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Assessment of Chemokine Motif Ligand 4 Gene Polymorphism in Rheumatoid Arthritis Patients and its Correlation with Disease Activity

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Abstract

Background **The recent development of biological-based antirheumatic therapies that target inflammatory pathways in RA has enabled increasing numbers of patients to achieve very low levels of disease activity, yet a substantial proportion of RA patients remain treatment-refractory. CCL4 SNP is suspected to be one of these aberrations as it has been related to other diseases yet its role in RA is not well studied.** ***Aim of work:*** **this study aimed to assess the association between the chemokine motif ligand 4 (CCL4) Gene Polymorphism and susceptibility to RA disease and its correlation to the activity of the disease.** ***Subjects and Methods*** **this is a case-control study where CCL4 polymorphisms (SNPs1719153) genotyping and its relation to RA activity were assessed by a real-time PCR in 50 RA patients in comparison with age and sex-matched healthy control.** ***Results:*** **The results of the present study showed a non-statistically significant difference between the patients and controls in the distribution of the genotypes (AA/AT) and alleles (A/T) frequency. And no significant differences between these genotypes in terms of medication use, clinical status, or disease activity.** ***Conclusion:*** **CCL4 gene SNP rs1719153 genotypes (AA and AT) as well as CCL4 SNP rs1719153 alleles (A and T) did not significantly differ between RA patients and normal controls and had no relation to disease activity. To the best of our knowledge, this study is one of few studies to identify the distribution of rs1719153 SNPs in RA in the Egyptian population.**

Keywords: Rheumatoid arthritis, CCL4, polymorphism, SNPs

Introduction

It is well-recognized that susceptibility to RA disease is influenced by genetic and environmental factors. The recent development of biological-based antirheumatic therapies that target inflammatory pathways in RA has enabled increasing numbers of patients to achieve very low levels of disease activity, yet a substantial proportion of RA patients remain treatment-refractory[(1,2)]. As genetic abnormalities account for almost 60% of susceptibility to RA and its development, it is of great importance to study these aberrations to be able to predict and design treatment protocols that suit each patient. CCL4 SNP is suspected to be one of these aberrations

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as it has been related to other diseases yet its role in RA is not well studied[3-6] Histological examination of rheumatoid joints revealed extensive CCL4 expression in sites of lymphocyte infiltration and cell proliferation, leading the study researchers to conclude that CCL4 may substantially mediate the trafficking of reactive molecules involved in RA inflammatory processes. Despite evidence inferring a role for CCL4 in the pathogenesis of RA and the involvement of CCL4 gene SNPs in various human diseases, few studies have investigated the relationship between CCL4 SNPs and risk of developing RA[7,8]. Chemokine (C–C motif) ligand 4 (CCL4), is macrophage inflammatory protein-1 (MIP-1b), which act as chemoattractant that plays a critical role in immune response, and inflammation. CCL4 has been found to be an important regulator for osteoclast migration. The CCL4 level was found to be raised in the early stages of RA in the patient's plasma and synovial fluid. The CCL4 protein acts as the chemokine being secreted under mitogenic signals and antigens and attracts monocytes, dendritic cells, natural killer cells, and other effector cells into the site of inflamed or damaged tissue. In an analysis of cartilage specimens from RA patients and multiorgan donors who served as controls, RT-PCR and flow cytometry revealed higher intracellular CCL4 expression levels among RA patients. Other researchers have also noticed higher CCL4 expression levels in T cells from RA patients[6,9-11]. Single nucleotide polymorphisms (SNPs) denote the single nucleotide variations occurring at specific sites in the genome with appreciable frequency within the population. Genotyping SNPs of a population and comparing the distribution frequency of SNPs among subgroups (e.g., controls and patients) are frequently utilized to examine disease risk and prognosis, including RA[11]. The present study is a case-control study, aimed to investigate whether the single nucleotide polymorphisms (SNPs) of the CCL4 gene can predict the risk of RA disease and whether the studied gene can predict disease activity

Subjects and Methods

This is a case-control study that included two groups: i) patients diagnosed with rheumatoid arthritis (n= 50), recruited from the Rheumatology department, Suez Canal University hospital; and ii) apparently healthy participants (n=50), who served as a normal control group, recruited from Blood Bank, Inclusion criteria of RA group were aged 18-65 years old diagnosed with Rheumatoid arthritis based on according to 2010 ACR-EULAR classification criteria. Patients were excluded from the study if they have a history of any of the following: Autoimmune diseases other than RA (i.e. SLE, Sjögren syndrome, etc.), or Chronic infections (i.e. hepatitis C virus, hepatitis B virus, or tuberculosis). Laboratory investigations were carried out in the Clinical Pathology department. History taking and clinical examination were carried out by a rheumatologist to confirm diagnosis according to 2010 ACR-EULAR classification criteria[12], and assess RA manifestations and disease activity using DAS 28 score [13]. Laboratory Investigations included Complete blood count (CBC), ESR, CRP, Autoantibody tests (ANA, and dsDNA) Anti-CCP by the automated chemical analyzer (COBAS e411 Roche diagnostics, Germany), Rheumatoid factor (RF) by the automated chemical analyzer (COBAS c501 Roche diagnostics, Germany). CCL4 polymorphisms (SNPs1719153) genotyping by real-time PCR. Genomic DNA was extracted from a peripheral whole blood sample using the

commercially QIAamp DNA Blood Mini Kit (Qiagen, Inc., Valencia, CA, USA). DNA purity and concentration were assessed spectrophotometrically using the Thermo Scientific NanoDrop™ Spectrophotometer (Thermo Fisher Scientific Inc. USA). CCL4 gene polymorphism (SNP rs1719153) were genotyped by: Sequence-specific forward and reverse primers to amplify the polymorphic sequence of interest. Real-time PCR was carried out. The PCR process consists of a series of temperature changes that are repeated 40 times. These cycles normally consist of three stages: the first, at 95°C for 10 min, allows the denaturation of the nucleic acids double chain; the second, at a temperature of 60°C for 1 min, allows the annealing of the primers with the DNA template; the third, at 72 °C for 1 min, facilitates the extension and final extension for 7 min at 72°C followed by cooling at 4°C. (TAGGGACTGTTGCACCGAGTTTCAC[A/T]GTTAAGGAAACAGAGGCACAG AGAG), Two TaqMan® MGB probes were used for each SNP: One probe labeled with VIC® dye detected the Allele 1 sequence. One probe labeled with FAM™ dye detected the Allele 2 sequence. In an appropriate tube, the components were combined: (TaqMan Universal Master Mix II, 2X), TaqMan genotyping assay mix (20X) and DNase-free water) Then the tubes were capped and vortexed to mix the solutions. Into each column, the PCR reaction mix volume were pipetted, the columns were sealed with a MicroAmp clear adhesive film, then centrifuged briefly to spin down the contents. 1 to 10 ng of genomic DNA or control DNA were needed for each reaction in the appropriate volume. The columns were sealed using MicroAmp Optical Caps and loaded into Rotor-Gene 6000 Real-Time rotary analyzer. After PCR amplification, end point plate readings were performed on the Rotor-Gene analyzer.

Statistical Analysis

Data were analyzed using SPSS program version 16 software. Data were presented as tables and graphs as appropriate. Quantitative data were expressed as mean and standard deviation, while qualitative data (for quantitative data) and chi square (for qualitative data). Significance was considered at *p* value of < 0.05 was expressed as number and percentage. Comparisons were performed using the T test.

Ethical Consideration

This study was approved by the faculty Ethical committee and informed consent was taken from every patient.

Results

The present study was a case-control study, aimed to investigate whether the single nucleotide polymorphisms (SNPs) of the CCL4 gene can predict the risk of RA disease and whether the studied gene can predict disease activity. The study included two groups, matched regarding age and sex. The majority of the patients group were females and the mean age was 47.5 ± 6.8 years (Table 1). Forty-six% of the patients had a history of other chronic illnesses. More than half of the patients were obese (52%). Moreover, the mean readings of patients' vital signs are within normal ranges. The mean age of onset of RA among patients was 45.3 ± 6.63 years old. The mean disease duration was 2.39 ± 1.21 years with a range from half a year to 7 years. About 64% of the patients were on Hydroquinone of dose 200 mg and 42% of them were on weekly methotrexate of dose 25 mg. All disease activity measures showed either moderate or severe disease activity with no disease remission (Tables 2&3).

Table 1: Demographic characteristics of the studied sample			
	RA group (n=50)	**Controls** (n=50)	***p-value***
Age *(mean ± SD)*	47.5 ± 6.8	46.2 ± 5.2	0.68 [a]
Gender, n (%)			
Male	15 (30)	10 (25)	0.24[b]
Female	35 (70)	40 (75)	

[a] *Mann-Whitney test.* [b] *Chi square test. Statistical significance at P < 0.05; Statistical significance at p < 0.05; RA: Rheumatoid Arthritis*

In this study, CCL4 gene polymorphism (SNP rs1719153) were compared in RA patients versus healthy control. The AT genotype carriers among the patients group were 24%, and 20% of the control group. Patients had AA genotype were 76%, while 80% of the control group had it. Moreover, those who carried the (A) alleles were 44% of the patients group and 45% in the control group. Regarding the (T) allele carriers they were 6% in the patients group and 5% in the control group. However, CCL4 gene SNP rs1719153 genotypes (AA and AT) as well as CCL4 SNP rs1719153 alleles (A and T) did not significantly differ between RA patients and normal controls (Table 4).

Table 2: Clinical Characteristics of RA patients	
Variables	**RA patients (n=50)**
Chronic illness, n (%)	
Absent	27 (54%)
Present	23 (46%)
Vital signs	
Systolic BP (mmHg)	110.2 ± 14.3
Diastolic BP (mmHg)	74.6 ± 10.1
Pulse pressure (mmHg)	33.4 ± 9.1
Heart rate	86.36 ±10.6
BMI (Kg/m²), mean ± SD	**30.87 ± 6.32**
Normal	9 (18%)
Overweight	15 (30%)
Obese	26 (52%)

RA: Rheumatoid Arthritis; BP: Blood Pressure BMI: Body Mass index

Based on disease activity scores, it was found that there was no significant difference between patients with moderate and severe disease activity regarding CCL4 SNP rs1719153 genotype expression.

Table 3: Disease characteristics of the RA patients	
Variables	**RA patients** (n=50)
Age of onset*	45.3 ± 6.63
Disease duration*	2.39 ± 1.21
Hydroquinone dose, n (%)	
200 mg	32 (64%)
400 mg	18 (36%)
Methotrexate dose, n (%)	
< 20 mg	16 (32%)
20 mg	13 (26%)
25 mg	21 (42%)
DAS/ESR*	**5.83 ± 1.05**
Moderate	23 (46%)
Severe	27 (54%)
DAS/ CRP*	**4.54 ± 0.92**
Moderate	29 (58%)
Severe	21 (42%)
CDAI*	**26.43 ± 10.3**
Moderate	25 (50%)
Severe	25 (50%)
HAQI*	1.64 ± 0.41

**Data are presented as* mean ± SD; *RA: Rheumatoid Arthritis; DAS: Disease Activity Score; ESR: Erythrocyte Sedimentation Rate; CRP: C-reactive protein; CDAI: Clinical Disease Activity Index; HAQI: Health assessment questionnaire index*

Likewise, there was no significant difference between patients with moderate and severe disease activity in the expression of allele (A) and allele (T) in SNP rs1719153. (Table 5). Table 6 compares CCL4 SNP rs1719153 genotypes in regard to patients' clinical characteristics. It was found that AA genotype was associated with higher age, chronic illness, and extra articular manifestations. Meanwhile, AT genotype was associated with obese patients. However, these associations are statistically insignificant. Table 7 compares CCL4 SNP rs1719153 genotypes in regard to patients' laboratory and disease activity parameter. It was found that AA genotype carriers had higher RF compared to AT genotype carriers with statistically significant difference. Moreover, AA genotype carrier had higher ESR and CRP compared to AT genotype carrier; however, this was statistically insignificant.

Discussion

RA genetics may help to facilitate risk prediction for individual patients and tailor their treatment accordingly[14]. SNP polymorphisms exist in considerable frequency among population, they can be used to detect risk and prognosis of several diseases including RA [1, 2, 7, 8, 15]This study aims to investigate whether the single nucleotide polymorphisms (SNPs) of the CCL4 gene can increase the risk of RA disease and whether the studied gene can affect disease activity. As CCL4 SNP is suspected to be one of these aberrations as it has been related to other diseases yet its role in RA is not well studied[3-6].

Table 4: CCL4 SNP rs1719153 polymorphism in RA patients *versus* healthy control

CCL4 SNP rs1719153	**RA** (n=50) **n (%)**	**Control** (n=50) **n (%)**	**OR (95 % CI)**	***P-value***
Genotypes (SNP)				
AA	38 (76)	40 (80)	Reference	0.63
AT	12 (24)	10 (20)	1.263 (0.460- 3.465)	
TT	0 (0)	0 (0)		
Alleles (n=200)				
A	88 (44)	90 (45)	Reference	0.65
T	12 (6)	10 (5)	1.227 (0.504 – 2.986)	

Statistical significance at P < 0.05; CI: Confidence interval; CCL4: Chemokine C-C motif Ligand 4; RA: Rheumatoid Arthritis; SNP: Single Nucleotide Polymorphism; OR: Odds Ratio

Table 5: CCL4 SNP rs1719153 polymorphism in RA patients according to activity Clinical Disease Activity Index (CDAI)

Variables	**RA Activity**		**P-value**
	Moderate (n=29)	**Severe** (n=21)	
Genotypes (SNP)			
AA	24 (82.8)	14 (66.7)	0.31 [a]
AT	5 (17.2)	7 (33.3)	
TT	0 (0)	0 (0)	
Alleles (n=100)	(n=46)	(n=54)	
A	43 (89.6)	39 (84.8)	0.55 [a]
T	5 (10.4)	7 (15.2)	

[a]*Chi square test; Statistical significance at P < 0.05; RA: Rheumatoid Arthritis; CCL4: Chemokine C-C motif Ligand 4, SNP: Single Nucleotide Polymorphism*

The results of the present study showed that there was non statistically significant difference between the patients and controls in the distribution of the genotypes (AA/AT) and alleles (A/T) frequency. Also TT genotype was not detected in both groups according to the open access ensembl.org, human (GRch38p.13, release 103) the rs1719153, has a minor allele frequency of 4% in the African population as represented in the 1000 genomes phase 3 samples in the country, the south Asian has a MAF of 59%, therefore it's hard to detect the mutant type in our ethnic group but it can be easily detected in the Asian population as the MAF represented more than half. Hu et al 2020 reported that in individuals carrying the T-containing genotype of the CCL4 rs1719153 polymorphism were not susceptible to RA in Asian population[16]. Kuo et al (2018) recruited between 2007 and 2015, 217 RA patients and 371 control participants. They found that those with the A/T genotype were less likely to develop RA. In addition, the GTEx database suggested that the T variant in the rs1719153 is associated with a trend towards a lower level of CCL4 expression as compared with the wild type A nucleotide. Both RF and anti-CCP are specific markers for RA. CRP is usually ordered along with ESR; although they are not specific tests, the flaring up of their values indicates the degree of inflammation in the cases [17]. Accordingly, this study assessed the CCL4 SNP rs1719153 genotypes and alleles in regard to patients' laboratory parameters and found that AA genotype carriers had higher RF compared to AT genotype carriers with statistically significant difference. When it was compared between CCL4 SNP rs1719153 alleles regarding patients' clinical characteristics.

Table 6: Association of CCL4 SNP rs1719153 genotype with disease characteristics of RA patients (n=50)

Variables	CCL4 SNP genotypes		P-value
	AT (n=12)	**AA** (n=38)	
Age of onset, mean ± SD	42.75 ± 6.96	36.14 ± 6.41	0.106 [a]
Disease duration, mean ± SD	2.08 ± 0.51	2.49 ± 1.35	0.259 [a]
Hydroquinone dose, n (%)			
200 mg	7 (58.3)	25 (65.8)	0.74 [b]
400 mg	5 (41.7)	13 (34.2)	
Methotrexate dose, n (%)			
< 20 mg	3 (25)	13 (37.1)	0.75 [b]
20 mg	3 (25)	7 (20)	
25 mg	6 (50)	15 (42.9)	
CDAI			
Moderate	5 (41.7)	19 (50)	0.45 [b]
Severe	7 (58.3)	19 (50)	
HAQI mean ± SD	1.8 ± 0.32	1.59 ± 0.43	0.102 [a]

[a] p-values are based on Mann Whitney U Test. Statistical significance at P < 0.05; [b] p-values are based on Fisher Exact Test. Statistical significance at P < 0.05; [c] p-values are based on Chi-Square Test. Statistical significance at P < 0.05; CCL4: Chemokine C-C motif Ligand 4; SNP: Single Nucleotide Polymorphism; RA: Rheumatoid Arthritis; CDAI: Clinical Disease Activity Index; HAQI: Health assessment questionnaire index

Table 7: Association of CCL4 SNP rs1719153 genotype with laboratory measures of RA patients			
Variables	**CCL4 SNP genotypes**		**P-value**
	AT (n=12)	**AA** (n=38)	
Anti-CCP U/ml	123.92 ± 135.92	179.05 ± 162.5	0.122 [a]
RF IU/ml	55.33 ± 29.03	79.13 ± 45.99	**0.045** [a]
ESR mm/hr	57.67 ± 24.66	61.16 ± 26.08	0.624 [a]
CRP mg/l	3.4 ± 2.5	3.43 ± 1.73	0.502 [a]
Hemoglobin (g/dl)	9.56 ± 0.96	10.09 ± 1.43	0.169 [a]
TLC ($x10^3/mm^3$)	6.23 ± 1.46	8.09 ± 8.84	0.413 [a]
Platelets ($\times10^3$)	407.17 ± 155.08	372.18 ± 139.61	0.474 [a]

[a]Mann Whitney U Test; [b]Chi-Square Test. Statistical significance at P < 0.05; CCL4: Chemokine C-C motif Ligand 4; SNP: Single Nucleotide Polymorphism; RA: Rheumatoid Arthritis; Anti CCP: Anti Cyclic Citrullinated Peptide; RF: Rheumatoid Factor; ESR: Erythrocyte Sedimentation Rate CRP: C-reactive protein; TLC: Total leucocytic count

It was found that (A) allele was associated with higher age of onset and disease duration, while (T) allele was associated with higher hydroquinone and methotrexate dose as well as higher Clinical Disease Activity Index (CDAI). However, with nonstatistically significant difference. Kuo et al (2018) made a similar analysis of SNP rs1719153 to evaluate the impact of the genotype on medication, RF clinical status, and serum inflammatory markers. Their results agreed with this study as they found no significant differences between these genotypes in terms of medication use, clinical status, or serum inflammatory markers. Limitation of our study is the small sample size, additional studies are needed in the future to validate these results by using a larger cohort of RA patients. This study assessed the genetic part only without comparing it with the phenotype, it would be valuable to work on both in future study. Up to our knowledge this study is the first in Egypt to assess the association between the CCL4 SNP rs1719153 and disease susceptibility and clinical activity in RA patients.

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