RESEARCH ARTICLE

Role of +405C>G and +936C>T Polymorphisms of the Vascular Endothelial Growth Factor Gene and Risk of Esophageal Cancer in the Kashmiri Population

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Abstract

Background: The gene for the vascular endothelial growth factor (VEGF), which promotes angiogenesis and permeability, is polymorphic. The aim of the present study was to evaluate the relationship between +936C>T and +404C>G polymorphism of VEGF with risk of esophageal cancer in the Kashmiri population in India. Materials and Methods: 150 esophageal cancer patients and 150 unrelated healthy controls were genotyped for two VGEF SNPs (+405C/G, and +936C/T) using DNA extracted from prospectively collected blood samples by the PCR-RFLP method. Results: For the VEGF +936C>T polymorphism a significant association of CT and combined CT+TT genotypes was observed with increased risk of esophageal cancer (p=0.021; 0.024). For the +405C>G polymorphism we observed significantly increased frequency of GG genotype in cases as compared to controls and also the +405 GG Genotype was observed to have a two fold risk(OR=2.7356; 95%CI=1.1409-6.5593; p=0.020). The combined genotypes of GG-CC and GG-CT of +405C>G and +936C>T were found to be significantly associated with increased risk of esophageal cancer (p=0.0376; 0.0099). Conclusions: From the results of the present study a significant association of +936C>T and +405C>G polymorphisms with increased esophageal cancer risk exists in the Kashmiri population.

Keywords: Esophageal cancer - VEGF - polymorphisms - angiogenesis - Kashmir - India

Asian Pac J Cancer Prev, **16 (1)**, 97-101

Introduction

Esophageal cancer is the eight most common cancer and sixth cause of cancer deaths worldwide, with the majority of cases occurring in developing countries. There are striking geographic variations in incidence. Very high incidence rates (over 40-50/100,000/year in males and in females) have been consistently reported in a region that extends from the Caspian Sea to Central Asia, defining the so called "Esophageal Cancer Belt"(Mir and Dar, 2009). Kashmir Valley, in Northern India, lies at the south border of this high incidence region. Although there is no continuous cancer registration in Kashmir, current observational studies indicate that Kashmir Valley is a region of high risk of esophageal cancer (Mattoo and Kaul, 1974) with incidence of 42 and 27/100.000/year for men and women, (Khuroo et al., 1992) Several previous studies have attributed high incidence of esophageal and gastric cancers in Kashmir to considerable amount of nitroso compounds in raw foodstuffs and use of hot salted tea (Khuroo et al.,1992; Siddiqi et al., 1992). Salted tea used in Kashmirvalley has considerable amounts of N-nitrosoproline (NPRO) (360 micrograms/kg) and N nitrosopipecolic acid (NPIC) (5870 micrograms/kg) which may impart risk for EC in this area (Siddiqi et al., 1988).

The incidence of esophageal cancer, particularly the S subtype, is rising at an alarming rate. the Kashmiri population has a distinct, social, culture and dietary habits and distinct climate. A study carried out by the Radio Oncology Dept. of SKIMS from 2007-2013 around 10000 patients with different cancers were registered with Esophageal cancer topping the list more than 1100 patients with esophageal cancers were registered. The esophageal cancer according to the study is still dominating all other types of cancers in the Kashmir valley In 2013,the number of new cases of esophageal cancer in the SKIMS to reach 2100, but the number of deaths is estimated to approach 1800, highlighting the poor prognosis of this disease. Multimodality treatment regimens, with chemo radiation and surgery, are frequently recommended, although the additional benefit from this approach is controversial. In addition, trimodality therapy is associated with significant side effects, and treatment delivery is challenging in this patient population in which co morbidities are common. Prognostic factors have the potential to improve the selection of patients for whom additional treatments are most beneficial

Angiogenesis is a regulated process of new vessel formation that has a role in a discrete number of normal physiological events, but is activated inappropriately in a

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range of diseases, and is an early event in carcinogenesis. (Ferrara, 2002). Vascular endothelial growth factor A (VEGF) is the most potent angiogenic factor. VEGF induces endothelial cell proliferation and migration, enhances vascular permeability, reduces endothelial cell apoptosis and promotes stromal proteolysis. (Ferrara and Davis-Smyth, 1997). As in other malignancies, VEGF is of interest in esophageal cancer as a potential prognostic and predictive marker. Multiple single nucleotide polymorphisms (SNP) have been identified within the VEGF gene, +405G/C and +936C/T are in linkage disequilibrium and are of interest due to their potential functional implications. Investigated, prospectively, in a large cohort of patients with esophageal cancer by evaluating the association of each SNP independently, (Chae et al., 2006).

Materials and Methods

Subjects

The study was approved by the institutional Ethical committee of sheri Kashmir institute of medical sciences Soura Srinagar Kashmir India. This study included 150 Esophageal cancer patients and 150 age, sex, dwelling and smoking matched controls. All the Esophageal cancer cases selected for study were histologically confirmed SCC and adenocarcinoma cases. Patients were recruited from OPD, Department of Medical Oncology and department of Radiation oncology who visited our institute from April 2010 to Nov 2013. All those Patients who had prior history of cancer other than esophageal cancer and patients who had received any chemotherapy/radiotherapy were excluded from our study. All participants of the control group were selected from individuals receiving routine medical examinations in the same hospital, with no history of other cancer, and no symptoms of other acute or chronic inflammations. The structured questionaire was used during an in person interview to get information on personal and disease history of the subject.

Genomic DNA extraction and genotyping

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, and Australia). Polymerase chain reaction (PCR) was performed using an iCycler Thermal Cycler (Bio- Rad, Hercules, CA, USA). *VEGF 936C>T* and VEGF 405C>G genotypes were determined using PCR-RFLP method. Amplifications were carried out and a PCR product was amplified using the specific forward and reverse primer. Primers were designed and selected using Primer3, version 0.4.0 software. The PCR reaction mixture consisted of Taq (1.5 U), sense and antisense primers (0.5 μmol/l), Mg_{2+} (50 mmol/l), dNTP (0.2mmol/l), and template (1 μg) and was subjected to an initial denaturing step of 5 min at 95°C, then 35 cycles of denaturing for 30 s at 95°C, annealing for 30s at 56°C, extension for 30 s at 72°C, and a final extension step of 10 min at 72°C. Digestion of the amplified products of *VEGF 936C>T* and VEGF -405 C>G was done by using 10 units restriction endonucleases *NlaIII* (fermentas) and *BsmFI* (fermentas) respectively and incubated at 37°C for 16 hours. The digested products were checked on 3% agaroses gel, the RFLP picture for VEGF 936 C>T genotype was identified as *(C/C genotype (size 208bp), C/T genotype (size 208, 122, 86bp) T/T heterozygous genotype (size 122, 86bp)* and VEGF 405 C>G was identified as *C/C genotype (size 282 bp)), G/G genotype (size 181bp+101bp) C/G heterozygous genotype (size 282, 181 & 101bp)*

Statistical analysis

The distribution of the *VEGF 936C>T and VEGF 405 C>G* genotypes in controls was compared using t-test for continuous or Chi-square test for categorical variables. Chi-square test was also used to evaluate deviations from Hardy-Weinberg equilibrium (HWE), compare genotype and Allelic differences and to assess the association of polymorphism with various parameters. Unconditional logistic regression was used to compute the odds ratios (ORs) and their 95% confidence intervals (CIs), with adjustments for age, sex dwelling and smoking. Fisher's exact test for calculating P values and unpaired t test was used to comapre the mean, SD. Significance level was taken at $p<0.05$. Statistical tests were performed using the software SPSS 8.0 (SPSS Inc., Chicago, Illinois).

Results

A total of 150 esophageal cancer cases and 150 healthy controls were successfully evaluated for genotyping of VEGF +936C>T and +405C>T. The frequencies of tested genotypes in cases and controls are given in Table

Table 1. Genotype and Allele Frequencies 0f 936C/T and 405C/G Polymorphism in Esophageal Cancer Patients and Controls

Genotype		Patientn=150	Controlsn=150	(95% CI)	Odds ratio	P value
VEGF 936C/T	CC	110 (73.3)	126 (76.6)	Ref		Ref
	CT	38 (24)	21 (16.6)	1.1477-3.7434	2.0727	0.021448
	TT	02 (2.6)	03 (2)	0.1253-4.6541	0.7636	1
	C	117 (88)	142 (92)			
	T	33 (22)	8 (5.3)	1.505-8.0898	3.4893	<.0001
VEGF405-C/G	CC	09 (6)	17 (11.3)	Ref		Ref
	CG	57 (38)	66 (44)	0.6751-3.9419	1.6313	0.27332
	GG	84 (56)	58 (38.6)	1.1409-6.5593	2.7356	0.020604
	C	39 (26)	62 (41.3)			
	G	111 (74)	88 (58.6)	1.2302-3.2686	2.0052	0.00721

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

1). The observed genotypes for the controls population was in complete accordance with the Hardy Weinberg equilibrium (P >0.05). The frequencies of CC, CG, GG genotype of VEGF+405 C>G polymorphism were CC= 6 *vs* 11.3 %, CG=38 *vs* 44 %, GG=56 *vs* 38.6% in cases and controls respectively. In this study we observed significantly increased frequency of GG Genotype in cases as compared to controls (56 *vs* 38.6%; p=0.020). Association of VEGF+405GG genotype was also observed with increased risk of esophageal cancer (OR=2.736 and 95%CI=1.149-6.553). In addition to that we also observed the combined genotype CC and GG was also associated with higher Esophageal cancer risk in homozygous co dominant model (OR=2.736, 95%CI;1.149-6.553).We also observed significantly increased frequency of G allele in patients which exhibited 2.00 fold higher risk to Esophageal cancer (OR=2.0052, 95%CI, 1.2302-3.2686, p=0.00721) Table 1. For VEGF+936C>T polymorphism the frequencies of CC, CT, TT Genotype were CC=73.3 *vs* 76%;CT=24 *vs* 16.6%;TT= 2.6 *vs* 2% in cases and controls respectively. There was significantly increased frequency of CT Genotype in Esophageal cancer patients as compared to controls (24 *vs* 16.6; p=0.021).Patients having CT genotype were associated with two fold risk to Esophageal cancer (OR=2.0727; 95%CI=1.1477-3.7434).In the dominant model individual carrying the combined CT+TT Genotype were significantly associated 1.901 fold risk for Esophageal cancer as compared to CC genotype (OR=1.901;95%CI=1.089-3.368; p=0.024). There was also highly significant differences between C and T allele frequencies in the Esophageal cancer patients and controls (p≤0.0001).T alleles were associated with high risk of developing esophageal cancer (OR=3.4893;95%CI=1.505-8.0898)

We also analyzed the combined genotype of two polymorphism and observed that Genotype combination CG-CT; GG-CC and GG-CT VEGF +936C>T and 405C>G polymorphism were more common in patients than controls (p≤0.05).Patients having GG-CT Genotype combination (p=0.0099) are more Susceptible to esophageal cancer Tables 2 and 3.

Table 2. Combined Genotype Frequencies in Esophageal Cancer Patients and Controls

VGEF+405C>G and 936+ C>T Genotype combination	Patients n(%)	Controls n(%)	95%CI	P value
CC - CC	7 (4.6)	15 (10)	Ref	Ref
CC - CT	2 (1.3)	2 (1.3)	0.2482-18.499	0.590
CG - CC	48 (32)	67 (44.6)	0.608-4.2484	0.466607
CG - CT	10 (6.6)	5 (3.3)	1.0578-17.3632	0.0367
CG - TT	2 (1.3)	4 (2.6)	0.1571-7.3076	1
GG - CC	66 (44)	52 (34.6)	1.0331-7.1602	0.037667
GG - CT	14 (9.3)	4 (2.6)	1.7981-31.2834	0.009971
GG - TT	1 (0.6)	1 (0.6)	0.1163-39.4714	1

*n-number of subjects; CI-confidence interval; p values calculated using χ^2 test, statistically significant P values are shown in Bold.

Discussion

Esophageal cancer is the eighth most common cancer worldwide, responsible for 462,000 new cases in 2002 (4.2% of the total), and sixth most common cause of death from cancer with 386,000 deaths (5.7% of the total) (Parkin et al., 2005). It also constitutes 7% of all gastrointestinal cancers and is one of the most lethal of all cancers. Cancer of the esophagus has a very poor survival: 16% of the cases in the United States and 10% in Europe survive at least five years (Sant et al., 2003). 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). VEGF protein expressions in esophageal cancer, analyzed their significance by combining clinical pathological features of esophageal cancer and investigated the influences of VEGF on tumor angiogenesis and the relations of them with tumorous invasion and metastasis (Tao Jiang et al., 2012). These VEGF variants might potentially contribute to inter individual 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 esophageal cancer risk.

For VEGF +405C>G polymorphism, we observed

Table 3. Association between Esophageal Cancer Patients and Polymorphisms

Genetic model	405 C>G		Genetic model	936 C>T	
	OR(95%CI)	P value		OR(95%CI)	P value
Dominant model:			Dominant model:		
CG+GG *vs* CC	2.118(0.908-4.917)	0.07634	CT+TT *vs* CC	1.901(1.089-3.368)	0.024203
Over dominant model:			Over dominant model:		
CG *vs* CC+GG	0.695(0.436-1.111)	0.16153	CT *vs* CC+TT	2.082(1.153-3.759)	0.013518
Recessive model:			Recessive model:		
GG *vs* CC+CG	1.625(1.016-2.612)	0.04334	TT *vs* CC+CT	0.662(0.109-4.028)	1
Homozygous co dominant:			Homozygous codominant:		
GG *vs* CC	2.736(1.149-6.553)	0.02064	TT *vs* CC	0.766(0.1253-4.651)	1
Heterozygous codominanat:			Heterozygous codominanat:		
CG *vs* CC	1.633(0.671-3.949)	0.27332	CT *vs* CC	2.077(1.147-3.744)	0.014469
Allele Contrast:			Allele contrast:		
G *vs* C	2.002 (1.232-3.266)	0.00721	T *vs* C	5.004(2.226-11.257)	<.0001

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

significant association of GG genotype and G allele with higher susceptibility for esophageal cancer. Similar to our findings, significant association of +405GG genotype has been reported with increased risk for pancreatic adenocarcinoma (Sivaprasad et al., 2013). 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 lipopolysacharide 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 +405 CC+CG genotype has been associated with higher susceptibility for Prostate, gastric (Sfar et al., 2006; Guan et al., 2009), and small cell lung carcinoma in males (Zhai et al., 2008). 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 esophageal cancer. 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., 2008) and glioma (Bao et al., 2011). 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 esophageal cancer might be due to variations of allele frequencies within different ethnic groups. 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 esophageal cancer. In the present study, we did not observe any significant association of VEGF +936C>T and +405C>G with the clinical stage of the esophageal cancer which might be due to less number of samples in the particular stage of the cancer. The findings of present study indicated a significant association of VEGF +936C>T and +405C>G polymorphisms with the increased esophageal cancer risk in Kashmir. Findings of SNPs influencing VEGF targeted therapies as a predictive marker would be of great help for physicians to tailor individual therapy.

Acknowledgements

We are grateful to the patients and controls for providing the blood samples. This study was supported by the ICMR grant as Senior Research fellowship (SRF FILE NO.3/2/2/100/2011/NCD-II and IRSD NO:2011-09740).

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