

Association study of CCL20 rs6749704 with Rheumatoid Arthritis in Iraqi population

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Abstract

Rheumatoid arthritis (RA) is a chronic auto immune and inflammatory disease of obscure etiology. However, factors of genetic, immunological and environmental have a pivotal role in disease development. This study was investigated the polymorphisms of SNP (rs6749704) of CCL20 gene and the serum level of the chemokine CCL20 its association with susceptibility to RA. Genotypes for the gene was determined using HRM (High Resolution Melting) genotyping assay by RT-PCR. The chemokine serum level was determine using ELISA analysis. Accordingly, a case-control analysis was carried out on 60 patients with RA and 30 control. The results are given in the following: The mean of CCL20 level was increased highly significant in the serum of RA patients than that of controls (300.42 ± 11.11 vs 108.55 ± 6.09 pg/ml, P value=0.0001**). The analysis of Logistic regression for CCL20 gene (rs6749704) revealed that CC genotype was significantly more frequent in RA patients [P = 0.000, OR=22.52, CI 95% (4.76–205.87)]. Also, C allele was significantly more frequent in RA patients than in the control group [70.83% versus 16.67%, P = 0.000, OR=12.14, CI 95% (5.57–27.36)], so the CC genotype and C allele and higher serum level of the chemokine CCL20 was associated with RA disease in Iraqi patients.

Keywords: HRM (High Resolution Melting), Rheumatoid arthritis (RA), ELISA analysis.

1. INTRODUCTION

Rheumatoid arthritis (RA) is a chronic, long-lasting inflammatory disease that mostly affects the joints and causes ongoing synovial inflammation that eventually destroys joints and causes deformity and disability (Suzuki et al., 2013; Sandoughi et al., 2017). RA was 2- to 3-fold more frequent in females than in males (Lin et al, 2017; Kato et al, 2017). Although the disease's etiologies and causes are not fully understood, it is likely that the interconnection of hereditary and environmental variables plays a vital role in RA pathogenesis (Ishikawa and Terao, 2020). A large number of genetic risk loci that are connected to RA have been discovered through extensive whole genome sequencing employing different cohorts throughout the world (Messemaker et al, 2015). RA pathogenesis is also influenced by genetic variants, most commonly reflected by single nucleotide polymorphisms (SNPs) (Stahl et al, 2010; Ishikawa and Terao, 2020). The chemokine CCL20, also known as Macrophage inflammatory protein 3 α (MIP-3 α) or liver activation regulated chemokine (LARC), is an 8-kDa protein whose gene is located on 2q33-37 (Lee and Körner, 2019). CCL20 gene contains four exons and three introns (Chen et al, 2020). The human CCL20 gene has an open reading frame of 291 bp, 4 exons, and 5 untranslated regions (UTRs) spaced by introns (Choi et al, 2005). The receptor of CCL20 known as CCR6 and accordingly this chemokine acts as a chemoattractant for CCR6-expressing cells, such as Th17 cells (Lubberts, 2015). CCL20 manifests physically in the skin, intestines, and liver. The CCL20-CCR6 axis has long been associated with inflammatory and infectious disorders, including rheumatoid arthritis and human immunodeficiency virus infections (Kadomoto et al, 2020). It has been suggested that CCL20 is an emerging player in the etiology of rheumatoid arthritis (Pathak et al, 2015). Endothelial cells, neutrophils, natural killer (NK) cells, TH17 cells, B cells, and a number of other immune cells (dendritic cells, DCs, Langerhan's cells (LCs), and macrophages) are among the inflammation-related cells that have been demonstrated to release or express CCL20 (Lee et al, 2013). Inflammatory bowel illnesses have been linked to polymorphisms in the CCL20 promoter area, such as the rs13034664 and rs6749704 SNPs (Choi et al, 2005). Early detection and effective treatment may reduce the risk of joint damage and improve long-term results (Moeze et al, 2013).

2. Methods

2.1. Subjects

In this study, the blood samples were collected from 60 patients who admitted the department of chronic arthritis diseases Baghdad teaching hospital / medical city / Baghdad / Iraq and 30 healthy control from Iraqi population that were selected randomly with no family history of RA or other autoimmune disease. A physical examination and medical history were checked in all patients. The excluded patient with hepatic disease, malignant illness, cardiac failure, renal failure, and psoriasis. Nearly five milliliters of blood were collected from each patient and separated into two parts. The first blood part was collected in a tubes containing EDTA (2 ml) to use for genetic analysis. While the second part collected in a gel tube to use in ELISA technique and for other laboratory analysis. An informed consent form was signed from each subject included in the study, and the study was approved by the Ethical committee of Department of life science, Iraq.

2.2 Laboratory Measurement

The method of Westergren was used to assess ESR. Latex agglutination test was employed to assess RFs (RF Latex Test Kit) and CRP (CRP Latex Test Kit) in serum of patients and controls.

2.3. DNA extraction and genotyping

The DNA extraction from the blood of patients and control subjects by Easy Pure Blood Genomic DNA kit. Then agarose gel electrophoresis was adopted to confirm the presence and integrity of the extracted DNA (Russell and Sambrook, 2001). In order to detect the genetic variation in the two studied groups and its different genotypes in CCL20 gene for SNP (rs6749704 T>C), HRM real-time polymerase chain reaction (HRM RT-PCR) were used. Primers used in this study were designed according to their reference sequence in the National Center for Biotechnology Information's database (NCBI), the primers that were used forward TCAACAATTCTGAGGCTCTATATTGA and reverse TCCTTACCTACCTTTAACTCTCCA, the two primers were used in Real Time PCR reaction mixture. The thermal profile of HRM genotyping: Enzyme activation at 94°C for 60 sec which was followed by 40 cycle of, Denaturation at 94°C for 5 sec, Annealing at 62 °C for 15 sec, Extension at 72 °C for 20 sec, HRM at temperature between 65-95 °C for 0.2 sec for 1 degree.

2.3 Measurement the serum level of the chemokine

The level of CCL20 and Anti-CCP in serum of RA patient and control was measured by using commercial ELISA kit from My Biosource company (USA) according to the manufacturer's procedure.

2.2. Statistical analysis

To identify the impact of various factors on study parameters, the Statistical Analysis System- SAS (2018) program was utilized. Chi-square test was performed to compare percentage (0.05 and 0.01 likelihood) in a significant way. Estimated correlation coefficient between the study's variables.

Both the odds ratio, which was evaluated using a unique 2 formula by Abramson in 2011 (Rodriguez et al. in 2009), and the P values computed with Fisher's exact test were statistically evaluated using the WINPEPI computer program (version 11.63). The chi squared test was used to examine the Hardy-Weinberg equilibrium utilizing studies from the OEGE (Online Encyclopedia for Genetic Epidemiology) database (Barret et al,2005).

3.Result

3.1. Clinical and demographic characteristics of RA patients and healthy controls.

The clinical and demographic characteristics of RA patients and healthy controls are listed in (Table1). Mean age of RA patients was highly significant increased compared with controls (46.23 ± 1.46 vs. 38.04 ± 2.07 year; $p = 0.001^{**}$). Females were more commonly occurring than males in RA patients (83.33 vs 16.67%) and control (74.07 vs 25.93%). The ESR mean in patient was highly significant increased than healthy control (41.73 ± 3.19 versus 9.2 ± 0.65 mm/hour, $P = 0.0001^{**}$). All healthy control individuals were seronegative for RF, while 70 % of RA patients were seropositive and 30% of patient were seronegative. All healthy control individuals were seronegative for CRP, while 48 % of RA patients were seropositive and 52% of patient were seronegative. All healthy control individual were seronegative for Anti-CCP antibody, while 48% of RA patients were seropositive and 52% of RA patient were seronegative.

Table 1 Clinical and demographic characteristics of RA patients and healthy controls

variable	patient	control	P value
Age in years Mean \pm SE	46.23 ± 1.46	37.433 ± 1.92	0.001**

Gender			
Male	16.67%	25.93%	0.383
Female	83.33%	74.07	
ESR			
Mean \pm SE	41.733 \pm 3.19	9.2 \pm 0.65	0.0001**
RF	70% seropositive 30% seronegative	0% seropositive 100% seronegative	_____
CRP	48% seropositive 52% seronegative	0% seropositive 100% seronegative	_____
Anti-CCP	48% seropositive 52% seronegative	0% seropositive 100% seronegative	_____

3.2 The serum level of CCL20 in Rheumatoid Arthritis patient.

Mean for CCL20 level in the serum of RA patients significantly higher than that of controls (300.42 \pm 11.11 vs 108.55 \pm 6.09 ng/ml ,P value=0.0001**) (Figure1).

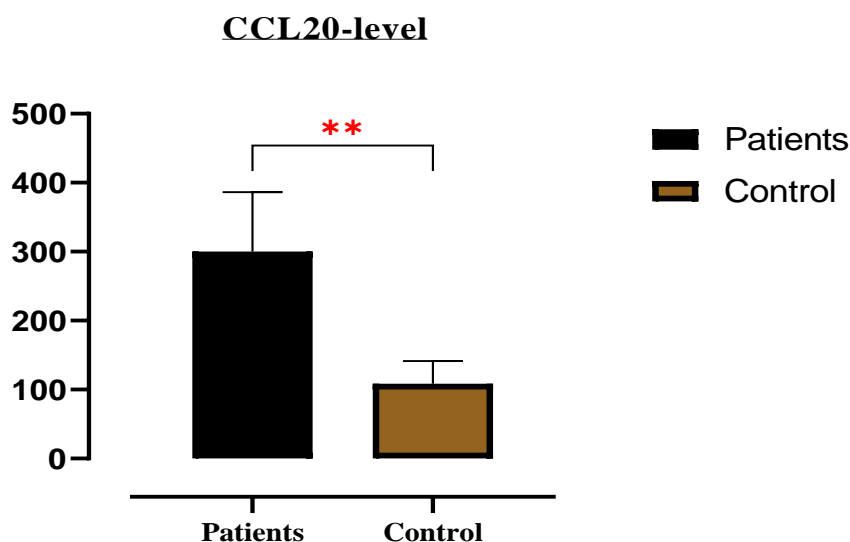


Figure (1) Serum Level of CCL20 in Rheumatoid Arthritis Patients and Control.

3.3. Association of CCL20 rs6749704 with Rheumatoid Arthritis.

The HRM analysis identified three genotypes: TT, TC and CC, which were correspondent to two alleles; T and C(Figure 2). Frequencies of CCL20 genotypes and alleles in patients and controls, including ORs and 95% CIs were reported in (Table 2). Revealing the (rs6749704) of CCL20 gene, showed that the wild genotype TT was present in 20% of RA patients versus 73.33% of controls [p=0.000 ,OR=0.09,CI 95%(0.03—0.26)] while the mutant homozygous genotype CC was found in 61.67% of RA patients versus 6.67% of control [p=0.000 ,OR=22.52 ,CI 95%(4.76—205.87)] and the heterozygous genotype TC had frequency 18.33% of RA patients versus 20% of controls [P=0.891 ,OR=0.90,CI 95%(0.30—2.91)]. The frequency of T allele in RA patients 29.17% versus 83.33% in control [p=0.000 , OR=0.08 ,CI(0.04--- 0.18)] while the frequency of C allele in RA patient 70.83% versus 16.67% in control [P=0.000 ,OR=12.14 ,CI 95% (5.57.—27.36)] the results showed a significant variation of both two alleles between RA patient and control. CC genotype was significantly more prevalence in RA patients [P = 0.000, OR=22.5 ,CI 95% (4.76—205.87)]. Additionally, the C allele was significantly more frequent in RA patients than in the healthy control group [70.83% vs 16.67%, P = 0.000, OR=12.14,CI 95% (5.57—27.36)]. So the individuals who have homozygous mutant CC genotype more likely to get the disease than others (22.52 fold), also individuals who have C allele appeared a significant predictor confirming a high risk (12.14 fold) for RA susceptibility (Table 2).The TT genotype was significantly more prevalence in healthy individuals (73.33% versus 20%).Also, T allele was significantly more prevalence in healthy control (83.33% versus 29.17%).So the TT genotype and T allele appeared a protective effect against the disease .TC genotype shows non significant difference between patient and healthy control (p=0.891).

Analysis of Hardy-Weinberg equilibrium (HWE) in control group and RA group revealed that the genotypes of healthy group was consistent with the equilibrium because a non significant variation between observed and expected genotype frequencies of the SNP rs6749704 ($p = 0.125$) was noticed in healthy control group while a significant differences was noticed between observed and expected genotype ($p < 0.05$) in RA group (Table 3), this revealed that RA group were inconsistent with equilibrium.

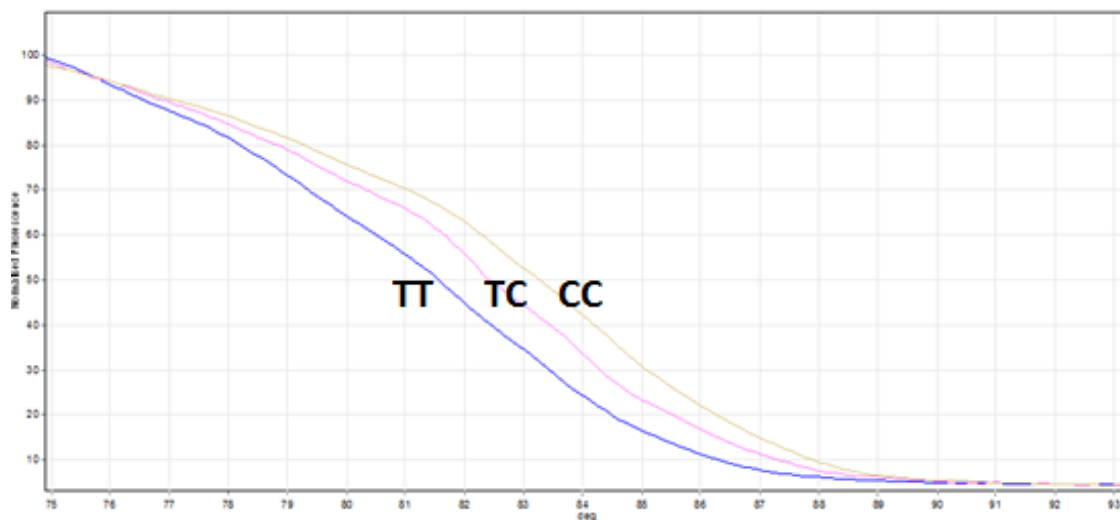


Figure (2) The result output of HRM for three genotypes in CCL20 SNP (rs6749704)

Table (2) The genotype and allele frequencies of CCL20 gene of Control group and RA group.

Genotype/ Allele	RA group N=60 N (%)	C group N=30 N (%)	P value	OR ratio	CI 95%
TT	12 (20%)	22 (73.33%)	0.000	0.09	(0.03-0.26)
TC	11 (18.33%)	6 (20%)	0.891	0.9	(0.30-2.91)
CC	37 (61.67%)	2 (6.67%)	0.000	22.52	(4.76-205.87)
T	35 (29.17%)	50 (83.33%)	0.000	0.08	(0.04-0.18)
C	85 (70.83%)	10 (16.67%)	0.000	12.14	(5.57-27.36)

Table (3) Numbers and Percentage Frequencies (observed and expected) of CCL20 gene (rs6749704) Genotypes and their Hardy-Weinberg Equilibrium (HWE) in RA Patients and Controls.

Genotype	Rheumatoid arthritis patients (No=60)				Control No=30			
	Observed		expected		observed		expected	
	No	%	No	%	No	%	No	%
TT	12	20%	5.1	8.51%	22	73.33%	20.83	69.44%
TC	11	18.33%	24.79	41.32%	6	20%	8.33	27.78%
CC	37	61.67%	30.1	50.17%	2	6.67%	0.83	2.78%
HWE Analysis	P=0.000				P=0.125			

4. Discussion

Our result revealed that the CCL20 level was highly significant in RA patient than healthy control. CCL20 affects immune cells in a variety of ways, and its dysregulation contributes to autoimmune and inflammatory conditions such psoriasis, inflammatory bowel disease, systemic lupus erythematosus, and rheumatoid arthritis (Schutyser et al, 2003; Koga et al, 2016). In an inflammatory response, CCL20 can also be found upregulated at a number of anatomical locations including the skin and intestine and has been demonstrated to be implicated in the cellular pathogenesis of diseases such as psoriasis and ulcerative colitis at these sites (Harper et al, 2009). Levels of CCL20 and its CCR6 receptor are elevated in many autoimmune diseases, which aids in attracting T helper (Th17) cells to the site of inflammation (El Sharkawi et al, 2019). In agreement with the current study, Pournazari and co-workers (2022) research showed that the CCL20 levels in serum were increased significantly in RA

patients compared to the healthy controls ($p < 0.0001$), Li and co-workers (2017) showed that the level of CCL20 in serum was increased significantly in MS patients compared with controls, Ikawa and co-workers (2021) research on systemic sclerosis patients also revealed that the CCL20 level in patient was significantly higher than healthy controls. The use of biological anti-TNF agents and antirheumatoid medications in the treatment of rheumatoid arthritis-like conditions has recently advanced, however there are certain disadvantages, such as treatment resistance and immune suppression. Therefore, researchers are focusing on certain other important elements, such as chemokines and chemokine receptors, which are involved in immune cell migration to the inflammatory milieu. Therefore, the two most significant chemokine receptors involved in immune cell migration are CCL20 and CCR6, respectively (Meitei et al, 2021).

In this study the association of CCL20 rs6749704 with RA was studied, revealing that the genotype CC and the allele C was associated with RA disease in Iraqi population.

A study made by El Sharkawi et al (2019) on the rs6749704 SNP T>C in CCL20 gene on Egyptian patient with MS autoimmune disease revealed that the TC genotype was significantly more prevalence in Multiple sclerosis patients [$P = 0.01$, OR =2.6 (95% CI 1.4–4.8)]. Also, C allele was significantly more frequent in MS patients than in the control group [27.7% versus 15.8%, $P = 0.006$, OR=2.0 95% CI (1.2–3.4)] which was agreed with the current study under allelic model and disagreed under genotyping model.

A study by Jafar Zadah and co-workers (2014) on CCL20 rs6749704 in MS patients showed that the genetic variants at rs6749704 did not significantly differ between the MS and healthy groups. In contrast to other MS patterns, SPMS patients had a much lower frequency of the TC genotype. Additionally, the frequency of TC genotype in patients with SPMS was lower than in the control group, with a borderline significance. These data indicate that rs6749704 may have a relationship with some patterns of MS disease.

The Study of -786T>C (rs6749704) SNP by Choi and co-workers (2005) on the Ulcerative colitis patients in Korean population showed that there was non significant difference between patient and control which was disagree with the current study.

5. Conclusions

In conclusion, it was the first association study of rs6749704 of CCL20 gene with Rheumatoid Arthritis in Iraqi population. A significant association of this SNP was found with RA. The analysis revealed that the allele C was a risk allele and the genotype CC was risk genotype. Also the serum level of CCL20 was highly significant increase in Rheumatoid Arthritis patients in comparison with control. Based on results and conclusions of the present study. More Studies are needed to assure the role of CCL20 in Rheumatoid Arthritis in Iraqi population. However, the CCL20 SNPs genotyping is an important aspect of research; a re-evaluation is required but on the basis of a molecular determination (DNA-sequencing). This will definitely help in giving better results.

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