

## RESEARCH ARTICLE

# SNP and Haplotype Analysis of Vascular Endothelial Growth Factor (VEGF) Gene in Lung Cancer Patients of Kashmir

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### Abstract

Vascular endothelial growth factor (VEGF) is a major mediator of angiogenesis involving tumor growth and metastasis. In this large case-control study, we investigated whether functional polymorphisms (+405C>G, +936C>T) in the VEGF gene are associated with the risk of lung cancer. The study investigates the association between variants of VEGF gene and lung cancer. We performed single nucleotide polymorphism (SNP), haplotype and linkage disequilibrium studies on 100 patients and 128 healthy controls with 2 SNPs in the VEGF gene. The results were analyzed using logistic regression models, adjusted for age and sex. No Significant association was detected between individual SNPs and lung cancer using all the models of inheritance (codominant, dominant, recessive, over dominant and additive) for finding an association between genotypes and the cancer risk. The P values obtained for two markers were non-significant (P>0.05). Haplotype analysis produced additional support for the non-association of individual haplotypes/all haplotypes with the cancer risk (Global association P=0.56). Our findings suggest the non-involvement of genetic variants (+405C>G, +936C>T) of the VEGF gene in the etiology of lung cancer.

**Keywords:** SNP- VEGF- haplotype-lung cancer

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### Introduction

Cancer is a result of multiple gene-environment interactions occurring over several decades. Certain chemical carcinogens may contribute to the carcinogenic process in lung epithelial cells by inducing genomic instability, either directly or indirectly, through inflammatory processes (Haugen A et al., 2000). The major risk factor for lung cancer is an excessive exposure to tobacco smoke. However, only about 11% of tobacco smokers ultimately develop lung cancer, suggesting genetic factors may influence risk for lung cancer among those who are exposed to carcinogens. After the effect of tobacco smoke was stratified, an approximately 2.5-fold risk was attributable to a family history of lung cancer (Amos CI et al., 1999). Therefore, it is rational to speculate that certain common genetic variants or polymorphisms may have an impact on lung cancer risk. VEGF, also called VEGF-A, belongs to the cysteine-knot superfamily of growth factors that are characterized by the presence of eight conserved cysteine residues (Ciulla TA et al., 1999; Muller YA et al., 1997). Vascular endothelial growth factor (VEGF) was first described as a vascular permeability factor in tumor cells because of its ability to generate

tissue edema (Ferrara N, 2004). In humans, it is expressed by different cell types, including smooth muscle cells, macrophages, neutrophils, and platelets (Hamamichi Y et al., 2001). Expression is regulated by low oxygen tension, growth factors such as transforming growth factor, and inflammatory cytokines such as interleukin-6 (Ferrara N et al., 2003). Besides its role in vascular permeability, VEGF is a multifunctional angiogenic regulator involved in blood vessel formation, mitogenesis, epithelial cell proliferation, and endothelial cell survival (Ferrara N et al., 2004). VEGF has multiple isoforms, whose distinct properties affect both availability and signaling function of this angiogenic factor. The gene encoding VEGF is located on the short arm of chromosome 6 (6p21.1) in humans (Tischer E et al., 1991). Human VEGF gene is composed of eight exons, separated by seven introns. Numerous SNPs in the promoter, 5'- and 3'- untranslated regions (UTRs) are present in VEGF. Genome-wide association studies (GWAS) of lung cancer identified inherited susceptibility variants on chromosome 15q25, 5p15 and 6p21 (Landi MT et al., 2009). Numerous single nucleotide polymorphisms (SNPs) identified in regions of human genome that associate with a particular trait or disease, genome-wide association studies (GWAS)

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compare the frequency of inherited variation between two groups of individuals. Comparing individuals with and without lung cancer, this approach identified several susceptibility regions of interest (Broderick P et al., 2009; Wang Y et al., 2008.). The 5'- and 3'- UTR contain key regulatory elements which are sensitive to hypoxia (Minchenko A et al., 1994), and contributes to high variability in VEGF production among tissues (Schultz A et al., 1999). For example -634G>C SNP in the 5'-UTR of VEGF affects the protein translation efficiency (Watson CJ et al., 2000) and 936C>T SNP in the 3'-UTR influences the circulating plasma concentrations (Renner W et al., 2000) and tumor tissue expression of VEGF (Koukourakis MI et al., 2004). However, it is likely that only a small number of these polymorphisms and haplotypes (linearly linked SNPs) actually have a functional effect on VEGF translation, whereas others act as proxies (Pander J et al., 2007). Therefore the aim of the present study was to investigate whether the two variants in the VEGF gene, either individually or as part of a haplotype have a role in making individuals prone to lung cancer.

## Materials and Methods

### Study population

The present study included 100 lung cancer patients and 128 healthy controls. Patients were recruited between June 2009 and December 2010 at SKIMS (Tertiary care 600 bed Hospital) India. Eligible cases, including any person over the age of 18 with incident primary lung cancer confirmed by hospital pathologists, were enrolled with no limitations on tumor histology, or stages. But the patients but patients with a prior history of cancer other than lung cancer, who received chemotherapy were excluded from the study. Control subjects were cancer-free individuals

Table 1. Characteristics of Patients and Healthy Controls

Variable	Cases	Controls	*P value
Total	100	128	
Gender			0.16
Male	84	98	
Female	16	30	
Age(Yrs.) Mean± SD	58±11.56	55±12.48	0.06
Smoking status			0.1
Smoker	79	89	
Non-smoker	21	39	
Histology			
Squamous cell carcinoma	65	NA	
Adenocarcinoma	8		
Large cell carcinoma	27		
Grade			
G1	57	NA	
G2	25		
G3	18		
Stage			
I and II	67	NA	
III and IV	33		

\*P value calculated by Pearson Chi-square statistic

randomly selected from the health examination clinics of the same hospital during the same time period of case recruitment. Controls with a prior history of cancer were excluded. The control subjects were frequency matched to the cases on the bases of age, gender and smoking as shown in Table 1. The study was performed after approval by the institutional review board. At recruitment, a trained research nurse was assigned to obtain informed consent for the collection of a blood sample and to administer a structured questionnaire. The questionnaire collected information about demographic characteristics, lifestyle factors (such as number of cigarettes smoked), medical history, and family history of cancer. For smoking status, a person who was then smoking at least once a day and had been doing so for more than 6 months was regarded as a smoker.

### Genotyping analysis

For the DNA polymorphism study, we used 100 lung cancer cases and 150 normal controls. DNA extraction was performed according to the manufacturer's protocol for Qiagen DNA extraction kits (Qiagen, Hilden, NRW, Germany). DNA content was quantified by spectrophotometric absorption (Nanodrop Spectrophotometer, BioLab, Scoresby, VIC, Australia). Polymerase chain reaction (PCR) was performed using an iCycler Thermal Cycler (Bio-Rad, Hercules, CA, USA). VEGFA +405C>G (rs2010963), +936C>T (rs3025039) and -634G>C (rs2010963) genotypes were determined using PCR-RFLP method. Amplifications were carried out and a PCR product was generated using the specific forward and reverse primers. Primers were designed and selected using Primer3, version 0.4.0 software. For +405C>G, forward primer 5'-CCACTTGAATCGGGCCGACG-3', reverse primer -5'-GTCTGTCTGTCTGTCCGTC-3', for +936C>T, forward primer 5'-AGGAAGAGGAGACTCTGCGCAGAGC-3', reverse primer 5'-CCTGTAGACACACCCACCA TACATACATTTA-3' and for -634G>C forward primer 5'-ATTTATTTTTGCTTGCCATT-3', reverse primer 5'-GTCTGTCTGTCTGTCCGTC-3'. The annealing temperatures for VEGFA +405C > G, +936C>T and -634G>C were 610C, 580C and 600C and the restriction enzymes used were BsmFI and NlaIII (New England Biolabs).

### Statistical analysis

Allele and haplotype frequencies were compared

Table 2. SNP +405C>G Genotype and Allele Frequencies (N=228)

	Total (%)	Controls (%)	Cases (%)
Genotype			
C/C	78(0.34)	36(0.31)	42(0.38)
C/G	130(0.57)	71(0.61)	59(0.53)
G/G	20(0.09)	09(0.08)	11(0.1)
Allele frequencies			
C	286(0.63)	143(0.62)	143(0.64)
G	170(0.37)	89(0.38)	81(0.36)

C, cytosine; G, Guanine

Table 3. SNP+405C>G Association with Disease (n=228, adjusted by Sex and Age)

Model	Genotype	Control	Case	aOR (95% CI)	*P-value	AIC(Akaike information criterion)
Co- dominant	C/C	36(31%)	42(37.5%)	1	0.41	322.1
	C/G	71(61.2%)	59(52.7%)	0.70 (0.40-1.24)		
	G/G	09(7.8%)	11(9.8%)	1.03 (0.38-2.79)		
Dominant	C/C	36(31%)	42(37.5%)	1	0.29	320.8
	C/G+G/G	80(69%)	70(62.5%)	0.74 (0.43-1.29)		
Recessive	C/C+C/G	107(92.2%)	101(90.2%)	1	0.6	321.6
	G/G	09(7.8%)	11(9.8%)	1.28 (0.51-3.25)		
Over-dominant	C/C+G/G	45(38.8%)	53(47.3%)	1	0.18	320.2
	C/G	71(61.2%)	59(52.7%)	0.70 (0.41-1.19)		
additive	-----	-----	-----	0.88 (0.57-1.35)	0.56	321.6

\*P-value calculated by the  $\chi^2$  test; aOR and 95%CI calculated by logistic regression, adjusted for age and sex; Major genotype is indicated as Reference.

Table 4. SNP 936C>T Genotype and Allele Frequencies (n=221)

	Total (%)	Controls (%)	Cases (%)
Genotype			
C/C	133 (0.6)	65 (0.58)	68 (0.62)
C/T	70 (0.32)	38 (0.34)	32 (0.29)
T/T	18 (0.08)	09 (0.08)	09 (0.08)
Allele frequencies			
C	336 (0.76)	168 (0.75)	168 (0.77)
T	106 (0.24)	56 (0.25)	50 (0.23)

C, cytosine; T, Thymine

between cases and controls using Fisher's exact test. The  $\chi^2$ -test was used to examine whether the genotype distributions were within the Hardy-Weinberg equilibrium. An exact test for Hardy-Weinberg equilibrium is performed, which was also used to examine the statistical significance of the differences in the allele frequency and genotype distribution between the groups. The odds ratios (ORs) and 95% confidence intervals (CIs) were obtained using logistic regression analysis. Since the EM algorithm does not accurately estimate haplotypes frequencies below 1%, haplotypes <1% in both groups were not considered. The analyses were conducted using SPSS version 11.5 (SPSS, Chicago, IL, U.S.A.). P values

< 0.05 were considered significant.

Calculation of pair wise LD between the SNPs was carried out using the ARLEQUIN 2.0 (Schneider S et al., 2000). We used D' to describe the magnitude of LD (Lewontin RC, 1988) and  $\chi^2$  square values for allelic association (calculated by the Arlequin program) to determine if the D'-value was statistically significant.

## Results

Among the recruited 100 lung cancer patients, 39 were classified as squamous cell carcinoma (SCC), 61 as adenocarcinoma and large cell carcinoma. There were no statistical differences between the cases and controls for the mean age, gender, or smoking status. To explore a possible association between lung cancer and the 2 SNPs (rs2010963 and rs3025039) of VEGF, the genotype distributions were compared between cases and controls and the effects of lung cancer risk factors were simultaneously adjusted in the multiple logistic regression models. We performed an association study with 2 SNPs in the VEGF gene and compared 100 patients suffering from lung cancer with 128 healthy controls. All SNPs were found to be in HWE ( $P > 0.05$ ) in both the cases and control samples except +405C>G genotype frequencies of control population as shown in Table 2 and Table 4.

Table 5. SNP+936C>T Association with Disease (N=228, Adjusted by Sex+Age)

Model	Genotype	Control	Case	a OR (95% CI)	*P-value	AIC(Akaike information criterion)
Co-dominant	C/C	65(31%)	68(37.5%)	1	0.79	314.1
	C/T	38(61.2%)	32(52.7%)	0.81 (0.45-1.46)		
	T/T	09(7.8%)	09(9.8%)	0.97 (0.36-2.60)		
Dominant	C/C	65(31%)	68(37.5%)	1	0.54	312.2
	C/T+T/T	47(69%)	41(62.5%)	0.84 (0.49-1.45)		
Recessive	C/C+C/T	103(92.2%)	100(90.2%)	1	0.94	312.6
	T/T	09(7.8%)	09(9.8%)	1.04 (0.39-2.73)		
Over dominant	C/C+T/T	74(38.8%)	77(47.3%)	1	0.49	312.1
	C/T	38(61.2%)	32(52.7%)	0.82 (0.46-1.45)		
additive	-----	-----	-----	0.91 (0.60-1.38)	0.66	312.4

\*P-value calculated by the  $\chi^2$  test; aOR and 95%CI calculated by logistic regression, adjusted for age and sex; Major genotype is indicated as Reference.

Table 6. D, D', R and P-Values for All Combinations of the VEGF Snps in Cases and Controls

	+405C>G	936C>T
+405C>G		-0.0716 0.801 -0.3468 0
936C>T	D D' r P	

Inter-marker LD

The standardized measure of LD denoted as D, D', r as well as the corresponding P-values were calculated for a pair of SNP in both the cases and controls. Table 6 shows this data. For a pair of SNP, the statistical significance of the D'-value was calculated. D'-value of the SNP combination was statistically significant with (p-value <0.05).

Single-marker/ Haplotype association analysis

Given that most individuals with lung cancer (especially male) are smokers in Kashmir, the risk of VEGF +405C>G and 936 C>T polymorphisms related to lung cancer was examined with stratification by gender and age. To increase statistical power, adjusted ORs were assessed using co-dominant, dominant, recessive, over-dominant and additive genetic models. The inheritance model with the lowest AIC (Akaike information criterion) is considered appropriate for the individual SNP data. The results of the single SNP association analysis are presented in Table 3 and Table 5. We found no statistically significant differences between the genotype distribution of VEGF +405C>G and 936C>T polymorphism and lung cancer. To better describe the contribution of the VEGF locus to the determination of lung cancer risk, we next studied genetic variants in the context of their haplotypes. The haplotype frequency is estimated via the EM Algorithm (Expectation Maximization algorithm) for two SNP's. Haplotype analyses also did not reveal any association with lung cancer risk like genotype analyses at each locus alone. Table 7 shows the haplotype analysis for the 2 SNPs. Haplotypes with <1% frequency were excluded from haplotype analysis. Haplotype frequencies were estimated from the genotyping data after stratified by

gender and age. Furthermore, to examine the effect of specific haplotypes involved in lung cancer, the software by default selects the most common haplotype as the reference group and estimate haplotype-specific ORs by the haplotype-based logistic regression method. The advantage of using a common haplotype as reference is that it is more homogenous than a pool of different haplotypes, i.e., each haplotype is compared with the same reference group for consistency. Table 9? demonstrates the frequencies for the estimated 2-marker haplotypes among patients and controls. We observed two major haplotypes that account for 76% of all possible combinations in the patient sample and 74% in the control sample. A non-significant evidence for association with lung cancer was provided by a P-value (Global-test) of 0.56. Individual haplotype tests by examining the distribution differences for each haplotype showed that distinct haplotypes did not differ significantly between cases and controls except the haplotype 4 which is six times as frequent in controls (P=0.64).

Discussion

In the present study, neither VEGF +405G>C and +936C>T polymorphisms nor haplotypes of the two SNPs significantly influenced susceptibility to NSCLC. This finding may be because each polymorphism alone is insufficient to influence the susceptibility to lung cancer, but that set of two polymorphisms (haplotype) effect on lung cancer risk due to a combined effect on gene function. Another possible explanation is that effect of VEGF haplotypes on lung cancer risk may be due to linkage disequilibrium with other functional variants in the VEGF gene (Shahbazi M et al., 2002; Stevens A et al., 2003). Thus, additional studies with more subjects are needed to confirm this finding. Previous studies have reported that +405C/G polymorphisms were associated with VEGF expression, production, and disease development. Individuals carrying the +405CC and CG genotypes were linked with higher VEGF expression and vascular density in the tumor of NSCLC (Koukourakis MI et al., 2004). (Sfar S et al., 2006) reported that the combined +405CC/CG genotype was associated with increased risk of prostate cancer. In Asian populations, the combined +405CC/CG genotype was associated with increased risk of small cell lung cancer (Lee SJ et al., 2005). The +405CC genotype was also reported to be associated with higher serum VEGF levels, increased risks of retinopathy and myocardial infarction in diabetes (Awata T et al., 2002;

Table 7. Haplotype Frequencies Estimation and Haplotype Association with disease (Adjusted by Age and Sex) [N=231]

+405C>G	936C>T	Total	Controls	Cases	Cumulative frequency	OR (95% CI) <sup>β</sup>	*P-value (χ <sup>2</sup> -test.)
C	C	0.4058	0.379	0.4333	0.4058	1.00(ref.)©	
G	C	0.3555	0.3712	0.339	0.7612	0.70 (0.40 - 1.21)	0.21
C	T	0.221	0.2364	0.2052	0.9823	0.75 (0.45 - 1.23)	0.26
G	T	0.0177	0.133	0.0225	1	1.55 (0.25 - 9.50)	0.64

<sup>β</sup>, All haplotypes were included in the same model and the OR (95%CI) of lung cancer associated with each haplotype estimated by comparison with the common reference haplotype; ©ref: reference group.

Awata T et al., 2005; Petrovic D et al., 2007), and higher tumor aggressiveness in breast cancer (Jin Q et al., 2005). There is debate on the exact function of the +405G>C polymorphism, some studies have shown that the +405C allele is associated with lower VEGF production and VEGF promoter activity (Watson CJ et al., 2000; Stevens A et al., 2003). In addition, some studies do not show a role of the +405G>C polymorphism in VEGF production or disease risk (Awata T et al., 2002; Petrovic D et al., 2007). In case of individual SNP analysis, we found a statistically non-significant association of genotypes of rs2010963 and rs3025039 with lung cancer risk in all the genetic models used for analysis (P values > 0.05). VEGF polymorphisms also showed non-significant association with the cancer risk, on analysis using multivariate analysis (P>0.05). The results of the present study suggest that polymorphisms in VEGF and distinct haplotypes of 2 SNPs did not represent risk factors in lung cancer. However, possibility of a susceptibility locus lies in other parts of VEGF needs to be examined with more polymorphisms. Based on the pathologic significance of VEGF in NSCLC and the potential biological effects of VEGF polymorphisms on VEGF production, we hypothesized that functional single nucleotide polymorphisms of the VEGF gene would be associated with differential risk of NSCLC. Although a number of epidemiologic studies have proved that incidence, risk, histology, and pathogenesis of lung cancer differed between women and men, the mechanisms driving these differences are largely unknown (Caracta CF, 2003). Genetic factors have been proposed to account for gender differences in lung cancer risks. For example, there was a higher frequency of tumor suppressor gene p53 mutations among women with NSCLC than among men with NSCLC; proto-oncogene K-ras gene mutations have been found to be more common in female patients with lung cancer who were smokers than among male smokers with lung cancer (Olak J et al., 2004). Both in vitro and in vivo studies have proved that androgen could up-regulate VEGF expression (Sordello S et al., 1998; Stewart RJ et al., 2001), whereas androgen ablation inhibited VEGF expression (Sibug RM et al., 2002). On the contrary, estrogen reduced VEGF expression (Joseph IB et al., 1997; Niklaus AL et al., 2002). The frequencies of haplotype +405C/936C, +405G/936C, +405C/936T and +405G/936T among the controls in the present study were 0.379, 0.371, 0.236, and 0.133, respectively. In the present study, variant allele frequency of +405C>G was 0.38 which was lower than (0.519) and 936C > T frequency was 0.25 which was higher than (0.20) among controls (Lee SJ et al., 2005). No significant association was detected in single-marker and haplotype tests, with statistically non-significant Global P-value based on permutation procedures that account for haplotypes tested (haplotype test, P=0.56). Since none of the haplotype is associated with lung cancer risk when analyzed independently through different inheritance models, this finding is consistent with the genotypic data.

In conclusion, results presented in the study revealed that genotypic association at a specific locus and haplotype association are in agreement with the idea that genetic variants of VEGF (+405C>G & 936C>T) did not play

significant role in the etiology of lung cancer. However, this study could not exclude the possibility that more than two SNPs could influence NSCLC carcinogenesis. Future studies on other VEGF sequence variants and on their biological functions are also needed to understand the role of the VEGF polymorphisms and haplotypes in determining the risk of lung cancer. Moreover, since genetic polymorphisms often vary between ethnic groups, further studies are needed to clarify the association between the VEGF polymorphism and lung cancer in diverse ethnic populations.

#### Conflict of Interest

None of the authors declared conflict of interest

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