Colorectal Cancer Risk in Relation to Hypoxia Inducible Factor-1α (Hif-1 α) and Von Hippel-Lindau (Vhl) Gene Polymorphisms

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ABSTRACT

Colorectal cancers (CRC) are among the four most frequently seen cancers in humans and are the second leading cause of cancer-related deaths. Hypoxia up regulates multiple genes involved in different steps of metastatic process, including angiogenesis, proliferation, migration, invasion, motility, adhesion and survival. Hypoxia Inducible Factor 1 (HIF-1) is a master regulator protein of cellular hypoxia-response and triggers the expression of above-mentioned metastatic-process genes. Von Hippel Lindau (VHL) is a protein that plays critical role in the response to hypoxia and product of a tumor suppressor gene. We studied three single nucleotide polymorphisms, rs11549465 (1772C > T), rs11549467 (1790G > A) in HIF-1α and rs779805 (5'UTR A > G) in VHL, and assessed their associations with CRC risk, clinicopathologic and demographic features and lifestyle, and tumor stage and grade of CRC patients and/or healthy controls. ARMS-PCR technique for genotyping of rs11549465 C > T and rs11549467 G>A and PCR-RFLP technique for genotyping of rs779805 A>G were used. CT/TT genotypes of HIF-1α 1772C > T polymorphism were found to increase the risk of colorectal cancer in patients (OR= 1.96, 95% CI= 1.02-3.77, p< 0.05). Additionally, it was demonstrated via statistical analyses that higher age, male gender, cancer history in family, co-existing diseases, and exposure to white soil stands to be risk factors of colorectal cancer (p< 0.05). No significant relation was found between patient's TNM stages and distributions of genotype (p> 0.05). The findings from our study demonstrates that, in addition to risk factors for colorectal cancer, scanning CT/TT genotypes of HIF-1α C1772T polymorphism can be advantageous in early-diagnosis of colorectal cancer.

Keywords: Colorectal cancer, HIF-1α, VHL, rs11549465, rs11549467

ÖZET

Kolorektal Kanser Riski ile Hipoksiyle İndüklenen Faktör-1 Alfa (HIF-1α) ve von Hippel-Lindau (VHL)

Kolorektal kanser insanda sık görülen dört kanser türü arasında yer alır ve ölüme götüren kanserler içerisinde ikinci sıradadır. Hipoksi, anjiogenez, proliferasyon, migrasyon, invazyon, adhezyon ve sağkalımı içeren metastatik süreçte rol alan pek çok geni kontrol eder. Birçok kanser türünün gelişmesinde etkili olduğu düşünülen HIF-1α gen proteini hücrenin hipoksiye cevabının anahtar regülatörüdür. VHL proteini tümör baskılayıcı bir genin ürünü olup, hipoksiye cevap yolağında önemli rol alır. Biz bu çalışmada, HIF-1α geninin rs11549465 (1772C > T), rs11549467 (1790G > A) polimorfizmleri ile VHL geninin rs779805 (5'UTR A > G) polimorfizmini ve ayrıca yaş, cinsiyet, ailede kanser öyküsü, sistemik hastalıklar, beyaz toprak maruziyeti, sigara ve alkol tüketimi ile CRC riski arasındaki ilişkiyi araştırdık. Çalışmaya, histolojik olarak teyit edilen CRC tanısı almış 92 hasta ile kontrol grubu olarak 101 birey katıldı. DNA izolasyonu için periferal kan kullanıldı. HIF-1α genindeki rs11549465 C > T ve rs11549467 G>A polimorfizm değişimlerini genotiplemek için ARMS-PZR; VHL rs779805 A > G polimorfizm değişimini genotiplemek için PZR-RFLP moleküler tanı yöntemleri kullanıldı. Hasta grubunda, HIF-1α1772C > T polimorfizminin CT/TT genotipleri CRC riski ile istatistiksel anlamda ilişkili bulundu (OR= 1.96, 95% CI= 1.02-3.77, p< 0.05). Ayrıca kanser öyküsü, ileri yaş, erkek cinsiyet, eşlik eden hastalıklar ve beyaz toprak maruziyetinin kolorektal kanser için birer risk faktörü olduğu istatistiksel analizlerle belirlendi (p< 0.05). Hastaların TNM evreleri ile genotip dağılımları arasında belirgin bir ilişkiye rastlanmadı (p> 0.05). Sonuç olarak, HIF-1α C1772T polimorfizminin CT/TT genotipleri, ailede kanser öyküsü ve beyaz toprak maruziyeti CRC için birer risk faktörüdür ve CT/TT genotipi kolorektal kanserin erken tanısında avantaj sağlayabilir bir risk belirteci olarak kullanılabilecektir.

Anahtar Kelimeler: Kolorektal kanser, HIF-1α, VHL, rs11549465, rs11549467

INTRODUCTION

Today, despite ongoing intensive investigation, colorectal cancer (CRC) continues to be a major health problem all over the world and the second deadliest cancer after lung cancer.[1] Risk factors for the development of CRC include old age, male gender, chronic inflammatory bowel disease, eating habits, tobacco and alcohol consumption and inheritage of critical cancer genes.[2,3]

Approximately 5% of patients with CRC suffer from hereditary cancer syndromes such as familial adenomatous polyposis (FAP) and hereditary non-polyposis colorectal cancer (HNPCC).[4] However, most of colon cancers are not a hereditary. As other cancers, it is believed that colorectal cancers are developed by acquired genetic instability and uncontrolled proliferation of initially a mutated somatic cell later with additional mutations.[5]

Clinical and experimental evidence suggests that tumor hypoxia is a critical micro environmental factor associated with poor prognosis, enhanced metastatic potential and resistance to therapy.[6,7] HIF-1α is a key regulator that controls cellular reaction to hypoxia and is over-expressed in most solid tumors in response to low oxygen concentrations.[8] Many physiologic events including cell proliferation, angiogenesis, metabolism, erythropoesis, and pathophysiologic events such as tumor invasion and metastasis were regulated by hypoxia.[9] HIF-1 that plays a critical role in the oxygen homeostasis is a heterodimeric protein consisting of HIF-1α ve HIF-1α subunits. While α subunit is found in the cytoplasm and functions as the regulator, nucleus-localized α subunit serves as a structural component.[10]

The outcome of the HIF-1α protein varies with normoxic and hypoxic conditions. In normoxia, HIF-1α is rapidly degraded. The VHL (Von-Hippel Lindau), is a tumor suppressor protein, plays a role in the realization of the degradation.[11] It has many functions in the cell metabolism; but the most well-defined one is the substrate recognizer role in the E3 ubiquitin ligase complex.[12] Under normal oxygen conditions, HIF-1α is hydroxylated at two proline residues and acetylated at a lysine residue in the oxygen-dependent degradation domain. These modifications facilitates binding of VHL to HIF-1α is further degraded by proteasome.[13] In hypoxia, because of blockage of hydroxylation, HIF-1α degradation does not occur. Thus HIF-1α accumulates and translocates to the nucleus. Here it binds to HIF-1α to generate the HIF-1 heterodimer. The adaptation of cell to hypoxia starts with binding of HIF-1α as a transcription factor to special DNA regions and transcribing of the target genes.[14]] Immuno-histo-chemical analysis has demonstrated that HIF-1α level is higher in human tumor tissue than in normal tissue. In in vivo and in vitro studies, it was shown that HIF-1α/VHL pathway associates with cell proliferation rate, tumor development, invasion and metastasis potential.[15] Because of the thought that the polymorphisms in HIF-1α and VHL genes could lead to individual differences in HIF-1α expression, these polymorphisms and transcriptional activities of target genes were examined in several disorders and situations, such as prostate cancer, breast cancer, renal cell cancer, peripheral artery diseases, epithelial to mesenchymal transition and fibronectin restructuring.[16-18]

In this study, it was investigated that whether 1772C>T (rs11549465) and 1790G > A (rs11549467) polymorphisms in oxygen-dependent degradation domain of HIF-1α gene, and rs779805 polymorphism in 5'UTR of VHL gene are risk factors for colorectal cancer.

This is the first report that evaluates the association of HIF-1α and VHL gene polymorphisms with colorectal cancer development in Turkish population.

MATERIALS AND METHODS

Study Population

The study subjects were consisting of 92 patients with CRC and 101 cancer-free, healthy controls from the Department of General Surgery of Necmettin Erbakan University, Meram School of Medicine. The local ethics committee of Meram School of Medicine approved the study (reference number: 2012-177) and all individuals in the study gave written informed consent. All subjects were interviewed face-to-face to collect individual demographic data and exposure information, including age, gender, body mass index (BMI), tobacco

Table 1. Primer sequences and reaction conditions for the SNPs analyzed in this study.

Gene	Primer Sequence	*Tm*	Fragment size
HIF-1α rs11549465(1772C>T) exon 12	Forward inner primer (T allele): 5'-TCCAGTTACGTTCCTTCGATCAGTTGTAAT-3' Reverse inner primer (C allele): 5'-AGGGCTTGCGGAACTGCTTTCTAAGGG-3' Forward outer primer (5'-3'): 5'-GCTGAAGACACAGAAGCAAAGAACCCAT-3' Reverse outer primer (5'-3'): 5'-TGTATGTGGGTAGGAGATGGAGATGCAA-3'	65.3°C	270 bp (C allele) 230 bp (T allele) 450 bp (two outer primers)
HIF-1α rs11549465(1790G>A) exon 12	Forward inner primer (G allele): 5'-TCAGTTGTCACCATTAGAAAGCAGTTACG-3' Reverse inner primer (A allele): 5'-TGAGGACTTGCGCTTTCAGGGCTGGT-3' Forward outer primer (5'-3'): 5'-GCTGAAGACACAGAAGCAAAGAACCCATTT-3' Reverse outer primer (5'-3'): 5'-ATGTGGGTAGGAGATGGAGATGCAATCA-3'	64.9°C	207 bp (G allele) 294 bp (A allele) 447 bp (two outer primers)
VHL rs779805, 5'-UTR (A>G)	Forward outer primer (5'-3'): 5'-CAGGGAGGTCAAGGCTGCAGTGAGCCAA -3' Reverse outer primer (5'-3'): 5'-CATTCCCTCCGCGATCCAGACCACC -3'	71.6°C	A allele 150+122+70+29 bp G allele 150+122+45+29+25bp

and alcohol use, cancer and systemic disease history, white-soil exposure, feeding habits. All of them were Turkish and genetically unrelated. Tumor stage and grade of patients were considered, as can be determined.

SNP Selection

SNPs in HIF-1α and VHL genes were selected based on HapMap data (http://hapmap.ncbi.nlm.nih.gov/)[19] and previous reports.[8,20,21] In identification of the potentially functional polymorphisms, the following criteria were used: (i) located in 5'UTR, 3'UTR or coding region of gene; (ii) minor allele frequency (MAF) >5% in population; and (iii) associated with cancer risk or survival in previous studies. According these criteria, two SNP in HIF-1α gene (rs11549465 and rs11549467) and a SNP in VHL gene (rs779805) were determined in the study.

Genotyping

Anticoagulated EDTA blood from total 193 persons was stored at -20°C. Genomic DNA was isolated by SDS-proteinase K method. Two HIF-1α SNPs were genotyped by Tetra-Primer ARMS-PCR method. Primers were designed by using the program at http://primer1.soton.ac.uk/primer1.html.[22] Primers we used for SNP detection are shown in Table 1. Each PCR reaction was carried out in total volume of 20 μl, containing 50-150 ng of template DNA, 25 pMol of inner and outer primers, 10 mMol dNTP, 1X PCR buffer, 2.5 μl Taq polymerase (Sibenzyme US; west Roxbury, MA, LLC (USA and Canada). PCR cycling conditions consistent of an initial denaturation step for 94°C for 8 min, followed by 30 cycle of 94°C for 1 min, 1 min at the annealing temperature appropriated in Table1, 72°C for 1 min and a final extension of 72°C for 8 min. VHL gene SNP was genotyped by PCR-RFLP method. Primers were designed by using the program at (http://primer3plus.com/web_3.0.0 /primer3web_input.htm).[23] The 25 μl PCR reaction contained 50-150 ng DNA, 10 pMol of each primers, 10 mM dNTP, 1X PCR buffer, 2.5 μl Taq polymerase (New England Biolabs, Inc., Ipswich, MA, USA). PCR cycling conditions consistent of an initial denaturation step for 95°C for

Table 2. Clinicopathologic characteristics of patients and control subjects.

Total	CRC No. (%) 92	Control No. (%) 101	p
Age (years)	63 (21-85) Median (Range)	48 (29-81) Median (Range)	0.055
Sex			
Male	48 (52.2)	54 (53.5)	0.886
Female	44 (47.8)	47 (46.5)	
BMI	26 (12,40-39,50) Median (Range)	25,05 (19,90-30,60) Median (Range)	0.072
Family history of cancer			
No	58 (63.0)	85 (84.2)	0.001
Yes	34 (37.0)	16 (15.8)	
Accompanying disease			
No	52 (56.5)	67 (66.3)	0.184
Yes	40 (43.5)	34 (33.7)	
Smoking status			
No	61 (66.3)	57 (56.4)	0.184
Yes	31 (33.7)	44 (43.6)	
Alcohol use			
No	85 (92.4)	86 (85.1)	0.173
Yes	7 (7.6)	15 (14.9)	
White soil exposure			
No	39 (42.4)	65 (64.4)	0.002
Yes	53 (57.6)	36 (46.6)	
Astler Coller Stage			
B1	6 (6.5)		
B2	17 (18.5)		
C1	2 (2.2)		
C2	19 (20.7)		
D	2 (2.2)		
Not known	46 (50.0)		
Grade			
Grade1	8 (8.7)		
Grade2	40 (43.5)		
Grade3	2 (2.2)		
Not known	42 (45.7)		

10 min, followed by 30 cycle of 94^{0}C for 1 min, 1 min at the annealing temperature appropriated in Table 1. 72^{0}C for 90 sec and a final extension of 72^{0}C for 10 min. The 10 l of PCR products were digested overnight with Hae III endonuclease (New England Biolabs, Inc., Ipswich, MA, USA) at 37 oC and digested DNA fragments were resolved on 5% agarose gel.

Statistical Analysis

Continuous variables were analyzed by Student's t-test and Chi-square test and presented as means±standart deviation (SD). The Fisher's exact test was used to compare the differences of ages and demographic characteristic distributions between healthy controls and CRC patients. For all SNPs, compliance with the Hardy–Weinberg equilibrium (HWE) was assessed using SNPstats software (http://bioinfo.iconcologia.net/snpstats/start.htm).[24] in patients and controls, and adjusted for age and sex. Multiple logistic regression models (co-dominant, dominant, and recessive) were applied to get odds ratio (OR), 95% confidence interval (CI) and P-value. All data analysis was performed using SPSS 18.0 software (SPSS Inc., Chicago, IL, USA). A P value of < 0.05 was considered as significant.

Table 3. Genotype and allele frequencies of SNPs in CRC and control subjects

SNP	Model	Genotype	CRC n (%)	Control n (%)	OR (95% CI)	Pa-value
rs11549465						
	Co-dominant	CC	62 (67.4)	81 (80.2)	Ref.	
		CT	27 (29.4)	16 (15.8)	2.20 (1.09-4.45)	0.078
		TT	3 (3.3)	4 (4)	0.98 (0.21-4.54)	
	Dominant	C/C	62 (67.4)	81 (80.2)	Ref.	
		C/T-T/T	30 (32.6)	20 (19.8)	**1.96 (1.02-3.77)**	0.042
	Recessive	C/C-C/T	89 (96.7)	97 (96)	Ref.	
		T/T	3 (3.3)	4 (4)	0.82 (0.18-3.75)	0.79
	Over-dominant	C/C-T/T	65 (70.7)	85 (84.2)	Ref.	
		C/T	27 (29.4)	16 (15.8)	**2.21 (1.10-4.43)**	0.024
	Allele	C	151 (82)	178 (88)	Ref.	
		T	33 (18)	24 (12)	1.621 (0.92-2.86)	0,11
rs11549467		G/G	91 (98.9)	98 (97)	Ref.	0.35
		G/A	1 (1.1)	3 (3)	0.36 (0.04-3.51)	
	Allele	G	183 (99)	199 (99)	Ref.	0.62
		A	1 (1)	3 (1)	2.759 (0.28-26.75)	
rs779805	Co-dominant	A/A	48 (52.2)	63 (62.4)	Ref.	0.28
		G/A	31 (33.7)	24 (23.8)	1.70 (0.88-3.25)	
		G/G	13 (14.1)	14 (13.9)	1.22 (0.52-2.83)	
	Dominant	A/A	48 (52.2)	63 (62.4)	Ref.	0.15
		G/A-G/G	44 (47.8)	38 (37.6)	1.52 (0.86-2.70)	
	Recessive	A/A-G/A	79 (85.9)	87 (86.1)	Ref.	0.96
		G/G	13 (14.1)	14 (13.9)	1.02 (0.45-2.31)	0.96
	Over-dominant	A/A-G/G	61 (66.3)	77 (76.2)	Ref.	0.13
		G/A	31 (33.7)	24 (23.8)	1.63 (0.87-3.06)	
	Allele	A	127 (69)	150 (74)	Ref.	0.26
		G	57 (31)	52 (26)	1.295 (0.83-2.02)	

RESULTS

Characteristics of CRC Patients and Controls

Frequency distribution of clinicopathologic characteristics of patients and controls are shown in Table 2. There were no significant differences between the patients and controls regarding age, sex, body mass index, drinking status, and smoking (all p> 0.05). In 58 patients, there was no cancer in their family, there was colorectal cancer in 17 patient's family and 17 patient's family had different cancer types. 16 individuals in control group had different cancer types in their relatives. The patient's family story of cancer frequency was different from control group (p= 0.001). There were more accompanying disease such as diabetes, hypertension, asthma and chronic obstructive pulmonary disease (COPD) among the patients (%43.5) than the controls (%33.7) but this was not statistically significant.

However, asbestos exposure ratio in patients with colorectal carcinoma was significantly higher (p< 0.05) than control subjects. Therefore, asbestos exposure may be considered as a risk factor for colorectal carcinoma. Clinicopathological data included cancer grade and stage was shown in Table 2.

The relation between HIF-1α (rs11549465 and rs11549467) and VHL (rs779805) polymorphisms and the CRC risk

Allele frequencies and genotype distributions of VHL and HIF-1α polymorphisms in patients and controls are shown in Table 3. HIF-1α 1772C>T polymorphism genotype frequencies were analyzed with co-dominant, dominant, recessive and over-dominant genetic models. While working out with genotype frequencies, less homozygous mutant genotypes were evaluated with heterozygous genotypes. In dominant model C/T-T/T genotype

was risk factor for CRC (p= 0.042). C/C genotypes were significantly less frequent than C/T-T/T genotype in CRC patients (OR= 1.96; %95 CI 1.02-3.77). CRC risk was increased for C/T genotypes in over-dominant model (p= 0.024) (OR= 2.21; %95 CI 1.10-4.43). No significant differences in allele frequencies were observed between patients and controls (p> 0.05). In dominant model there was no statistically significant differences in genotype and allele frequencies between patients and controls (p> 0.05).

We analyzed genotype frequencies of HIF-1α 1790G>A (A588T) polymorphisms but there was no statistically significant differences in genotype and allele frequencies between patients and controls (p> 0.05).

VHL rs779805 polymorphism genotype frequencies were analyzed with co-dominant, dominant, recessive and over-dominant genetic models. Heterozygous (AG) and homozygous mutant (GG) genotypes were rather frequent in this polymorphism but no statistically significant differences were found in genotype and allele frequencies between patients and controls (p> 0.05). Genotypes were determined successfully in all subjects and did not deviate from the Hardy–Weinberg equilibrium among patients or controls.

DISCUSSION

Hypoxia-inducible factor-1 (HIF-1) regulates the genes that are involved in cell adhesion, angiogenesis, erythropoesis, apoptosis, and glucose metabolism.[25] In the present study, we investigated between CRC and 1772C>T (P582S) and 1790G>A (A588T) polymorphisms which are located within the oxygen-dependent degradation domain of the HIF-1α gene and rs779805 polymorphism in 5'UTR of VHL gene. CT/TT genotype was an important risk factor in HIF-1α 1772C>T polymorphism for CRC patients (p= 0.042). In dominant model CT/TT genotype was more frequent in CRC patients (OR= 1.96; %95 CI 1.02-3.77). In dominant model there was no statistically significant differences in genotype and allele frequencies between patients and controls (p> 0.05). CRC risk was increased for C/T genotypes in over-dominant model (p= 0.024) (OR= 2.21; %95 CI 1.10-4.43).

Numerous investigators studied on the association between HIF-1α polymorphisms and cancer risk but the results were various. Tanimoto et al reported that CT or GA genotypes correlate with significantly elevated transcription activity in head and neck cancer patients.[8] Foley et al were the first investigators that reveal the HIF-1α 1772C>T polymorphism to increase the risk in the prostate cancer patients.[26] Kim et al. determined that HIF-1α 1772C>T polymorphism and expression of HIF-1α relationship could be accepted as a risk factor for cancer development and predictive marker of poor prognosis in breast cancer patients.[27] Conversely Apaydin et al suggest that neither 1772C>T nor 1790G>A polymorphism of HIF-1α gene influence susceptibility to sporadic breast cancer.[28] Fransen et al reported that CT/TT and GA genotype displayed a significantly higher risk to developing ulcerative colorectal cancer.[29] In our study, genomic DNA extracted from the peripheral blood was used and showed that CT/TT genotype of the HIF-1α 1772C>T polymorphism was the risk factor for the CRC.

In our study we analyzed genotype frequencies of HIF-1α 1790G>A (A588T) polymorphisms but there was no statistically significant differences in genotype and allele frequencies between patients and controls (p> 0.05). Knechtel et al could not found risk association for HIF-1α functional polymorphisms (P582S, A588T) and CRC but A588T allele was associated with tumor localization and tumor size.[30] Some studies emphasized that G1790A may be a marker of disfavorable prognosis in early stages of oral cancer.[31]

We studied rs779805 polymorphism in 5'UTR of VHL gene but no significant differences in genotype and allele frequencies were observed between patients and controls (p> 0.05). Chen et al observed that the rs779805 A>G polymorphism was significantly associated with risk for prostate cancer. The AG and AG/GG genotypes were associated with decreased risk of prostate cancer. Further, this decreased risk was more distinct in the groups of nonsmokers, nondrinkers and patients without family history of cancer.[16]

Some studies determined that tobacco and alcohol consumption significantly increase the HIF-1α

gene expression and could be a risk factor to develop hepatocellular carcinoma.[32] No positive results were observed in our study. But to understand the mechanism of the influence of the tobacco and alcohol consumption to the cancer formation, there must be detailed description of the patient's habits.

Buyukdogan et al emphasized white soil exposure increases the CRC risk and our study was consistent with this result and also this was a good example for the environmental factors associated with the CRC.[33]

As a result we investigated the associations between the VHL and HIF-1α functional polymorphisms and CRC. We asked for the patients and controls group's demographic data such as age, gender, family history of cancer and environmental factors such as white soil exposure, consumption of tobacco and alcohol, and tumor stage and grade of CRC patients. CT/TT genotypes of HIF-1α1772C>T polymorphism, cancer history in family and white soil exposure were found to increase the risk of CRC. Prospective studies are needed to learn more about the role of HIF-1α polymorphisms in cancer.

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