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Association of *VEGFA* polymorphisms with the risk of oesophageal cancer in Punjab, India: A case-control study

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***Background & objectives*: Vascular endothelial growth factor (*VEGF*) is one of the most important angiogenic factors which stimulates tumour progression induction of endothelial cell migration and division, inhibition of the apoptosis of endothelial cells, induction of serine protease activity and enhancement of vascular permeability. This study aimed to investigate the correlation of *VEGF*+405G/C,-7C/T and+936C/T polymorphisms with oesophageal cancer risk.**

***Methods*: DNA samples of 464 subjects (231 sporadic oesophageal cancer affected individuals and 233 controls) were genotyped for*VEGF*+936C/T,+405G/C and-7C/T polymorphisms. *VEGF*+936C/T and +405G/C polymorphisms were genotyped by PCR-RFLP method whereas *VEGF*-7C/T polymorphism was genotyped using Amplification refractory mutation system-polymerase chain reaction (ARMS-PCR).**

***Results*: CT genotype of *VEGF*-7C/T polymorphism was significantly associated with reduced risk of oesophageal cancer. *VEGF*-7C/T polymorphism was significantly associated with reduced risk of oesophageal cancer underdominant, co-dominant, over dominant and log-additive genetic models in total patients and in the female group. $C_{+936}G_{+405}T_{-7}$ haplotype was significantly associated with decreased risk (*P*=0.01)of oesophageal cancer in total patients and also in the male group (*P*=0.02).**

***Interpretation & conclusions*: In future, replication of the findings of the present study in a larger sample from different ethnic groups, along with functional analysis, may be insightful for the role of *VEGFA* polymorphisms in the pathogenesis of oesophageal cancer. Identification of the correlation of *VEGF* variants with specific therapy in oesophageal cancer may help in better selection of patients and monitoring treatment response in *VEGF*-therapy.**

Key words Angiogenesis - oesophageal cancer - polymorphism - untranslated regions - *VEGF*

Various angiogenic factors secreted by tumour cells stimulate the microvessel formation around the tumour cells[1].Vascular endothelial growth factor (VEGF) is one of the most important angiogenic factors which stimulates tumour progression by the induction of endothelial cell migration and division, inhibition of the apoptosis of endothelial cells, induction of serine protease activity and enhancement

of vascular permeability[2].*VEGF* has been reported to be implicated in lymphatic metastasis and dysplastic lesions in oesophageal carcinoma[3] and over-expression of *VEGF* has been reported in about 30–60 per cent of oesophageal cancer (EC) patients.

VEGF or *VEGFA* is localized on 6p21.3, having a 14kb coding region and comprises eight exons and seven introns. *VEGFA* is highly polymorphic with several potentially functional single nucleotide polymorphisms (SNPs) in the 5′ untranslated region (UTR), 3′ UTR and promoter. UTRs inhabit genomic regions that may serve as one of the key mediators of cancer pathogenesis by causing post-transcriptional deregulation[4]. Functional genetic variations in the *VEGF* have been reported to be involved in the progression and development of EC, due to which it can be used as a potential prognostic and predictive marker[5]. It has been documented that *VEGF* +405G/C (*VEGF* -634G/C) 5′ UTR polymorphism affects the translation efficiency of *VEGFA* and is correlated with microvessel density and prognosis of many cancers[6]. Association of GG genotype of *VEGF* +405G/C polymorphism with increased risk of EC has been reported in Kashmiri patients from north India[7].

The association of *VEGF* -7C/T polymorphism located in 5′ UTR has been explored in gastrointestinal tract (GIT) cancers such as gastric cancer in northern Chinese[8], colorectal in Japanese[9], hepatocellular in Korean[10] and Italian population[11]. So far, no study has been published in the literature on *VEGF* -7C/T polymorphism in EC.

The 3′UTR polymorphism *VEGF* +936C/T affects VEGF plasma levels by reducing the activity of activator protein (AP-4) in tumour tissue[12]. Correlation of *VEGF* +936C/T polymorphism with EC risk has been studied in different populations. Association of *VEGF* +936T allele with better survival was reported in Caucasian EC patients[5]. Increased risk of EC was observed in Caucasians[13] and north Indian[7] patients carrying the combined CT+TT genotype.

The prognostic and predictive significance of some *VEGF* polymorphisms have been documented in oesophageal cancer. CC and CG genotype of *VEGF* +405G/C polymorphism was associated with improved overall survival time in Japanese oesophageal squamous cell carcinoma patients treated with 5-fluorouracil/cisplatin[14]. GG genotype of *VEGF* +405GG genotype showed improved overall survival in Taiwanese EC patients treated with cisplatin[14]. In the German population, *VEGF* +936CT and TT genotypes were reported to influence the response to cisplatin or oxaliplatin with worse event-free survival in oesophagogastric junction adenocarcinoma patients[16].

As per author's knowledge, there is only one study on *VEGF* +936C/T and *VEGF* +405G/C polymorphisms in oesophageal cancer from north India[7]. *VEGF* is an important target in anticancer therapy, and identification of the role of *VEGF* variants in oesophageal cancer will help physicians to tailor therapy and for monitoring the therapy response. Therefore, the present study aimed to investigate the correlation of *VEGF* +405G/C (rs2010963), -7C/T (rs25648) and +936C/T (rs3025039) polymorphisms with EC risk in patients from Punjab, northwest India. As far as we know, this is the first study reporting the combined role of these three *VEGF* variants in EC.

Material & Methods

This case-control study was conducted in the Department of Human Genetics, Guru Nanak Dev University, Amritsar, Punjab, India from October 2020-March 2022. The study was carried out according to ICMR's Ethical guidelines. The study protocol was approved by the institutional ethics committee.

Study subjects: A total of 464 individuals, including 231 sporadic EC affected individuals and 233 controls, were investigated in this case-control study. The study participants were clinically diagnosed at Sri Guru Ram Das Institute of Medical Sciences and Research, Amritsar, Punjab. The control group comprised randomly selected healthy, unrelated gender and age-matched individuals without any malignant and chronic disease from the same geographical area as EC affected individuals. Characteristics including gender, age, habits, habitat, family and disease history of all participants were recorded on proforma. From each study participant, a 5 ml blood sample was collected in an EDTA-coated vial after obtaining their signed informed consent. The blood sample was stored at -20°C till further use.

Genomic DNA isolation and genotyping of VEGF+936C/T, +405G/C and -7C/T polymorphisms: From the EDTA anticoagulated blood sample, the genomic DNA was extracted using the standard phenol-chloroform method. The quality and quantity of DNA samples were checked on 1% agarose gel. Genotyping of *VEGF* +936C/T and +405G/C polymorphisms was

Table I. Baseline data of oesophageal cancer affected individuals and healthy controls

Variable	Affected individuals n=231, n(%)	Controls n=233, n(%)
Gender		
Males	94 (40.7)	94 (40.34)
Females	137 (59.3)	139 (59.66)
Age		
≤50 yr	84 (36.36)	88 (37.77)
>50 yr	147 (63.64)	145 (62.64)
Total (Mean±SD)	56.83±12.55	54.15±13.51
Males (Mean±SD)	58.46±12.94	56.07±12.63
Females	55.77±12.24	53.921±13.05
Pathological type		
Squamous cell carcinoma	214 (92.64)	-
Adenocarcinoma	17 (7.36)	-

done using the polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) method. The PCR reaction for *VEGF* +405G/C and +936C/T polymorphisms was carried out in atotal volume of 15 µl having 50 ng of DNA sample, 1X *Taq* buffer with 1.5 mM $MgCl_2$, 0.3 µl of dNTPs mix, 5 pmol of forward and reverse primer (Sigma-Aldrich, Bangalore) and 1U of *Taq*DNA polymerase (Sigma-Aldrich, Bangalore). The details of screening conditions and genotyping methodology have been described in Supplementary Table. The PCR products of *VEGF* +405G/C (304 bp) and +936C/T (207 bp) polymorphisms were digested overnight with *BsmF*I and *Nla*III restriction enzymes, respectively, as per manufacturer's instructions (New England Biolabs, Beverly, MA). The genotying detail and gel photographs showing pattern of genotyping has been mentioned in Supplementary Table and Supplementary Figure 1 and 2, respectively.

Genotyping of *VEGF*-7C/T polymorphism was done by using amplification refractory mutation system-PCR (ARMS-PCR). Two different PCR master mixes were prepared in separate tubes with C and T allele-specific reverse primers. The PCR reaction was carried out in a 10 µl volume containing 50 ng genomic DNA, 1X *Taq* buffer with 1.5 mM $MgCl_2$, 0.15 µl dNTPs mixture, 4 pmol of each primer (Sigma-Aldrich, Bangalore) and 1U *Taq* DNA polymerase (Sigma-Aldrich, Bangalore). A negative control without a DNA sample was included in each batch of reaction. The amplified PCR products were separated on 2.4% agarose gel. A sharp band of size 183bp represents C and T alleles while a band size of 425 bp represents control (Supplementary Table and Supplementary Fig. 3). To ensure quality control, genotyping was carried out without knowing the status of the case/control. Data were revalidated by reanalysing 10per cent of samples and both analyses' results were 100 per cent concordant.

Statistical analysis: The data were presented as percentages or as mean±standard deviation. The allele and genotype frequencies of *VEGF* +936C/T, +405G/C and -7C/T polymorphisms in EC affected individuals and controls were tested for deviation in Hardy-Weinberg equilibrium (HWE) by using the Chi-square test. The strength of the association of alleles and genotypes with EC risk was measured by overall odds ratio (OR) with a 95% confidence interval (95% CI). Lastly, we investigated the associations of haplotypes of *VEGF* +936C/T, +405G/C and -7C/T polymorphisms with EC risk using online SNP stats software (*https://www.snpstats.net/start.htm*). A probability value of $P<0.05$ was considered statistically significant. The age, gender, diet, smoking status and alcohol consumption were selected as adjustment variables.

Results

Demographic characteristics: In total, 464 study participants comprising 231 sporadic EC affected individuals (94 males and 137 females) and 233 controls (94 males and 139 females) were included in the present study. The number of female cancer patients (n=137) was higher in comparison to male (n=94). The mean age (±SD) of EC affected individuals at diagnosis was 56.83±12.55 yr and that of the controls was 54.15 ± 13.51 yr. About 64 per cent of affected individuals developed cancer of oesophagus after the age of 50 yr. The demographic characteristics of EC affected individuals and controls are given in Table I.

VEGF +936C/T, +405G/C and -7C/T Polymorphisms and risk of oesophageal cancer: The genotype distributions of *VEGF* +405G/C and -7C/T polymorphisms were in HWE in the control group ($P > 0.05$). The distribution of genotypes and alleles of *VEGF* polymorphisms is given in Table II. A statistically significant association of CT genotype of *VEGF*-7C/T polymorphism with reduced risk of EC was observed in total study participants (OR: 0.54, 95% CI: 0.34–0.85; $P=0.02$) and also in the female group (Table III). T allele of *VEGF*-7C/T

Table II. Association of *VEGF* +936C/T, +405G/C and -7C/T polymorphisms with oesophageal cancer risk

	Affected individuals (n=231) n(%)	Controls (n=233) n(%)	OR (95% CI)	*P* value[#]
VEGF +936C/T (rs3025039)				
Genotype				
CC	183 (79.22)	192 (82.4)	Reference	
CT	47 (20.35)	41 (17.6)	1.11 (0.68–1.82)	0.65
TT	1 (0.43)	0	-	-
Allele				
C	413 (89.39)	425 (91.2)	Reference	
T	49 (10.61)	41 (8.8)	1.23 (0.79–1.9)	0.35
VEGF +405C/G (rs2010963) [HWE: Affected individuals (*P*=0.63); Controls (*P*=0.29); Both (*P*=0.23)]				
Genotype				
CC	17 (7.36)	20 (8.58)	Reference	
CG	98 (42.42)	110 (47.21)	1.05 (0.52–2.11)	0.23
GG	116 (50.22)	103 (44.21)	1.32 (0.66–2.66)	0.43
Allele				
C	132 (28.57)	150 (32.19)	Reference	
G	330 (71.43)	316 (67.81)	1.19 (0.89–1.57)	0.23
VEGF-7C/T (rs25648) [HWE: Affected individuals (*P*=0.51); Controls (*P*=0.22); Both (*P*=0.69)]				
Genotype				
CC	182 (78.79)	162 (69.53)	Reference	
CT	45 (19.48)	68 (29.18)	0.54 (0.34–0.85)	0.02[*]
TT	4 (1.73)	3 (1.29)	1.19 (0.26–5.4)	0.82
Allele				
C	409 (88.53)	392 (84.12)	Reference	
T	53 (11.47)	74 (15.88)	0.69 (0.47–1)	0.05

P[*]<0.05 significance. [#]Adjusted for age, gender, diet, alcohol and smoking status. OR, odds ratio; CI, confidence interval

polymorphism was marginally associated with reduced risk of EC in total study participants (*P* =0.05) and the female group (*P*=0.06). *VEGF*-7C/T polymorphism was significantly associated with reduced risk of oesophageal cancer under dominant, co-dominant, over-dominant and log-additive genetic models in total study participants and also in the female group (Table III). No significant difference was found in genotype, allele frequencies (Table II) and genetic models (Table IV) of *VEGF* +405G/C and +936C/T polymorphisms in total EC affected individuals and also in male and female groups (*P*>0.05).

Association of VEGF haplotypes with oesophageal cancer risk: Analysis of haplotypes of *VEGF* +936C/T, +405G/C and -7C/T polymorphisms showed that $C_{+936}G_{+405}T_{-7}$ haplotype was significantly associated with decreased risk (*P*=0.01)of EC in total study participants (Table V). Haplotype $C_{+936}C_{+405}C_{-7}$ was marginally associated (*P*=0.07) with decreased EC risk in total study participants. When we performed a gender-stratified analysis, a significant association of $C_{+936}G_{+405}T_{-7}$ haplotype (*P*=0.02) with decreased risk of EC was observed in the male group (Table V). There was no association between other haplotypes and EC risk.

Discussion

In the present case-control study, we evaluated the association between *VEGF* +936C/T, +405G/C and -7C/T functional polymorphisms and the risk of EC. An association of *VEGF*-7C/T polymorphism with reduced risk of EC was found. In this study, no

Table III. Gender-wise stratification analysis on the association between *VEGF* polymorphisms and oesophageal cancer risk.

	Males Affected individuals (94); Controls (94)				Females Affected individuals (137); Controls (139)			
Genotype	Affected individuals n(%)	Control n(%)	OR 95% CI)	*P* value[#]	Affected individuals n(%)	Control n(%)	OR (95% CI)	*P* value[##]
				VEGF +936C/T				
Genotype								
CC	72 (76.6)	75 (79.79)	Reference		111 (81.02)	117 (84.17)	Reference	
CT	22 (23.4)	19 (20.21)	1.04 (0.46–2.35)	0.93	25 (18.25)	22(15.83)	1.17 (0.62–2.21)	0.52
TT	0	0	-	-	1(0.73)	-	-	-
Allele								
C	166 (88.3)	169 (89.89)	Reference		247 (90.15)	256 (92.09)	Reference	
T	22 (11.7)	19 (10.11)	1.18 (0.61–2.25)	0.62	27 (9.85)	22 (7.91)	1.27 (0.7–2.3)	0.42
				VEGF+405C/G				
Genotype								
CC	3 (3.19)	7 (7.45)	Reference		14 (10.22)	13(9.35)	Reference	
CG	45 (47.87)	45 (47.87)	2.33 (0.57–9.59)	0.24	53 (38.69)	65(46.76)	0.76 (0.33–1.75)	0.51
GG	46 (48.94)	42 (44.68)	2.56 (0.62–10.52)	0.19	70 (51.09)	61(43.88)	1.06 (0.46–2.44)	0.88
Allele								
C	51 (27.12)	59 (31.38)	Reference		81 (29.56)	91 (32.73)	Reference	
G	137 (72.87)	129 (68.69)	1.23 (0.79–1.92)	0.36	193 (70.44)	187 (67.27)	1.16 (0.8–1.66)	0.42
				VEGF-7C/T				
Genotype								
CC	70 (74.47)	64 (68.09)	Reference		112 (81.75)	98(70.5)	Reference	
CT	21 (22.34)	27 (28.72)	0.66 (0.3–1.42)	0.56	24 (17.52)	41(29.5)	0.49 (0.28–0.88)	0.02*
TT	3 (3.19)	3 (3.19)	-	-	1 (0.73)	-	-	-
Allele								
C	161 (85.64)	155 (82.45)	Reference		248 (90.51)	237 (85.25)	Reference	
T	27 (14.36)	33 (17.55)	0.79 (0.45–1.37)	0.39	26 (9.49)	41 (14.75)	0.6 (0.36–1)	0.06

*P**<0.05 singnificance. [#]Adjusted for age, diet, alcohol and smoking status; [##]adjusted for age, diet and smoking status

significant correlation of *VEGF* +405G/C and +936C/T polymorphisms with EC risk was found. As per authors knowledge, there is no reported study on *VEGF*-7C/T polymorphism in EC and limited data on *VEGF* +936C/T and +405G/C polymorphism. Our results agreed with the case-control study conducted by Gu *et al*[17] in a Chinese population in which no association between *VEGF* +936C/T polymorphism and EC risk was found. Combined CT+TT genotype of *VEGF* +936C/T polymorphism was associated with increased risk of EC in Caucasian patients[13]. Our results are contrary to the previous two reports from north India. A study from north India on Kashmiri patients reported an association of the CT genotype and T allele of *VEGF* +936C/T polymorphism with an increased risk of EC[7]. In another study, the *VEGF* +936CT genotype was reported to be associated with an increased risk of oral cancer in north Indian patients[18]. The currently available results on *VEGF* +936C/T polymorphism in GIT cancers are inconsistent. A meta-analysis[19] of 29 studies including 13,293 cases and 12,308 control individuals reported a significant association of the T allele of *VEGF* +936C/T polymorphism with increased risk of oral cancer. A significant association of *VEGF* +936TT genotype with advanced stage of disease and distant metastasis has been reported in Korean colorectal patients[20]. Correlation of T allele of *VEGF* +936C/T polymorphism with improved overall survival has been reported in Caucasian EC patients[5]. Association

Table IV. Genetic models of *VEGF* polymorphisms and oesophageal cancer risk

Polymorphism	Total		*P* value[#]	Males		Females	
	Model	OR (95% CI)		OR (95% CI)	*P* value[##]	OR (95% CI)	*P* value[###]
VEGF +936C/T	Co-dominant	1.11 (0.68–1.82)	0.65	-	-	1.27 (0.62–2.21)	0.52
	Dominant	1.13 (0.69–1.84)	0.63	-	-	1.21 (0.64–2.27)	0.55
	Over dominant	1.11 (0.68-1.81)	0.68	-	-	1.16 (0.62–2.19)	0.64
	Log-additive	1.14 (0.71–1.85)	0.59	-	-	1.24 (0.67–2.30)	0.48
VEGF +405C/G	Dominant	0.80 (0.54–1.19)	0.55	1.24 (0.76–2.02)	0.38	0.73 (0.44–1.21)	0.44
	Dominant	0.81 (0.55–1.19)	0.28	2.44 (0.61–9.74)	0.19	0.77 (0.48–1.25)	0.29
	Recessive	0.96 (0.48–1.94)	0.91	1.14 (0.64–2.02)	0.66	1.36 (0.5–2.58)	0.72
	Over dominant	0.82 (0.56–1.20)	0.31	1 (0.56–1.77)	1.0	0.73 (0.45–1.18)	0.2
	Log-additive	0.87 (0.64–1.18)	0.37	0.78 (0.48–1.27)	0.32	0.89 (0.62–1.28)	0.52
VEGF-7C/T	Co-dominant	0.54 (0.34–0.85)	0.02*	0.66 (0.3–1.42)	0.56	0.49 (0.28–0.88)	0.02*
	Dominant	0.56 (0.36–0.88)	0.01*	0.67 (0.32–1.4)	0.29	0.52 (0.29–0.92)	0.02*
	Recessive	1.54 (0.29–8.22)	0.61	0.87 (0.12–6.32)	0.89	-	-
	Over dominant	0.53 (0.34–0.84)	0.01*	0.66 (0.31–1.43)	0.29	0.49 (0.27–0.87)	0.01*
	Log-additive	0.63 (0.42–0.95)	0.03*	0.73 (0.39–1.39)	0.34	0.56 (0.32–0.98)	0.04*

*P**<0.05 singnificance. [#]Adjusted for age, gender, diet, alcohol and smoking status; [##]adjusted for age, diet, alcohol and smoking status; [###]adjusted for age, diet and smoking status

Table V. Haplotype frequencies of *VEGF* polymorphisms in oesophageal cancer affected individuals and controls

Haplotype	Affected individuals frequency	Controls frequency	OR (95% CI)	*P* value[#]
Total				
$C_{+936}G_{+405}C_{-7}$	0.547	0.476	Reference	
$C_{+936}C_{+405}C_{-7}$	0.26	0.301	0.71 (0.5–1.02)	0.07
$C_{+936}G_{+405}T_{-7}$	0.087	0.134	0.52 (0.31–0.85)	0.01*
$T_{+936}G_{+405}C_{-7}$	0.06	0.047	0.89 (0.4–2)	0.79
$T_{+936}G_{+405}T_{-7}$	0.02	0.02	0.60 (0.15–2.33)	0.46
$T_{+936}C_{+405}C_{-7}$	0.018	0.016	1.32 (0.28–6.26)	0.73
Males				
$C_{+936}G_{+405}C_{-7}$	0.531	0.47	Reference	
$C_{+936}C_{+405}C_{-7}$	0.249	0.261	0.64 (0.32–1.31)	0.23
$C_{+936}G_{+405}T_{-7}$	0.103	0.168	0.39 (0.18–0.87)	0.02*
$T_{+936}G_{+405}C_{-7}$	0.055	0.041	0.55 (0.11–2.73)	0.46
$T_{+936}C_{+405}C_{-7}$	0.022	0.053	0.37 (0.05–2.63)	0.32
Females				
$C_{+936}G_{+405}C_{-7}$	0.556	0.497	Reference	
$C_{+936}C_{+405}C_{-7}$	0.268	0.317	0.73 (0.48–1.11)	0.15
$C_{+936}G_{+405}T_{-7}$	0.078	0.107	0.6 (0.31–1.17)	0.13
$T_{+936}G_{+405}T_{-7}$	0.005	0.035	0.06 (0–21.01)	0.34
$T_{+936}G_{+405}C_{-7}$	0.066	0.033	1.68 (0.63–4.45)	0.3

*P**<0.05 significance. [#]Adjusted for age, gender, diet, alcohol and smoking status in total; for age, diet, alcohol and smoking status in males and for age, diet and smoking status in female subjects

of *VEGF*+936TT genotype with worse overall survival was reported in Korean gastric cancer[21], with lower overall survival in Greek colorectal adenocarcinoma[22] and with increased overall survival in Spanish colorectal cancer patients[23]. In bevacizumab-treated metastatic colorectal cancer patients, the +936TT genotype was significantly associated with better time to treatment failure as compared to CC and CT genotype [24].

The present study has observed no association of VEGF+405C/G (-634 C/G) polymorphism with EC risk. Similarly, no association of *VEGF*+405C/G polymorphism with EC risk has been reported in Caucasian patients[12]. A significant association of GG genotype and G allele of *VEGF* +405C/G polymorphism with increased risk of EC has been reported in north Indian Kashmiri patients[7]. The *VEGF* +405C/G polymorphism has been studied in various GIT cancers with mixed results. *VEGF* +405CC genotype was associated with larger tumour size and advanced stage of disease in Greek gastric cancer patients[25]. A meta-analysis[26] of six studies on *VEGF*-634C/G polymorphism involving 1504 cases and 1707 controls reported that the-634GG genotype was significantly associated with reduced risk of gastric cancer in Europeans, whereas the -634GC genotype was associated with increased gastric cancer risk in Asians. No association of *VEGF* +405C/G polymorphism with gastric cancer risk was reported in south Indian patients [27].

The association of *VEGF* -634CC genotype with decreased survival rate has been reported in Greek gastric cancer[25] and colorectal adenocarcinoma patients[22]. A case-control study on gastric cancer in the Omani population reported that patients with *VEGF* +405GG genotype had a lower survival rate as compared to patients with combined *VEGF*+405CC+CG genotype[28].

In the present case-control study, we found that the CT genotype of *VEGF*-7C/T polymorphism was significantly associated with reduced risk of EC in the total patients and also female group. As per authors knowledge, there is no published study on *VEGF*-7C/T polymorphism in EC so far. There was no association of *VEGF* -7C/T polymorphism with colorectal cancer in Japanese[9], hepatocellular cancer in Korean[10] and gastric cancer in the Chinese[8] population.

Furthermore, we evaluated the combined effects of *VEGF* +936C/T, +405C/G and -7C/T polymorphisms and observed that $C_{+936}G_{+405}T_{-7}$ haplotype was significantly associated with reduced risk of EC in total study participants and also in the male group. Association of CGT haplotype of *VEGF* -460T/C, +405C/G and +936C/T polymorphisms with increased risk of oesophageal adenocarcinoma has been reported in Caucasians[13]. Association of CGC haplotype of *VEGF* -460T/C, +405C/G and +936C/T polymorphisms with reduced overall survival has been documented in Caucasian oesophageal cancer patients[5]. TCT haplotype of *VEGF* -460T/C, +405C/G and +936C/T polymorphisms were associated with decreased risk of gastric cancer in Korean patients[29]. In surgically resected Korean gastric adenocarcinoma patients, the CACC haplotype of *VEGF* -460T/C, -116G/A, +405C/G and +936C/T polymorphisms were significantly associated with worse overall survival and disease-free survival [21]. Haplotype -634C/+936C and -634G/+936T were associated with decreased risk to colorectal cancer in Korean patients[20]. Romanian surgically treated colorectal adenocarcinoma patients with -2578A/-634G/+936T haplotype had worse overall survival ratesin comparison to cases with -2578C/-634G/+936C haplotype[30]. Haplotypes -2578C/ -460T/ +405C/ +936C and -2578C/-460T/+405C/+936T have been reported to be associated with inferior response rate in metastatic colorectal cancer[31]. In Chinese stage I and II gastric cancer patients, -460T/+405G/-7C and -460C/+405G/-7C haplotypes were associated with poor survival[8]. Haplotype CCGAGCCC of *VEGF*-2578/-1203/-1190/-1179/-1154/-634/-7/+936 polymorphisms was associated with smaller tumour size in Korean hepatocellular cancer patients[10]. There was no significant association of genotype, allele and haplotype of *VEGF* -460T/C, +405G/C and +936C/T polymorphisms with gastric cancer risk in Omani patients[28].

In the present study, we observed a gender-specific association of *VEGF* polymorphisms with EC risk. CT genotype of *VEGF*-7C/T polymorphism was significantly associated with decreased risk of EC in total study participants and also in female affected individuals, whereas the $C_{+936}G_{+405}T_{-7}$ haplotype was significantly associated with reduced risk of EC in total affected individuals and the male group. Gender-specific differences in the regulation of vascular remodelling have been demonstrated in the perigonadal adipose tissue of mice subjected toa high-fat diet[32]. The investigators reported higher levels of *VEGFA* and *VEGFR2* and high vascular density in the visceral adipose tissue of female mice as compared to male mice in response to a high-fat diet. Out of 137 female patients, 107 (78%) were postmenopausal in the present

study. It has been documented that oestradiol, the main sex hormone in females, modulates angiogenesis *via* its effects on endothelial cells and regulates *VEGFA* expression in adipose tissue[33]. In non-small cell lung cancer, it has been reported that elevated circulating oestrogen and progesterone in females may promote VEGF secretion and tumour angiogenesis[34]. Significantly higher adverse effects of chemotherapy have been reported in female oesophagogastric cancer patients in comparison to male patients[35]. Recently, Hall *et al*[36] suggested that gender-based analysis must be included in cancer research for appropriate interpretation and implication of results in real-world practice.

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Supplementary Table. Detail of *VEGF* polymorphisms and screening conditions

Polymorphism	Location	Annealing temperature	Size of amplicon	Fragment size after digestion	Primer sequences Reference
VEGF +405C/G (-634C/G) (rs2010963)	5′ UTR	53°C	304bp	C allele -304bp G allele -203bp & 101bp	Gentilini *et al*[1] (2008)
VEGF -7C/T (+1032C/T) (rs25648)	5′ UTR	59°C	C or T allele-183bp Control-425bp	-	Tavakkoly-Bazzaz *et al*[2] (2010)
VEGF +936C/T (rs3025039)	3′ UTR	59°C	207bp	C allele -207bp T allele -122bp & 85bp	Lachheb *et al*[3] (2008)

bp, base pair

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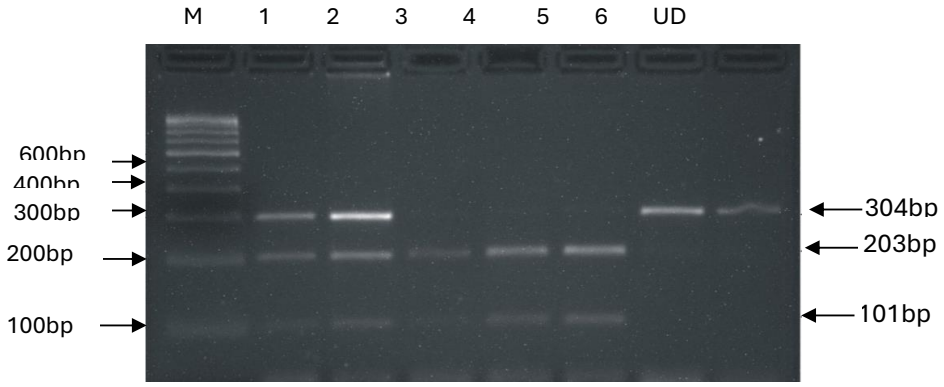
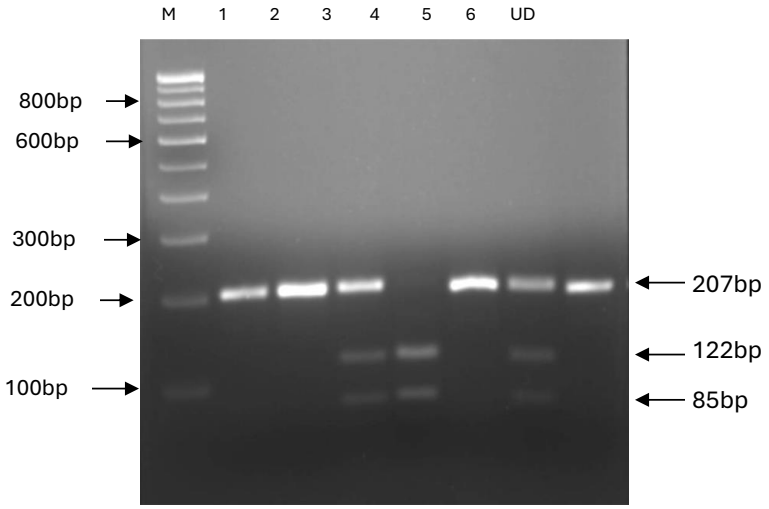
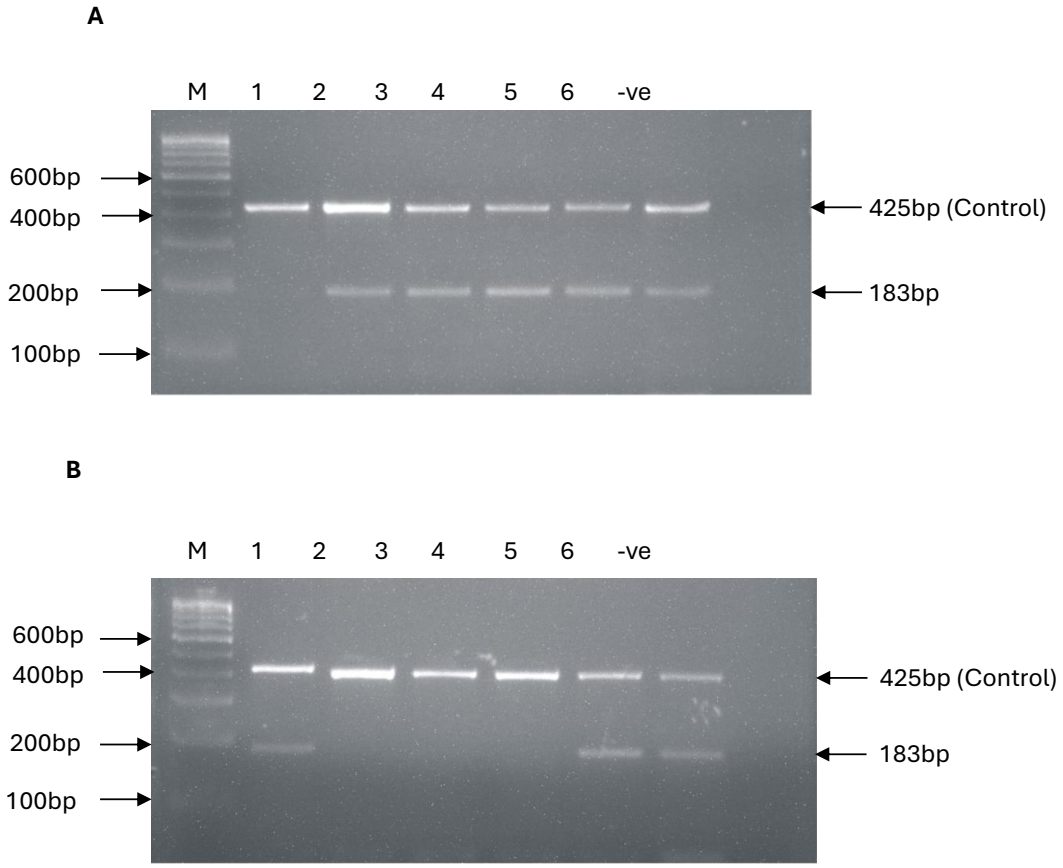


Supplementary Fig. 1. An agarose gel showing different genotypes of *VEGF* +405G/C polymorphism. Lane 1 and 2 represent individuals with CG genotype, lanes 3-5 represents individuals with GG genotype and lane 6 represents individuals with CC genotype. M, 100bp molecular weight marker; UD, undigested sample; bp, base pairs.



Supplementary Fig. 2. An agarose gel showing different genotypes of *VEGF* +936C/T polymorphism. Lanes 1, 2 and 5 represent individuals with CC genotype; lanes 3, 6 represent individuals with CT genotype and lane 4 represents individuals with TT genotype. M, 100bp molecular weight marker; UD, undigested sample; bp, base pairs.



Supplementary Fig. 3. Agarose gel showing different genotypes of *VEGF* -7C/T polymorphism expression using (A) C allele and (B) T allele specific primers. In both panels, lane 1 represents individuals with TT genotype, lanes 2–4 represent individuals with CC genotype and lanes 5 and 6 represent CT genotype, -ve represents negative control. M, 100bp molecular weight marker; bp, base pairs.