Breast Cancer Res Treat (2008) 109:297–304
DOI 10.1007/s10549-007-9655-z

PRECLINICAL STUDY

Genetic polymorphisms in the vascular endothelial growth factor gene and breast cancer risk. The Austrian "tumor of breast tissue: incidence, genetics, and environmental risk factors" study

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Received: 6 June 2007 / Accepted: 7 June 2007 / Published online: 17 July 2007
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Abstract
Purpose Vascular endothelial growth factor (VEGF) is a key regulator of tumor-induced angiogenesis and is required for growth of tumors. We tested the hypothesis that VEGF gene polymorphisms may be associated with breast cancer.
Experimental design We performed a case–control study including 804 female incident breast cancer patients and 804 female age-matched healthy control subjects. We selected seven VEGF candidate polymorphisms and determined genotypes by 5′-nuclease (TaqMan) assays. Furthermore, VEGF plasma levels and genotypes were analyzed in a group of 81 healthy volunteers (64 men and 17 women).
Results Haplotype analysis showed two separate blocks of high-linkage disequilibrium, formed by five polymorphisms upstream of the coding sequence (promoter and 5′ untranslated region) and two polymorphisms downstream of the coding sequence. None of the single polymorphisms or haplotypes was significantly associated with the presence of breast cancer. After Bonferroni correction for multiple testing, only one statistical signifcant association between VEGF genotypes and haplotypes and tumor characteristics was observed (-634C allele and small tumor size; $p < 0.001$). In a multivariate regression analysis including sex, age, VEGF genotypes, and haplotypes as covariates and VEGF plasma level as dependent variable, none of the VEGF polymorphism or haplotypes was a significant predictor of VEGF plasma levels.
Conclusions Our findings do not support the hypothesis that VEGF polymorphisms are associated with breast cancer risk. The association of the VEGF -634C allele with small tumor size is in clear contrast to a previous publication and should be interpreted with caution until replicated by additional studies.

Keywords Breast cancer · Vegf polymorphisms · Haplotypes · Genetics · Epidemiology · Vegf plasma levels

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Introduction

Tumor growth and progression requires the formation of new blood vessels, a process called angiogenesis. Angiogenesis is a complex multifactorial process involving a variety of proangiogenic and proteolytic enzyme activators and inhibitors [1]. The most important regulator of angiogenesis is vascular endothelial growth factor (VEGF), which is overexpressed in several tumor tissues. VEGF is a disulfide-bonded dimeric glycoprotein, sharing close sequence homology with placenta growth factor, VEGF-B and VEGF-C, and lower sequence homology with platelet-derived growth factor (PDGF) [2]. VEGF plasma levels are highly predictive for tumor growth and survival rate of breast cancer patients [3, 4] and therapeutic strategies blocking VEGF action successfully inhibited tumor growth [4].

Several single nucleotide polymorphisms (SNPs) have been described in the VEGF gene, some of them have been associated with VEGF expression and/or clinical phenotypes. We have previously reported a significant association of one variant, VEGF 936C>T (rs3025039), with decreased risk for breast cancer [5]. This result was subsequently confirmed by one study [6], but refuted by two other studies [7, 8].

In order to replicate and expand previous data on the role of VEGF polymorphisms in breast cancer risk, we have determined seven SNPs and haplotypes of the VEGF gene in 804 incident breast cancer patients and 804 healthy age-matched population-based control subjects.

Materials and methods

Subjects

The Austrian "tumor of breast tissue: incidence, genetics, and environmental risk factors" (TIGER) study is an ongoing study investigating risk factors for breast cancer. TIGER consists of 804 consecutive female patients with histologically confirmed incident breast cancer without any other cancer diagnosis beside breast cancer. All patients were recruited between January 2000 and September 2004 from

Table 1 Primer and probe sequences for 5′-exonuclease assays

Polymorphism	Primer/probe	Sequence (5′–3′)
–2578C>A	Forward primer	CAGAGGCTATGCCAGCTGTAG
	Reverse primer	GTGGGACCAGTCAGTCTGATTATC
	A-probe	VIC-CACCCAGATCTTGCCAG-NFQ
	C-probe	FAM-ACCCAGATCGTGCCAG-NFQ
–2489C>T	Forward primer	CCTCAGAGCCCCAACTTTGTT
	Reverse primer	TGCATATAGGAAGCAGCTTGGAAA
	–2489C probe	VIC-CCAGCCAGGAATT-NFQ
	–2489T probe	FAM-AAACCAGCTAGGAATT-NFQ
–1498C>T	Forward primer	GTGTGGGTGAGTGAGTGTGT
	Reverse primer	GTGACCCCTGGCCTTCTC
	–1498T probe	VIC-CTCCAACACCCTCAAC-NFQ
	–1498C probe	FAM-CCAACGCCCTCAAC-NFQ
–634G>C	Forward primer	GAGAGAAGTCGAGGAAGAGAGAGA
	Reverse primer	CCCAAAAGCAGGTCACTCACTT
	–634G probe	VIC-CCCTGTCCCTTTCG-NFQ
	–634C probe	FAM-CCTGTCGCTTTCG-NFQ
–7C>T	Forward primer	CCGAGCCGGAGAGGGA
	Reverse primer	GCACCCAAGACAGCAGAAAGT
	–7C probe	VIC-CATGGTTTCGGAGGCC-NFQ
	–7T probe	FAM-ATGGTTTCAGAGGCC-NFQ
936C>T	Forward primer	ACTCCGGCGGAAGCATTC
	Reverse primer	AGCAAGAAAAATAAAATGGCGAATCCA
	936C probe	VIC-CAAGAGGGACCGTGCTG-NFQ
	936T probe	FAM-AAGAGGGACCATGCTG-NFQ
1612G>A	Forward primer	GCTTACTCTCACCTGCTTCTGA
	Reverse primer	TCTTCGCCGGGACATCTG
		VIC-CCAGGAGGCCACTG-NFQ
		FAM-CCCAGGAGACCACTG-NFQ

NFQ non-fluorescent quencher. Underlined nucleotides indicate the site of the polymorphism

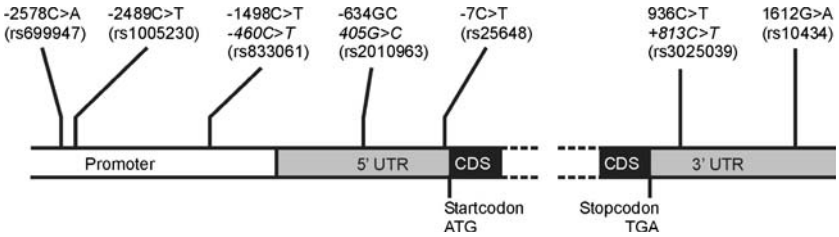

Fig. 1 Structure of the VEGF gene and position of candidate gene polymorphisms. Position of polymorphism are relative to the translation start, *italic positions* indicate alternative designations. *Dashed lines* indicate 13 kb region between upstream polymorphisms and downstream polymorphisms, containing the coding sequence (*CDS*) and seven introns

patients attending the Division of Oncology, Department of Internal Medicine, Medical University Graz, Austria. Patients were included in the aftercare measures program of the Division of Oncology Graz, providing follow-ups in regular intervals (3 months interval in years 1–3, 6 months interval in years 4–5, and 12 months interval in years 6–15 after diagnosis). Follow-up investigations included clinical check-up, laboratory (including CEA and CA15-3), radiological (bone scan, liver scan, chest X-ray, and mammograms), and gynecological analyses.

The majority of the participants of TIGER had not participated in any genetic association study before, a small fraction of TIGER participants ($n = 18$) were also included in a previous case–control study from the same Department [5].

For each patient of the TIGER study, one healthy female age-matched (±2 years) control subject was enrolled. Control subjects were recruited from local health screening studies, the presence of known current or previous malignant disease was excluded anamnestically.

The study was performed according to the Austrian Gene Technology Act and has been approved by the Ethical Committee of the Medical University Graz. Written informed consent was obtained from all participating subjects. All study participants (patients and controls) were Caucasians (Table 1).

Selection of VEGF polymorphisms

With the use of the public NCBI SNP database and available literature [9, 10–12], we selected VEGF candidate polymorphisms with a minor allele frequency of at least 0.10 and location in the promoter region, coding region or untranslated region of the VEGF gene. Using this approach, seven common VEGF polymorphisms were chosen for further analysis (Fig. 1).

DNA isolation and genotyping assays

Genomic DNA was isolated by standard procedures. VEGF genotypes were determined between November 2005 and August 2006 using 5′-nuclease assays (TaqMan). Reaction conditions were as described previously [13]. Primers and probe sets are summarized in Table 2. The laboratory staff responsible for genotyping were blinded for case/control status.

Determination of VEGF plasma levels

The VEGF plasma levels were determined in 81 healthy volunteers (64 men and 17 women) using a commercially available enzyme immunoassay (human VEGF Quantikine, R&D Systems, Wiesbaden, Germany) as described previously [10]. All reactions were performed in duplicate. The assay was specific for VEGF165 and did not detect related molecules, e.g., PDGF or placental growth factor.

Construction of haplotypes and statistical analysis

Haplotypes and linkage disequilibrium were determined using the Haploview program (Version 2.05, http://www.broad.mit.edu/personal/jcbarret/haploview/). Assignment of individual haplotype pairs was performed by the PHASE Version 2.1 software [14]. Statistic analysis was done using SPSS 14.0 for Windows. Numeric values were analyzed by Student's *t*-test, proportions of groups were compared by chi-squared test. Odds ratio (OR) and 95% confidence interval (95% CI) were calculated by logistic regression analysis. Threshold for significance was $p < 0.05$.

Due to the fact that precise frequencies of VEGF genotypes and haplotypes were not at the planning phase of the study, an a priori Power analysis was performed assuming a frequency of 0.1 for a hypothetical genetic risk marker. Using these condidtions, the present study had a Power of 0.99, 0.95 or 0.75 to detect or exclude an OR of 2.0, 1.7 or 1.5 for breast cancer. The statistical Power increased with higher frequencies and/or higher ORs, and decreased with lower frequencies and/or lower ORs of genetic markers.

Results

Tumor characteristics of TIGER participants are presented in Table 2. VEGF genotypes did not deviate from the Hardy Weinberg equilibrium in patients or controls.

Table 2 Characteristics of breast cancer patients enrolled in the TIGER study

Characteristic	Data available (%)		Breast cancer patients (%)
n			804
Age at diagnosis	804 (100)	Years	58.0 ± 12.2
Regional lymph node metastases	727 (90.4)	No	359 (49.4)
		Yes	368 (50.6)
Tumor size	772 (96.0)	≤20 mm	629 (81.5)
		>20 mm	143 (18.5)
Histological grade	767 (95.4)	1–2	431 (56.2)
		3–4	336 (43.8)
Children	794 (98.8)	0	125 (15.7)
		1	184 (23.2)
		2	272 (34.3)
		3 or more	213 (26.8)
Her2neu overexpression	485 (60.3)	No	317 (65.4)
		Yes	168 (34.6)
Estrogen receptor	783 (97.4)	Negative	162 (20.7)
		Positive	621 (79.3)
Progesteron receptor	779 (96.9)	Negative	262 (33.6)
		Positive	517 (66.4)

Haplotype analysis showed two separate blocks of high-linkage disequilibrium, formed by five polymorphisms upstream of the coding sequence (promoter and 5′ untranslated region) and two polymorphisms downstream of the coding sequence, respectively (Fig. 2). None of the single polymorphisms or haplotypes was significantly associated with the presence of breast cancer (Table 3).

Tumor characteristics of breast cancer patients stratified by VEGF genotypes and haplotypes are summarized in Tables 4 and 5. Five associations were below the significance treshold of 0.5. Applying Bonferroni correction for multiple testing, only the association of the -634G>C polymorphism with tumor size remained statistically significant. VEGF genotypes or haplotypes were furthermore

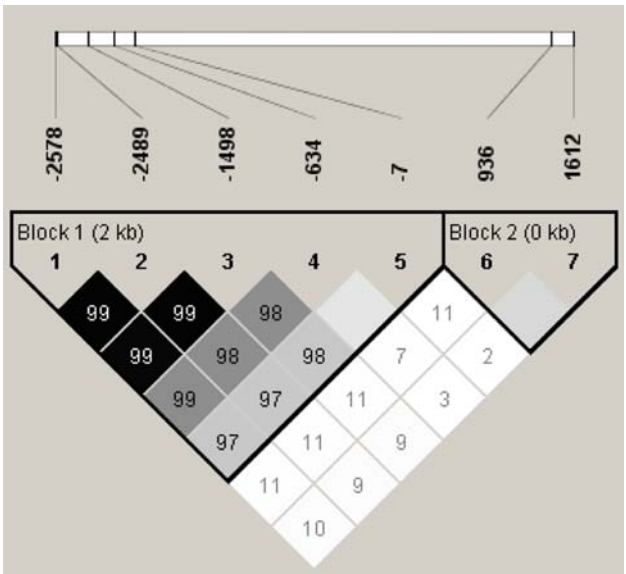

Fig. 2 Linkage disequilibrium of VEGF polymorphisms. *Values in squares* are LD′ between single markers. *Dark squares* indicate high r^2 and *bright squares* indicate low r^2 values

Table 3 Allele frequencies and association of VEGF single markers and haplotypes with breast cancer

Allele/haplotype	Frequency among breast cancer patients	Frequency among control subjects	*p*
Single markers			
–2578A	0.481	0.465	0.36
–2489T	0.480	0.466	0.42
–1498T	0.479	0.465	0.44
–634C	0.335	0.337	0.88
–7T	0.166	0.166	0.99
936T	0.145	0.150	0.69
1612A	0.471	0.459	0.51
Upstream haplotypes (–2578C>A, –2498C>T, –1498C>T, –634G>C, –7C>T)			
CCCCC	0.334	0.337	0.87
ATTGC	0.312	0.297	0.37
CCCGC	0.182	0.198	0.268
ATTGT	0.163	0.165	0.891
Downstream haplotypes (936C>T, 1612G>A)			
CA	0.471	0.460	0.52
CG	0.383	0.390	0.70
TG	0.146	0.150	0.74

Haplotype frequencies were derived using the PHASE software

not associated with HER2neu overexpression, estrogen receptor status or progesteron receptor status (data not shown).

The potential association of VEGF polymorphisms and haplotypes with VEGF plasma levels were determined in a multivariate linear regression analysis. Polymorphisms and haplotypes were entered assuming codominant effects (0 = polymorphism/haplotype not present; 1 = one copy present; 2 = two copies present). None of the VEGF polymorphism or haplotypes was a significant predictor of VEGF plasma levels. This did not change when sex and age were entered in the model as additional covariates (Table 6).

Discussion

Aim of the present study was to re-evaluate the association of VEGF gene polymorphisms and their haplotypes with breast cancer risk in a large case–control study including incident patients and population-based control subjects. No significant differences in allele, genotype, and haplotype distribution of the VEGF gene polymorphisms between breast cancer cases and controls were detected. Our study does not support the notion that VEGF polymorphisms do modify the risk of breast cancer.

Our data are in contrast to a case–control study we had performed previously [5]. In that study, including 500 Caucasian breast cancer cases and 500 controls, we observed a decreased risk for breast cancer in carriers of VEGF 936T allele. VEGF genotypes in that study were determined by a PCR-RFLP, which may be more error-prone than the TaqMan assay used in the present study. Furthermore, the present study included only incident breast cancer patients, whereas in the previous study incident and as well as prevalent patients had been included.

Table 4 Relationship between VEGF genotypes and breast cancer characteristics

Polymorphism	Genotype	Age at onset	Regional lymph node metastases		Tumor size		Histological grade	
		Years	No (%)	Yes (%)	≤20 mm (%)	>20 mm (%)	1–2 (%)	3–4 (%)
–2578A	GG	57 ± 13	81 (47.6)	89 (52.4)	138 (78.4)	38 (21.6)	100 (57.1)	75 (42.9)
	GA	58 ± 12	172 (50.3)	170 (49.7)	304 (81.7)	68 (18.3)	214 (57.4)	159 (42.6)
	AA	58 ± 12	98 (50.0)	98 (50.0)	173 (84.8)	31 (15.2)	108 (54.8)	89 (45.2)
	p	0.63		0.85		0.27		0.83
–2489T	CC	58 ± 12	100 (50.5)	98 (49.5)	175 (85.0)	31 (15.0)	108 (54.0)	92 (46.0)
	CT	58 ± 12	172 (49.3)	177 (50.7)	308 (80.8)	73 (19.2)	216 (56.5)	166 (43.5)
	TT	57 ± 12	83 (48.8)	87 (51.2)	139 (79.4)	36 (20.6)	102 (58.3)	73 (41.7)
	p	0.58		0.94		0.33		0.70
–1498T	CC	58 ± 12	101 (50.8)	98 (49.2)	176 (85.0)	31 (15.0)	108 (54.0)	92 (46.0)
	CT	58 ± 12	172 (49.1)	178 (50.9)	308 (80.8)	73 (19.2)	218 (56.9)	165 (43.1)
	TT	57 ± 12	82 (48.8)	86 (51.2)	138 (79.3)	36 (20.7)	100 (57.5)	74 (42.5)
	p	0.65		0.92		0.31		0.75
–634C	GG	57 ± 12	150 (46.9)	170 (53.1)	258 (76.8)	78 (23.2)	195 (58.0)	141 (42.0)
	GC	59 ± 12	153 (50.0)	153 (50.0)	281 (84.4)	52 (15.6)	187 (56.7)	143 (43.3)
	CC	58 ± 13	50 (59.5)	34 (40.5)	79 (91.9)	7 (8.1)	43 (51.2)	41 (48.8)
	p	0.059		0.12		0.001		0.52
–7C>T	CC	58 ± 12	239 (48.9)	250 (51.1)	428 (82.5)	91 (17.5)	275 (53.5)	239 (46.5)
	CT	58 ± 12	97 (50.5)	95 (49.5)	166 (81.4)	38 (18.6)	128 (62.7)	76 (37.3)
	TT	57 ± 14	9 (47.4)	10 (52.6)	12 (60.0)	8 (40.0)	10 (50.5)	10 (50.0)
	p	0.90		0.92		0.039		0.069
936C>T	CC	58 ± 12	256 (48.8)	269 (51.2)	449 (79.5)	116 (20.5)	302 (54.5)	252 (45.5)
	CT	60 ± 12	92 (52.6)	83 (47.4)	156 (86.7)	24 (13.3)	114 (61.3)	72 (38.7)
	TT	60 ± 14	9 (42.9)	12 (57.1)	19 (90.5)	2 (9.5)	14 (66.7)	7 (33.3)
	p	0.13		0.56		0.054		0.17
1612G>A	GG	58 ± 12	106 (50.5)	104 (49.5)	186 (83.4)	37 (16.6)	132 (59.5)	90 (40.5)
	GA	58 ± 12	170 (49.9)	171 (50.1)	288 (79.6)	74 (20.4)	203 (56.2)	158 (43.8)
	AA	57 ± 13	81 (47.9)	88 (52.1)	151 (83.9)	29 (16.1)	92 (52.0)	85 (48.0)
	p	0.60		0.88		0.35		0.33

Table 5 Relationship between VEGF haplotypes and breast cancer characteristics

Haplotype Upstream haplotypes	Copies	Age at onset	Regional lymph node metastases		Tumor size		Histological grade	
		Years	No (%)	Yes (%)	≤20 mm (%)	>20 mm (%)	1–2 (%)	3–4 (%)
CCCCC	0	57 ± 12	170 (46.7)	194 (53.3)	297 (77.7)	85 (22.3)	213 (56.1)	167 (43.9)
	1	60 ± 12	149 (50.3)	147 (49.7)	269 (83.5)	53 (16.5)	179 (56.1)	140 (43.9)
	2	57 ± 12	40 (59.7)	27 (40.3)	63 (92.6)	5 (7.4)	39 (57.4)	29 (42.6)
	p	0.004		0.14		0.007		0.98
ATTGC	0	58 ± 12	179 (48.5)	190 (51.5)	313 (81.1)	73 (18.9)	219 (57.5)	162 (42.5)
	1	58 ± 12	156 (51.8)	145 (48.2)	272 (82.7)	57 (17.3)	181 (55.2)	147 (44.8)
	2	59 ± 12	24 (42.1)	33 (57.9)	44 (77.2)	13 (22.8)	31 (53.4)	27 (46.6)
	p	0.57		0.36		0.59		0.75
CCCGC	0	58 ± 12	267 (51.3)	253 (48.7)	458 (82.8)	95 (17.2)	309 (56.1)	242 (43.9)
	1	57 ± 12	84 (45.4)	101 (54.6)	152 (77.9)	43 (22.1)	108 (56.0)	85 (44.0)
	2	58 ± 13	8 (36.4)	14 (63.6)	19 (79.2)	19 (79.2)	14 (60.9)	9 (39.1)
	p	0.56		0.18		0.31		0.90
ATTGT	0	58 ± 12	258 (48.9)	270 (51.1)	459 (82.0)	101 (18.0)	298 (53.7)	257 (46.3)
	1	58 ± 12	94 (51.1)	90 (48.9)	160 (81.6)	36 (18.4)	125 (63.5)	71 (36.2)
	2	56 ± 14	7 (46.7)	8 (53.3)	10 (62.5)	6 (37.5)	8 (50.0)	8 (50.0)
	p	0.83		0.85		0.14		0.044
Downstream haplotypes								
CA	0	58 ± 12	105 (51.0)	101 (49.0)	183 (83.6)	36 (16.4)	131 (60.1)	87 (39.9)
	1	58 ± 12	169 (49.9)	170 (50.1)	286 (79.4)	74 (20.6)	203 (56.5)	156 (43.5)
	2	57 ± 13	81 (47.9)	88 (51.2)	151 (83.9)	29 (16.1)	92 (52.0)	85 (48.0)
	p	0.55		0.84		0.32		0.27
CG	0	58 ± 13	130 (47.8)	142 (52.2)	241 (84.6)	44 (15.4)	161 (56.5)	124 (43.5)
	1	58 ± 12	179 (51.7)	167 (48.3)	297 (80.7)	71 (19.3)	204 (55.6)	163 (44.4)
	2	57 ± 12	46 (47.9)	50 (52.1)	82 (77.4)	24 (22.6)	61 (59.8)	41 (40.2)
	p	0.56		0.58		0.21		0.75
TG	0	58 ± 12	255 (48.9)	266 (51.1)	447 (79.7)	114 (20.3)	300 (54.5)	250 (45.5)
	1	59 ± 12	92 (53.2)	81 (46.8)	155 (87.1)	23 (12.9)	113 (61.4)	71 (38.6)
	2	60 ± 14	8 (40.0)	12 (60.0)	18 (90.0)	2 (10.0)	13 (65.0)	7 (35.0)
	p	0.15		0.43		0.052		0.20

Table 6 Multivariate linear regression of VEGF plasma levels

Parameters		Coefficient	Standardized coefficient	*p*
Sex (male = 1, female = 2)		26.06	0.414	<0.001
Age (years)		0.09	0.058	0.62
Single nucleotide polymorphisms	VEGF –1498T	8.42	0.216	0.76
	VEGF –634C	–1.61	–0.031	0.90
Upstream haplotypes (–2578, –2498, –1498, –634; –7)	CCCCC	12.18	0.290	0.65
	CCCGC	7.55	0.178	0.77
	ATTGT	–5.69	–0.095	0.65
Downstream haplotypes (936, 1612)	CA	1.38	0.039	0.73
	TG	–3.66	–0.063	0.59

Genetic data were coded assuming co-dominant effects (0 = polymorphism/haplotype not present; 1 = one copy present; 2 = two copies present)

The following genetic parameters were excluded from the model because of collinearity with parameters inside the model: –2578A, –2489T, –7T, 936T, 1612A, upstream haplotype ATTGC, downstream haplotype CG

The fact that the significant association between the VEGF 936C>T polymorphism and breast cancer was not replicated in the present study remains puzzling. On the other hand, non-replication of significant primary genetic association results is a well-known phenomenon in the field of genetic epidemiology. Consequently, a number of methodical papers on genetic association studies have stressed the importance of studies confirming (or confuting) results of primary reports [15–17]. Replication of association studies is imperative to draw firm conclusions about the role of genetic risk factors.

Recently, Jin and co-workers investigated the association between four VEGF polymorphisms (-2578C>A, -1154G>A, -634G>C, and 936C>T) and breast cancer risk [8]. As main result of their study, no association between VEGF polymorphisms or haplotypes and the presence of breast cancer was observed. This is in line with our findings. However, Jin and co-workers reported an association of the VEGFR -634CC genotype and the -2578/-634 CC haplotype with high-tumor aggressiveness (large tumor and high-histologic grade). Interestingly, the same genotype was associated with smaller tumor size and had no effect on histologic grade in the present study. These opposing results underline again the utmost importance of replication of genetic studies.

In a study by Jacobs and co-workers, VEGF alleles -2578C and –1154G were associated with invasive, but not with in situ breast cancer. VEGF polymorphisms –634G>C and 936C>T were not related to breast cancer susceptibility [7]. Kataoka and co-workers reported that breast cancer risk was influenced by the VEGF 936C>T polymorphism, but not by the –1498T>C or the –634G>C polymorphism [6]. Taken together, on the basis of currently available data, a clear effect of VEGF genotypes on breast cancer risk is unlikely.

The hypothesis that polymorphisms of the VEGF might influence breast cancer risk has been built upon the notion that VEGF gene polymorphism are associated with altered VEGF gene expression. In the present study, we were unable to detect any clear effects of VEGF genotypes on VEGF plasma levels. This is in line with a recent publication from Berrahmoune and co-workers, who reported that plasma VEGF concentrations were under strong genetic control in healthy families, but not influenced by VEGF genotypes at positions –1498, –634 or 936 [18]. It is likely that substantial genetic determinants of vascular growth might be found in other candidates genes, such as those for hypoxia inducable factor (HIF1), VEGF receptor 1 (Kinase Insert Domain Receptor; KDR) or VEGF receptor 2 (FMS-Related Tyrosine Kinase 1; FLT [19]).

Acknowledgment This study was supported by the Anniversary Fund of the Österreichische Nationalbank (Project Nr. 10609).

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