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CCR4 C1014T and *CCL22* C16A Genetic Variations in the Iranian Patients with Colorectal Adenocarcinoma

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ABSTRACT

C-C motif chemokine 22 (*CCL22*) C16A genetic variation (rs4359426) and C-C chemokine receptor type 4 (*CCR4*) C1014T variation (rs2228428) have been suggested to affect the expression level of the cognate proteins. Here we tried to investigate the plausible association of these polymorphisms with development of colorectal cancer.

165 patients with colorectal adenocarcinoma (age 54.4±13.4) and 150 age- and sex-matched healthy individuals were enrolled. Genotyping was performed by PCR-RFLP methods. Results indicated the frequency of 16A allele in *CCL22* gene to be 31/330(9.4%) and 33/300(11%) in patients and controls, respectively (p=0.59). The frequencies of CC, CA, and AA genotypes at this locus were not significantly different between patients and controls (135/165; 81.8%, 29/165; 17.6%, 1/165; 0.6% in the patients and 121/150; 80.1%, 25/150; 16.6% and 4/150; 2.6% in the control group, p= 0.34). At the locus 1014 in *CCR4*, T allele was observed with the frequency of 107/330 (32.4%) and 83/300 (27.7%) in patients and controls, respectively (p=0.22).

Analyses indicated no significant differences in the frequencies of CC, CT and TT genotypes at this locus between patients and controls (77/165; 46.7%, 69/165; 41.8% and 19/165; 11.5%; versus 83/150; 55.0%, 51/150; 33.8% and 16/150; 10.6%, respectively, p= 0.29). The presence of individual genotypes was not associated with clinicopathological characteristics of the disease, including tumor size, tumor grade and LN involvement (all with p>0.05).

These findings collectively suggested that *CCR4* C1014T and *CCL22* C16A genetic variations were neither associated with the risk, nor with the progression of colorectal cancer in Iranian population.

Keywords: CCL22; CCR4; Chemokine; Colorectal cancer; Iranian population

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INTRODUCTION

Colorectal cancer is one of the most common causes of cancer related deaths in men and women mainly in the industrial world. The great majority of mortality are due to the tumor metastases to distant organs. If metastasis occurrs, the five year survival rate after surgical intervention falls from 95% to less than 10%.[1,2] The metastatic potential is highly dependent on the ability of cancer cells to interact and communicate with the tumor microenvironment. Chemokines and their receptors are key players in this process.[3-5]

CCL22 is a Chemokine produced mainly by macrophages. It can also be secreted from Dendritic cells, as well as many types of tumor cells.[6,7] CCR4 which is the prominent receptor of CCL22 belongs to the G-protein coupled receptor family of proteins and is also the receptor for CCL2, 3, 5 and CCL17.[8-10] The expression of this receptor has been reported on the surface of several malignant cells.[11-15] Production of CCL22 in tumor microenvironment, beside the selective expression of CCR4 on the surface of Th2 and Treg cells, preferably recruits these immune-inhibitory cells to the site of tumor, and leads to a condition which has been shown to affect the prognosis of cancer patients.[16-18]

The C16A single nucleotide polymorphism (SNP) in *CCL22* gene (rs4359426) has been suggested to affect the mRNA level.[19] This mutation occurs within the first exon of the *CCL22* gene and causes Asp to Ala substitution in the signal peptide-encoding region. Thus, it is suggested that this mutation may also alter the processing efficiency or releasing kinetics of nascent CCL22 protein in endoplasmic reticulum, and increases the secretion level of this Chemokine.[20] More over the C1014T SNP of *CCR4* gene (rs2228428) has been suggested to affect mRNA stability which in turn can increase the expression level of this receptor on the cell surface.[21] Based on the previously suggested immune-suppressive roles of CCR4 and CCL22 in several cancers, reports on high level of CCL22 expression by gastrointestinal cells especially in the pathological situations,[22-24] and regarding the potential gain of function effect of the aforementioned genetic polymorphisms on final products, we designed the present study to investigate the plausible association of these genetic variations with colorectal cancer and the prognostic factors of the disease.

MATERIALS AND METHODS

Our study group consisted of 165 patients with colorectal carcinoma (mean age 54.4±13.4 years) whose cancers were verified after pathological evaluation of the excised tumor mass. The patients' pathological and clinical information were obtained from their medical files. The control group consisted of 150 age- and sex-matched healthy individuals (mean age 52.4±12.0 years) with no personal or immediate-relatives history of malignancies or autoimmune diseases. All the patients and controls were from the southern area of Iran. This study was approved in the ethics committee of Shiraz University of Medical Sciences, and the informed consent was obtained from all participants before sample collection.

Genomic DNA was extracted from peripheral blood leukocytes by salting out method as previously described by Miller et al.[25] The amount and the purity of DNA for each sample were determined by measuring the optical density at 260 and 280 nm wavelengths using a spectrophotometer (Eppendorf, Germany). DNA samples were stored at -20°C until use. Genotyping was performed using Polymerase Chain Reaction-Restriction Fragment Length Polymorphism (PCR-RFLP) reactions, previously introduced by Tsunemi et al.[21] and Wang et al.[20] with slight changes in annealing temperatures. *CCR4* and *CCL22* gene-specific primers, annealing temperatures, restriction enzymes and length of digested fragments are illustrated in table 1.

Table 1. Primers, PCR annealing temperatures and restriction enzymes (REs) in genotyping C1014T SNP in *CCR4* gene and C16A SNP in *CCL22*

Locus	Primer	Primer sequence	Ref.	Annealing	RE*	Length of digested fragments
CCR4 C1014T	Forward	5'-TGTGGGCTCCTCCAAATGTA-3'	1	55.0°C	*RsaI*	T allele: 187bp and 19bp
	Reverse	5'-TGTAAGCCTTCCTCCTGACA-3'				C allele: 206bp
CCL22 C16A	Forward	5′-TGGGAGGTAGTTCTTCTTTTGA-3	2	58.5°C	*MboI*	A allele: 168bp and 47bp
	Reverse	5′-CCACAGCAAGGAGGACGA-3′				A allele :215bp

*RE: restriction enzyme

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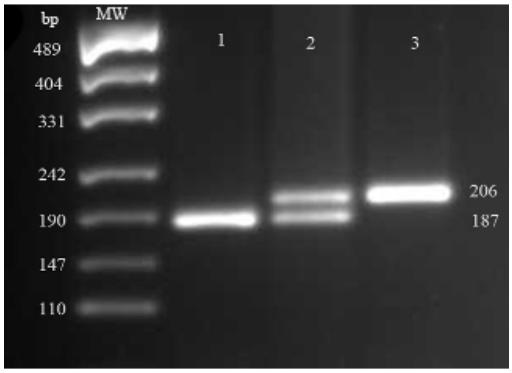

Figure 1. Results of Restriction Fragment Length Polymorphism (RFLP) using *RsaI* for detection of 1014C/T genetic variation in *CCR4* gene on agarose gel 2.5%. TT homozygote, CT heterozygote, and CC homozygote samples have been run in lane 1,2, and 3, respectively. MW: molecular size marker

Three different genotypes of *CCL22* C16A SNP were identified after 2.5%-agarose gel electrophoresis followed by gel staining with GelRed (Biotium,USA) as previously published.[26] Different genotypes of *CCR4* C1014T SNP were identified after 2.5%-agarose gel electrophoresis and staining as illustrated in figure 1.

Statistical analyses were performed with SPSS 11.5 (SPSS Inc, Chicago, IL, USA) and EpiInfo 2002 (CDC, Atlanta, Georgia, USA) software packages. Hardy-Weinberg equilibration was calculated by using the population-genetics software package; Arlequin 3.1.[27] Fisher exact test, X^2 test and non-parametric correlation tests were used according on the requirement. *P* values less than 0.05 were considered statistically significant.

RESULTS

165 patients with colorectal cancer (all pathologically assigned to have colorectal cancer) and 15، age- and sex-matched healthy individuals were genotyped for C1014T (rs2228428) and C16A (rs4359426) SNPs, in *CCR4* and *CCL22* genes, respectively. As presented in table 2, all patients were diagnosed to have adenocarcinoma. Only in 34.1% of reported cases, lymph nodes were involved by the tumor cells. Information regarding distant metastasis at the time of sampling was not available and was accordingly not taken into analysis in the present study.

Table 3 shows the distribution of genotypes and alleles in the populations of our study. Distribution of the observed and the expected genotypes were analyzed by Arlequin and no significant deviation was found from Hardy-Weinberg equation (p>0.05). The frequency of variant allele A at the locus C16A in *CCL22* gene was 31/330 (9.4 %) in patients and 33/300 (11%) in controls, respectively (p=0.59). The frequencies of CC, CA, and AA genotypes at this locus were respectively 135/165 (81.8%), 29/165 (17.6%), 1/165 (0.6%) in the patients, and 121/150 (80.7%), 25/150 (16.7%) and 4/150 (2.6%) in the control group. Statistical analysis indicated no differences in the distribution of genotypes at this locus between patients and controls (p=0.34). The frequency of variant allele T at the locus C1014T in *CCR4* gene was 107/330 (32.4 %) and 83/300 (27.7%) in patients and controls, respectively (p=0.22). The frequencies of CC, CT, and TT genotypes at this locus were respectively 77/165 (46.7%), 69/165 (41.8%), 19/165 (11.5%) in the patients with colorectal cancer, and 83/150 (55.4%), 51/150 (34%) and 16/150 (10.6%) in the control group. Statistical analysis indicated no differences in the distribution of genotypes at this locus between patients and controls (p =0.29).

We then performed the analysis for association between the clinicopathological factors and the inherited genotypes in *CCL22* and *CCR4* genes in patients with colorectal cancer. The missing data regarding tumor stage/sub-stage, tumor size and lymph node (LN) involvement were not taken into account in the study. The presence of individual genotypes was not observed to be significantly associated with clinicopathological characteristics of the disease, including tumor size, tumor grade, LN involvement and tumor stage based on TNM staging system (all with p>0.05).

Table 2. The clinicopathological information of 165 patients with colorectal cancer

Variables	Categories	Value	Valid Percent
Age (Mean ± SD)	-	54.4±13.4	-
Sex	Male	81	49.1
	Female	84	50.9
Tumor type	Adenocarcinoma	165	100
Histological grade	Well differentiated	105	63.6
	Moderately differentiated	50	30.3
	Poorly differentiated	10	6.1
Tumor site	Colorectal region	161	97.6
	Anal canal	2	1.2
	Appendix	2	1.2
Tumor Stage	I	25	26.1
	II	36	37.6
	III	28	29.1
	IV	7	7.2
	Missing	69	-
Tumor Sub-stage	I	26	27.1
	IIA	32	33.4
	IIIA	6	6.1
	IIB	3	3.1
	IIIB	11	11.5
	IIIC	11	11.5
	IV	7	7.3
	Missing	69	-
Tumor size	< 2cm	7	8
	2-5cm	57	65
	> 5cm	24	27
	Missing	77	-
Lymph-node involvement	Free	54	65.85
	Involved	28	34.15
	Missing	83	

Table 3. The frequencies of genotypes and alleles at positions C16A in *CCL22* gene and C1014T in *CCR4* in patients with Colorectal cancer and control group

Locus			Patients (%)	Control (%)	*P* value
CCL22 C16A	Genotypes	CC	135(81.8)	121(80.1)	0.34
		CA	29(17.6)	25(16.6)	
		AA	1(0.6)	4(2.6)	
		Total(N)	165	150	
	Alleles	C	299 (90.6)	267 (89.0)	0.59
		A	31 (9.4)	33 (11.0)	
		Total (2N)	330	300	
CCR4 C1014T	Genotypes	CC	77(46.7)	83(55.0)	0.29
		CT	69(41.8)	51(33.8)	
		TT	19(11.5)	16(10.6)	
		Total (N)	165	150	
	Alleles	C	223 (67.6)	217 (72.3)	0.22
		T	107 (32.4)	83 (27.7)	
		Total (2N)	330	300	

DISCUSSION

The immune-subversive behavior of several tumors by exploiting the CCR4/CCL22 proteins may help the malignant cells to escape immunity and metastasize to other parts of the body.[14,16,28] This behavior is highly dependent on recruiting the $FOXP3^+$ Treg cells that have a powerful immune-suppressive function.[15,17,29-32] On the other hand, several studies have shown that through the process of differentiation of Th0 to Th2 cells, the expression level of the CCR4 on the surface of these cells increases.[16,17] Expression of CCR4 by Th2 and Treg cells and production of CCL22 by different immune and tumor cells at the site of tumor causes these inhibitory cells to be recruited at tumor site and promotes tumor progression.[16-18] Previous investigations have indicated a high profile of CCL22 protein production by the cells of gastrointestinal tract, especially in the pathological situations. This can lead to the accumulation of immune-inhibitory CCR4 bearing cells, within the intestinal microenvironment.[22-24] High expression of CCR4/CCL22 within the tumor microenvironment has been indicated to be associated with poor prognosis in several types of malignancies including adult T-cell leukemia/lymphoma, diffuse large B-cell lymphoma, and peripheral T-cell lymphoma .[12,13,33,34]

In a study by Hirota et al., 39 SNPs have been reported in *CCL22* gene. The C16A SNP is located within the first exon of the *CCL22* gene and has been suggested to affect the mRNA level by the change in the sequence of protein signal peptide.[19,20] By using allele-specific transcript quantification (ASTQ), this group indicated the effect of C16A mutation on mRNA level in Epstein-Barr virus transformed lymphoblastoid cells.[19] The C1014T SNP is located in the tail of CCR4 protein and has been reported to be associated with increased expression level of this receptor on cell surface.[21]

In the present study, we could not find any association between colorectal cancer neither with C16A SNP in *CCL22* gene, nor with C1014T SNP in *CCR4* gene. The analysis in the patients also indicated no association between the inherited genotypes with the cancer prognostic factors.

Previous investigations on these target polymorphisms indicated controversial results. While the *CCL22* C16A genetic variation has been indicated to be associated with higher susceptibility to pathological conditions like atopic dermatitis[19] and higher risk of developing Helicobacter pylori infection-related gastric carcinoma,[20] Tsunemi et al. indicated that C1014T SNP in *CCR4* gene is not associated with susceptibility to atopic dermatitis, in a population with Japanese origin.[21] In a recent study in our lab, we were unable to indicate the association of C16A SNP in CCL22 with predisposition to breast cancer.[26] The discrepancies between these investigations might be due to the differences both in the sample size and genetic background of the investigated populations, and/or may be due to the differences in the molecular pathogenesis of different cancers.

The findings of the present study collectively suggests that *CCR4* C1014T and *CCL22* C16A genetic variations are neither associated with the risk, nor with the prognostic factors of colorectal cancer in our target population. Increasing the sample size and including other genetic variations of *CCL22* and *CCR4* in other studies are required to completely rule out the CCR4 and CCL22 as the genetic susceptibility factors in colorectal cancer.

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