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VEGF gene +936C/T polymorphism decreases the risk of colorectal cancer

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Summary. *Background/Aims:* Vascular endothelial growth factor (VEGF) is a signal protein and plays a major role in angiogenesis and metastasis of tumors. Some variation in the 3'untranslated region (3'UTR) of *VEGF* gene could alter the expression of this gene. In this study we have evaluated the possible association between +936C/T (rs3025039) polymorphism located in 3'UTR of *VEGF* gene and the risk of colorectal cancer (CRC) in an Iranian population. *Methods:* In the present case-control study, *VEGF* genotypes of +936C/T polymorphism were determined in 200 patients with CRC and 200 control individuals. DNA was extracted from blood specimens and genotypes were analyzed by the polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) method. *Results:* A significant correlation was found between +936C/T variant in the *VEGF* gene and CRC risk. Frequency of the TT genotype was 16% in controls and 2% in the case study. There was a statistically significant difference in TT genotype frequency between CRC patients and controls (adjusted OR= 0.229, 95% CI= 0.058-0.899; *P*=0.035). *Conclusions:* These results suggest that the TT genotype may have a protective effect against CRC in an Iranian population.

Key words: VEGF, angiogenesis, 3'UTR, colorectal cancer, SNP, genotypes

«Il polimorfismo +936C/T del gene VEGF abbassa il rischio di insorgenza del cancro colonrettale»

Riassunto. Il fattore di crescita endoteliale (VEGF) è una proteina segnale e partecipa con un ruolo importante nei processi di angiogenesi e metastatizzazione dei tumori. Alcune varianti nella regione 3' non-tradotta (3' UTR) del gene *VEGF* può alterare l'espressione del gene. In questo studio abbiamo valutato la possibile associazione tra il polimorfismo +936C/T (rs3025039) localizzato nella 3' UTR del gene *VEGF* ed il rischio di insorgenza del cancro colonrettale (CRC) nella popolazione iraniana. *Metodi:* Nel presente studio caso-controllo, il genotipo di *VEGF* del polimorfismo + 936C/T è stato determinato in 200 pazienti con CRC ed in 200 individui di controllo. Il DNA è stato estratto da campioni di sangue ed i genotipi sono stati analizzati attraverso il metodo della *polymerase chain reaction-fragment lenght polymorfism* (PCR-RFLP). *Risultati:* Una significativa correlazione è stata trovata tra la variante + 936 C/T del gene *VEGF* ed il rischio di CRC. La frequenza del genotipo TT è stata del 16% nei controlli e del 2% nei casi dello studio. C'è stato una differenza statisticamente significativa nella frequenza dei genotipi TT tra i pazienti con CRC ed i controlli (OR aggiustato = 0.229, 95% CI =0.058-0.899; P=0.035). *Conclusioni:* Questi risultati suggeriscono che il genotipo TT può avere effetti protettivi contro il CRC nella popolazione iraniana.

Parole chiave: VEGF, angiogenesi, cancro colonrettale, SNP, genotipi

1. Introduction

Angiogenesis, the formation and generation of new blood vessels from endothelial precursors, is a prerequisite for growth and metastasis of solid malignancies. The vascular endothelial growth factor (VEGF) superfamily has been recognized as critically penetrating tumor-related angiogenesis (1-6). VEGF is one of the important mitogens and plays a major role in angiogenesis (4, 7). An irregularity in the production of VEGF is involved in the pathogenesis of several diseases (8). A clinical investigation has shown that overexpression of VEGF is related with angiogenesis and prognosis of solid tumors (3). VEGF particularly binds to tyrosine kinase transmembrane receptor (VEGFR) on the endothelial cell surface. VEGF/VEGFR interaction starts intracellular signal transduction pathways that mediate angiogenesis and vascular permeability (9).

Single nucleotide polymorphisms (SNPs) are the most prevalent resources of human genetic alternations, and they may contribute to an individual's susceptibility to cancer (10). *VEGF* gene which has been mapped to chromosome 6p12-p21 has important multiple SNPs such as -2578C/A, -1154G/C, -634G/C and +936C/T that are related with alternation of expression (11-14). One of the important SNPs which has been studied is +936C/T located in the 3'untranslated region (3'UTR) of *VEGF* gene (15).

We conducted a case-control study to investigate the association between +936C/T polymorphisms and the risk of colorectal cancer in an Iranian population.

2. Materials and methods

2.1. Study population

Between 2009 and 2011, there were a total of 400 individuals (including 200 sporadic colorectal cancer (CRC) patients and 200 healthy controls) recruited at the Research Center for Gastroenterology and Liver Disease at Taleghani Hospital, Tehran. All subjects were Iranian and signed a consent form before donating blood. Colonoscopy for patients was performed by gastroenterologists and diagnosis was confirmed by pathologists. All patients had pathological and clinical symptoms indicative of colon cancer and control individuals were healthy volunteers. This study was conducted under the approval of the Ethics Committee of the Research Institute for Gastroenterology and Liver Diseases, Shahid Beheshti University of Medical Sciences, Tehran, Iran.

2.2. Genotyping

Total genomic DNA was extracted from peripheral blood by the salting out method (16). The +936C/T polymorphism was chosen based upon previous published studies (10, 17, 18). Specific primers for PCR that covered the selected SNP were forward 5'ATACAGAACGATCGATACAG3', reverse 5'ACAGCAGTCAAATACAT3'. Initial denaturation was carried out at 94°C for 10 min, and then continued as follows: 35 cycles at 95°C for 45 sec, 61°C for 30 sec, 72°C for 40 sec, followed by a final extension at 72°C for 10 min. PCR products were digested by NlaIII restriction enzyme (New England Biolabs) at 37°C for 5 h. The digested PCR products were run on 2.5% agarose gel and stained with ethidium bromide for visualization under UV light. The fragment lengths for CC, CT and TT genotypes were 424bp, 424+171+253bp and 171+253bp, respectively. The length of the genotype bands can be seen in Figure 1. To confirm the polymerase chanin reaction-restriction fragment lenght polymorphism (PCR-RFLP) procedure, 10% of the PCR products were sequenced using an ABI PRISM 3130xL Genetic Analyzer (Applied Biosystems®, Invitrogen Life Technologies, Carlsbad, CA, USA) and the chain termination method.

2.3. Statistical analysis

Unconditional logistic regression analysis was conducted to estimate the adjusted and unadjusted odds ratio (OR) and 95% confidence interval (CI) to determine association of +936C/T with the risk of colorectal cancer (CRC). OR and 95% CI were adjusted for age, sex, Body Mass Index (BMI) and smoking status. In CRC patients the correlation between the clinical pathology features and polymorphism was examined using the chi-square test. Data were considered significant when the statistical p value was <0.05.

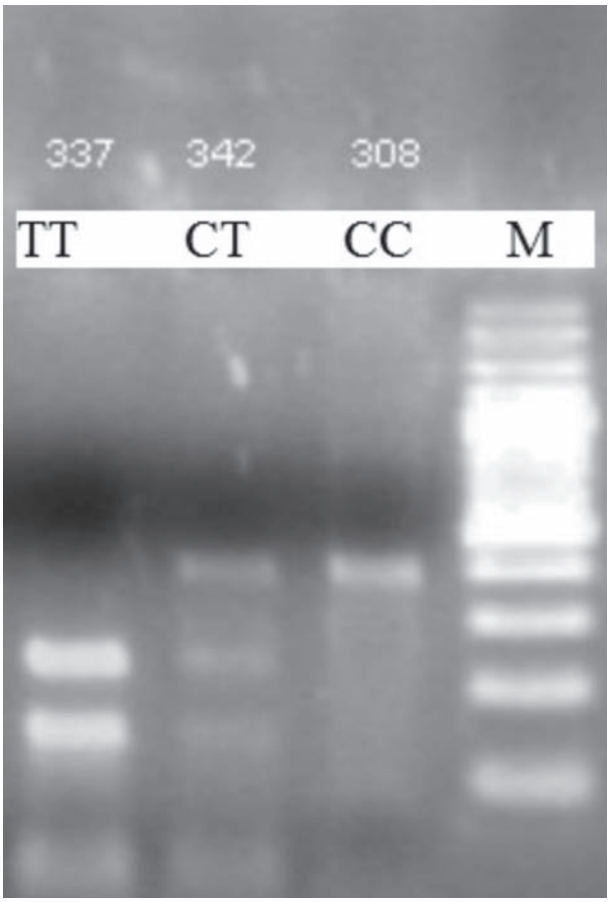

Figure 1. Electrophoresis digested products with NlaIII restricted enzyme on agarose gel showed different bonds. The marker (M) used was 100 base pairs.

All the statistical analyses were carried out using SPSS (version 13).

3. Results

In this study, we examined 200 healthy individuals and 200 patients. Age and BMI average were 43.32±15.04, 24.75±3.81 in controls and 58.31±13.13, 25.80±3.91 in cases, respectively. The demographic characteristics (age, sex, smoking and BMI) of the two groups and the clinical stage of the patients in the current study are shown in Table 1. A comparison of genotype frequencies of VEGF+936C/T polymorphisms between patients and controls is shown in Table 2. The frequencies of the CC, CT and TT genotypes of VEGF+936C\T polymorphism were found to be

Table1. Demographic characteristics of the study population.

Variable	Control	CRC patient
Age (y), (mean± SD)	43.32 ± 15.04	58.31 ± 13.13
BMI (mean± SD)	24.75 ± 3.81	25.80 ± 3.91
Sex (%)		
Male	83 (41.5%)	109 (54.5%)
Female	117 (58.5%)	91 (45.5%)
Smoking (%)		
Yes	21 (10.5%)	36 (18%)
No	179 (89.5%)	164 (82%)
Clinical stage (%)*		
Stage 0, I and II	_	78 (50.6%)
Stage III and IV	_	76 (49.4%)

* Stage is missing in 46 colorectal cancer patients.

75.5%, 17% and 7.5% in controls, and 74.5%, 23.5% and 2% in cases, respectively. Carriers of the TT genotype showed a decrease in colorectal cancer risk (OR, 0.229; 95% CI, 0.058-0.889; p=0.035). The frequencies of C and T alleles were 84% and 16% in controls, and 83.3% and 13.8% in cases, respectively. No significant differences were observed in allele frequencies between the case and control studies (OR, 0.837; 95% CI, 0.566-1.237; p=0.371). The relationship between the genotype and stages of disease is shown in Table 3. There was no significant association between the stage and the genotype (p=0.682).

4. Discussion

VEGF exerts its effects on angiogenesis, invasion and metastasis of tumor cells and is one of the most important factors in regulated angiogenesis (4, 19). Polymorphisms play a key role in the variance between individuals in susceptibility and intensity of disease (17). There are many polymorphisms in *VEGF* gene associated with an increased risk and survival for several tumors including CRC (3, 20-23). To our knowledge, this is the first report of an investigation into association between *VEGF* +936C/T and the risk of colorectal cancer in an Iranian population. In our study, the frequency of the TT genotype was found to be significantly different between patients and controls. The TT genotype is more frequent in control subjects

Table 2. The genotype and allele frequencies of VEGF +936 C\T polymorphism among CRC patients and controls.

Genotypes	Controls n=200(%)	CRC patients n=200(%)	Unadjusted OR (95%CI)	Adjusted OR (95%CI)	p
CC	151 (75.5%)	149 (74.5%)	1.00 (Ref)		
CT	34 (17%)	47 (23.5%)	1.401 (0.853 - 2.3)	1.332 (0.746 - 2.379)	0.332
TT	15 (7.5%)	4 (2%)	0.27 (0.088 - 0.833)	0.229 (0.058 - 0.899)	0.035
Alleles	Controls n=200(%)	patients n=200(%)	OR (95%CI)		P
C	336 (84%)	345 (83.3%)	1.00 (Ref)		
T	64 (16%)	55 (13.8%)	0.837 (0.566 - 1.237)		0.371

Data were adjusted for age, sex and BMI

Table 3. The tumor-stage specific distribution of VEGF +936 C/T polymorphism genotypes among CRC patients

Genotype	Stage 0	Stage I	Stage II	Stage III	Stage IV	P value
CC	5 (4.5%)	13 (11.7%)	41 (36.9%)	38 (34.2%)	14 (12.6%)	0.682
CT	2 (5.0%)	3 (7.5%)	12 (30.0%)	14 (35.0%)	9 (22.5%)	
TT	0 (-)	0 (-)	2 (66.7%)	0 (-)	1 (33.3%)	

* Stage is missing in 46 colorectal cancer patients

Table 4. Comparing frequency of 936 T allele and TT genotype distribution in colorectal cancer patients in previous studies

Authors	Year	Country	P value	T Frequency in controls	T Frequency in cases	TT Genotype in controls	TT Genotype in cases
Bae *et al.* (17)	2008	Korea	0.458	0.138	0.193	3	9
Chae *et al.* (33)	2008	Korea	0.059	0.209	0.202	12	16
Dassoulas *et al.* (4)	2009	Greece	0.001	0.354	0.402	79	74
Hofmann *et al.* (23)	2008	Austria	0.152	0.152	0.122	11	8

than CRC patients (P=0.035). According to a previous study, Lee *et al.* reported that +936CT+TT reduces the risk of lung cancer (24). However, Kataoka *et al.* have shown that the +936TT genotype is similar in breast cancer patients and controls (25). Dassoulas *et al.* have reported that there is no significant association between +936C/T polymorphism and CRC (4). Thus past studies evaluating the risk of +936C/T polymorphism with different cancer types have shown conflicting results. Many studies have demonstrated that *VEGF* expression could be associated with poor prognosis of several solid tumors including esophageal cancer (26), gastric cancer (27), ovarian cancer (28) and CRC (29). Some polymorphisms located in specific regions (promoter, 5' and 3'UTR) of the *VEGF* gene are related to a change of expression level of this gene (30-32). Krippl and colleagues have demonstrated that allele +936T decreases the VEGF plasma level and reduces the risk of breast cancer (32). One possible explanation of the results concerning +936C/T polymorphism is that the change of C to T allele causes an alternation in the binding site of transcription factor and subsequently the transcription level and ultimately protein production level is reduced (8). In the present study allele frequency is found to be similar between cases and controls (T allele frequency was 0.16 and 0.138 in controls and cases, re-

spectively). Again, Chae *et al.* reported that there is no association of allele frequency between CRC patients and healthy controls (33). By contrast, Bae *et al.* reported that +936T allele is associated with an increased risk of colorectal cancer (17). Comparative frequencies of T allele and TT genotype in +936C/T polymorphisms among different studies are shown in Table 4. Perhaps the reason for these differences could be ethnic heterogeneity, genotype distributions, interactions between environmental factors or genes and different sample sizes (34). VEGF protein is one of the most important factors which is involved in metastasis and advance of colorectal cancer; hence, we analyzed the role of +936C/T polymorphism on the development of tumor stages. Our results show that there is no association between genotypes and tumor stage. In line with our results, Hofmann *et al.* report having found no correlation between genotypes and tumor stage in colorectal cancer (23). In summary, our results in this study support the idea that the *VEGF* +936TT genotype may be a genetic marker for decreased risk of colon cancer in the Iranian population.

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