Association of –2549 Insertion/Deletion Polymorphism of Vascular Endothelial Growth Factor with Breast Cancer in North Indian Patients

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Aims: The aim of the present study was to assess the role of the vascular endothelial growth factor (VEGF) –2549 insertion/deletion (I/D) polymorphism in susceptibility to sporadic breast cancer. **Methods:** DNA samples of 94 breast cancer patients and 94 unrelated healthy control individuals with matched age and gender from the same geographical region of Punjab, North West India were screened for the –2549 I/D polymorphism. Serum VEGF-C (sVEGF-C) levels of breast cancer patients and healthy controls were measured using an enzyme-linked immunosorbent assay. **Results:** The frequency of the II, ID, and DD genotype was 23.40 versus 10.64%, 48.94 versus 52.13%, and 27.66 versus 37.23%, in patients and controls, respectively. A statistically significant difference was observed for genotype distribution among the patients and controls ($\chi^2 = 6.039$, $p = 0.049$). There was a significant increase in the I allele frequency in the patients as compared with controls (47.86 versus 36.70%, $p = 0.028$). The sVEGF-C levels were also considerably higher in patients as compared to healthy controls ($p < 0.01$). **Conclusions:** The VEGF –2549 I/D polymorphism has a role in the susceptibility to breast cancer in the Amritsar region of Punjab, India.

Introduction

BREAST CANCER IS A HETEROGENEOUS DISEASE composed of multiple different subtypes with distinct molecular features and clinical behavior (Polyak, 2011). Although germline mutations in *BRCA1* and *BRCA2* have been identified as major susceptibility genes in high-risk families (Claus *et al.*, 1991; Easton *et al.*, 1995), much of the genetic variability in breast cancer especially sporadic cancer might be due to the combination of low-penetrance variants that tend to be more frequently found in selected populations (Stacey *et al.*, 2007). The growth of solid tumors, including breast tumors, depends on angiogenesis, the process by which new blood vessels develop from the endothelium of a pre-existing vasculature. It is a crucial factor for local tumor growth and for distant metastasis in breast cancer (Folkman, 1995; Carmeliet, 2005; Kerbel, 2008). Tumors release angiogenic factors that stimulate endothelial cell migration, proliferation, and capillary formation. There is heterogeneity in the degree of tumor angiogenesis and breast cancers with more robust angiogenic profiles having a worse outcome. The vascular endothelial growth factor (VEGF), an endothelial cell-specific mitogen, is a critical regulator of normal and pathologic angiogenesis

(Ferrara and Henzel, 1989; Plouet *et al.*, 1989; Phillips *et al.*, 1990). The actions of the VEGF family has been reported to be crucial for tumor growth and metastasis as tumors cannot grow beyond the initial size without establishment of a blood supply for acquiring nutrients and carrying away debris (Roy *et al.*, 2006; Roskoski, 2007). It has been reported that VEGF contributes to cancer development by promoting cell growth and enhancing cell survival (Byrne *et al.*, 2005; Takahashi and Shibuya, 2005; Breen, 2007; Epstein, 2007). Women with advanced breast cancer who received anti-angiogenesis therapy showed improvement in outcome (Schneider and Sledge, 2007).

VEGF is a soluble, secreted growth factor and is expressed in cancers of many different histological types. VEGF levels have been correlated with prognosis of patients with breast cancer (Berns *et al.*, 2003), colorectal carcinoma (Lee *et al.*, 2000), prostate cancer (George *et al.*, 2001), lung cancer (Fontanini *et al.*, 1997), pancreatic cancer (Fujimoto *et al.*, 1998), melanoma, and gastric cancer (Maeda *et al.*, 1996; Takahashi *et al.*, 1996). VEGF has been reported as a strong independent predictor of relapse-free (Gasparini *et al.*, 1997; Eppenberger *et al.*, 1998) and overall survival (Gasparini *et al.*, 1997; Linderholm *et al.*, 1998) in node-negative breast cancer

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patients. It has also been reported that VEGF expression not in tissues, but in a serum sample was correlated with tumor vascularity, and high serum VEGF (sVEGF) levels could predict poor prognosis in cancer patients (Kido *et al.*, 2001; Karayiannakis *et al.*, 2002).

VEGF (MIM 192240) is localized on 6p21.3 (Vincenti *et al.*, 1996) and consists of eight exons exhibiting alternate splicing to form a family of proteins (Tischer *et al.*, 1991). DNA sequence variations in the VEGF may alter VEGF production, thereby causing inter individual differences in the susceptibility to cancer due to their effects on the pathways of tumor angiogenesis. VEGF-A is highly polymorphic in the promoter and 5' untranslated region and single-nucleotide polymorphisms in this region have been reported to regulate VEGF expression via alternative initiation of transcription and internal initiation of translation (Akiri *et al.*, 1998; Huez *et al.*, 1998). Polymorphisms of VEGF have been studied with disparate results in various diseases like type 2 diabetes (Awata *et al.*, 2002; Buraczynska *et al.*, 2007), giant cell arteritis (Rueda *et al.*, 2005), renal complications in Henoch-Schonlein purpura (Rueda *et al.*, 2006), hepatocellular carcinoma (HCC) (Kong *et al.*, 2007), prostate cancer (Onen *et al.*, 2008), familial endometriosis (Zhao *et al.*, 2008), ectopic pregnancy (Elito *et al.*, 2010), glomerulonephritides (Safrankova *et al.*, 2011), hypertension (Kim *et al.*, 2012), age-related macular degeneration (Lu *et al.*, 2012), and breast cancer (Lu *et al.*, 2005; Jacobs *et al.*, 2006; Langsenlehner *et al.*, 2008; Schneider *et al.*, 2008; Rodrigues *et al.*, 2012).

The insertion/deletion (I/D) polymorphism (rs35569394) in the promoter region of VEGF at the position -2549 relative to the translation start site has been linked to an increased transcriptional activity (Yang *et al.*, 2003). Several studies have analyzed the -2549 I/D polymorphism in the context of susceptibility to various diseases like Behcet's disease (BD) (Salvarani *et al.*, 2004), asthma (Lachheb *et al.*, 2008), HCC (He *et al.*, 2010), recurrent miscarriages (Aggarwal *et al.*, 2011), and end-stage renal disease (Prakash *et al.*, 2012). Association of the DD genotype of the -2549 I/D polymorphism has been reported with diabetic retinopathy (Awata *et al.*, 2002; Buraczynska *et al.*, 2007), diabetic nephropathy (Yang *et al.*, 2003), peripheral artery disease (Bleda *et al.*, 2012), and end-stage renal disease (Prakash *et al.*, 2012). A high frequency of the ID genotype has been reported in systemic sclerosis (Allanore *et al.*, 2007), prostate cancer (George and Mittal, 2011), and type 2 diabetic patients with peripheral artery disease (Bleda *et al.*, 2012).

In the Punjab state of North India, the cancer incidence is reportedly increasing (The Tribune, 2010). An increase in the frequency of sporadic breast cancer has been observed at the Sri Guru Ram Das (SGRD) Rotary Cancer Hospital, Vallah, Amritsar in the state of Punjab, North West India (personal communication). To identify the genetic reasons for the increase in breast cancer, the present study was aimed to evaluate the association of the -2549 I/D polymorphism in the promoter region of VEGF and sVEGF-C levels with a breast cancer risk. There is no published data on the VEGF -2549 I/D polymorphism in breast cancer from this region. To the best of our knowledge, it is the first study in breast cancer on VEGF, especially the -2549 I/D polymorphism in this population.

Materials and Methods

Study design and collection of genetic material

The present study was conducted in accordance with the principles of the Declaration of Helsinki and was approved by the ethics committee of the Guru Nanak Dev University, Amritsar, Punjab, India. Ninety-four (2 males and 92 females) clinically confirmed cases of sporadic breast cancer were selected from the SGRD Institute of Medical Sciences and Research, Vallah, Amritsar, Punjab. The patients who had not received chemotherapy, radiotherapy, or blood transfusion before surgery were included in this study. Ninety-four unrelated healthy control individuals with matched age and gender were also analyzed in the study. The control individuals with no history of any cancer or other chronic disease for the last three generations and not on regular medications for at least 2 years from the date of sampling were recruited from the same geographic area as that of patients. The demographic characteristics, a detailed family history, the reproductive history, and a previous disease history of all subjects were recorded on the pretested structured questionnaire. After informed consent, 8 mL blood sample of each subject was collected in sterile tubes. The serum was separated and stored at -20°C. Genomic DNA was extracted from blood using a standard phenol chloroform method.

Screening of -2549 I/D polymorphism (rs35569394) of VEGF

The I/D polymorphism localized in the promoter region of VEGF was screened by direct polymerase chain reaction (PCR) using published primer sequences (Buraczynska *et al.*,

FIG. 1. A photograph of a 2.3% ethidium bromide-stained agarose gel showing the insertion/deletion (I/D) polymorphism of VEGF at -2549 position. Lane M represents the 100-bp molecular weight marker, and lane 16 represents -ve control. Lane 1, 2, 12, and 13 represents individuals with the II genotype, lane 3 to lane 10 represents individuals with the ID genotype, and lane 11, 14, and 15 represents individuals with the DD genotype.

TABLE 1. CHARACTERISTICS OF BREAST CANCER PATIENTS AND CONTROL INDIVIDUALS

	Patients n (%)	Controls n (%)
Gender		
Female	92 (97.87%)	92 (97.87%)
Male	2 (2.13%)	2 (2.13%)
Mean age	49.62 ± 10.98	42.78 ± 13.11
Range (years)	25–75	23–76
Mean age at menarche	14.46 ± 1.78	14.5 ± 1.46
Mean age at menopause	46.74 ± 5.98	46.54 ± 7.29
Menstrual history		
Premenopausal	35 (38.04)	64 (69.56)
Postmenopausal	57 (61.96)	28 (30.44)
Early menarche (≤ 12 years)	7 (7.61)	5 (5.43)
Late menopause (≥ 55 years)	4 (4.35)	2 (2.17)
Stage		
I	11 (11.70)	—
II	47 (50.00)	—
III	25 (26.60)	—
IV	11 (11.70)	—

2007). Genomic DNA was amplified in a final reaction volume of 15 µL. The PCR conditions were initial denaturation at 95°C for 5 min followed by 35 cycles with denaturation at 95°C for 45 s, annealing at 55°C for 30 s, extension at 72°C for 45 s, and final extension at 72°C for 10 min in a Mastercycler gradient (Eppendorf). The amplified products were analyzed on 2.3% ethidium bromide-stained agarose gel. A band of 229 bp represents an 18-bp insertion (I allele), and the band of 211 bp represents an 18-bp deletion (D allele) (Fig. 1). Genotyping was performed without knowledge of the case or control status.

Analysis of sVEGF-C

sVEGF-C levels were measured using a commercially available human VEGF quantitative enzyme-linked immunosorbent assay (ELISA) kit (RayBiotech, Inc.), according to the manufacturer's instructions. All analyses were performed in duplicate and mean values were used for statistical calcu-

lations. The minimal detectable amount of VEGF-C by this kit was <15.0 pg/mL, and the maximum amount detected was 10,000 pg/mL.

Statistical analysis

The data were analyzed using SPSS (version 16.0; SPSS, Inc.) and expressed as mean ± standard deviation for quantitative variables, number, and percentage for qualitative values. Deviations from the Hardy–Weinberg equilibrium (HWE) were tested using the χ^2 goodness-of-fit test. Difference of genotype and allele frequencies between breast cancer patients and controls was assessed by the χ^2 test and an odds ratio (OR) with 95% confidence interval (CI) was used as a measure of strength of association. Correlation between sVEGF-C levels with a clinical stage and the –2549 I/D polymorphism was assessed using the Mann–Whitney U-test and the Kruskal–Wallis test. Probability values less than or equal to 0.05 were considered statistically significant.

Results

Characteristics of the patients and controls

Characteristics of the breast cancer patients and controls are listed in Table 1. The mean age of patients was 49.6 years (range 25–75 years). Of the 94 patients, 11 patients had stage I, 47 had stage II, 25 had stage III, and 11 had stage IV cancer. The mean age of controls was 42.78 years.

Analysis of –2549 I/D polymorphism

Genotype analysis revealed no significant deviation from the HWE in the patients and control groups ($p=0.85$ in cases, 0.237 in controls, and 0.489 in both). The frequency of II, ID, and DD genotype was 23.40 versus 10.64%, 48.94 versus 52.13%, and 27.66 versus 37.23%, in patients and controls, respectively (Table 2). A statistically significant difference was observed for genotype distribution in the patients and controls ($\chi^2=6.039$, $p=0.049$). A significantly increased frequency of I allele was also observed in patients (47.86%) as compared to the control group (36.70%) ($\chi^2=4.81$, $p=0.028$). The OR of the DD genotype versus II genotype between the breast cancer patients and controls was 0.34 (95% CI 0.14–0.83), while the OR of D versus I allele was 0.63 (95% CI 0.42–0.95).

TABLE 2. GENOTYPIC AND ALLELIC DISTRIBUTION OF –2549 INSERTION/DELETION POLYMORPHISM OF VEGF IN BREAST CANCER PATIENTS AND CONTROLS

	Patients n (%)					Controls n (%)	OR (95% CI)	χ^2 (p-value)
	Stage I n=11	Stage II n=47	Stage III n=25	Stage IV n=11	Total n (%)			
Genotypes								
II	2 (2.13)	12 (12.77)	4 (3.19)	4 (4.25)	22 (23.40)	10 (10.64)	1.00 (Reference)	6.039 (0.049)
ID	9 (9.57)	20 (21.28)	12 (12.77)	5 (5.32)	46 (48.94)	49 (52.13)	0.43 (0.18–1.0)	
DD	—	15 (15.96)	9 (9.57)	2 (2.13)	26 (27.66)	35 (37.23)	0.34 (0.14–0.83)	
Alleles								
I allele	13 (6.91)	44 (23.40)	20 (10.64)	13 (6.91)	90 (47.86)	69 (36.70)	1.00 (Reference)	4.81 (0.028)
D allele	9 (4.79)	50 (26.60)	30 (15.96)	9 (4.79)	98 (52.14)	119 (63.30)	0.63 (0.42–0.95)	

Figures in parentheses represents frequency of each genotype and allele; statistically significant p -values ($p<0.05$) are indicated in bold. HWE ($\chi^2=0.036$, $p=0.85$ in patients, $\chi^2=1.397$, $p=0.237$ in controls and $\chi^2=1.433$, $p=0.489$ in both).

n, number of subjects; sVEGF, serum vascular endothelial growth factor; OR, odds ratio; CI, confidence interval.

TABLE 3. CORRELATION BETWEEN sVEGF-C LEVELS AND VEGF –2549 INSERTION/DELETION POLYMORPHISM IN BREAST CANCER PATIENTS AND CONTROLS

Study group	No. of subjects	sVEGF-C Mean $\pm$ SEM, pg/mL	sVEGF-C Range, pg/mL	p-Value
Controls	94	318.19 $\pm$ 39.24	13.96–1500.26	<0.01 ^a
Patients	94	1026.71 $\pm$ 86.49	29.74–3634.29	
Patients				0.002 ^b
Stage I	11	343.13 $\pm$ 95.56	32.68–1045.06	
Stage II	47	1009.05 $\pm$ 108.3	57.87–3556.18	
Stage III	25	1358.04 $\pm$ 193.52	29.74–3634.29	
Stage IV	11	865.45 $\pm$ 280.70	98.84–3199.2	
– 2549 I/D polymorphism				
Patients				
Genotype				0.842 ^b
II	22	1147.68 $\pm$ 203.74	57.87–3199.2	
ID	46	949.42 $\pm$ 114.13	32.68–3556.18	
DD	26	1044.55 $\pm$ 168.85	29.74–3634.29	
Controls				
Genotype				0.186 ^b
II	10	463.42 $\pm$ 124.03	20–1248.49	
ID	49	323.58 $\pm$ 55.98	13.96–1500.26	
DD	35	269.14 $\pm$ 60.85	14.43–1445.38	

Statistically significant *p*-values (*p* < 0.05) are indicated in bold.

^aMann–Whitney U-test.

^bKruskal–Wallis test.

SEM, standard error of mean; I/D, insertion/deletion.

sVEGF-C analysis

The mean sVEGF-C concentration was found to be 1026.71 $\pm$ 86.49 pg/mL in breast cancer patients and 318.19 $\pm$ 39.24 pg/mL in controls, but there was a wide variation in the range (Table 3). There was a significant difference in sVEGF-C levels between cancer patients and healthy controls (*p* < 0.01). A significant association between sVEGF-C levels and the clinical stage was also observed (*p* = 0.002). Higher values of sVEGF-C levels were observed in clinical stage III patients, followed by stage II, stage IV, and stage I. The mean sVEGF-C concentrations for II, ID, and DD genotype of the VEGF –2549 I/D polymorphism was 1147.68 $\pm$ 203.74 versus 463.42 $\pm$ 124.03 pg/mL, 949.42 $\pm$ 114.13 versus 323.58 $\pm$ 55.98 pg/mL, and 1044.55 $\pm$ 168.85 versus 269.14 $\pm$ 60.85 pg/mL in patients and controls, respectively. Breast cancer patients with the II genotype had higher sVEGF-C levels as compared to the ID and DD genotypes, but the difference was not statistically significant (*p* = 0.842).

Discussion

In the present case–control study, 94 sporadic breast cancer patients (92 females and 2 males) and 94 unrelated healthy control individuals (92 females and 2 males) were analyzed. A significantly higher number of patients were postmenopausal (61.96%) as compared to controls (30.44%). In previous reports, an increased risk of breast and endometrial cancers has been reported in postmenopausal obese women in the Western (Reeves *et al.*, 2007), Korean (Song *et al.*, 2008), as well as Asian population (Kuriyama *et al.*, 2005).

VEGFs and their receptors play an important role in the formation of a vascular network. The vascular endothelial growth factor C (VEGF-C) and D (VEGF-D), key regulators of lymphangiogenesis, stimulate the proliferation, migration, and

survival of endothelial cells *in vitro*. They have been shown to induce lymphangiogenesis and facilitate metastases in transgenic mice and in other *in vivo* models (Mandriota *et al.*, 2001; Kleespies *et al.*, 2005). VEGF expression has been correlated with poor prognosis in many cancers, including carcinomas of the breast, kidney, colon, brain, ovary, cervix, thyroid, bladder, esophagus, and prostate, and in osteoid and soft tissue sarcomas and pediatric tumors (Liu *et al.*, 1995). Significantly elevated levels of sVEGF have also been reported in esophageal (Krzystek-Korpaczka *et al.*, 2007; Kimura *et al.*, 2008) and gastric cancer patients (Wang *et al.*, 2007; Seo *et al.*, 2010) than healthy controls. The measurement of the circulating soluble marker of angiogenesis has more advantages over other subjective approaches like immunohistochemical assessments or immunoassays. In the present study, we observed a significant elevation of sVEGF-C levels in breast cancer patients as compared to controls (*p* < 0.01). A significant association of sVEGF-C levels with the clinical stage has also been observed (*p* = 0.002). Several studies have reported higher sVEGF levels in patients with breast cancer and many other types of cancer, with higher levels in metastatic disease than in localized disease or in progressive disease during treatment (Yamamoto *et al.*, 1996; Salven *et al.*, 1997, 1999; Kraft *et al.*, 1999; Adams *et al.*, 2000; Heer *et al.*, 2001; Ali *et al.*, 2011). In a previous study from India, significantly higher sVEGF levels have been reported in breast cancer patients as compared to controls (Pande *et al.*, 2011).

Inter individual differences in the susceptibility and severity of a disease might be explained by the effect of functional polymorphisms that have effect on the regulation of gene expression. A number of functional polymorphisms in the VEGF have been reported and have been associated with an increased risk for several tumors (Krippel *et al.*, 2003; Lin *et al.*, 2003). The –2549 I/D polymorphism has been reported

to be in perfect linkage disequilibrium with the –2578 C/A polymorphism (rs699947) (Jin *et al.*, 2005; Nakanishi and Watanabe, 2009; Yuan *et al.*, 2009). Individuals with the –2578A allele have an 18-bp insertion, while those with the –2578C allele have a deletion. Association of the –2578A allele has been reported with the risk of breast cancer (Schneider *et al.*, 2008) and with thyroid cancer development and regional lymph node metastasis in men (Hsiao *et al.*, 2007).

In the present study, DD and ID genotypes and the D allele frequency of the –2549 I/D polymorphism were lower in breast cancer patients as compared to controls. The *in vitro* functional studies reported that the presence of the D allele at –2549 position in the promoter region leads to enhanced expression of VEGF (Cooper *et al.*, 1999). It has been reported that constructs containing the 18-bp deletion had a 1.95-fold increase in the transcriptional activity compared with those containing the insert (Yang *et al.*, 2003). Contrary to previous reports of ID or DD genotypes being associated with systemic sclerosis in the European Caucasians (Allanore *et al.*, 2007), HCC in the Chinese Han population (He *et al.*, 2010), and in recurrent miscarriages in the North Indian population (Aggarwal *et al.*, 2011), in the present study, frequencies of the II genotype and I allele were significantly higher in breast cancer patients than controls thus indicating a probable decreased transcriptional activity of VEGF in patients.

The DD genotype of the –2549 I/D polymorphism has been associated with diabetic retinopathy (Awata *et al.*, 2002; Buraczynska *et al.*, 2007), diabetic nephropathy in type 1 diabetic patients (Yang *et al.*, 2003), and peripheral artery disease in type 2 diabetic patients (Bleda *et al.*, 2012). The ID genotype has been described as a potential risk marker for diabetic retinopathy in Caucasian patients of Spanish origin (Bleda *et al.*, 2012).

In previous studies from North India, the DD genotype has been associated with end-stage renal disease (Prakash *et al.*, 2012), while the ID genotype has been associated with greater than twofold risk of prostate cancer (George and Mittal, 2011). For the –2549 I/D polymorphism, association of the I allele has been previously reported with BD in Italian patients (Salvarani *et al.*, 2004) and with its severity in Tunisian patients (Kamoun *et al.*, 2008).

In the present study, the population in Amritsar has a racial mixture of Indo-Scythian and Caucasian (Bhasin *et al.*, 1992). The breast cancer patients of Amritsar have a significantly higher frequency of the II genotypes and I allele. To the best of our knowledge, there are no previous reports on the association of the II genotype and I allele with breast cancer from India. Thus, the –2549 I/D polymorphism is among the genetic factors responsible for predisposition to breast cancer. A higher level of sVEGF-C was observed in the breast cancer patients, but a clear association of the sVEGF levels with a particular genotype of the –2549 I/D polymorphism and the clinical stage was not apparent probably due to the small sample size. Therefore, further studies are required to assess its relation with disease progression and survival post-therapy in breast cancer. A follow up of the patients has been initiated to compare the pre- and post-therapy sVEGF-C level to assess the efficacy of therapy.

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