifying prognostic markers in esophageal cancer has clinical applicability. The difficulty of accurately staging esophageal cancer has led to aggressive treatment being recommended to patients across a range of stages of the disease. This poses a challenge in the design of clinical trials, as trials of novel agents may fail at an early stage, as benefit in subgroups of patients may be masked by inclusion of patients who may have either a particularly good or poor prognosis disease at the outset. The use of prognostic factors to stratify patients more precisely into at risk groups, and guide clinical trial design has the potential to avoid these issues. Further, as anti-angiogenic targeted therapies are now being evaluated in the treatment of esophageal cancer it is timely to evaluate the prognostic impact of germline variation in the pathway in patients receiving standard therapies.

There are limitations to this study. Although others have correlated these *VEGF* SNPs with plasma VEGF levels (9,19), due to the lack of available tissue samples, we were unable to correlate *VEGF* genotype with VEGF mRNA or protein expression within tumours. In any case, the association between genotype and VEGF expression may be technically challenging as no association was found for lung cancer, despite identifying positive genotype-survival outcomes (unpublished supplementary data to Heist et al. (9)). The mechanism by which altered VEGF expression from germline variation impacts on outcome, may arise early in the disease process, through the promotion of early vascularisation of metastases, or reflect an interaction between the tumor and the cellular environment which also bears the same germline variation. This may not be reflective in the assessment of VEGF expression from available (sometimes archival) primary tumor samples. Further, this analysis will also be compounded by VEGF expression from the accumulation of somatic VEGF genetic aberrations. Secondly, the sample size of 361 is very large for esophageal cancer, but is only average for all studies evaluating *VEGF* polymorphisms and cancer outcomes (median sample size 413, range 100–1193) (10–17). Finally, we utilized a candidate polymorphism approach which allows us to compare with studies of other disease sites and focuses on functional variants, but therefore will not evaluate the entirety of polymorphic variation across this gene.

In conclusion, this is the first study to evaluate the prognostic significance of *VEGF* polymorphisms in a large cohort of esophageal cancer patients. We report the variant allele of *VEGF 936C/T* is associated with an improved overall survival compared with the wildtype genotype. Further study and validation is necessary to determine the potential clinical impact of these findings.

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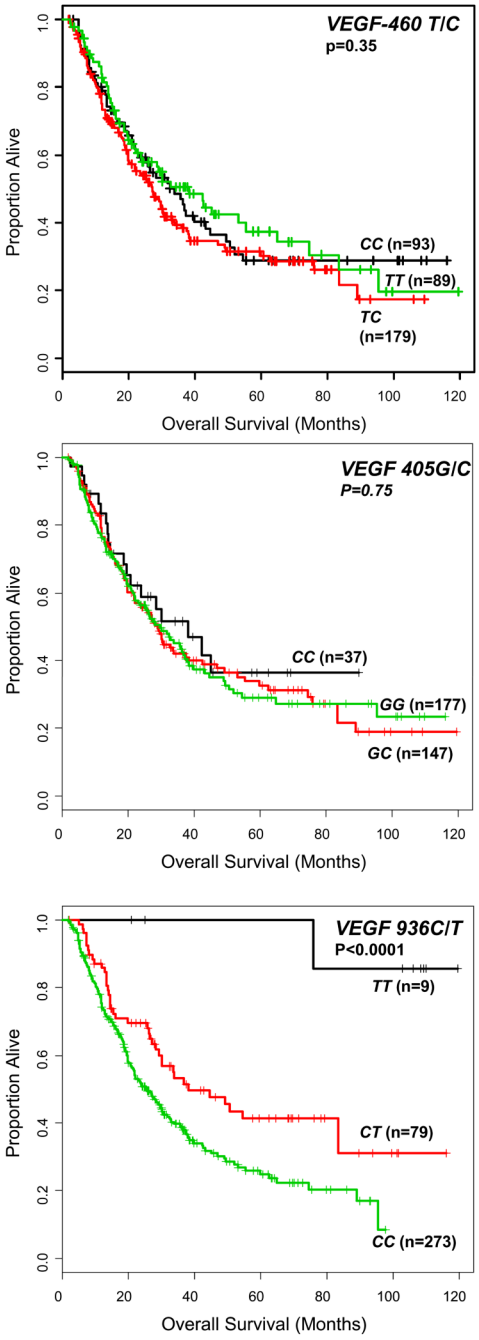

Figure 1.
Kaplan Meier Curves of Vascular Endothelial Growth Factor (*VEGF*) *−460T/C, +405G/C and 936C/T* and Overall Survival (OS). Log-Rank p values displayed

Table 1

Patient demographics and treatment characteristics

Characteristic	Result (%)
Gender	
Male	312 (86)
Female	49 (14)
Age	
Median (range)	64 (21–91)
Ethnicity	
Caucasian	348 (96)
Other	13 (4)
Performance Status	
0–1	310 (86)
2	42 (12)
3	9 (2)
Smoking Status[a]	
Never Smoker	72 (20)
Ever Smoker	190 (53)
Current Smoker	96 (27)
Stage[*a]	
Lymph Node Negative (I-IIA)	102 (28)
Lymph Node Positive (IIA-IVA)	192 (53)
Metastatic (IVB)	66 (18)
Tumor Site[a]	
Mid-upper esophagous	39(13)
Distal esophagous	192 (62)
Gastro-esophageal	78 (25)
Histology	
Adenocarcinoma	293 (81)
Squamous	56 (16)
Poorly Differentiated/Other	12(3)
Treatment[*]	
Surgery alone	61 (17)
Tri-modality (chemo-radiation and surgery)	189 (52)
Adjuvant chemo-radiation	16 (4)

Characteristic	Result (%)
Neoadjuvant chemotherapy	3 (1)
Adjuvant chemotherapy	7 (2)
Definitive chemo-radiation	38 (11)
Chemotherapy alone	35(10)
Radiation therapy alone	5 (1)
Photodynamic Therapy	2 (0.5)
Other	5 (1)

[*] May not add up to 100% due to rounding

[a] = unknown in 3 cases for smoking status, 1 case for disease stage and 55 cases for tumor site

Table 2

Association between Vascular Endothelial Growth Factor (*VEGF*) *−460T/C*, *VEGF 405G/C* and *936C/T* and Overall Survival (OS).

			Overall Survival	
Genotype	**Number**	**Deaths**	**Median (Months)**	**AHR* (95% CI), p value**
VEGF −460T/C				
TT	89	50	38	Reference
TC	179	110	27	1.29 (0.91–1.83),0.15
CC	93	53	34	1.20 (0.80–1.81),0.38
VEGF 405G/C				
GG	177	100	30	Reference
GC	147	94	29	0.93 (0.69–1.25), 0.63
CC	37	19	38	0.76 (0.46–1.25), 0.28
VEGF 936C/T				
CC	**273**	**171**	**25**	**Reference**
CT	**79**	**41**	**38**	**0.70 (0.49–0.99), 0.04**
TT	**9**	**1**	**NR**	**0.11 (0.02–0.82), 0.03**

Abbreviations: AHR- adjusted hazard ratio. CI- confidence interval. NR- not reached

*Adjusted for age, gender, performance status and disease stage.

Table 3

Association between Vascular Endothelial Growth Factor (*VEGF*) *−460T/C*, *VEGF 405G/C* and *936C/T* and Overall Survival (OS) in patient subgroups defined by histology and disease stage.

Genotype	Adenocarcinoma	Squamous Carcinoma	Lymph Node Negative	Lymph Node Positive/Metastatic
	AHR* (95%CI),p value	AHR* (95%CI),p value	AHR* (95%CI),p value	AHR* (95%CI),p value
Overall Survival				
VEGF −460T/C				
TT	Reference	Reference	Reference	Reference
TC	1.34 (0.90–1.98),0.15	0.94 (0.39–2.28),0.89	0.85 (0.44–1.66),0.64	1.32 (0.89–1.95),0.17
CC	1.24 (0.78–1.97),0.37	1.32 (0.44–3.95),0.62	0.24 (0.03–1.87),0.17	1.28 (0.80–2.05),0.30
VEGF 405G/C				
GG	Reference	Reference	Reference	Reference:
GC	0.94 (0.68–1.30), 0.70	0.76 (0.33–1.78),0.53	1.11 (0.52–2.37),0.79	0.91 (0.65–1.26),0.57
CC	0.80 (0.46–1.41),0.45	0.73 (0.21–2.56),0.62	0.91 (0.38–2.15),0.82	0.79 (0.47–1.34),0.38
VEGF 936C/T				
CC	Reference	Reference	Reference	Reference:
CT	**0.67 (0.45–0.99), 0.04**	0.93(0.38–2.26),0.87	0.54 (0.25–1.16),0.11	**0.63 (0.42–0.93),0.02**
TT	**0.13 (0.02–0.94), 0.04**	**	0.15 (0.02–1.18),0.07	**
T/-	**0.60 (0.41–0.89), 0.01**	0.93(0.38–2.26),0.87	**0.44 (0.21–0.92),0.03**	**0.56 (0.38–0.84),0.005**

Abbreviations: AHR- adjusted hazard ratio. CI- confidence interval

*Adenocarcinoma and Squamous carcinoma subgroups adjusted for age, gender, performance status, and disease stage. Lymph node negative and lymph node positive/metastatic subgroups adjusted for age, gender, performance status.

**Too few patients/events to analyze