Population-Based Case-Control Study of *VEGF* Gene Polymorphisms and Breast Cancer Risk among Chinese Women

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Abstract

Vascular endothelial growth factor (VEGF) is a major angiogenic factor involved in a number of pathologic processes, including neovascularization, a crucial step in the development of solid malignancies. Using data and specimens collected in the Shanghai Breast Cancer Study, a population-based case-control study conducted in urban Shanghai, China from 1996 to 1998, we evaluated the association of *VEGF* gene polymorphisms with breast cancer risk. Included in this study were 1,093 cases and 1,184 age-matched controls who had completed an in-person interview and donated a blood sample to the study. Polymorphisms in the promoter region (T−460C), 5′ untranslated region (C+405G), and 3′untranslated region (C936T) were genotyped using the Taqman allelic discrimination assay. No statistically significant case-control difference was found for the C+405G and T−460C polymorphisms. However, the C936T polymorphism was associated with a reduced risk of breast cancer. Compared with CC genotype carriers, women who had the TT genotype showed a decreased risk [odds ratio (OR), 0.65; 95% confidence interval (95% CI) 0.41-1.02], and the inverse association was restricted to premenopausal women (OR, 0.45; 95% CI, 0.25-0.79). Six common haplotypes were identified. Compared with the most common haplotype (−460T/405C/936C), the −460T/405G/936T haplotype was associated with a reduced risk of breast cancer (OR, 0.67; 95% CI, 0.43-1.04), particularly in premenopausal women (OR, 0.47; 95% CI, 0.27-0.81). Our study suggests that the *VEGF* C936T polymorphism might be a susceptibility factor for breast cancer among Chinese women. (Cancer Epidemiol Biomarkers Prev 2006;15(6):1148–52)

Introduction

Vascular endothelial growth factor (VEGF) is an endothelial cell-specific mitogen, an angiogenic inducer, and a mediator of vascular permeability (1). New blood vessels (neovascularization) are required by most solid tumors not only to supply metabolic demands but also to provide potential routes for tumor dissemination and metastasis (2). In breast cancer tissue, *VEGF* mRNA expression is increased compared with adjacent normal breast tissue (3). Moreover, high tissue VEGF levels seem to correlate with poor prognosis and decreased overall survival for breast cancer patients (4). Currently, targeting VEGF pathways is one of the strategies in the treatment of cancer (5).

The human *VEGF* gene is located on chromosome 6p21.3 and contains eight exons, separated by seven introns, and its coding region spans ~14 kb (6, 7). The promoter region and 5′-untranslated region of the *VEGF* gene were first screened for polymorphisms by Watson et al. (8). Fifteen novel polymorphisms were identified, and +405GG genotype of C+405G polymorphism was significantly associated with increased peripheral blood mononuclear cell VEGF protein production stimulated by lipopolysaccharide (8). Haplotype analyses of single nucleotide polymorphisms (SNP) in these regions showed that carriage of the −460C and +405C alleles significantly alters VEGF promoter activity and responsiveness (9). Renner et al. reported three novel polymorphisms (C702T, C936T, and G1612A) in the 3′-untranslated region and found that carriers of the 936T allele had significantly lower VEGF plasma levels than noncarriers (10).

Several studies have investigated the association of *VEGF* gene polymorphisms with diseases in which angiogenesis plays a major role in pathogenesis, such as diabetic retinopathy (11), renal cell carcinoma (12), acute renal allograft rejection (13), prostate cancer (14), and malignant melanoma (15). The results, however, were mixed. Thus far, three studies have investigated the association of *VEGF* polymorphisms with breast cancer risk with inconsistent results. Krippl et al. (16) reported that the T allele of VEGF 936C/T was associated with decreased breast cancer risk. Recently, Jin et al. (17) reported that the *VEGF* polymorphisms −2578C/A, −1154G/A, and 936C/T were not associated with breast cancer risk. Smith et al. (18) also reported that the −1154G/A polymorphism was not associated with breast cancer risk. We focused this study on the investigation of three functional SNPs: G+450C, C−460T, and C936T. Based on the data reported thus far regarding the functionality of these SNPs, we hypothesized that *VEGF* −406C, +405G, and 936C alleles might be associated with increased breast cancer risk and evaluated this hypothesis in the Shanghai Breast Cancer Study, a large population-based case-control study conducted among Chinese women in Shanghai.

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Materials and Methods

Study Subjects. Subjects included in this study were those recruited from 1996 to 1998 for the Shanghai Breast Cancer Study, a population-based case-control study. Details of the study methodology have been described elsewhere (19). Briefly, cases were identified through the population-based Shanghai Cancer Registry that ascertains virtually all cancer

cases diagnosed among residents of urban Shanghai. The controls were randomly selected from the general female population in Shanghai and frequency matched to cases by age (5-year interval). A structured questionnaire was used during an in-person interview to elicit information on demographic features, menstrual and reproductive history, sex hormone use, dietary habits, prior disease history, physical activities, tobacco and alcohol use, weight, and family history of cancer. Menopause was defined as a cessation of the menstrual cycle for 12 months or longer, excluding those periods caused by pregnancy or breast-feeding. In-person interviews were completed for 1,459 (91.1%) of the 1,601 eligible breast cancer cases newly diagnosed in the region during the study period and 1,556 (90.3%) of the 1,724 eligible controls. Of those who completed the in-person interviews, 2,503 [1,193 (81.8%) cases and 1,310 (84.2%) controls] donated blood samples. The buffy coats were stored at −70°C for subsequent DNA isolation. Cancer diagnoses for all patients were confirmed by two senior study pathologists through a review of tumor slides.

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 by 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 probes. The wild-type Taqman Minor Groove Binder 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 of 92°C for 15 seconds and 60°C for 1 minute. Fluorescent signals were measured at 60°C. Primers and probes for G+405C (rs2010963, Assays-on-Demand), C-460T (rs833061, Assays-by-Design), and T936C (rs3025039, Assays-by-Design) were obtained 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%.

Statistical Analysis. The χ^2 test was used to evaluate case-control differences in the distribution of allele types and genotypes. Breast cancer risk associated with each *VEGF* genotype was estimated using logistic regression models conditioned on age to accommodate the age frequency-matched study design. Odds ratios (OR) and their 95% confidence intervals were used to measure the strength of the association between *VEGF* gene polymorphisms and breast cancer risk (20, 21). Evaluated as potential confounders in the study were those that were independently related to breast cancer risk, such as family history of breast cancer, history of fibroadenoma, menopausal status, physical activity, age at menarche, age at first birth, body mass index, and waist-to-hip ratio. The former five variables were included in the model as categorical variables, whereas the latter four variables were included in the model as continuous variables. Adjusting for these factors, however, did not appreciably change the ORs. Therefore, we only present the age-adjusted ORs. The presence of interaction was assessed using the likelihood ratio test by comparing the model including the main effects only, with that including both the main effects and the possible interactive variables. We employed the software PHASE (version 2.1), which used a Bayesian statistical method (22), to derive haplotypes for the *VEGF* gene. The association between haplotypes and breast cancer risk was evaluated using logistic regression models. All statistical tests were based on two-tailed probability.

Results

The distribution of selected demographic characteristics and major risk factors for breast cancer in the Shanghai Breast Cancer Study have been previously reported (19) and is presented briefly in Table 1. Breast cancer cases and controls were comparable in age and education levels. An elevated risk of breast cancer was observed for all known major breast cancer risk factors, including a prior history of breast fibroadenoma, physical inactivity, higher waist-to-hip ratio, higher body mass index, early onset of menarche, late onset of menopause, and late age at first live birth. Participants in the current study were similar to those of parent study with regard to all abovementioned factors (data not shown).

Allele frequencies for the individual polymorphisms among controls were similar to those reported previously in other Asian populations (11, 23). Minor alleles (frequency) among controls were C (0.26) in T−460C, C (0.40) in C+405G, and T (0.19) in C936T polymorphisms (Table 1). No statistically significant differences were found in allele distributions between cases and controls. Hardy-Weinberg equilibrium was observed for all polymorphisms in controls ($P > 0.05$).

The frequencies of *VEGF* genotypes by case-control status and the association between *VEGF* genotypes and breast cancer risk are presented in Table 2. The homozygous TT genotype of the C936T polymorphism was associated with a decreased breast cancer risk (OR, 0.65; 95% confidence interval, 0.41-1.02) compared with the CC genotype. The inverse association was confined to premenopausal women (OR, 0.45; 95% confidence interval, 0.25-0.79). No statistically significant association was found for the other two SNPs, and no apparent interaction was found for these SNPs in relation to breast cancer risk (data not shown). In addition, no apparent interaction was found for any of these SNPs with estrogen-related factors, such as age at menarche, age at first live birth, body mass index, and waist-to-hip ratio.

Associations of breast cancer risk with *VEGF* haplotypes are shown in Table 3. Six common haplotypes were identified to account for >99% of all haplotypes tagged by these three SNPs. Consistent with the genotype analysis, two (CGT and TGT) of three haplotypes that contain the T allele at the C936T were associated with a reduced risk of breast cancer in premenopausal women. In particular, an OR of 0.47 (95% confidence interval, 0.27-0.81) was statistically significant for the −460T/405G/936T haplotype in premenopausal women.

Discussion

Angiogenesis is essential for tumor growth and 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. The association of *VEGF* gene polymorphisms with disease risk attracts a great deal of attention because VEGF is one of the most potent angiogenic factors and plays a significant role in the development of solid tumors. As mentioned previously, several studies have evaluated the association of VEGF polymorphisms with breast cancer risk (16-18). The results, however, have been inconsistent. In this study, we found that

Table 1. Comparison of cases and controls by selected demographic characteristics, major risk factors for breast cancer in the Shanghai Breast Cancer Study

Subject characteristics*	Case (n = 1,133)	Control (n = 1,233)	P
Demographic factors			
Age (y)	47.6 ± 8.0	47.2 ± 8.7	0.213
Education ≥ high school (%)	43.5	42.9	0.765
Major risk factors			
Breast cancer in first-degree relatives (%)	3.4	2.4	0.113
Ever had breast fibroadenoma (%)	9.7	5.1	<0.001
Age at menarche (y)	14.5 ± 1.6	14.7 ± 1.7	0.002
Age at the first birth (y) †	26.8 ± 4.1	26.2 ± 3.8	<0.001
Age at menopause (y) ‡	48.2 ± 4.6	47.5 ± 5.0	0.042
Postmenopause (%)	32.9	36.1	0.108
Hormone replacement therapy (%)	2.6	2.6	0.962
No regularly physical activity (%)	80.7	74.1	<0.001
Body mass index (kg/m^2)	23.5 ± 3.4	23.2 ± 3.4	0.028
Waist-to-hip ratio	0.81 ± 0.06	0.80 ± 0.06	0.004
VEGF polymorphisms§			
T−460C (rs833061, promoter)			
T	1,650 (73.5)	1,809 (74.0)	0.67
C	596 (26.5)	635 (26.0)	
C+405G (rs2010963, 5′UTR)			
C	892 (40.7)	962 (40.2)	0.69
G	1,298 (59.3)	1,434 (59.8)	
C936T (rs3025039, 3′UTR)			
C	1,822 (82.1)	1,937 (81.0)	0.34
T	396 (17.9)	453 (19.0)	

Abbreviation: UTR, untranslated region.
*Values are presented as means ± SD among cases and controls unless otherwise noted.
† Among parous women.
‡ Among postmenopausal women.
§Values are presented as number of chromosomes and (%) among cases and controls.

women who carry the TT genotype in the C936T polymorphism had a decreased risk of breast cancer among premenopausal women.

The C936T polymorphism has been reported to be associated with lower VEGF plasma levels (10, 20). Those who are homozygous for TT have lower VEGF production compared with the CC genotype, which, in turn, may decrease the risk of tumor development (10). This laboratory observation supports the finding from our study of an inverse association of the 936T allele with breast cancer risk. Consistent with our study, Krippl et al. have recently reported that carriers of a 936T allele had a decreased risk of breast cancer (16). The 936T allele was also found to be associated with a reduced uptake of ^{18}F-fluorodeoxyglucose, used for detection and staging of breast cancer (24). On the other hand, a recently published study showed no association of breast cancer risk with several *VEGF* SNPs, including the C936T variant (17). The number of subjects with the 936T/T genotype, however, was small in that study that included cases and controls recruited from multiple sources in Poland, Germany, and Sweden.

Table 2. Association of *VEGF* polymorphisms and breast cancer risk by age at diagnosis and menopausal status (the Shanghai Breast Cancer Study, 1996-1998)

Genotype	All subjects		Menopausal status			
			Premenopausal		Postmenopausal	
	Cases/controls	OR (95%CI)*	Cases/Controls	OR (95% CI)*	Cases/Controls	OR (95% CI)*
T−460C						
TT	616/665	1.00 (reference)	410/410	1.00 (reference)	203/254	1.00 (reference)
TC	418/479	0.94 (0.79-1.12)	288/316	0.92 (0.75-1.14)	128/160	1.01 (0.75-1.37)
CC	89/78	1.23 (0.89-1.70)	54/53	1.06 (0.70-1.59)	35/25	1.69 (0.98-2.93)
P_{trend}	0.775		0.629		0.201	
			$P_{interaction}$ † = 0.410			
C+405G						
CC	192/182	1.00 (reference)	118/109	1.00 (reference)	72/72	1.00 (reference)
CG	508/598	0.81 (0.64-1.02)	350/375	0.90 (0.66-1.21)	156/222	0.74 (0.50-1.09)
GG	395/418	0.90 (0.70-1.14)	266/287	0.90 (0.65-1.21)	128/129	1.00 (0.66-1.51)
P_{trend}	0.670		0.439		0.602	
			$P_{interaction}$ = 0.173			
C936T						
CC	744/793	1.00 (reference)	507/501	1.00 (reference)	234/290	1.00 (reference)
CT	334/351	1.01 (0.85-1.21)	215/225	0.93 (0.74-1.17)	117/124	1.17 (0.86-1.59)
TT	31/51	0.65 (0.41-1.02)	18/41	0.45 (0.25-0.79)	13/10	1.60 (0.69-3.73)
P_{trend}	0.392		0.041		0.179	
			$P_{interaction}$ = 0.041			

Abbreviations: OR, odds ratio; 95% CI, 95% confidence interval.
*The odds ratios, 95% confidence interval, and Ps for trend test were derived from logistic models, adjusting for age.
† $P_{interaction}$ of T−460C, C+405G, and C936T with menopausal status.

Table 3. Associations between *VEGF* haplotype and breast cancer risk

Haplotype*	All subjects (%)			Premenopause			Postmenopause		
	Cases (%)	Controls (%)	OR (95% CI)	Cases (%)	Controls (%)	OR (95% CI)	Cases (%)	Controls (%)	OR (95% CI)
T-C-C	34.1	33.6	1.00 (reference)	33.3	31.6	1.00 (reference)	35.2	36.7	1.00 (reference)
T-G-C	29.6	29.8	0.97 (0.84-1.11)	30.4	29.9	0.96 (0.80-1.14)	28.4	29.8	0.97 (0.77-1.24)
C-G-C	17.7	17.0	1.02 (0.86-1.21)	18.7	17.8	1.01 (0.82-1.25)	15.7	15.7	1.01 (0.75-1.36)
C-G-T	8.2	8.4	0.96 (0.78-1.18)	7.0	8.6	0.80 (0.61-1.03)	10.3	7.5	1.37 (0.97-1.95)
T-C-T	5.9	6.0	0.98 (0.76-1.25)	6.0	6.3	0.96 (0.71-1.31)	5.6	5.8	0.96 (0.63-1.48)
T-G-T	3.9	4.6	0.67 (0.43-1.04)	3.9	5.0	0.47 (0.27-0.81)	3.7	3.7	1.07 (0.65-3.16)

Abbreviations: OR, odds ratio; 95% CI, 95% confidence interval.
*In the order of T−460C, C+405G, and C936T. Haplotype frequency was derived using the program PHASE.

We found in this study that the association of the *VEGF* 936TT genotype with breast cancer risk was restricted to premenopausal women who have a much higher estrogen level than postmenopausal women. Estrogen exposure plays a central role in the development and progression of breast cancer (25, 26). Estrogen modulates angiogenesis, under both physiologic and pathologic conditions, mainly via effects on endothelial cells (27). In addition, estrogen has been reported to increase *VEGF* mRNA expression and protein in breast cancer cells (28) and isolated endometrial cells (29, 30). Recently, a positive correlation between estradiol and VEGF was shown in normal human breast tissue *in vivo* with microdialysis system (31). These data suggest that VEGF plays a significant role in the development of breast and other hormone-related cancers.

The mechanism by which the *VEGF* 936T allele leads to lower VEGF plasma levels is currently unknown. Several potential mechanisms have been suggested: (*a*) the C-to-T transition may lead to the loss of a potential binding site for AP-4, a transcription factor; (*b*) this polymorphism may be in linkage disequilibrium with another unknown polymorphism elsewhere; and (*c*) the C-to-T transition may lead to a change of mRNA structure (10).

The rate of type I errors is a major concern in association studies of genetic factors. Wacholder et al. (32) proposed to evaluate type I errors using the false-positive report probability method. For prior probabilities of 0.10 and 0.25, we obtained values for the false-positive report probability of 0.089 and 0.031, respectively, for the positive association of 936TT genotype with breast cancer risk observed in the study. These false-positive report probability values are below the 0.20 level proposed by Wacholder et al.'s criterion (32) as acceptable, providing some assurance for the validity of the positive association observed in this study.

We did not detect statistically significant associations of breast cancer with the two SNPs (C−460T and C+405G) in the promoter region and 5′-untranslated region of the *VEGF* gene. As mentioned previously, there is evidence suggesting that these two SNPs may be of functional significance (8, 9). Previous studies on the association of polymorphisms in the promoter and 5′-untranslated region with disease susceptibility produced mixed results; some showing an association [diabetic retinopathy (11) and acute renal allograft rejection (13)] and some showing no association [malignant melanoma (15), preterm delivery (33), and prostate cancer (14)]. The contribution of these polymorphisms, therefore, may vary by disease or organ. Intriguingly, we reported very recently that carrying either the −460C or the +405G allele was associated with decreased overall survival, whereas the C936T polymorphism was not related to survival (34). Further investigation to explain this discrepancy may be needed.

The participation rate in this study was high, minimizing the potential selection bias that is common to many case-control studies. Chinese women living in Shanghai are relatively homogenous in ethnic background; >98% of them are in a single ethnic group (Han Chinese). The sample size of this study is large, which provides a stable estimate of the association of breast cancer risk with *VEGF* gene polymorphisms. The strong methodology of the study, along with biological plausibility of the findings, lends support to the theory that *VEGF* C936T polymorphisms may play an important role in breast cancer development.

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