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CCR4 1014C/T and CCL22 16C/A Genetic Variations in Iranian Patients with Thyroid Cancer

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Abstract

Background: The aim of this study was to investigate the association between thyroid cancer and 16C/A single nucleotide polymorphism (SNP) in C-C motif chemokine 22 (CCL22) as well as 1014C/T SNP in C-C chemokine receptor type 4 (CCR4).

Method: In this case-control study, polymerase chain reaction restriction-fragment length polymorphism (PCR-RFLP) was performed for 113 thyroid cancer patients and 112 age-sex matched healthy controls to investigate the genotype distribution.

Results: At position 16C/A in CCL22, 95 patients (84.3%) were found to have CC genotype, while 17 individuals (14.8%) inherited CA genotype and 1 (0.9%) had AA genotype. In the control group, 92 volunteers (82.1%) inherited CC genotype, 18 individuals (16.1%) had CA genotype, and 2 (1.8%) had AA genotype. The frequency of CC, CT, and TT genotypes of 1014C/T SNP in CCR4 gene was 60 (53.1%), 43 (38.1%), and 10 (8.8%) in the patients, and 57 (53.3%), 43 (40.2%), and 7 (6.5%) in the control group, respectively. There were no statistically significant differences between the patients and controls in terms of 16C/A polymorphism in CCL22 (P = 0.816) and 1014C/T SNP in CCR4 1014C/T gene position (P = 0.801). Nevertheless, the study of their association indicated that inheriting the CC genotype of CCR4 was significantly associated with higher stages (stages 3 and 4) in thyroid cancer.

Conclusion: 1014C/T genetic variation in CCR4 and 16C/A polymorphism in CCL22 were not found to have a role in genetic susceptibility to thyroid cancer. Inheriting CC genotype at 1014 locus in CCR4 may; however, affect cancer progression in patients with thyroid cancer.

Keywords: CC chemokine receptor 4, CCL22 chemokine, Genetic variations, Single nucleotide polymorphism, Thyroid cancer, Cancer progression

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Introduction

Thyroid cancer (TC) is the most prevalent endocrine malignancy with complex etiology.[1] The number of individuals affected by this cancer has increased over the recent years.[2] The prevalence of TC in women is three times higher than that in men.[3] The role of genetic variations in TC incidence has been previously studied.[4] Nevertheless, most of the TC-related genetic variations have not been described.[1] The association between the immune response and development of TC has been shown to be controversial.[5] The potential of cancer cells in communicating with the tumor microenvironment could influence the metastasis ability. Chemokines and their receptors have an important role in this process.[6] Chemokines are a family of proteins that are secreted by numerous cells, such as tumor and inflammatory cells, and can contribute to the occurrence and development of cancers.[7] According to the sequence of their conserved N-terminal cysteine residues, they are categorized into four subfamilies: CXC, CC, C, and CX3C.[8] Through binding to their receptors, chemokines affect the fate of the tumor cells, including proliferation, apoptosis, and metastasis.[9] Macrophages produce and secret Cys-Cys (CC) chemokines, such as macrophage-derived chemokine (MDC/CCL22).[10] The CCL22 gene is mapped on the short arm of chromosome 16 (q16). The specific receptor of CCL22 is CCR4 which also acts as a receptor for other CC chemokines.[10,11] Overexpression of certain chemokine receptors has been found to be associated with cancer metastasis, for instance, CXCR4 in breast cancer, prostate cancers, and gastric carcinoma[12-14] and CCR6 in pancreatic cancer.[15] Furthermore, the 1014C/T single nucleotide polymorphism (SNP) of CCR4 gene (rs2228428) has been proposed to affect the stability of mRNA, which can subsequently enhance the expression level of the receptor on the cell surface.[16] The association between these genetic variations with lung cancer and colorectal adenocarcinoma was investigated by Erfani et al.[17,18] To the best of our knowledge, this is the first time that the CCR4/CCL22 polymorphism is evaluated in thyroid cancer. Considering the immune-suppressive roles of CCR4 and CCL22 in several cancers,[19-21] we aimed to study the association of these genetic variations with thyroid cancer.

Subjects and Methods

Subjects

In this case-control study, 113 patients affected by thyroid malignancy with a mean age of 40.52 ± 14.61 years were enrolled. The specimens were obtained from the hospitalized patients whose malignancy and type of tumor tissues were confirmed by the pathological evaluation following surgery. The inclusion criterion for the patient group was pathological and clinical confirmation of thyroid cancer. Medical history of other malignancies, autoimmune disorders, and immune deficiencies were considered as the exclusion criteria. The control group with the mean age of 40.58 ± 14.51 years were selected from the Blood Transfusion Center and DNA samples banked at the center. They had no history of cancer and autoimmune diseases in their first-degree relatives. The information of the patients, including age, tumor type, grade of tumor, and metastasis was extracted from medical records and pathology reports. Initially, both groups (patient and control) were provided with informed

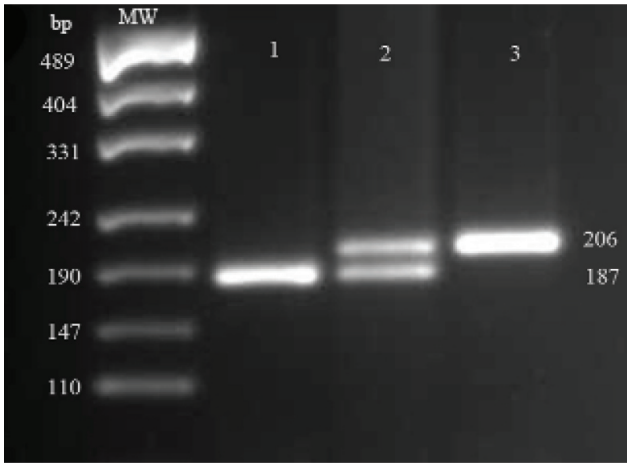

Figure 1. Results of restriction fragment length polymorphism (RFLP) using RsaI for detection of 1014C/T genetic variation in the CCR4 gene on agarose gel 2.5%. CC homozygote, CT heterozygote, and TT homozygote samples were run in lanes 1,2, and 3, respectively.
MW: Molecular size marker

consent forms to be signed prior to the experiment. The study was approved by the Ethics Local Committee at Shiraz University of Medical Sciences, Shiraz, Iran (IR.SUMS.REC.1390.2558).

DNA extraction and genotyping

Peripheral blood lymphocytes were obtained from the participating individuals and their DNA was extracted using salting out method. PCR-RFLP method was employed to identify the polymorphism in CCL22 gene at 16C/A and 1014C/T in the CCR4 gene.[16,22] Briefly, for CCL22 gene at 16C/A position, PCR reaction was performed in the presence of genomic DNA and specific primers and the amplification was done with annealing the temperature of 58.5ºC for 35 cycles in thermo-cycler (Techne Fleigene, England). The amplified products were treated with 5 IU of Mbol restriction enzyme (Fermentas, Lithuania) overnight at 37ºC (See table1). Digested DNA was separated applying agarose gel electrophoresis.[23] Accordingly, the samples were run in 2.5% agarose gel and then visualized under UV light after staining with Gel Red (Biotium, USA). If A allele existed, the 212 bp product was cut into 165 and 47 bp fragments. However, if C allele was present at this position, it was remained undigested. Similarly, for 1014C/T in the CCR4 gene, PCR reaction was amplified with the annealing temperature of 55ºC for 35 cycles in thermo-cycler (Techne Fleigene, England). RsaI restriction enzyme (Fermentas, Lithuania) was used to detect genotypes in this position (Figures 1 and 2). Table 1 represents the feature of the primers and restriction enzymes used in the experiment. We previously verified the data from RFLP reaction through direct sequencing of PCR products on the ABI 310 genetic analyzer (ABI, USA).[23]

Statistical analysis

In this study, the data were analyzed utilizing IBM© SPSS© Statistics V. 18.0. Prior to conducting statistical analysis, the study groups were evaluated in terms of compliance with Hardy Weinberg equilibrium.[24] T-test was used to verify the compliance of age. Chi-square test or Fisher Exact Test and NPar Tests were employed to evaluate the differences and calculate the *P* value. The data were presented as mean ± SD, and *P* values lower than 0.05 were considered as statistically significant.

Results

In general, 115 patients participated in this investigation. Two individuals were excluded due to the unavailability of their pathological and clinical information. Hence, the study continued with 113 patients with a mean age of 40.52 ± 14.61 years and 112 normal individuals with a mean age of 40.58 ± 14.51. The patient and control groups were matched in terms of sex and age. Table 2 illustrates the clinical and pathological information of the patients. Based on the data analysis, papillary TC accounts for the majority of TC (76.1%).

After genotyping of both groups, it was revealed that the distribution of the genotypes among both patient ($P = 0.56$ for 16C/A CCL22 and $P = 0.64$ for 1014C/T CCR4) and control ($P = 0.28$ for 16C/A CCL22 and $P = 1$ for 1014C/T CCR4) groups was in accordance with Hardy Weinberg equilibrium.

16C/A CCL22 polymorphism

Table 3 illustrates the frequencies of genotypes and alleles at position 16C/A CCL22 among the patients with TC and healthy control groups. At

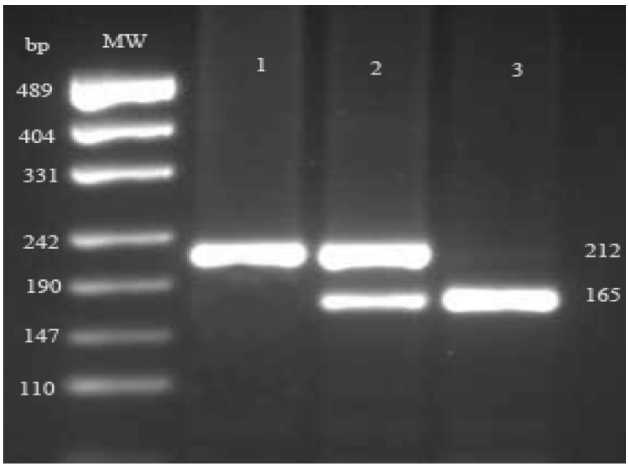

Figure 2. Results of restriction fragment length polymorphism (RFLP) using MboI for detection of 16C/A genetic variation in the CCL22 gene on agarose gel 2.5%. CC homozygote, AC heterozygote, and AA homozygote samples were run in lanes 1, 2, and 3, respectively.
MW: Molecular size marker

Table 1. The features of the primers and restriction enzymes used

Locus	Primer	Primer sequence	Ref.	Annealing	RE	Length of digested fragments
CCR4 1014C/T	Forward Reverse	5′-TGTGGGCTCCTCCAAATGTA-3′ 5′-TGTAAGCCTTCCTCCTGACA-3′	16	55.0ºC	*RsaI*	TT: 206bp CT: 206,187,19bp CC: 187,19bp
CCL22 16C/A	Forward Reverse	5′-TGGGAGGTAGTTCTTCTTTTGA-3′ 5′-CCACAGCAAGGAGGACGA-3′	22 and 23	58.5ºC	*MboI*	CC: 212bp CA: 212,165,47bp AA: 165,47bp

*RE: Restriction enzyme

this locus, 95 patients (84.3%) were observed to have CC genotype, while 17 individuals (14.8%) inherited CA genotype, and 1 person (0.9%) had AA genotype. In the control group, 92 volunteers (82.1%) inherited the CC genotype, 18 individuals (16.1%) had the CA genotype, and 2 subjects (1.8%) had the AA genotype. No significant differences were observed between the patients and control groups in terms of CCL22 16C/A genotypes ($P = 0.816$). Based on the statistical analysis, allele C, the wild type allele, was the dominant allele in the patients. The frequency of C allele was not significantly different between the control group and patients ($P = 0.72$).

1014C/T CCR4 polymorphism

Table 3 demonstrates the frequencies of genotypes and alleles at position 1014C/T locus in the CCR4 gene among the patients with TC and healthy control groups. The frequency of CC, CT, and TT genotypes of 1014C/T SNP in the CCR4 gene was 60 (53.1%), 43 (38.1%), and 10 (8.8%) in patients, and 57 (53.3%), 43 (40.2%), and 7 (6.5%) in the control group, respectively. There were no statistically significant differences between the patients and controls in terms of CCR4 1014C/T genotypes ($P = 0.801$). C allele, the wild type allele, was the dominant allele in both groups with no statistically meaningful differences ($P = 0.85$) (Table 3).

Correlation between the genotypes and clinical and pathological factors

Statistical analyses indicated that inheriting 1014 CC genotype of CCR4 was significantly associated with higher stages (stage 3 and 4) in TC (P value < 0.05). No significant associations were observed between other genotypes with clinical and pathological factors of the disease, including tumor type, tumor grade, and tumor classification (P value > 0.05).

Discussion

Given that the genetic polymorphisms in the genes encoding chemokines and their receptors are associated with malignancies,[18] we investigated the association of CCR4 and CCL22 polymorphisms with TC in this study. Statistical analysis of 16 C/A SNP in CCL22 gene indicated no significant associations between the gene polymorphism and thyroid cancer. The results of our study revealed that at this position, CC genotype and C allele were dominant in the population, with minor allele frequency (MAF) of 0.1. The frequency of minor allele was not significantly different between the patient and control groups. No correlations were also observed between the genotypes at this position with clinical and pathological factors of the disease, including tumor type, tumor grade, and tumor classification.

The mutation at 16 C/A position, which occurs within the first exon of the CCL22 gene, leads to substitution of Asp with Ala in the signal peptide coding region; this may in turn alter the processing efficiency or releasing feature of newly-synthesized CCL22 protein in endoplasmic reticulum, thereby increasing the secretion level of this Chemokine.[22] In line with the finding of a previous work of ours on breast cancer in a population from southern Iran, this study was not able to indicate the association between this polymorphism and the progression and susceptibility to thyroid cancer.[23] Similarly, we observed no significant associations between this polymorphism and clinical-pathological symptoms of the patients compared to the control group in

Table 2. Clinical and pathological characteristics of patients with thyroid cancer

Clinicopathological characteristic	No. out of 113	Statistics
Age (years)	112	Mean ± SD:40.52 ± 14.61
		Minimum:14
		Maximum: 87
Tumor type	102	PTC (%76.1)
		MTC (%8.8)
		FTC (%4.4)
		HTC (1%)
		Unknown (9.7%)
TNM stage	97	Stage I :(52.5%)
		Stage II :(15.5 %)
		Stage III: (16.5%)
		Stage IV: (15.5%)
Histological grade	15	Grade 1(Well-Differentiated): 80%
		Grade 2 (Moderately-Differentiated): 6.7%
		Grade 3 (Poorly-Differentiated): 13.3%

PTC: Papillary thyroid cancer; MTC: Medullary thyroid cancer; FTC: Follicular thyroid cancer; HTC: Hurthle cell thyroid cancer

colorectal adenocarcinoma.[17] In an investigation conducted by Jafarzade et al., the serum level of CCL22 was measured in patients with breast cancer. They evaluated the polymorphism of CCL22 2030G/C (rs223818) in these patients. Their results indicated that the serum level of CCL22 was higher in the patients than that in the control group. In addition, evaluation of the mentioned polymorphism showed that the frequency of allele C and CC genotype was higher in the patients than that in the controls. Furthermore, the serum level of CCL22 increased in higher stages. Interestingly, in both groups, the serum level of CCL22 was higher in the individuals with the CC genotype than that in those with the GG genotype. Collectively, this group suggested the influence of CCL22 2030G/C (rs223818) polymorphism on the serum level of CCL22, thereby having a role in susceptibility to breast cancer.[25] Hirota et al., showed a correlation between 16C/A polymorphism in CCL22 and susceptibility to atopic dermatitis.[26] In a study performed on Italian patients with multiple sclerosis, no significant associations were reported between the CCL22 16 C / A polymorphism and the disease.[27] The reported controversy may be due to the genetic differences in populations under the study and the variation in the molecular pathogenesis of different types of diseases.

Our observation regarding CCR4 1014C/T SNP revealed no significant associations between this SNP and susceptibility to thyroid cancer. The distribution of CC genotype and C allele at this locus was observed to be dominant in the population, with a MAF of 0.3. The frequency of minor allele was not significantly different between the patients and controls. Despite these observations, the study of associations indicated that inheriting the CC genotype of CCR4 was significantly correlated with higher stages (stage 3 and 4) in thyroid cancer. CCR4 1014C/T SNP occurs in the tail of CCR4 protein, which causes the overexpression of this receptor through influencing CCR4 mRNA.[16] Jones et al. suggested that CCR4 expression by tumor cells is related to large cell transformation in most common types of cutaneous non-Hodgkin lymphoma.[28] Lee et al., reported that overexpression of CCR4 was correlated with tumor recurrence and down survival of gastric cancer.[29] The study of the association between CCR4 1014C/T SNP and cancer is scarce. In two other studies by our group, no statistically significant associations were found between CCR4 1014C/T SNP and colorectal and lung cancer.[17,18] Consistently, Tesunemi et al., reported that CCR4 gene polymorphism at 1014C/T does not influence the susceptibility to dermatitis in the Japanese population.[16]

Observing no differences in the distribution of the genotype between patients and controls in addition to the association of CCR4 1014 CC genotype with higher stages (stage 3 and 4) in patients suggests that although CCR4 1014C/T

Table 3. The frequency of the genotypes and alleles at position 16C/A in CCL22 gene, as well as 1014C/T in CCR4 gene among the patients with thyroid cancer and the healthy control group

		Controls (N=112, 2N=224, Valid %)	Patients (N=113, 2N=226, Valid %)	*P* value
CCL22 16 C/A (rs4359426)				
Genotypes	CC	92 (82.1%)	95 (84.3%)	0.83
	CA	18 (16.1%)	17 (14.8%)	0.98
	AA	2 (1.8%)	1 (0.9%)	0.62
Alleles	C	202 (90.2%)	207 (91.6%)	0.72
	A	22 (9.8%)	19 (8.4%)	
CCR4 1014C/T (rs2228428)				
Genotypes	CC57	(53.3%)	60 (53.1%)	0.91
	CT	43 (40.2%)	43 (38.1%)	0.85
	TT7	(6.5%)	10 (8.8%)	0.7
	Missing	5	0	
Alleles	C	157 (73.4%)	163 (72.1%)	0.85
	T	57 (26.7%)	63 (27.9%)	
	Missing	10	0	

SNP may not have a role in the incidence of thyroid cancer, it may alter the cancer progression in patients who suffer from this disease, the finding which merits further investigation.

Inheriting CC genotype at 1014 locus in CCR4 may; however, affect cancer progression in patients with thyroid cancer. However, our study only investigated two SNPs in CCR4 and CCL22 genes. Investigation of other CCR4 and CCL22 genetic variations as well as protein level is of great necessity to completely elucidate the role of these molecules in thyroid cancer. Small sample size in each group was another limitation of our study. Further studies are suggested with bigger sample sizes on different ethnic groups to explore the possible association between investigated SNPs and genetic susceptibility to thyroid cancer.

Conclusion

In conclusion, this investigation did not reveal a significant association between 1014C/T polymorphism in CCR4, as well as 16C/A polymorphism in CCL22 and susceptibility to TC in the southern Iranian population.

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Conflict of Interest

None declared.

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