Association of Genetic Polymorphisms in the *VEGF* Gene with Breast Cancer Survival

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Abstract

The vascular endothelial growth factor (VEGF) is an important regulator of angiogenesis and vascular permeability. *VEGF* overexpression has been associated with advanced stage and poor survival of several cancers. We evaluated the association of functional polymorphisms in the *VEGF* gene with breast cancer survival in a cohort of 1,193 breast cancer patients who were recruited as part of a population-based case-control study in Shanghai, China from 1996 to 1998 and followed for cancer recurrence and mortality between March 2000 and December 2002. Included in the study were three functional polymorphisms (*C−460T*, *G+405C*, and *C+936T*) in the *VEGF* gene. Carrying the *−460C* or *+405G* allele was associated with decreased overall survival. The age-adjusted hazard ratios (HR) were 1.5 [95% confidence interval (95% CI), 0.9-2.5] for *−460CC* genotype carriers and 1.6 (95% CI, 1.0-2.5) for *+405GG* genotype carriers compared with noncarriers. Further analyses showed that the *−460T/+450C/+936C* haplotype was related to increased survival (HR, 0.57; 95% CI, 0.4-0.9), whereas the *−460C/+405G/+936T* haplotype was associated with nonsignificantly decreased survival (HR, 2.1; 95% CI, −0.9 to 4.7). The *C+936T* polymorphism alone was not related to overall or disease-free survival. This study suggests that *VEGF* polymorphisms may be a significant genetic marker for breast cancer prognosis. (Cancer Res 2005; 65(12): 5015-9)

Introduction

The growth of solid tumors, including breast cancer tumors, depends on angiogenesis, the process by which new blood vessels develop from the endothelium of a preexisting vasculature. Tumors promote angiogenesis by secreting or activating angiogenic factors that stimulate endothelial migration, proliferation, and capillary morphogenesis. Newly formed blood vessels supply the tumor with nutrients and oxygen, dispose of the metabolic waste products of tumor cells, generate paracrine stimuli, and provide potential routes for tumor dissemination. Thus, tumor-induced angiogenesis plays a pivotal role in cancer progression and metastasis (1, 2).

The vascular endothelial growth factor (VEGF) is one of the most potent endothelial cell mitogens and plays a critical role in angiogenesis. VEGF specifically binds to two transmembrane VEGF receptor tyrosine kinases on endothelial cells to initiate intracellular signal transduction pathways that mediate angiogenesis and vascular permeability (3). Several lines of compelling evidence from *in vitro* and *in vivo* experiments have shown that increased *VEGF* expression is associated with tumor growth and metastasis, whereas the inhibition of VEGF signaling results in suppression of both tumor-induced angiogenesis and tumor growth (4). Clinically, high levels of blood, urinary, or intratumoral VEGF and increased microvessel density in tumors have been observed to be associated with advanced stage disease and worse prognosis for several types of tumors, including breast cancer (2, 5–8).

The *VEGF* gene is located on chromosome 6p21.3. At least 30 single-nucleotide polymorphisms (SNP) in this gene have been described in the literature. Of particular note are three SNPs (*G+405C* in the 5′-untranslated region, *C−460T* in the promoter region, and *C+936T* in the 3′-untranslated region) that are common and are related to VEGF protein production (9–12). The *+936T* allele has been related to lower levels of plasma VEGF in healthy men (9) and women (10), whereas the *+405G* allele has been shown to significantly increase lipopolysaccharide-stimulated VEGF protein production in peripheral blood mononuclear cells (11). Furthermore, carrying a haplotype containing the *+405G* and *−460C* polymorphisms was found to significantly increase basal VEGF promoter activity and phorbol ester–induced responsiveness (12). These SNPs have been implicated in the risk for several types of tumors and other diseases with a putative angiogenic basis (10, 13–15). However, the effect of these polymorphisms on breast cancer prognosis has not been evaluated. Based on the biological and pathologic significance of VEGF, it is possible that functional genetic variations in the *VEGF* gene may contribute to the progression of breast cancer. To test this hypothesis, we evaluated the effects of these three functional polymorphisms (*G+405C*, *C−460T*, and *C+936T*) in the *VEGF* gene on breast cancer survival in a cohort of breast cancer patients who had participated in a population-based case-control study, the Shanghai Breast Cancer Study (16).

Materials and Methods

Participants. Of the 1,459 breast cancer patients who were recruited into the Shanghai Breast Cancer Study from August 1996 to March 1998 and ages 25 to 64 years, 1,455 were included in the survival study. Four subjects were excluded due to lack of adequate information for follow-up. The initial institutional diagnoses of breast cancer were confirmed by independent review of pathologic slides by two senior pathologists. A peripheral blood sample (10 mL from each woman) was obtained from 1,193 (82%) study participants at recruitment. The blood samples were typically processed within 6 hours of collection and stored at −70°C until the relevant bioassays were conducted. Information on cancer diagnosis, disease stage (tumor-node-metastasis stage, TNM stage), cancer treatments, and estrogen and progesterone receptor (ER/PR) status was abstracted from medical charts using a standard protocol.

Patients were followed for cancer recurrence and mortality between March 2000 and December 2002 with a combination of active follow-up and record linkage to the death certificates kept by the Vital Statistics Unit of the Shanghai Center for Disease Control and Prevention (17). Of the 1,455

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Table 1. Overall survival by demographics and known breast cancer prognosis factors, the Shanghai Breast Cancer Study

Covariables	Levels	All cases (n = 1,455)				Subjects with genotype information (n = 1,119)			
		No. cases	No. deaths	5-y survival, %*	P	No. cases	No. deaths	5-y survival, %*	P
Age at diagnosis	<40	211	39	83.5	<0.01	174	32	83.4	0.03
	40-49	700	98	86.1		553	75	86.7	
	50-59	374	80	78.8		265	56	79.1	
	60-64	170	23	86.7		127	18	86.2	
TNM	0-I	358	23	94.2	<0.01	282	18	94.1	<0.01
	IIa	508	59	88.5		396	45	88.9	
	IIb	320	63	80.8		247	50	80.5	
	III-IV	165	69	58.6		123	49	60.2	
ER	Positive	647	99	85.1	0.47	496	75	85.5	0.44
	Negative	371	62	83.5		285	44	84.6	
	Unknown	437	79	82.4		338	62	81.7	
PR	Positive	634	95	85.3	0.42	497	75	85.2	0.42
	Negative	368	64	82.9		275	42	85.0	
	Unknown	453	81	82.8		347	64	82.5	
Surgery	Yes	1,446	235	84.2	—	1,113	178	84.5	—
	No	1	1	—		0	0	—	
	Unknown	8	4	50.0		6	3	50.0	
Chemotherapy	Yes	1,367	223	84.1	0.01	1,046	167	84.6	0.08
	No	70	10	85.3		59	9	84.4	
	Unknown	18	7	60.9		14	5	64.3	
Radiotherapy	Yes	566	136	76.2	<0.01	425	103	76.0	<0.01
	No	690	75	89.9		533	57	90.2	
	Unknown	199	29	85.3		161	21	86.9	
Total		1,455	240	83.9		1,119	181	84.3	

*Proportion of survival derived from Kaplan-Meier analysis.

eligible patients, 1,290 (88.4%) were followed-up via in-person contact or by phone from March 2000 to December 2002. Among them, 200 patients were deceased. Through interview of patients, or next of kin for deceased patients, we obtained information on disease progress, recurrence, quality of life, and cause of death (if deceased). Survival status for the remaining 165 participants was established in June 2003 by linkage to the death registry. Through the linkage, 40 deaths were identified; information on the date of death and cause of death was obtained. One hundred twenty-six subjects had no match in the death registry and were assumed to be still living. Their date of last contact was assigned to be December 30, 2002, 6 months before our search of the vital statistics registry, to allow for a possible delay of entry of the death certificates into the registry. This study was approved by the Institutional Review Board of Vanderbilt University and all other participating institutes; consent was obtained for all study participants.

DNA isolation and genotyping assays. Genomic DNA was extracted from buffy coat fractions using a Puregene DNA Purification kit (Gentra System, Minneapolis, MN) following the manufacturer's protocol. DNA concentration was measured using a PicoGreen dsDNA Quantitation Kit (Molecular Probes, Eugene, OR). The allelic discrimination of the *VEGF* gene polymorphisms was assessed with the ABI PRISM 7900 Sequence Detection Systems (Applied Biosystems, Foster City, CA), using the fluorogenic 5′ nuclease assay with Taqman Minor Groove Binder (MGB) probes. The wild-type Taqman MGB probes were FAM labeled and the mutant probes were VIC labeled. The final volume for each reaction was 5 μL, consisting of 2.5 μL Taqman Universal PCR Master Mix (Applied Biosystems), 0.6 μL of each primer, 0.2 μL of each Taqman probe, and 2.5 ng genomic DNA. The PCR profile was an initial denaturation step at 95°C for 10 minutes and 40 to 55 cycles with 92°C for 15 seconds and 60°C for 1 minute. Fluorescent signals were measured at 60°C. The primers and probes for the *G+405C* (rs2010963) and the *C−460T* (rs833061) polymorphism assays (assay on demand), as well as for the *C+936T* (rs3025039) polymorphism assay (Assay-by-Design), were ordered from Applied Biosystems.

The laboratory staff was blind to the identity of the subjects. Quality control samples were included in the genotyping assays. Each 384-well plate contained four water, eight CEPH 1347-02 DNA, eight blinded quality control samples, and eight unblinded quality control samples. The blinded and unblinded quality control samples were taken from the second tube of study samples included in the study. The concordance for the blinded samples was >97%. Genotypes for polymorphisms of *G+405C, C−460T,* and *C+936T* in the *VEGF* gene were successfully determined for 1,091, 1,119, and 1,105 patients, respectively.

Statistical analysis. The primary outcomes used for this study were overall survival and disease-free survival. The end point for the overall survival analysis was any death and for the disease-free survival analysis was cancer recurrence/metastasis or death related to breast cancer. Survival time was calculated as the time from cancer diagnosis to the end points of the study, censoring at the date of last contact or noncancer death (for disease-free survival only). Due to the lack of disease relapse information, the 165 patients whose survival status was established via linkage with the death registry were excluded from the disease-free survival analysis. The Kaplan-Meier method was used to compute 5-year survival rates and the log-rank test was applied to test the differences in survival across different genotypes. The Cox regression model was applied to evaluate the effect of the *VEGF* genotype on overall survival and disease-free survival with adjustments for age. Analyses were also conducted by stratifying the data by characteristics of breast cancer to examine the potential interactive effects.

We employed the software PHASE (version 2.1), which uses a Bayesian statistical method (18), to derive haplotypes for the *VEGF* gene. The

association of haplotypes and breast cancer survival was evaluated using a Cox model by treating each haplotype as a continuous variable with probabilistically assigned values, as recommended by Zaykin (19). All tests were based on two-sided probability.

Results

Table 1 shows selected demographic factors and known prognostic factors of breast cancer and the corresponding 5-year survival rates for the whole cohort of 1,455 breast cancer patients and the 1,119 subjects who were included in the genotype association study. As expected, older age and TNM stage were the major prognostic factors for survival. Virtually, all (99%) patients received surgery and 94% of patients received chemotherapy. Radiotherapy was related with poorer prognosis, an effect reflecting late stage at diagnosis. ER/PR status was not predictive of survival in this population. However, information regarding ER/PR status was missing for a sizable number of subjects. The results were similar for the entire cohort and the subgroup of patients included in the current analysis.

The frequencies of the genotypes were 36.1% (*GG*), 46.4% (*GC*), and 17.5% (*CC*) for *G+450C;* 7.9% (*CC*), 37.2% (*CT*), and 54.9% (*TT*) for *C−460T;* and 67.1% (*CC*), 30.1% (*CT*), 2.8% (*TT*) for *C+936T;* all followed the Hardy-Weinberg equilibrium. The *VEGF G+405C* and *C−460T* polymorphisms were in strong linkage disequilibrium (correlation coefficient, $R = 0.47$; Lewontin's D', $D' = 0.94$) in the study population, whereas their linkage with the *T+936C* polymorphism was much weaker ($R = 0.08$; $D' = 0.21$ and $R = 0.04$; $D' = 0.27$, respectively).

Table 2 shows the association of VEGF genotypes with overall and disease-free survival. During the study period, there were 181 deaths among subjects who were included in the genotype study. Of these, 165 (91%) died of breast cancer. The *−460CC* genotype was found to be associated with lower overall survival and disease-free survival rates. The age-adjusted hazards ratios (HR) associated with the *−405CC* genotype were 1.5 [95% confidence interval (95% CI), 0.9-2.5] and 1.5 (95% CI, 1.0-2.4), respectively, for overall survival and disease-free survival compared with the −406TT genotype. Patients with the *+405GG* genotype had a significantly lower overall survival rate (80.3%) than those with the *+405CC* genotype (87.8%). The age-adjusted HRs associated with the *+405GG* genotype as compared with the *+405CC* genotype were 1.6 (95% CI, 1.0-2.5). This polymorphism, however, was not related to disease-free survival (HR, 1.0; 95% CI, 0.7-1.5 for the +405GG genotype compared with the *+405CC* genotype.) The Kaplan-Meier survival curves by genotypes of *G+405C* and *C−460T* are presented in Fig. 1*A* and *B*, showing a clear association of overall survival after diagnosis of breast cancer with the genotypes defined by these SNPs. No clear association was observed between *C+936T* polymorphism and overall survival or disease-free survival.

Haplotype analyses were conducted to evaluate the combined effect of the three polymorphisms on breast cancer survival (Table 3). The three most common haplotypes in this cohort of breast cancer patients were *−460T/+450G/+936C, −460T/+405C/+936C,* and *−460C/+405G/+936C,* with the respective frequencies being 30.0%, 32.5%, and 17.8%. The *−460T/+405/+936C* haplotype was related to increased overall survival (HR, 0.57; 95% CI, 0.4-0.9). The *−460G/+405G/+936T* (HR, 2.1; 95% CI, −0.9 to 4.7) and *−460C/+405G/+936C* (HR, 1.4; 95% CI, 0.8-2.4) haplotypes were associated with decreased overall survival, although the point estimates were not statistically significant. No significant associations between haplotypes and disease-free survival were observed.

The associations of the *VEGF G+405C* and *C−460T* genotypes with tumor characteristics at diagnosis were examined. We found that these polymorphisms were unrelated to the TNM, ER, and PR status of the cancer at diagnosis. Additional adjustment for those

Table 2. G+405C, C−460T, and C+936T polymorphisms of *VEGF* gene in association with breast cancer survival, the Shanghai Breast Cancer Study

VEGF	Cases	Overall survival			Disease-free survival		
Genotype		Events	5-y survival %*	HR† (95% CI)	Events	5-y survival %*	HR†,‡ (95% CI)
C−460T							
TT	614	92	85.7	1.0	107	80.8	1.0
CT	417	70	83.4	1.2 (0.8-1.6)	74	79.5	1.1 (0.8-1.4)
CC	88	19	78.7	1.5 (0.9-2.5)	22	72.3	1.5 (1.0-2.4)
P_{trend}			0.19	0.11		0.16	0.14
G+405C							
CC	191	26	87.8	1.0	39	77.5	1.0
GC	506	77	85.3	1.2 (0.8-1.8)	78	82.2	0.7 (0.5-1.1)
GG	394	78	80.3	1.6 (1.0-2.5)	83	76.7	1.0 (0.7-1.5)
P			0.03	0.02		0.10	0.48
C+936T							
CC	741	114	85.0	1.0	133	80.2	1.0
CT	333	59	83.1	1.2 (0.9-1.6)	60	79.3	1.0 (0.8-1.4)
TT	31	6	79.8	1.3 (0.6-2.9)	4	83.4	0.8 (0.3-2.2)
P_{trend}			0.46	0.28		0.80	0.98

*Proportion of survival derived from Kaplan-Meier analysis.
†Adjusted for age.
‡Subjects with missing information on disease relapse ($n = 165$) were excluded from this analysis.

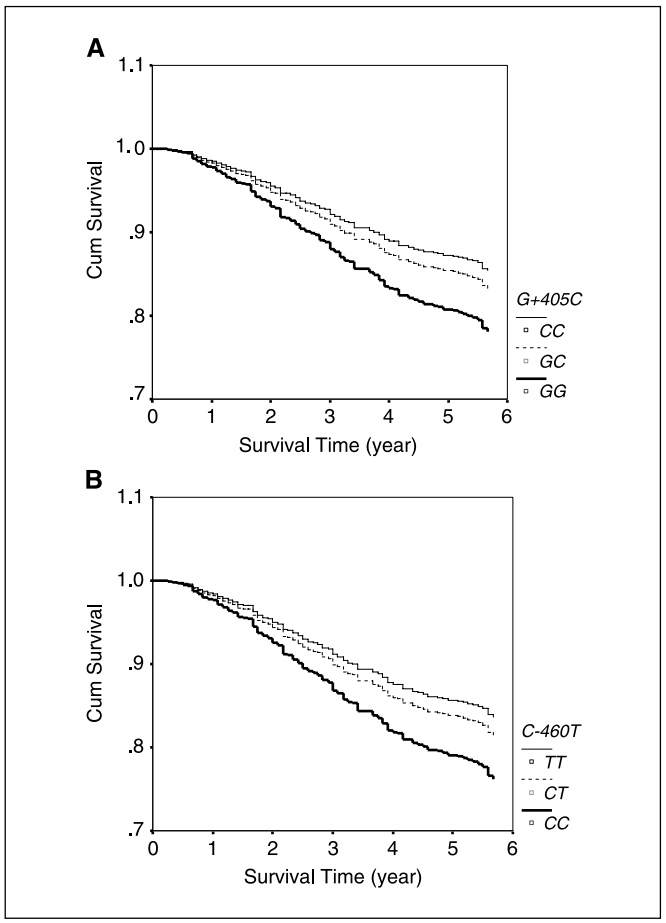

Figure 1. Overall survival of patients after diagnosis of breast cancer by *VEGF G+405C* and *C−460T* polymorphisms. *A,* patients with the *+405GG* genotype had a lower survival rate than those with the +405CC genotype. *B,* patients with the *−460CC* genotype had a lower survival rate than those with the *−460TT* genotype.

tumor characteristics in the Cox regression model did not materially change the genotype-survival associations (data not shown). We found no major modifying effect of these tumor characteristics on the gene-survival associations (data not shown).

Discussion

Angiogenesis is not only essential for tumor growth but also plays a critical role in the invasion and metastasis of tumor cells. Angiogenesis is regulated by many growth factors, among which VEGF plays a central role and serves as an important prognostic factor in a variety of tumors, including breast cancers. A number of functional polymorphisms in the *VEGF* gene have been reported and have been associated with increased risk for several tumors (10, 14). To our knowledge, no previous study has reported an association of *VEGF* gene polymorphism with breast cancer survival.

The genotype frequencies of the *G+405C* and *C+936T* polymorphisms in the current study population are similar to those reported in Japanese and European populations (9, 10, 13). The frequency of the *−460TT* genotype (54.4%) in our study population, however, is higher than those reported in two previous, smaller studies (30% and 36.1%, based on <200 subjects; refs. 14, 15). We found that *G+405C* and *C−460T* polymorphisms are in strong linkage disequilibrium in our study population, consistent with findings from an earlier study (11). Survival after breast cancer diagnosis was lower among patients with the *−460CC* and *+405GG* genotypes and higher among subjects with the *−460T/+405C/+936C* haplotype. These findings are biologically plausible, given the pivotal role the *VEGF* gene plays in tumor growth and progression and the functional significance of these SNPs. It is well documented that overexpression of the *VEGF* gene and high blood or tumor levels of VEGF is associated with poor breast cancer progression (2, 8) and high levels of intratumoral VEGF predicts poor response to systemic therapy in advanced breast cancer (6, 7). The *G+405C GG* genotype has been previously linked to significantly increased VEGF production (11). The *+936T* allele of *VEGF* has also been reported to be associated with lower VEGF plasma levels in healthy subjects (9, 10) and with decreased risk for breast cancer (10). In a recent *in vitro* study, carrying a haplotype containing the *+405G* and *−460C* polymorphisms was found to significantly increase basal *VEGF* promoter activity and phorbol ester–induced responsiveness (12). Several VEGF inhibitors have currently progressed into cancer clinical trials in different tumor types, including breast cancer, and preliminary data are very encouraging (20). If the individual angiogenic potential could be predicted on the basis of *VEGF* genotypes, the efficacy of

Table 3. Association of VEGF haplotype and breast cancer survival, the Shanghai Breast Cancer Study

Haplotypes (*C−460T/G+405C/C+936T*)	Haplotype frequency*	Overall (*n* = 1,129)		Disease-free (*n* = 1,006)	
		HR (95% CI) †	*P*	HR (95% CI)	*P*
TGC	30.0	1.2 (0.7-1.9)	0.54	0.8 (0.5-1.3)	0.44
TGT	5.1	1.4 (0.4-4.6)	0.59	0.7 (0.2-2.8)	0.63
TCC	32.5	0.6 (0.4-0.9)	0.02	1.0 (0.6-1.5)	0.87
TCT	6.0	0.9 (0.3-2.5)	0.76	0.6 (0.2-1.7)	0.30
CGC	17.8	1.4 (0.8-2.4)	0.23	1.3 (0.8-2.2)	0.25
CGT	6.4	2.1 (0.9-4.7)	0.08	1.6 (0.8-3.4)	0.20
CCC	1.8	0.3 (0.0-7.5)	0.48	0.3 (0.0-17.7)	0.56
CCT	0.4				

*Haplotype frequency was derived using the program PHASE.
†HRs were derived from the Cox regression model by treating the probability of each haplotype as a continuous independent variable.

antiangiogenic treatment in solid tumors, including breast cancer, could be further enhanced.

It is worth mentioning that the associations in this study between the single SNPs or haplotypes of the *VEGF* gene and disease-free survival were not as clear as those observed for overall survival. However, the information on disease relapse and cause of death in this study was based on self-reports or reports from next of kin (for the deceased subjects). The misclassification in ascertaining the outcomes for disease-free survival is most likely to be undifferential, and thus could have biased results toward null. In addition, information on relapse was missing for 165 subjects whose survival status was established via linkage with the death registry. The reduced sample size in the disease-free survival analysis compromised the statistical power of this analysis.

In summary, we found that the *+450G* and *−460C* alleles in the *VEGF* gene were significantly associated with poorer survival after diagnosis of breast cancer, and that the *−460T/+405C/+936C* haplotype was associated with more favorable survival outcomes. These findings were based on a large cohort of breast cancer patients who were systematically identified and recruited with a high response rate, which minimized potential selection bias. Given the homogeneous ethnic background of Chinese women living in Shanghai (>98% belong to a single ethnic group), any potential confounding effect due to ethnicity is likely to be small. The association found in this study is biologically plausible and may have significant implications for breast cancer treatment.

Acknowledgments

Received 8/4/2004; revised 11/15/2004; accepted 2/3/2005.

Grant support: National Cancer Institute grants USPHS RO1CA64277 and RO1CA90899.

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We thank Drs. Qi Dai and Fan Jin for their contributions in coordinating data and specimen collection in Shanghai, Bethanie Hull for her assistance in the preparation of this article, and all the study participants and research staff of the Shanghai Breast Cancer Study for support.

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Cancer Res 2005;65:5015-5019.