CCL22 16C/A Genetic Variation is not Associated with Breast Carcinoma in Southern Iranian Population

Nasrollah Erfani[1], Faezeh Moghaddasi-Sani[2], Mahboubeh Razmkhah[1], Mohammad Reza Haghshenas[1], Abdolrasoul Talei[3], Abbas Ghaderi[1,4*]

[1]Cancer Immunology Research Group, Shiraz Institute for Cancer Research, School of Medicine, Shiraz University of Medical Sciences, Shiraz, [2]Department of Biology, Faculty of Sciences, Islamic Azad University-Science and Research Branch, Tehran, [3]Department of Surgery, [4]Department of Immunology, School of Medicine, Shiraz University of Medical Sciences, Shiraz, Iran

ABSTRACT

Background: CCL22/MDC is a CC chemokine with a critical role in regulation of the immune balance in physiological condition. CCL22/CCR-4 ligation has been documented to participate in the migration of regulatory T (Treg) cells and Th2 lymphocytes to the site of breast tumors; circumstances that are known to be associated with poor prognosis. **Objective:** To investigate the association of a single nucleotide polymorphism (SNP) in CCL22 gene; 16C/A (rs4359426; Asp2Ala), with susceptibility to breast cancer in a sample of Iranian population. **Methods:** 161 patients with pathologically confirmed breast carcinoma (mean age 49.3 ± 11.5 yrs) and 178 age-matched healthy women (mean age: 49.3 ± 12.9 yrs) were studied. CCL22 genotypes were investigated by the Polymerase Chain Reaction-Restriction Fragment Length Polymorphism (PCR-RFLP) method. Data was verified by direct automated sequencing. Arlequin analysis showed no deviation from Hardy-Weinberg equilibrium. **Results:** The most frequent genotype in both patient and control groups was wild type CC genotype with frequency of 146 out of 161 (90.7%) among patients and 153 out of 178 (86.0%) in control group (p=0.24). The frequency of CA genotype was 15 (9.3%) and 23 (12.9%) in patients and controls, respectively (p=0.38). No AA genotype was observed among patients but this genotype was observed with the frequency of 2 out of 178 (1.1%) in control subjects. The minor allele frequency (MAF) was 0.07 in the population. **Conclusion:** No correlation was found between the investigated genotypes and clinicopathological characteristics of the patients. Conclusively, results of this investigation do not support the association of 16C/A SNP (rs4359426; Asp2Ala) in CCL22 gene with susceptibility to, and progression of, breast cancer in Iranian population.

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Keywords: Breast Cancer, CCL22/MDC, Chemokine, Polymorphism, Tumor Immunology

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*Correspondence author: Dr. Abbas Ghaderi, Shiraz Institute for Cancer Research, School of Medicine, Shiraz University of Medical Sciences, Shiraz, Iran, Tel: (+) 98 711 2303687, Fax: (+) 98 711 2304952, e-mail: ghaderia@sums.ac.ir

INTRODUCTION

The etiopathology of breast cancer is extremely multi-parametric; involving numerous genetic, endocrine and external environmental factors (1,2). Production of chemokines by tumor cells, as well as tumor infiltrative immune cells has been indicated to recruit different leukocytes and macrophages to the site of tumor (3). It has been recently found that breast carcinoma cells express the chemokine C-C motif ligand (CCL)-22/ Macrophage-derived Chemokine (MDC) (4), specific ligand for chemokine receptor CCR-4. CCR-4 is selectively expressed by regulatory T (Treg) cells as well as Th2 lymphocytes (5-7). Investigations indicated the recruitment of these cells to the tumor microenvironment under the influence of CCL22/CCR-4 ligation (6,8,9). Recruitment of Treg cells and Th2 lymphocytes, which are the main attenuators of cellular immunity, may provide a favorable microenvironment for tumor growth, and have been widely reported to be associated with bad prognosis in cancer patients (8-11).
The gene for CCL22 chemokine has been mapped on chromosome 16q13 (4) with several single nucleotide polymorphisms (SNPs) in both coding and noncoding sequences, of which 16C/A (rs4359426); affects the amino acid sequence of signal peptide, with a potential effect of final protein expression (12). Considering CCL22 as one of the key regulators of Th2 and Treg cell trafficking, the effects of immune-inhibitory cell recruitment in breast cancer prognosis, and the functional effect of CCL22 16C/A SNP (GAT to GCT coding change which causes a 2 aspartate (2Asp) to 2 alanine (2Ala) substitution in the CCL22 protein (12), we hypothesized that this SNP might render susceptibility to breast carcinoma. Accordingly, we aimed to investigate, in the present study, the association of this SNP with the susceptibility to breast cancer in a population from the south of Iran.

MATERIALS AND METHODS

Subjects. Our study group consisted of 161 patients with breast carcinoma (mean age 49.3 ± 11.5 years) whose cancer was 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 178 age-matched healthy women, with no personal and familial history of cancer or autoimmune disease (mean age 49.3 ± 12.9). All patients and controls were from the south area of Iran and the controls were selected among the volunteers from the university staff, as well as, people who referred to the Shiraz hospital for no-invasive, non-autoimmune, non-infectious diseases. This study was approved in ethical committee of Shiraz University of Medical Sciences and informed consent was obtained before sample collection.
DNA Extraction and Genotype Analysis. DNA was extracted from peripheral blood leukocytes by salting out method as previously described by Miller et al. (13). The amount and the purity of DNA for each sample were determined by measuring the optical density at 260 and 280 nm wavelengths using spectrophotometry (Ependorf, Germany). DNA samples were stored at -20°C until use.
Genotypes at position 16C/A in CCL22 (rs4359426; Asp2Ala) was determined by Polymerase Chain Reaction-Restriction Fragment Length Polymorphism (PCR-RFLP) method. The forward (5′-Tgg gAg gTA gTT CTT CTT TTg A-3′) and reverse primers (5′-CCA CAG CAA ggA ggA CgA-3′) (12) were used to amplify the region around

16C/A SNP. PCR reaction was performed in a mix containing 0.3 µg genomic DNA, 0.6 pmol/L each primers (Takapozist, Iran), 0.6 mmol/L each dNTP (CinnaGen, Iran), 0.45 mmol/L MgCl2(CinnaGen, Iran), 2 units Taq DNA polymerase (CinnaGen, Iran), 1X PCR buffer (CinnaGen, Iran) and ddH2O to a final volume of 15µL. The samples were amplified for 35 cycles in thermo-cycler (Techne Fleigene, England), with annealing temperature of 58.5°C.

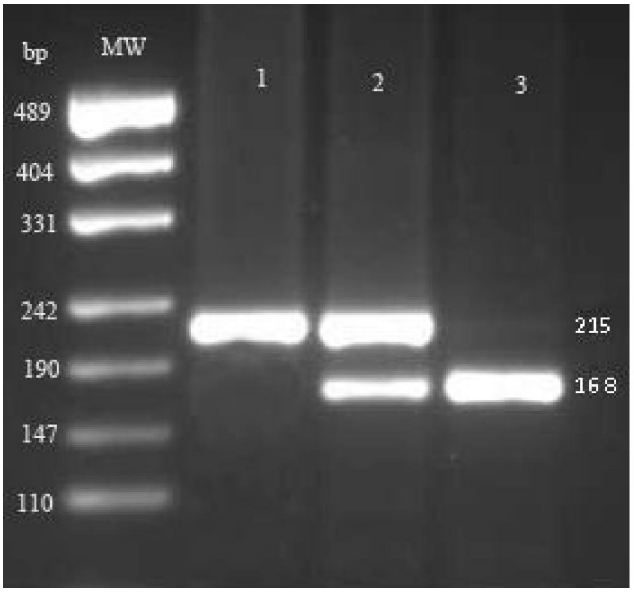

Figure 1. Polymerase Chain Reaction-Restriction Fragment Length Polymorphism (PCR-RFLP) of 16C/A genetic variation (rs4359426; Asp2Ala) in the CCL22 gene Lane 1: CC homozygous, Lane 2: heterozygous, Lane3: AA homozygous, Note that A allele was digested to into 168 and 47 bp fragments, with the later band leaving the gel very fast, not present in the photo.

The amplified products were then underwent restriction reaction in the presence of 5 IU of *Mbo*I restriction enzyme (Fermentas, Lithuania) overnight at 37°C. The samples were then run in 2.5% agarose gel under electrophoretic field and the resulted bands were visualized under UV light after staining with GelRed (Biotium, USA). The 215 bp product from 16C/A loci was cut into 168 and 47 bp fragments when A allele existed, but remained undigested if C allele was present at this position (Figure 1). The data from RFLP reaction was verified by direct sequencing of PCR products on the ABI 310 genetic analyzer (ABI, USA). Figure 2 illustrates a part of sequence data from a sample with heterozygote genotype.

Statistical analyses were performed by SPSS 11.5 (SPSS Inc, Chicago, IL, USA) and Epi Info 2002 (CDC, Atlanta, Georgia, USA) software packages. Hardy-Weinberg equilibration was calculated by using the population genetics software package; Arlequin 3.1 (14). Fisher exact test, X^2 test and non-parametric correlation tests were used based on the requirement. P value less than 0.05 was considered statistically significant.

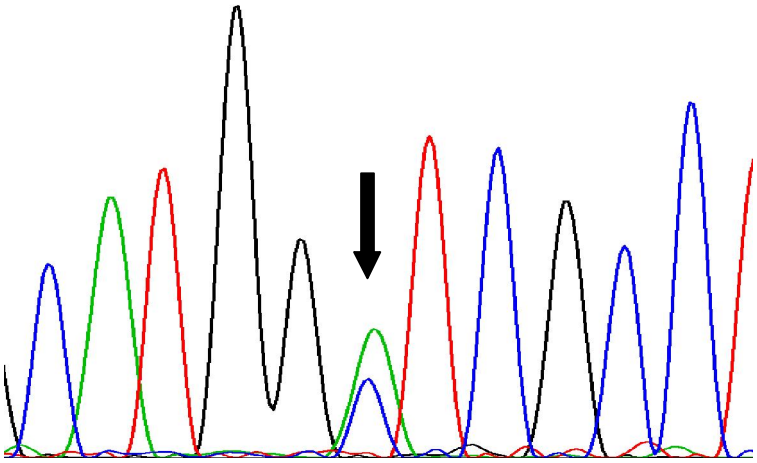

Figure 2. Verifying the heterozygote genotype at position 16C/A in CCL22 gene (rs4359426; Asp2Ala) by direct automated sequencing. The heterozygote peak is pointed by arrow.

RESULTS

One-hundred sixty one patients with breast carcinoma and 178 age-matched healthy control women were genotyped for the 16C/A SNP (rs4359426; Asp2Ala) in the coding sequence of CCL22. As illustrated in Table 1, the tumor type in 90.7% of the patients was Conventional-Infiltrative Ductal Carcinoma (IDC). Most of the patients (70.4%) were in clinical stage II and 56.1% had at least one lymph node involved by tumor cells at the time of surgery.

Only 1.3% of the patients were diagnosed to have distant metastasis at the time of sampling. Arlequin analysis showed no deviation of genotype frequencies from Hardy-Weinberg equilibrium, neither in the patients nor in the controls. Table 2 summarizes the frequencies of genotypes and alleles at position 16C/A in CCL22 gene in patients with breast cancer and healthy control group. As illustrated, the frequencies of CC, CA and AA genotype in patients and control group were respectively 146 (90.7%) versus 153 (86.0%), 15 (9.3%) versus 23 (12.9%) and 0 (0.0%) versus 2 (1.1%). The frequencies of C and A alleles at this position in patient and control groups were respectively 307 (95.3%) versus 392 (92.4%) and 15 (4.7 %) versus 27 (7.6%) respectively. Statistical analysis revealed no significant differences in the frequencies of genotypes and alleles between patients and controls.

Statistical analysis also indicated no correlation between genotypes at position 16C/A (rs4359426; Asp2Ala) with the clinicpathological characteristics of the patients including tumor type, tumor size, clinical stage, histological grade, LN involvement, lymphovascular invasion (LVI), distant metastasis and Nottingham prognostic index (NPI) ($p>0.05$).

Table 1. Clinicopathological features of patients with breast cancer.

Clinicopathological characteristic	No. out of 161	Statistics
Age (years)	161	Mean ± SD: 49.3 ± 11.5, Min: 25, Max: 82
Tumor type*	161	IDC-NOS (90.7%), MC (4.3%), Met. C (1.9%), Other-IDC (1.9%), ISDC (0.6%), Unknown (0.6%)
TNM stage	159	Stage I: 17.0%, Stage II: 70.4%, Stage III: 11.3, Stage IV 1.3%
Lymph node (LN) status	155	Free: 43.9%, Involved: 56.1%
Lymphovascular invasion (LVI)	130	Negative: 16.2%, Positive: 83.8%
Tumor size (cm)	150	Size ≤2: 32.7%, 2 < Size ≤ 5: 59.3% Size >5: 8.0%
Histological grade	118	Grade 1(Well Differentiated): 33.1% Grade 2 (Moderately Differentiated): 54.2% Grade 3 (Poorly Differentiated): 12.7%
Distant metastases at the time of diagnosis	160	Negative: 98.8% Positive:1.3 %
ER expression	137	Negative: 42.3% Positive: 57.7%
PR expression	137	Negative: 43.1% Positive: 56.9%
Nottingham Prognostic Index	110	NPI ≤ 3.4 (Good prognosis):22.7% 3.4 < NPI ≤ 5.4, (Moderate prognosis):54.5% NPI > 5.4, (Poor prognosis):22.7%

*Abbreviations: IDC-NOS: Infiltrative Ductal Carcinoma -Not Otherwise Specified (Conventional IDC), MC: Medullary Carcinoma, Met.C: Metaplastic Carcinoma, Oth-IDC: Other IDC subtypes, ISDC: Ductal Carcinoma In situ "Except for tumor type, all other percentages in this table are valid percentage, i. e. excluding missing data.

Table 2. Allele and genotype frequencies of 16C/A CCL22 gene polymorphism in breast cancer patients and controls.

Locus			Patients (n=161, 2n=322)	Healthy controls, (n= 178, 2n=356)	P Value	Odds ratio (OR)	95% Confidence interval (CI) for OR
CCL22 16C/A*	Genotype	CC	146 (90.7%)	153 (86.0%)	0.24	1.59	0.77-3.31
		CA	15 (9.3%)	23 (12.9%)	0.38	0.69	0.33-1.45
		AA	0 (0.0%)	2 (1.1%)	-	-	
	Allele	C	307 (95.3%)	329(92.4%)	0.16	1.68	0.84-3.38
		A	15 (4.7%)	27(7.6%)			

*rs4359426; Asp2Ala

DISCUSSION

In the present study we investigated the impact of CCL22 16C/A (rs4359426; Asp2Ala) polymorphism on susceptibility to breast carcinoma in a population from the south of Iran. Results indicated that CC genotype is the most frequent genotype among patients and controls; with no significant different distribution between these two groups (146 out of 161 (90.7%) and 153 out of 178 (86.0%), respectively; p=0.24). The frequencies of less abundant genotypes CA and AA in patients and controls were also not significantly different (p>0.0.05). Data analysis also indicated no correlation between CCL22 16C/A genotypes and clinicopathological characteristics of the disease. The minor allele frequency (MAF) in our population was 0.07.

It has already been suggested that C to A substitution at position 16 in exon 1 of CCL22 results in the substitution of 2 aspartate (2Asp) to 2 alanine (2Ala) in the N-terminal of the signal peptide of the CCL22 protein (15). Substitution of Asp by Ala at the N-termial end of signal peptide has been suggested to increases the net positive charge of the n-terminus, which potentially led to a higher level of CCL22 expression (16-17), although the direct effect of this SNP in CCL22 gene on final protein concentration remains to be elucidated.

By affecting the trafficking of two important immune cell types, T helper type 2 (Th2) and Treg cells, CCL22 might negatively affect tumor immunity. The function of Th2 cells suppresses cellular immunity to tumor tissue. Furthermore, Treg cells which are crucial in maintaining immune tolerance in physiological situation, unenthusiastically affect the magnitude of antitumor immune responses. Accumulation of Th2 lymphocytes, as well as, Treg cells at the tumor site has been widely reported. This cell migration has been indicated to be associated with poor prognosis in breast carcinoma and other types of cancer (8-11). Investigations indicated that part of this preferable cell-recruitment is regulated by the CCL22 chemokine which is produced in the tumor microenvironment (6,8,9). CCL22 is not only produced by different immune cell types, including macrophages and monocyte-derived dendritic cells (18), but also is expressed by several types of tumor cells (19-21). A recent study by Faget et al. indicated that human breast carcinoma cell lines produce significant level of CCL22 in response to inflammatory signals (TNF-α, IFN-γ, and interleukin (IL)-1β) (21). Since the CCL22

receptor; CCR4, is selectively expressed by Th2 (6), as well as, Treg cells (5,22), this situation may preferably recruit members of immune-regulatory army, resulting in the repression of immune responses at the in the tumor microenvironment.

In contrast to our study, a study in a population from China indicated a 2.3 times increase in the risk of gastric carcinoma among individuals who inherited Ala/Ala (AA) genotypes at position 16C/A in CCL22 gene in comparison to other genotypes (12). Minor allele frequency (MAF) in this study was 0.16 which indicate considerable higher frequency of the mutant allele in Chinese population than our population (MAF=0.07). Another study in Italian patients with Multiple Sclerosis (MS) was not able to indicate significant association of 16 C/A with the disease, although, in the context of a haplotype with CCL17 promoter polymorphism, AT haploytype (CCL22 A, CCL17 T) observed with significant lower frequency in MS patients than controls (23). Minor allele frequency (MAF) in this Italian sample was 0.04. Janssen et al. was not able to indicate the association of CCL22 polymorphism with respiratory syncytial virus bronchiolitis in a population from The Netherlands (24). The discrepancy between the data may come from the differences in the etiopathology of the investigated diseases, the differences in the study design and sample size, and finally the different genetic background of populations which affects the minor allele frequency (MAF) in each population.

We could not find any association between genotypes at position 16C/A in CCL22 (rs4359426; Asp2Ala) and the clinicpathological characteristics of the cancer including tumor type, tumor size, clinical stage, histological grade, LN involvement, lymphovascular invasion (LVI), distant metastasis and Nottingham prognostic index (NPI). This finding suggests that Asp2Ala change in CCL22 protein has no significant effect on the progression of the breast cancer in the study population.

As a limitation in our study, CCL22 expression on breast cancer tumor cells was not evaluated. Despite no differences in the frequencies of genotype/alleles between healthy controls and cancer patients, the expression of CCL22 may still be different between the two groups. Furthermore, from ethnicity point of view, although all of our participants were from the south area of Iran and most of them disclosed to be “Fars”, it should not be ignored that our population was not completely homogenous and people from different ethnic groups (Lor, Ghashghaiee, etc.) were also included.

Our study, to the best of our knowledge, is the first to investigate the association of CCL22 gene with susceptibility to breast cancer. The results, conclusively, do not support the association of 16C/A SNP (rs4359426; Asp2Ala) in CCL22 gene with susceptibility to, and progression of, breast cancer in Iranian population. Investigation of other CCL22 SNPs and haplotypes may be required to completely rule out the association of CCL22 gene variants with breast cancer.

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