Prevalence of +405G>C, –1154G>A Vascular Endothelial Growth Factor Polymorphism in Breast Cancer

Rani James · G. Ramesh · Lakshmi Krishnamoorthy ·
Rahul Bhagat · Shilpa Chadaga ·
Vijayalaxmi Deshmane · Girija Ramaswamy

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Abstract Vascular endothelial growth factor (*VEGF*) plays an important role in the development of Breast Cancer. The aim of this study was to investigate the association of polymorphisms in the *VEGF* gene on prognosis of Breast Cancer patients. This study comprised 200 patients with histologically confirmed cases of Breast cancer and 200 controls. Genotyping of the *VEGF* gene polymorphisms at +405G>C, –1154G>A, were performed by PCR-RFLP analysis. Preoperative plasma *VEGF* levels were determined by ELISA. Amongst both cases and controls, the genotypic distribution of the individual SNPs were all in Hardy–Weinberg equilibrium. Mean *VEGF* level was significantly elevated in cases compared to controls ($t = 8.248$; $P < 0.001$). No significant association was found between +405G>C, –1154G>A *VEGF* polymorphism and Breast Cancer. Logistic regression analysis revealed that 405GG & 1154GG were associated with higher levels of *VEGF*.

Keywords Breast cancer · *VEGF* polymorphism · Genotype · Plasma *VEGF* · ELISA

Introduction

Breast cancer is the most frequently diagnosed cancer and the leading cause of cancer death among females,

accounting for 23 % of the total cancer cases and 14 % of the cancer deaths [1]. It is estimated that presently nearly one million new cancer cases are being detected annually in the country [2]. Presently, 75,000 new cases of breast cancer occur in Indian women every year. The breast is the second most common site of cancer in women after the cervix uteri [3]. Results from experimental studies suggest that tumor progression and metastasis in breast cancer are angiogenesis dependent [4].

A key mediator in neoangiogenesis is vascular endothelial growth factor (*VEGF*) as it stimulates endothelial cell proliferation and migration, and increases vascular permeability; [5, 6] it is therefore regarded as a marker of tumor invasion and metastasis [7]. The *VEGF* gene is located on chromosome 6p21.3 and consists of eight exons that exhibit alternate splicing to form a family of proteins [8]. Purified *VEGF* is an approximately 46-kDa protein and it dissociates upon reduction into two apparently identical 23-kDa subunits [9].

There is considerable variation between individuals in *VEGF* expression, and analysis of the 5' flanking region of the gene has shown the presence of many polymorphisms [10–12]. G → C transition at +405 in the 5'-untranslated region has been implicated in a number of diseases, especially those with angiogenic basis [13, 14]. *VEGF* protein production was found to be associated with +405 polymorphism in healthy subjects [12]. Association has been reported in case–control studies between *VEGF* polymorphisms and diseases such as diabetic retinopathy, [13] ovarian cancer, [15] and endometriosis [16].

Analysis of the *VEGF* gene promoter region revealed a single major transcription start, which lies near a cluster of potential Sp1 factor binding sites. The promoter region also contains several potential binding sites for the transcription factors AP-1 and AP-2 [9]. The –1154G>A transition lies

R. James (✉) · G. Ramesh · L. Krishnamoorthy · R. Bhagat ·
S. Chadaga · G. Ramaswamy
Department of Biochemistry, Kidwai Memorial Institute of
Oncology, Dr. M.H. Marigowda Road, Bangalore 560029, India
e-mail: meranijames@yahoo.co.in

V. Deshmane
Department of Surgical Oncology, Kidwai Memorial Institute
of Oncology, Bangalore 560029, India

Table 1 Characteristic of cases & controls

Prognostic factor	Number of patients (%) (n = 200)	Number of controls (%) (n = 200)
Age		
≤40 years	54 (27 %)	49 (24.5 %)
40–55 years	82 (41 %)	88 (44 %)
≥55 years	64 (32 %)	63 (31.5 %)
Menopausal status		
Premenopausal	73 (36.5 %)	77 (38.5 %)
Postmenopausal	127 (63.5 %)	123 (61.5 %)
Nodal status		
Positive	113 (56.5 %)	–
Negative	87 (43.5 %)	–
Stage		
I	19 (9.5 %)	–
II	77 (38.5 %)	–
III	92 (46 %)	–
IV	12 (6 %)	–
Estrogen receptor		
Positive	105 (52.5 %)	–
Negative	95 (47.5 %)	–
Progesterone receptor		
Positive	102 (51 %)	–
Negative	98 (49 %)	–
Metastasis (37) (18.5 %)		
Skeletal	19 (9.5 %)	–
Node	13 (6.5 %)	–
Hepatic	3 (1.5 %)	–
Other sites	2 (1 %)	–

in the promoter region of the *VEGF* gene [17]. The –1154AA and GA genotypes were associated with low *VEGF* expression in lung cancer [18]. Jacobs et al. [19] have reported that one or two copies of –1154A allele resulted in lower risk of invasive breast cancer compared with zero-copy carriers. In another study by Schneider et al. [20] among American population, showed an increase in overall survival in breast cancer patients that carry

–1154G>A polymorphism. In contrast carriers of 1154GG genotypes had increased *VEGF* secretion in human peripheral blood mononuclear cells compared with carriage of a rare allele [21, 22].

In spite of the importance of angiogenesis in Breast cancer, there have been no published studies on the association of *VEGF* gene polymorphism and Breast cancer among Indians. This case control study was undertaken to study the prevalence of *VEGF* polymorphisms among Breast cancer patients and its association on the prognosis of Breast cancer. We also studied *VEGF* plasma levels in 200 healthy subjects and 200 patients to assess possible functional relevance of the *VEGF* polymorphisms.

Materials and Methods

Study Population

Histologically confirmed cases of Breast cancer samples were obtained from 200 patients registered (2008–2009) in the Breast service unit. Patient's age, stage, grade, nodal status, estrogen receptor (ER) and progesterone receptor (PR) status were noted from the case files. Age matched (± 5 years) healthy female controls (200) were selected from patient relatives.

The study protocol was approved by the Institutional Ethical Committee and Informed consent was obtained from the patients/relatives of the subjects under study. All the participants of the study belonged to the same ethnic group.

Blood Collection and DNA Extraction

About 5 ml of blood was collected from each subject after an overnight fast by routine venipuncture. The blood sample was then distributed into different tubes for the assays of various biochemical parameters. DNA for PCR–RFLP analysis was extracted from leukocytes isolated from whole blood by using DNA extraction kit (Genei).

Table 2 Genotype frequencies of +405G>C polymorphisms

405G>C	Cases (n = 200)		Controls (n = 200)	
	Observed	Expected	Observed	Expected
GG	89	88.4	85	83.9
GC	88	89.1	89	91.3
CC	23	22.4	26	24.9
Hardy–Weinberg equilibrium	$\chi^2 = 0.031$; $P = 0.860$		$\chi^2 = 0.126$; $P = 0.721$	
Variance of allele frequency	0.34		0.35	
Inference	Consistent with HWE		Consistent with HWE	

Table 3 Genotype frequencies of −1154G>A polymorphisms

1154G>A	Cases (<i>n</i> = 200)		Controls (<i>n</i> = 200)	
	Observed	Expected	Observed	Expected
GG	95	93.8	85	85.8
GA	84	86.3	92	90.4
AA	21	19.8	23	23.8
Hardy–Weinberg equilibrium	$\chi^2 = 0.143$; $P = 0.705$		$\chi^2 = 0.063$; $P = 0.801$	
Variance of allele frequency	0.32		0.35	
Inference	Consistent with HWE		Consistent with HWE	

Genotyping of *VEGF* Polymorphisms

Genotyping of the +405G>C polymorphisms in the 5' UTR of the *VEGF* gene was determined by PCR/RFLP analysis. The method described by Buraczynska et al. [23]

was used for the detection of +405G>C polymorphism. PCR was carried out in a total volume of 30 μ l, containing 200 ng genomic DNA, 1.5 μ mol of each primer (Sigma, USA), IX Taq polymerase buffer (1.5 mM MgCl₂) and 1 U of Taq DNA polymerase (Bangalore Genie, India).

Fig. 1 a PCR amplicons of +405G>C polymorphism. **b** Restriction digested products of +405G>C polymorphism. Line 1 is marker, lane 2 and 3 are homozygous (CC) and line 4 and 5 are heterozygous. Line 6 & 7 are wild type (GG)

Fig. 2 a PCR amplicons of –1154G>A polymorphism. **b** Restriction digested products of –1154G>A polymorphism. Line 1 is marker, line 2 & 3 PCR amplicons, line 4 is homozygous (AA) and line 5 is heterozygous (GA). Line 6 & 7 are wild type (GG)

Table 4 The associations of the *VEGF* +405G>C and –1154G>A polymorphisms with tumor characteristics (Node)

VEGF genotypes	Node (<i>n</i> = 200)		OR	<i>P</i> value
	Negative (<i>n</i> = 87)	Positive (<i>n</i> = 113)		
405G>C				
GG	37 (42.5 %)	52 (46 %)	1.00	–
GC	41 (47.1 %)	47 (41.6 %)	0.34	<0.001**
CC	9 (10.3 %)	14 (12.4 %)	0.23	0.004**
1154G>A				
GG	44 (50.6 %)	51 (45.1 %)	1.00	–
GA	33 (37.9 %)	51 (45.1 %)	0.14	<0.001**
AA	10 (11.5 %)	11 (9.7 %)	0.014	<0.001**

** Strongly significant (P value: $P \leq 0.01$)

For the +405G>C polymorphism the forward and reverse primers were 5'-ATTTATTTTTTGCTTGCCATT-3' and 5'-GTCTGTCTGTCTGTCCGTCA-3' respectively. PCR amplification was performed in a programmable thermal cycler gradient PCR system (Eppendorf AG, Hamburg, Germany). The PCR product was digested with the *BsmFI* restriction endonuclease. (New England Biolabs, USA) at 65 °C overnight for the +405G>C polymorphism, separated by 2 % agarose gel electrophoresis, and identified using ethidium bromide staining. For the *VEGF* +405 polymorphism the uncut fragment was 304 bp (C allele) and digestion products were 193 and 111 bp (G allele).

The method described by Han et al. [24] was used for the detection of –1154G>A polymorphisms. For the –1154G>A polymorphism the forward and reverse

Table 5 Comparison *VEGF* values between controls

<i>VEGF</i> pg/ml	Cases (<i>n</i> = 200)	Controls (<i>n</i> = 200)
Min–max	31–876.9	9–136.54
Mean $\pm$ SD	126.58 $\pm$ 16.23	71.76 $\pm$ 5.33
95 % CI	114.22–139.56	66.23–77.50
Inference	Mean <i>VEGF</i> level is significantly elevated in cases compared to control with <i>t</i> = 8.248; <i>P</i> < 0.001**	

** Strongly significant (*P* value: *P* $\leq$ 0.01)

Fig. 3 ROC curve

primers were 5'-TCCTGCTCCCTCCTCGCCAATG-3' and 5'-GCGGGGACAGGCGAGCCTC-3' respectively. Genomic DNA was amplified in a final volume of 30 μ l using the following conditions: initial denaturation at 95 °C for 10 min followed by 35 cycles at 95 °C for 45 s, 62 °C for 45 s, and 72 °C for 30 s. A final extension was at 72 °C for 10 min.

The resulting PCR product was digested with the *MnII* restriction endonuclease. (New England Biolabs, USA) at 37 °C overnight for the –1154G>A polymorphism. The PCR product of –1154G>A polymorphism has 206 base

Table 7 Levels of plasma *VEGF* in controls and cases according to +405G>C polymorphism

<i>VEGF</i> (pg/ml)	Cases (<i>n</i> = 200)	Controls (<i>n</i> = 200)	<i>P</i> value	Effect size
405G>C				
GG	161.18 $\pm$ 16.68	77.52 $\pm$ 5.11	<0.001**	6.77
GC	103.47 $\pm$ 14.61	71.06 $\pm$ 5.34	<0.001**	2.94
CC	94.48 $\pm$ 6.44	54.78 $\pm$ 4.99	0.002**	6.88

** Strongly significant (*P* value: *P* $\leq$ 0.01)

pairs. RFLP results of –1154G>A was 150 + 34 + 22 for the 1154G allele and 184 + 22 for the 1154A allele and 184 + 154 + 34 + 22 bp for the heterozygotes.

Plasma *VEGF* levels were determined among 200 patients using commercially available enzyme-linked immunoassay (ELISA) (Quantikine, R&D Systems Minneapolis, MN). The assay exhibits no significant cross-reactivity with other angiogenesis factors and has a sensitivity of 9.0 pg/ml. Optical density was measured at 450 nm using a microtitre plate reader (TECAN ELISA READER). Sample concentrations were assessed using a 1:2 dilution of a standard stock of *VEGF* (2,000 pg/ml) to 174, 1000, 500, 250, 125, 62.5, and 31.2 pg/ml.

Quantitation of Plasma *VEGF* Levels Using ELISA Assay

Aliquot 0.1 ml per well of the 2000, 1000, 500, 250, 125, 62.5, 31.2 pg/ml human *VEGF* standard solutions into the precoated 96-well plate. Add 0.1 ml of the sample diluent buffer into the control well (Zero well). Add 0.1 ml of each properly diluted sample of human sera, plasma, body fluids, tissue lysates or cell culture supernatants to each empty well. Seal the plate with the cover and incubate at RT for 2 h. Remove the cover, discard plate content and wash the plate three times with wash buffer. Add 0.1 ml of biotinylated anti-human *VEGF* antibody working solution into each well and incubate the plate at RT for 2 h. Wash the plate three times with wash buffer. Add 0.2 ml of prepared Substrate solution into each well and incubate the plate at RT for 25 min. Wash plate 3 t with times with wash buffer. Add 0.2 ml of prepared stop solution into each well. The color changes into yellow immediately. Absorbance was measured at 450 nm in a microplate reader.

Table 6 ROC curve analysis of *VEGF* pg/ml

	Cut-off	Sensitivity	Specificity	PPV	NPV	Accuracy	<i>P</i> value
<i>VEGF</i> (pg/ml)	>123.66	51.76	89.04	84.43	63.64	70.21	<0.001**

** Strongly significant (*P* value: *P* $\leq$ 0.01)

Table 8 Levels of plasma *VEGF* in controls and cases according to –1154G>A polymorphism

<i>VEGF</i> (pg/ml)	Cases (<i>n</i> = 200)	Controls (<i>n</i> = 200)	<i>P</i> value	Effect size
1154G>A				
GG	177.89 ± 18.77	90.52 ± 4.04	<0.001**	6.42
GA	98.30 ± 5.14	61.29 ± 5.11	<0.001**	7.21
AA	50.28 ± 5.14	46.51 ± 2.93	0.681	0.90

Results are mean ± SD for *VEGF* pg/ml

** Strongly significant (*P* value: *P* ≤ 0.01)

Statistical Analysis

OR and 95 % CI were estimated as a measure of association between the genotype and Breast cancer. The genotypic distribution amongst subjects was tested for Hardy–Weinberg equilibrium using Fisher’s exact test. Results on continuous measurements were presented on Mean ± SD (Min–Max) and results on categorical measurements were presented in Number (%). ROC curve analysis was performed to find the cut-off value *VEGF*—ELISA and Diagnostic value. Logistic regression analysis has been carried to assess the association of predictors of elevated *VEGF* (pg/ml) in cases. All statistical analysis was performed using SPSS 16.0. Statistical significance was assessed with a cut-off level of *P* < 0.05.

Results

The patients characteristics are described in Table 1. Clinically healthy individuals were used as controls. Amongst both cases and controls, the genotypic distributions of the individual SNPs (+405G>C, –1154G>A) did not significantly deviate from the Hardy–Weinberg equilibrium (Tables 2, 3). The +405 Genotype frequencies amongst the 200 cases were GG = 44.5 %, GC = 44 % and CC = 11.5 %. The +405 Genotype frequencies amongst the 200 controls were GG = 42.5 %, GC = 44.5 % and CC = 13 %.

GC = 44.5 % and CC = 13 %. PCR product of +405G>C SNP has 304base pairs. For the *VEGF* +405 polymorphism the uncut fragment was 304 bp (C allele, mutant allele) and digestion products were 193 and 111 bp (G allele, wild type) (Fig. 1a, b).

The genotype frequency for the homozygous wild-type and heterozygous and homozygous variant for –1154G SNP amongst cases & controls were as follows; 47.5, 42, 10.5 and 42.5, 46, 11.5 % respectively. PCR product of –1154G>A polymorphism has 206 base pairs. RFLP results of 1154G>A was 150 + 34 + 22 for (1154G allele) and 184 + 22 for (1154A allele) and heterozygous having 184 + 154 + 34 + 22 bp (Fig. 2a, b).

VEGF Sequence Variants and Breast Cancer

We examined the effect of the *VEGF* +405G>C and –1154G>A polymorphisms in relation to Breast cancer. In the present study a significant association was observed between *VEGF* polymorphism and nodal status. +405GC, –1154GA & AA genotypes were significantly less in node positive patients while carriers of the 405CC allele tended to have more node positive tumors than carriers of other genotype, which was significantly associated (0.004**). No other significant associations was found between the simultaneous carriage of the +405CC & –1154AA homozygous genotypes and tumor characteristics such as TNM stage, Grade, ER/PR status of the patients at the time of diagnosis. The results are shown in Table 4.

Plasma *VEGF* levels ranged from 31 to 876 pg/ml among breast cancer patients and among controls a range of non detectable level, (i.e. <9 pg/ml) to 136.0 pg/ml. Mean *VEGF* level was significantly elevated in cases compared to control (126.58 vs. 71.76 pg/ml, *P* < 0.001) (Table 5). Statistical cut off was determined using ROC curve (Fig. 3; Table 6). Baseline concentrations of plasma *VEGF* were elevated (≥123 pg/ml) in 52 % (103) of the 200 evaluable patients.

Next, we studied *VEGF* plasma levels and *VEGF* genotypes in Breast cancer patients and healthy subjects to assess possible functional relevance of the *VEGF* polymorphisms. The highest production was observed for the

Table 9 Assessment of association of predictors of elevated *VEGF* (pg/ml) in cases using Logistic regression analysis

Variables	<i>VEGF</i> (pg/ml) (<i>n</i> = 200)		<i>P</i> value	OR	95 % CI
	<123.66 (<i>n</i> = 97)	>123.66 (<i>n</i> = 103)			
CC405	–	–	–	–	–
GC405	52 (55.9 %)	36 (34 %)	0.103	2.74	0.82–9.21
GG405	29 (31.2 %)	60 (56.6 %)	0.002**	6.89	1.98–23.99
AA1154	–	–	–	–	–
GA1154	55 (59.1 %)	28 (26.4 %)	0.015*	14.36	1.67–123.5
GG1154	21 (22.6 %)	74 (69.8 %)	<0.001**	133.11	14.74–1202.37

* Moderately significant
(*P* value: 0.01 < *P* ≤ 0.05)

** Strongly significant (*P* value:
P ≤ 0.01)

405GG genotype, the intermediate production for the 405GC genotype, and the lowest production was observed for the 405CC genotype (Table 7).

For 1154G>A polymorphism, the highest production was observed for the GG genotype, the intermediate production for the GA genotype, and the lowest production was observed for the AA genotype. Patients with –1154AA allele were showing lowest *VEGF* production suggesting a protective role of this allele but this was not statistically significant ($P = 0.681$) (Table 8). By Logistic regression analysis we found that +405GG & –1154GG genotypes were associated with higher levels of *VEGF* (Table 9).

Discussion

Several studies have evaluated the association of *VEGF* polymorphisms with breast cancer risk [25–27]. The results, however, have been inconsistent. Studies have shown that several polymorphisms were associated with the production of the *VEGF* protein, and suggested that the regulation of *VEGF* expression occurs primarily at a transcriptional level [28]. The +405G>C transition lies in 5' untranslated region of the *VEGF* gene [17]. Watson et al. [12] reported that a G allele at position +405, which probably lies within the myeloid zinc finger protein (MZF1) binding site, affects transcriptional activity and increases *VEGF* production in peripheral blood mononuclear cells in response to lipopolysaccharide. We also observed that the +405C haplotype, which is associated with lower promoter activity, was more common in controls than in affected women. In contrast Awata et al. [13] reported that individuals with the +405CC genotype had a higher fasting serum *VEGF* level than those with other genotypes, and that they carried an increased risk of diabetic retinopathy. Stevens et al. [29] also reported that haplotype +405G has a higher promoter activity than haplotype +405C.

We have found that *VEGF* levels were significantly increased ($P < 0.001$) in patients compared to controls. The highest production was observed for the 405GG genotype, and the lowest for 405CC genotype (Table 7). The sensitivity of plasma *VEGF* in detecting breast cancer was 51.76 %, and the specificity was 89 % using a *VEGF* level of 123 pg/ml as the cutoff value. With the use of a highly sensitive enzyme immunoassay, we found that most of the primary invasive breast cancers had detectable concentrations of *VEGF* protein.

Several studies analyzed the effect of –1154G>A polymorphism among Breast cancer patients. A smaller study from the UK, including 144 cases and 263 controls, evaluated the effect of SNPs in the promoter regions of various cytokines, including the *VEGF* –1154G>A

transition, on risk and prognosis of breast cancer [27]. Schneider et al. [30] in a case control study evaluated the effect –1154G>A polymorphism in cancer patients reported an improved median overall survival in patients with *VEGF*-1154AA genotypes. In conclusion *VEGF* –1154G>A had no relation with overall risk of breast cancer, [19, 26, 27, 30] however the G allele was related with increased risk of invasive disease [19, 27, 30]. The *VEGF* polymorphism at position –1154 located within the promoter region has been linked to alterations in *VEGF* level [21, 22]. Pander et al. [31] reported that the variant allele for –1154G>A results in lower *VEGF* expression. A case–control study, with 571 breast cancer patients, evaluated the role of the germline *VEGF* –1154G>A SNP in German individuals, found that –1154GG has been associated with a higher *VEGF* production [26].

In our study, for 1154G>A polymorphism, the highest production was observed for the GG genotype, and patients with –1154A allele were showing lowest *VEGF* production (Table 8). –1154GA, AA genotypes were significantly less in node positive patients showing an OR 0.14 & 0.014 respectively (Table 4).

In the present study, the genotype distribution of the +405G>C, –1154G>A *VEGF* gene polymorphism was not significantly different between the cases and the controls. For +405G>C and 1154G>A, polymorphism, the highest production of plasma *VEGF* was observed for the GG genotype. 1154GA, AA genotypes were significantly less in node positive cases. Furthermore patients with –1154A allele were showing lowest *VEGF* production suggesting a protective role of this allele but this was not statistically significant. However, further studies on larger populations may be necessary to confirm these observations.

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