RESEARCH ARTICLE

Association of +405C>G and +936C>T Polymorphisms of the Vascular Endothelial Growth Factor Gene with Sporadic Breast Cancer in North Indians

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Abstract

Background: Vascular endothelial growth factor (*VEGF*), an endothelial cell specific mitogen, has been implicated as a critical factor influencing tumor related angiogenesis. The aim of present study was to evaluate the relationship between *VEGF* +936C>T and +405C>G polymorphisms of *VEGF* with risk of breast cancer in Punjab, India. Materials and Methods: We screened DNA samples of 192 sporadic breast cancer patients and 192 unrelated healthy, gender and age matched control individuals for VEGF +936C>T and +405C>G polymorphisms using the PCR-RFLP method. Results: For the *VEGF* +405C>G polymorphism, we observed significantly increased frequency of GG genotype in cases as compared to controls and strong association of +405GG genotype was observed with three fold risk for breast cancer (OR=3.07; 95%CI 1.41-6.65; p=0.003). For the +936C>T polymorphism, significant associations of CT and combined CT+TT genotypes were observed with elevated risk of breast cancer (p=0.021; 0.023). The combined genotype combinations of GG-CC and GG-CT of +405C>G and +936C>T polymorphisms were found to be significantly associated with increased risk of breast cancer (p=0.04; 0.0064). Conclusions: The findings of the present study indicated significant associations of *VEGF* +936C>T and +405C>G polymorphisms with increased breast cancer risk in patients from Punjab, North India.

Keywords: Breast cancer - *VEGF* - polymorphisms - angiogenesis - Punjab, North India

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Introduction

Angiogenesis, an essential process in tumor growth provides potential routes for tumor dissemination and metastasis (Folkman, 2002; Kerbel, 2008). Vascular endothelial growth factor (*VEGF*) is a potent endothelial cell-specific regulator of vasculogenesis and angiogenesis (Carmeliet, 2005; Roy et al., 2006). *VEGF* has been associated with both lymph node and visceral metastasis in variety of cancers (Ishigami et al., 1998; Kawakami et al., 2003; Peyromaure et al., 2007). Breast and gynaecologic cancers use lymphangiogenesis and *VEGF* has been described as major mediator of breast cancer angiogenesis (Schoppmann et al., 2002; Morabito et al., 2004).

The human *VEGF*-A (OMIM 192240) has been mapped to 6p21.3 and comprises a 14-kb coding region with eight exons (Tischer et al., 1991). *VEGF*-A is highly polymorphic with several polymorphisms reported in the promoter, 5´- and 3´-untranslated regions (UTRs). A common polymorphism +936C>T (rs3025039) located in 3´ UTR has been associated with different *VEGF* plasma levels (Renner et al., 2000; Krippl et al., 2003). This C to T substitution has been reported to alter binding of the transcription factor activating enhancer binding protein 4 (AP-4) which might affect mRNA structure (Renner et al., 2000). *VEGF* +936C>T polymorphism has been described as a potential predictive marker for clinical outcomes in gastric (Ruzzo et al., 2006; Tzanakis et al., 2006; Kim et al., 2007), breast (Lu et al., 2005) and ovarian cancer (Hefler et al., 2007). Another polymorphism +405C>G or -634C>G (rs2010963) located in potential binding site for MZF1 transcription factor in the 5´ UTR of *VEGF* has been significantly correlated with *VEGF* protein production (Watson et al., 2000). It has been demonstrated that this polymorphism effects expression at the post transcriptional level by altering the activity of the internal ribosome entry site (IRES)-B domain critical for expression of large *VEGF*A isoform (Huez et al., 2001; Bastide et al., 2008). Several studies have documented the association of *VEGF* +405C>G polymorphism with various cancers including Prostate lung (Lee et al., 2005), (Sfar et al., 2006), gastric (Chae et al., 2006; Tzanakis et

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al., 2006), colorectal (Chae et al., 2008; Maltese et al., 2009) and breast cancer (Oliveira et al., 2011) in diverse populations.

No association studies of *VEGF* +405C>G and +936C>T polymorphisms with breast cancer have been reported in North Indians, despite the increasing incidence of this cancer in this region. Since *VEGF* plays a significant role in the angiogenesis of various tumor types including breast cancer, hence the current study was proposed to evaluate the relationship between +405C>G and +936C>T polymorphisms of *VEGF* with the breast cancer risk in sporadic breast cancer patients from Punjab, North India. To the best of our knowledge, this is the first study on *VEGF* +405C>G and +936C>T polymorphisms in breast cancer from India.

Materials and Methods

Selection of subjects

The study was approved by the institutional Ethical Committee of Guru Nanak Dev University, Amritsar, Punjab, India. The breast cancer patients were recruited from Sri Guru Ram Das Institute of Medical Sciences and Research, Vallah, Amritsar, Punjab (India). In the present case control study a total of 384 subjects, consisting of 192 histopathologically confirmed sporadic breast cancer patients (4 males and 188 females) and 192 healthy control individuals (4 males and 188 females) were recruited as study subjects. The patients who had received chemotherapy, radiotherapy, or blood transfusion before surgery were excluded from the study. The control group consisted of gender and age matched unrelated healthy individuals recruited from the same geographical area as that of the patients. The selection criteria for the controls were no evidence of family history of any cancer or other chronic disease for the last three generations and not on regular medications for atleast two years from the date of sampling. A structured questionnaire was used during an in-person interview to elicit information on personal and disease history of the subjects. A written informed consent was obtained from all the participants.

Genomic DNA extraction and genotyping

Five millilitres of peripheral blood of each subject was collected in 0.5M EDTA. Genomic DNA was extracted using standard phenol chloroform method (Adeli and Ogbonna, 1990). The specific fragment of *VEGF* containing the single nucleotide polymorphisms +405C>G and +936C>T was amplified using previously published primer sequences (Gentilini et al., 2008; Lacheb et al., 2008). Each PCR reaction with a total volume of 15μl, contained 50ng of genomic DNA, 1X Taq buffer with 1.5mM $MgCl_2$ (Bangalore GeNei), 6 picomoles of each primer (Sigma), 33.3μM of dNTPs mix (Bangalore GeNei) and one unit of Taq polymerase (Bangalore GeNei). To monitor PCR contamination, one sample without DNA template was used as negative control in each assay.

For +405C>G analysis, PCR was performed using an initial denaturation at 95˚C for 5 min, followed with a denaturation at 95˚C for 45 sec, annealing at 53˚C for 1 min and an elongation at 72˚C for 45 sec for 35 cycles with final extension at 72˚C for 10 min in a Mastercycler gradient (Eppendorf, Germany). The amplified PCR products of 304bp were digested with BsmFI restriction enzyme (New England Biolabs, Beverly, MA) at 65°C for three hours and digestion reaction products were analysed on 2.3% ethidium bromide stained agarose gel. Two fragments of 203bp and 101bp indicates +405G allele whereas undigested fragment of 304bp represents +405C allele.

For +936C>T analysis, PCR was performed using an initial denaturation at 95˚C for 5 min, followed with a denaturation at 95˚C for 45 sec, annealing at 59˚C for 30 sec and an elongation at 72˚C for 45 sec for 35 cycles with final extension at 72˚C for 10 min in a Mastercycler gradient (Eppendorf, Germany). The amplified PCR products of 217bp were digested with NlaIII restriction enzyme (New England Biolabs, Beverly, MA) at 37°C for overnight and digestion reaction products were analysed on 2.3% ethidium bromide stained agarose gel. Two fragments of 132bp and 85bp indicates +936T allele whereas the undigested fragment of 217bp represents +936C allele. Genotyping was performed without the knowledge of subject status and randomly 10% of the samples were repeated with 100% concordance.

Statistical analysis

Baseline characteristics of patients and controls were compared using t-test for continuous variables or a chi-square (χ^2) test for categorical variables. Chi square test was also used to evaluate deviations from Hardy-Weinberg Equilibrium, compare genotype and allelic differences and to assess the association of polymorphisms with various parameters. Odds ratio (OR) and 95% confidence interval (CI) was used to assess the strength of association between the polymorphisms and the risk of cancer using various genetic models. p values were corrected (pc) in case of multiple comparisons using Bonferroni correction. The analyses was done using SPSS, Version 16 (SPSS Inc, Chicago, IL, USA). Probability value <0.05 was considered statistically significant.

Results

Characteristics of subjects

The baseline characteristics of patients and controls are summarized in Table 1. The mean age of the breast cancer patients and controls was 48.77±11.66 and 48.76±11.67 years respectively. Of the 192 breast cancer patients, 49 (25.52%) were of stage I, 91 (47.40%) of stage II, 40 (20.83%) of stage III and 12 (6.25%) of stage IV cancer.

Association of VEGF +405C>G and +936C>T polymorphisms with the risk of breast cancer

The genotype and allele frequencies of *VEGF* +405C>G and +936C>T polymorphisms are shown in Table 1. The genotype distribution of both the studied polymorphisms were in Hardy-Weinberg Equilibrium in both patients and control groups (p>0.05). The frequencies of CC, CG and GG genotypes of *VEGF* +405C>G polymorphism were 5.73 vs 12.5%, 40.10 vs 48.96% and 54.17 vs 38.54% in cases and controls respectively. We observed significantly increased frequency of GG genotype in cases as compared to controls (54.17 vs 38.54%; p=0.003). A strong association of +405GG genotype was observed with increased risk for breast cancer (OR=3.07; 95% CI, 1.41-6.65). In addition, combined CG and GG genotype was also associated with higher breast cancer risk in dominant genetic model (OR=2.35; 95% CI, 1.12-4.95). We also observed significantly increased frequency of G allele in patients which revealed 1.69 fold higher risk to breast cancer (OR=1.69; 95% CI, 1.24-2.30, p=0.0008) (Table 2).

For *VEGF* +936C>T polymorphism, the frequencies of CC, CT, TT genotypes were 80.73 vs 89.06%, 18.75 vs 10.42%, 0.52 vs 0.52% in patients and controls respectively. There was significantly increased frequency of CT genotype in breast cancer patients as compared to controls (18.75 vs 10.42%; p=0.021). Individuals carrying CT genotype were associated with two fold risk to breast cancer (OR=1.99; 95% CI, 1.10-3.58). We observed that in the dominant model, individuals carrying the combined CT+TT genotype were significantly associated with 1.94 fold risk for breast cancer compared to CC genotype (OR=1.94; 95% CI, 1.09-3.46, p=0.023). There was also significant difference in the C and T allele frequencies between breast cancer patients and the control individuals (p=0.031). Significantly higher frequency of T allele was observed in breast cancer patients (9.90%) as compared to controls (5.73%) and individuals carrying T allele were associated with increased risk of developing breast cancer (OR=1.81; 95% CI, 1.05-3.12) (Table 2).

We also analyzed the distributions of combined genotypes of two polymorphisms and observed that genotype combination CG-CT, GG-CC and GG-CT of *VEGF* +405C>G and +936C>T polymorphisms were significantly more common in patients as compared to controls (p<0.05). However, after applying Bonferroni correction of multiple variables, significant association to increased breast cancer risk remained with GG-CC (p=0.04) and GG-CT (p=0.0064) genotype combination. Carriers of respective genotype combination had 3.33 and 7.12-fold higher risk for breast cancer (Table 3). In addition, we also stratified our study subjects to investigate the relationship of two studied polymorphisms with the gender, age, menopausal status and clinical stage but we did not observe any significant association (p>0.05) (data not shown).

Table 1. Demographic, Clinical, Genotype and Allele Frequencies of *VEGF* +405C>G and +936C>T Polymorphisms in Breast Cancer Patients and Controls

Indices		Patients n=192	Controls n=192	p value
Gender	Males	4 (2.08)	4 (2.08)	1.0**
	Females	188(97.92)	188(97.92)	
Age in years	<40	43(22.40)	43(22.40)	1.0**
	≥40	149(77.60)	149(77.60)	
	Mean±SD	48.77±11.66	48.76±11.67	0.993#
Menopausal status				
	Premenopausal	76(40.43)	80(42.55)	
	Postmenopausal	112(59.57)	108(57.45)	0.675**
Clinical stage	I	49(25.52)		
	II	91(47.40)	-	-
	III	40(20.83)		
	IV	12 (6.25)		
Genotype and allele Frequencies				
+405C>G (rs2010963)				
	CC	11 (5.73)	24(12.5)	
	CG	77 (40.10)	94(48.96)	0.138**
	GG	104 (54.17)	74(38.54)	**0.003****
	C allele	99 (25.78)	142(36.98)	
	G allele	285 (74.22)	242(63.02)	**0.0008****
+936C>T (rs3025039)				
	CC	155 (80.73)	171(89.06)	
	CT	36 (18.75)	20(10.42)	**0.021****
	TT	1 (0.52)	1 (0.52)	0.945**
	C allele	346 (90.10)	362(94.27)	
	T allele	38 (9.90)	22 (5.73)	**0.031****

*Data are presented as number (%); significant p values are shown in bold: **p values calculated using χ^2 test; #p value calculated using t test

Discussion

Breast cancer is a most common cancer amongst females worldwide and is also the leading cause of cancer-related mortality (Jemal et al., 2011; American cancer society, 2011). In this malignancy, mutations in the BRCA1 and BRCA2 are inherited in an autosomal dominant manner and confer a high disease risk (Miki et al., 1994; Wooster et al., 1995) but account for only a few percent of breast cancer cases (Blackwood and Weber, 1998). However, low-penetrance genes contribute

Table 3. Combined Genotype Frequencies in Breast Cancer Patients and Controls

VEGF +405C>G and +936C>T	Patients n (%)	Controls n (%)	OR (95%CI)	p value
Genotype combination				
CC-CC	8 (4.17)	21(10.94)	Reference	
CC-CT	3 (1.56)	3 (1.56)	2.62(0.43-15.81)	0.2817
CG-CC	62 (32.29)	83(43.23)	1.96(0.81-4.72)	0.128
CG-CT	14 (7.29)	10 (5.21)	3.67(1.16-11.60)	**0.024**[a]
CG-TT	0 (0)	1 (0.52)	NC	NC
GG-CC	85 (44.27)	67(34.90)	3.33(1.39-7.99)	**0.005**[b]
GG-CT	19 (9.90)	7 (3.64)	7.12(2.17-23.40)	**0.0008**[c]
GG-TT	1 (0.52)	0 (0)	NC	NC

*n-number of subjects; OR-odds ratio; CI-confidence interval; NC-not calculated; Statistically significant p values are shown in bold; Bonferroni corrected p value (pc)=0.192[a], 0.04[b], 0.0064[c]; p values calculated using χ^2 test

Table 2. Association Analyses between Breast Cancer Patients and Controls

Genetic Model	+405C>G (rs2010963) OR(95% CI)	p value	Genetic Model	+936C>T (rs3025039) OR(95% CI)	p value
Dominant model: CG+GG vs CC	2.35(1.12-4.95)	**0.02**	Dominant model: CT+TT vs CC	1.94 (1.09-3.46)	**0.023**
Over dominant model: CG vs CC+GG	0.69(0.47-1.05)	0.081	Over dominant model: CT vs CC+TT	1.98 (1.10-3.57)	**0.021**
Recessive model: GG vs CC+CG	1.88(1.26-2.83)	**0.002**	Recessive model: TT vs CC+CT	1.00 (0.06-16.10)	1
Homozygous codominant: GG vs CC	3.07(1.41-6.65)	**0.003**	Homozygous codominant: TT vs CC	1.10 (0.07-17.79)	0.945
Heterozygous codominant: CG vs CC	1.79(0.82-3.88)	0.138	Heterozygous codominant: CT vs CC	1.99 (1.10-3.58)	**0.021**
Allele contrast: G vs C	1.69(1.24-2.30)	**0.0008**	Allele contrast: T vs C	1.81 (1.05-3.12)	**0.031**

*OR-odds ratio; CI-confidence interval; significant p values are shown in bold; p values calculated using χ^2 test

Table 4. Summary of Reported Studies Showing Relationship of *VEGF* +936C>T and +405C>G Polymorphisms with Breast Cancer Risk in Different Ethnic Groups

Variant	Cases/ Controls	Method	Population/ Ethnicity	Inference	Reference
+936C>T (rs3025039)					
	192/192	PCR-RFLP	North Indian	Association of CT and CT+TT genotype with increased breast cancer risk	Present study
	500/500	PCR-RFLP	Austrian	Carriers of T allele associated with the decreased breast cancer risk.	Krippl et al., 2003
	412/422	PCR-RFLP	Polish	No significant association	
	153/163	PCR-RFLP	German	No significant association	Jin et al., 2005
	924/934	Taqman	Swedish	No significant association	
	501/504	Taqman	-	Association of CC genotype with decreased risk for in situ breast cancer	Jacobs et al., 2006
	1109/1195	Taqman	Chinese	Association of TT genotype with the decreased breast cancer risk	Kataoka et al., 2006
	848/702	Taqman	Caucasians	No significant association	Balasubramanian et al., 2007
	500/500	PCR-RFLP	Austrian	Association of T allele with decreased breast cancer risk	Gerger et al., 2007
	60/60	SSCP	Turkish	Significantly increased frequency of CT genotype among patients	Eroglu et al., 2008
	319/290	PCR-RFLP	Polish	Association of CT+TT genotype with decreased breast cancer risk	Jakubowska et al., 2008
	804/804	Taqman	Austrian	No significant association	Langsenlehner et al., 2008
	520/715	Taqman	Caucasians	No significant association	Schneider et al., 2008
	235/235	PCR-RFLP	Brazilian	No significant association	Oliveira et al., 2011
	1918/1819	Taqman	Chinese	No significant association	Zhang et al., 2011
	453/461	Taqman and PCR-RFLP	Spanish	Association of CT+TT genotype with decreased breast cancer risk	Rodrigues et al., 2012
	680/680	PCR-RFLP	Chinese	Association of T allele with decreased breast cancer risk	Luo et al., 2013
+405C>G (rs2010963)					
	192/192	PCR-RFLP	North Indian	Association of GG and CG+GG genotype with increased breast cancer risk	Present study
	501/504	Taqman	-	No significant association	Jacobs et al., 2005
	936/941	Taqman	Swedish	No significant association	Jin et al., 2005
	1095/1198	Taqman	Chinese	No significant association	Kataoka et al., 2006
	490/498	Taqman	Caucasians	No significant association	Balasubramanian et al., 2007
	804/804	Taqman	Austrian	No significant association	Langsenlehner et al., 2008
	520/715	Taqman	Caucasians	No significant association	Schneider et al., 2008
	235/235	PCR-RFLP	Brazilian	Association of CC genotype with the increased breast cancer risk	Oliveira et al., 2011
	680/680	PCR-RFLP	Chinese	No significant association	Luo et al., 2013

*PCR-RFLP: Polymerase chain reaction-restriction fragment length polymorphism; SSCP: Single strand conformation polymorphism

to cancer susceptibility in a larger population of patients and are therefore responsible for a greater proportion of the disease burden (Wooster et al., 1995; Blackwood and Weber 1998; Nathanson and Weber, 2001; Pharoah et al., 2002). Single nucleotide polymorphisms (SNPs) represent the largest class of genetic variation within the tumor population and it has been suggested that most of population attributable cancer heritability is not related to rare deleterious gene defects but due to polymorphic variations in DNA sequence (Ponder, 2001; Zhao et al., 2003).

Vascular endothelial growth factor is one of the most important activators of tumor associated angiogenesis (Hicklin and Ellis, 2005). Different *VEGF* polymorphisms have been reported to result in different VEGF protein expression in cancer cells and tumor angiogenic activity (Koukourakis et al., 2004). These *VEGF* variants might potentially contribute to interindividual variation in the risk and progression of tumors (Jain et al., 2009). We examined the possible association between *VEGF* +936C>T and +405C>G polymorphisms with breast cancer risk. In the present study, we observed that women aged >40 years formed greater part of breast cancer population. Globally 89% of breast cancers are diagnosed from the age of 40 onwards (Ferlay et al., 2010).

For *VEGF* +405C>G polymorphism, we observed significant association of GG genotype and G allele with higher susceptibility for breast cancer. Similar to our findings, significant association of +405GG genotype has been reported with increased risk for pancreatic adenocarcinoma (Sivaprasad et al., 2013). Apart from cancer, significant association of GG genotype with albuminuria (Nikzamir et al., 2012) and hypertension (Hamedian et al., 2012) has been reported in Iranian diabetic patients. The GG genotype has also been reported to associated with higher risk for endometriosis in South Indian women (Bhanoori et al., 2005). Correlation of +405G allele has also been reported with increased risk for the coronary artery lesions in the Kawasaki disease (Kariyazono et al., 2004) and progressive retinopathy of prematurity (Cooke et al., 2004). The +405G allele has been shown to increase transcriptional activity and lipopolysaccharide stimulated *VEGF* production in peripheral blood mononuclear cells (Watson et al., 2000; Stevens et al., 2003). Contrary to our findings, association of CC genotype has been reported with increased risk for gastric cancer (Tzanakis et al., 2006), pancreatic adenocarcinoma (Talar-Wojnarowska et al., 2010) and breast cancer (Oliveira et al., 2011). Combined +405CC+CG genotype has been associated with higher susceptibility for Prostate (Sfar et al., 2006), gastric (Guan et al., 2009) and small cell lung carcinoma in males (Zhai et al., 2008a). Whereas, no association of *VEGF* +405C>G polymorphism was observed in Swedish (Jin et al., 2005), Chinese (Kataoka et al., 2006; Luo et al., 2013) and Austrian (Langsenlehner et al., 2008) breast cancer patients. A study from North India on Kashmiri lung cancer patients did not show any significant difference in genotype distribution of +405C/G polymorphism between cases and controls (Naik et al., 2012). Association of CG genotype has been reported with significantly reduced risk to colorectal cancer in Italian patients (Maltese et al., 2009).

For *VEGF* +936C>T polymorphism, we observed a significant association of CT genotype and T allele with increased risk for breast cancer. Our results are concordant with few other studies that also documented significant risk association. In Turkish population, increased frequency of CT genotype has been reported in breast cancer patients (Eroglu et al., 2008). Association of +936T allele with higher risk has been reported in different cancers including oral (Yapijakis et al., 2007), stomach (Bae et al., 2008) esophageal adenocarcinoma (Zhai et al., 2008b) and glioma (Bao et al., 2011). The +936T allele has also been reported to be associated with a reduced uptake of 18F-fluorodeoxyglucose used for detection and staging of breast cancer (Wolf et al., 2004). Association of +936C allele with reduced risk for in situ breast cancer has been documented in postmenopausal patients (Jacobs et al., 2006). In contrary to our results, protective effect of T allele has been reported in Austrian (Krippl et al., 2003; Gerger et al., 2007), Chinese (Kataoka et al., 2006; Luo et al., 2013), Polish (Jakubowska et al., 2008) and Spanish (Rodrigues et al., 2012) breast cancer patients. However, no significant association of *VEGF* +936C/T polymorphism has been reported with lung cancer risk in Kashmiri population from North India (Naik et al., 2012). The discrepancies of genotype/allele frequencies of *VEGF* +405C>G and +936C>T polymorphisms and their association with breast cancer might be due to variations of allele frequencies within different ethnic groups (Table 4).

For *VEGF* +405C>G and +936C>T polymorphism, we observed that individuals carrying GG-CC and GG-CT genotype combinations were significantly associated with increased risk for breast cancer. In Brazilian population, significantly higher frequency of the 936CC-405CC genotype combination was observed in breast cancer patients as compared to controls (Oliveira et al., 2011). In the present study, we did not observe any significant association of *VEGF* +936C>T and +405C>G with the clinical stage of the breast cancer which might be due to less number of samples in the particular stage of the cancer. Association of CC genotype of +936C/T polymorphism has been reported with tumor aggressiveness in Swedish breast cancer patients (Jin et al., 2005). The +405C allele has also been reported to associated with small breast tumor size in Austrian population (Langsenlehner et al., 2008). The findings of present study indicated a significant association of *VEGF* +936C>T and +405C>G polymorphisms with the increased breast cancer risk in Punjab. To advance these findings, we are currently screening other functional polymorphisms of *VEGF* to elucidate the role of these variants in pathology of breast cancer. Findings of SNPs influencing *VEGF* targeted therapies as a predictive marker would be of great help for physicians to tailor therapy in an individual manner.

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