A NOVEL FINDING OF IL-9 GENETIC POLYMORPHISM (RS17317275) AND SERUM LEVEL IN RHEUMATOID ARTHRITIS PATIENTS INFECTED WITH CMV

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Abstract

RA is a complex chronic disease, primarily affects the lining of the synovial joints and can cause progressive disability, premature death, and socioeconomic burdens. The clinical manifestations of symmetrical joint involvement include arthralgia, swelling, redness, and even limiting the range of motion. The pathogenesis is not known therefore the study included Rheumatoid arthritis (RA) is one of systemic inflammatory diseases that characterized by a progressive disabling course. The study included patients suffering from RA and as healthy controls. immunological and genetic factor were evaluated in each subject by using serum level of cytokine and genetic factor. The etiopathology of the disease is not well known for this reason the study was done.

Keyword: cytokine, rheumatoid arthritis, single nucleotide polymorphism.

Introduction

Rheumatoid arthritis (RA) is a chronic, symmetrical, inflammatory autoimmune disease that impacts the small joints initially, then progressing to the larger joints, and eventually demonstrate on the skin, eyes, lungs, heart and kidneys. Oftentimes, the cartilage and bone of joints are destroyed, while the tendons and ligaments weakened (Lee *et al.,* 2017). The aetiology and pathogenesis of several autoimmune diseases remain unknown. It appears that the main relationship between environmental triggers and the genetic factors is in charge of losing immunological tolerance (Miller *et al.*, 2012). In addition to these effectors, degradation of cartilage in RA happens when some cytokines such as tumor necrosis factor-alfa (TNF-α) (Golbargi *et al.*, 2019), interleukin-1 (IL-1) and interleukine-6 (IL-6) activate synoviocytes, leading to secretes the matrix metalloproteinases (MMPs), cathepsins and the proteinases into the synovial fluid (Snijesh *et al.*, 2018). Also, cytokines activate the chondrocytes, resulting in the direct release of additional MMPs inside the cartilage (Snijesh *et al.*, 2018; Strand and Kavanaugh, 2004).

In addition to the interaction between these effectors and some personal lifestyle factors that influenced the development of RA, it included age, gender, socioeconomic factor, hormonal factors and ethnicity (Salloom and Hatem, 2019; Tobón *et al.*, 2010). Some studies referred that Half risk for RA is believed to be genetically, and it is strongly related with the inherited tissue type Human Leukocyte Antigen (HLA)such as HLA-DRB1 including *0401 and *0404, and PTPN22, PADI4 genes (Goeldner *et al.*, 2010; Chou *et al.*, 2007).

Interleukin-9 (IL-9) is a member of a gamma-chain family of cytokines located at chromosome 5 5q31–35 which associated with RA, described for the first time as a member of the cytokines that have pivotal roles in the development, proliferation, survival and differentiation of multiple cell lineages of both innate and adaptive immunity that identified originally as a T cell growth factor belonging to the common γ-chain receptor cytokine family (Kaplan *et al.*, 2015; Rochman *et al.*, 2009). IL-9 signals via a heterodimeric receptor composed through a specific IL-9 receptor chain (IL-9Rα) and the common γ-chain that is also known as IL-2Rγ) (De Smedt *et al.*, 2000). In contrast, Cytomegalovirus (CMV) considered as one of the environmental factors that have a critical role in the etiopathogenesis and triggering of RA. It is related to opportunistic infections in immunocompromised patients with autoimmune rheumatic diseases (AIRD) (Azuma *et al.* 2009).

These pieces of information encouraged us to develop our knowledge about the relation between IL-9 genotyping and serum level in RA patients and the risk of CMV infection. The present study investigated the IL-9 at the serological level and molecular genotyping of IL-9 rs17317275 SNP by polymerase chain reaction (PCR). The obtained data were correlated with some demographic and clinical manifestation. Further, anti-CMV IgG antibody was also determined among the studied groups.

Materials and Methods

Samples investigated

A total sample of 156 Iraqi volunteers (76 rheumatoid arthritis patients: 20 males and 56 females who were already diagnosed by the physicians compared to 80 apparently healthy control individuals: 28 males and 52 females healthy volunteers) were enrolled in a case-control investigation during the period from March to June 2019 after obtaining the approval of Ethical Committee at the University of Baghdad, College of Sciences, Biology Department and the Iraqi Ministry of Health. The written informed consent was possessed by all volunteers. The study was accomplished in accordance with the Ethics Code of the World Medical Association (Declaration of Helsinki) (World Health Organization, 2013).

Serum anti-CMV IgG and anti-CCP antibodies status, and IL-9 level

Sera of RA patients and control groups were qualitatively screened for anti-CMV IgG antibody by using the commercial ELISA kit (Human Company, Germany). The sera were also quantitatively assessed for anti-cyclic citrullinated protein (anti-CCP) antibody by using the commercial ELISA kits (Demeditec Diagnostics, Germany). In addition, IL-9 serum level was evaluated in each group by using the commercial ELISA kit (MyBioSource Inc., USA).

The instructions recommended by the manufacturers were followed.

IL-9 single nucleotide polymorphism

The DNA was extracted from whole blood of all participants through employing the standard procedure recommended by the manufacturer (TONK BIOscience, USA). IL-9 genotyping of rs17317275 SNP (resulted from changing *A* allele to *G* allele at the forward DNA strand) was subsequently performed using allele-specific polymerase chain reaction (AS-PCR) described by Ye *et al.* (2012). This PCR system includes using two forward primers have the same nucleotides sequence instead of a targeted single nucleotide at the first position of the primer, and a common reverse primer. The applied primers (Integrated DNA Technologies, Inc., USA) were designated using the NCBI Primer-Blast website. The first forward primer sequence (sense primer) was: 5'-AAACTTCAGCTCTGTCCCTG-3', while the second forward primer sequence (sense primer) was: 5'-GAACTTCAGCTCTGTCCCTG-3', the common reverse primer sequence (antisense primer) was: 5'-GCCGGTTGTGGTGTCAAT-3'. The PCR reaction mixture (25 µL) consisted of two Eppendorf tubes, each Eppendorf tube involved 1 µL of one of the forward primers (10 pmol/ml) and 1 µL of common reverse primer (10 pmol/ml), 12.5 µL of Taq PCR Master Mix (Promega, USA), 1.5 µL of DNA, and 9 µL of free-nuclease water (Promega, USA). The PCR cycling was performed using two steps, the first initial denaturation step at 94 °C for 5 minutes, followed by 35 cycles included: second initial denaturation at 94 °C for 30 seconds, annealing at 54 °C for 30 seconds and elongation at 72 °C for 30 seconds. Single-cycle of final elongation at 72 °C was followed for 10 minutes. the PCR products were electrophoresed on 1.5% agarose gel at 100 V/cm2 for 30 minutes stained with Red Safe stain (Intron Biotechnology Inc., Korea). IL-9 genotyping was determined by electrophoresed the two PCR product for the same sample in neighboring wells. If an only single band presented in one of the two neighboring wells, referred to the homozygous genotype (AA or GG genotype). while, when the bands presented in the two neighboring wells, it referred to the heterozygous genotype (AG genotype). the PCR product size was determined using a Universal DNA Ladder (KAPPA TM, Korea).

Statistical analysis

The data of IL-9 serum level and anti-CCP antibody were analyzed for linearity, homogeneity and normal distribution using IBM SPSS statistical package version 25.0 (IBM Corp. Released 2017). The mean, standard error and the probability were calculated to determine the statistically significant differences. While in non-parametric data (anti-CMV IgG antibody status and IL-9 genotyping), Pearson's chi-square test and Fisher's exact probability was used to calculate the statistically significant differences. Also, the odd ratio and Fishers' exact probability of the genotyping and alleles frequencies were calculated by WinPepi program version 11.65 (Abramson, 2011). In addition, Hardy-Weinberg online calculator was used to calculate the probability of genotyping and alleles frequencies. A corrected probability value (*p*-value) using False discovery rate (FDR) used to correct the *p*-value, the corrected *p*-value < 0.05 was considered significant.

Results and Discussion

A total of 156 contributors were participated in the present study included 76 rheumatoid arthritis patients and 80 individuals considered as healthy controls. RA patients group included 20 RA males and 56 RA females' patients who were already diagnosed by the physicians. In contrast, control group included 28 males and 52 females' volunteers. The present results showed a non-significant difference between RA group age mean and controls (Table 1). Also, the current study included RA patients with age range 23 – 68 years, the highest age group was at the 55 – 61 years age group (28.95%), followed by 62 – 68 years age group (27.63%). While the lowest age group was at 23 – 30 years (7.89%) (Figure 1).

Table 1: Demographic aspects data of RA patients and control groups

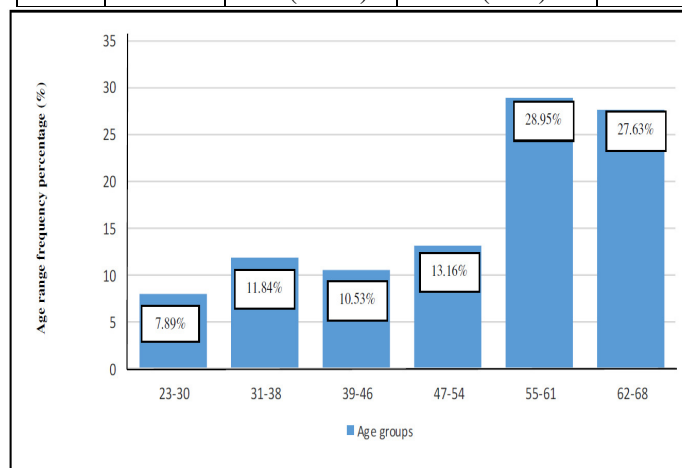| Demographic Age (mean ± SE years)		RA group (n= 76)	Control group (n= 80)	*P*-value
Gender	Male	20 (26.32)	28 (35.0)	0.298
	Female	56 (73.68)	52 (65.0)	

Fig. 1: Age range frequency at RA patients' group

These findings were in agreement with another previous study reported that RA can be detected at all age groups, but often between 40 and 60 years of age (Nodongo *et al.*, 2014). There is wide variability in RA presentation forms. The onset age of this disease appears to be a pivotal factor in its clinical spectrum (Soubrier *et al.*, 2010). It is considered that the patients suffering from elderly onset rheumatoid arthritis (EORA) when the disease initiated at the age of ≥60 years. This form of RA constitutes a ratio of 10 - 33% of RA cases (Tutuncu *et al.*, 2006, Olivieri *et al.*, 2005).

Also, the results of gender at patients group referred that the frequency percentage of RA was higher in females than males (73.68 vs. 26.32%, respectively) (Table 1). In addition, the female: male ratio in the present study was 2.9: 1. In agreement with such findings, previous researchers reported that the frequency percentage of RA was higher in females than males (Sokka *et al.*, 2009; Pandey *et al.*, 2017). Such, Myasoedova *et al.* (2010) were reported that RA affects females more often than males with ratio (3:1). This ratio was higher for 4 to 5 times in females with age less than 50 years old, but the ratio differed when the age was more than 60 years old, it became approximately 2: 1 (female: male) (Kvien *et al.*, 2006). In Latin America, the ratio prevalence of this disease was 5.2: 1 (female: male) in

Colombia and Argentina (Barragán-Martínez *et al.*, 2012; Spindler *et al.*, 2002), and 5.5: 1 (female: male) in Cuba (Lesuis *et al.*,2012).

The results in Table 2 showed the detected frequency percentage of anti-CMV IgG antibody between the studied groups. It showed that the anti- CMV IgG antibodies

seropositive frequency percentage was 65.7% in RA patients' group, while the seropositive frequency percentage in healthy control group was 2.5% with significant differences ($P <$ 0.001) between the compared groups. In addition, the results showed a high OR ratio (75.0).

Table 2 : Anti-CMV IgG antibody frequency percentage in the studied groups

Patients group					Control group					OR	*P*-value
Positive		Negative		Total	Positive		Negative		Total		
No.	%	No.	%		No.	%	No.	%			
50	65.7	26	34.2	76	2	2.5	78	97.5	80	75.0	1.7×10^{-9}

CMV has a critical role in the etiopathogenesis of RA, there were several mechanisms inducing the immune cells as a result to damaging the cells and tissues, leading to pro-inflammatory cytokines production and the mimicry to some self-antigens in the human body. The immunization with a small cytomegalovirus-specific peptide leads to multiple autoreactive antibodies, possibly through the molecular

mimicry and the epitope spreading, especially in the genetically predisposed persons (Aiello *et al.*, 2017).

The results in Table (3) showed the anti-CCP antibody serum level in RA patients' group compared to controls. There was a significant increased level of anti-CCP antibody in RA group compared to control group (341.6 ± 26.6 *vs.* 103.0 ± 11.0 U/ml, respectively) as in the table (3).

Table 3 : Anti-CCP antibody level in the studied groups

Groups	Patients group		Control group		*P-value*
	Mean	SE	Mean	SE	
Anti-CCP Ab Level (U/ml)	341.6	26.6	103.0	11.0	5.1×10^{-13}

The appeared results in compatible with other previous studies referred to significantly increased level (P<0.05) of anti-CCP antibody in RA patients than control group (Hanan *et al.,* 2011; Iman *et al.*, 2009). Because that anti-CCP antibody concentration one predict factor for inflammation such as RA. The anti-CCP antibodies appear in response to an epitope modified by a hormonally controlled enzyme, peptidylarginine deiminase, which converts arginine to citrulline (van Venrooij and Pruijn, 2000). Within rheumatoid joints arginine residues on fibrin and fibrinogen may be favored sites for citrullination process (Kinloch *et al.,* 2008). Also, the RA associated HLA-DRB1*0404 allele is associated with production of antibodies to citrullinated fibrinogen and the proliferation of T cell in response to

fibrinogen peptides is frequent in RA patients but rare in controls (Auger *et al.,* 2005). All these findings explain the high value of anti-CCP antibodies detected in RA patients compared with control. Anti-CCP is highly important for earlier and more accurate diagnosis of disease, improved prognostic information, and have been a role in RA pathogenesis, since these anti-CCP antibodies activate the complement system in vitro via the classical and alternative pathways and this a strong indication of their pathophysiologic involvement (Trouw *et al.,* 2009).

In addition to what was mentioned previously, the results of IL-9 serum level showed a significant increased level of IL-9 in RA group compared to control group (29.2 ± 3.7 *vs.* 10.4 ± 1.3pg/ml respectively) (Table 4).

Table 4: IL-9 serum level in the studied groups

Groups	Patients group		Control group		Probability
IL-9 level (pg/ml)	Mean	SE	Mean	SE	
	29.2	3.7	10.4	1.3	6.0×10^{-6}

Other previous studies showed that results indicate that the IL-9 serum levels were significantly higher in RA patients and in SLE patients than in healthy individuals (p < 0.001) (Goswami and Kaplan, 2011; Barra *et al.*, 2014; Dantas *et al.*, 2015). Furthermore, IL-9, and IL-6 with one other, considered the most responsible in the calculated cytokine score by summing all cytokines/chemokines level, weighted by their regression coefficients for the autoantibody association of RA (Hughes-Austin *et al.*, 2013). Also, IL-9 level was significantly increased in the synovial fluid of rheumatoid arthritis patients compared to osteoarthritis (OA), promoting the proliferation and the survival of synovial fluid of CD3(+) T cells in RA patients, the proliferation of these T cells being depended upon the PI3K/Akt/mTOR signalling pathway (Kundu Raychaudhuri *et al.*, 2016).

The genetic variations of IL-9 single nucleotide polymorphisms (SNPs) were investigated; rs17317275 (changing *A* allele to *G* allele at the nucleotide position 2 of the forward DNA strand). They were genotyped polymerase chain reaction with allele specific primer (PCR-ASP) technique (Ye *et al.,* 2012). The molecular size of rs17317275 on 1.5% agarose gel was 218 base pair (bp) as shown in figure (2).

The results of IL-9 genotyping and alleles frequency in table (5), showed that the data of patients group did not match Hardy-Weinberg equilibrium, while control group matched the equilibrium. The GG, AG / G allele (Genotyping /allele) showed a significantly elevated frequency in the patient's group compared to the control group. While in control groups, the AA/A (Genotyping /allele) showed a

significantly elevated frequency compared to patients' group (Table 5). In addition, the GG, AG/G (Genotyping /allele) showed an elevated value of the odds ratio (6.79 and 1.98) referring to be a risk factor, while, AA/ A showed a lessened value (0.13) referring to be a protective fraction (Table 5).
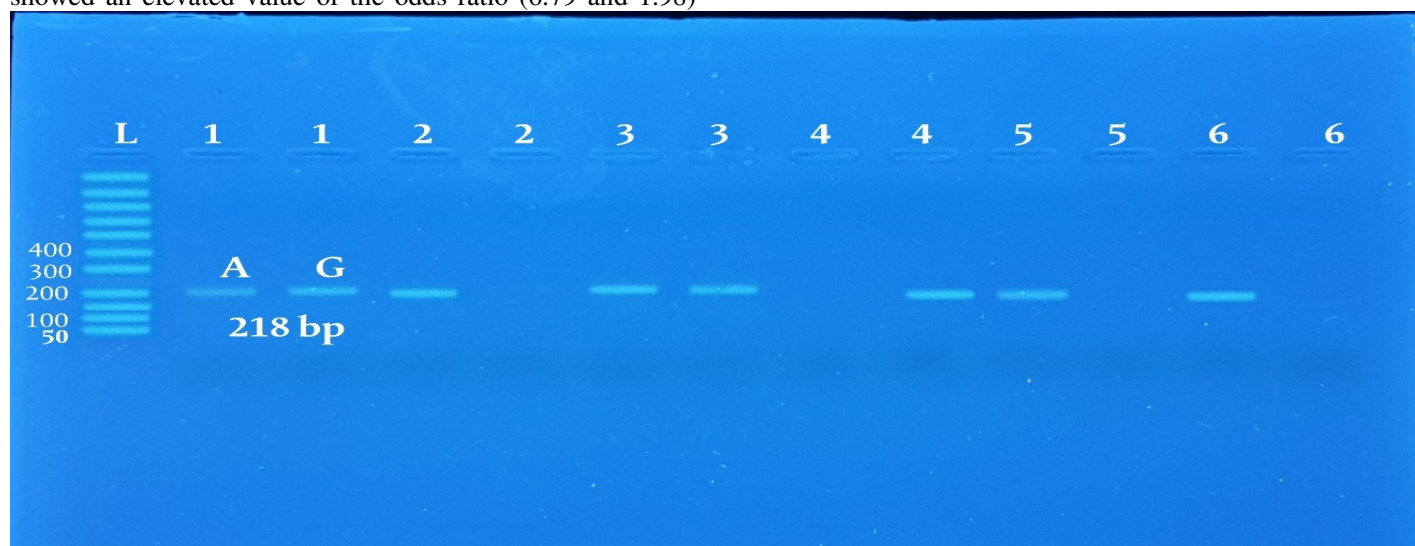

Fig. 2 : IL-9 rs17317275 gel electrophoresis on 1.5% Agarose gel stained with red safe stain. The molecular size of the resulted PCR product was 218 bp. L line: DNA ladder (50 bp); 1, 2, 3, 4, 5 and 6 Lines: the tested samples. The homozygous genotype was presented with a single band of one of A or G alleles for each sample, while heterozygous genotype was presented by two bands for each sample (two lines) (AG).

Table 5 : IL-9 genetic polymorphisms rs17317275 in patients group compared to control group

Genotyping	Patients' group (n=76)				Control group (n=80)				OR	P
	Observed		Expected		Observed		Expected			
	No.	%	No.	%	No.	%	No.	%		
AA	9	11.84	13.90	18.29	40	**48.75**	41.33	51.66	0.13	3.0×10^{-7}
AG	47	**61.84**	37.20	48.95	36	46.25	32.34	40.43	**1.98**	**0.038**
GG	20	**26.32**	24.90	32.76	4	5.0	6.33	7.91	**6.79**	2.6×10^{-4}
Total	76	100.0	76.0	100.0	80	100.0	80.0	100.0		
P-HWE	0.022				0.198					
										Allele frequency
A	43				72				0.29	5.5×10^{-5}
G	**57**				28				**3.41**	5.5×10^{-5}

OR: odd ratio, *P*: probability (two tailed), *P*-HWE: probability of Hardy-Weinberg equilibrium.

Interleukin (IL)-9, a member of the IL-2 cytokine family (Dardalhon *et al.*, 2008), is secreted by naïve CD4+ T cells in response to TGF-β and IL-4 (Th9 pathway) (Beriou *et al.*, 2010). However, IL-9 is also produced by activated Th2 lymphocytes and is involved in Th2-associated diseases (Goswami and Kaplan, 2011). Moreover, IL-9 is a growth factor for mast cells and T cells that help facilitate the Th9 immune response of allergic inflammatory diseases including asthma (Angkasekwinai *et al.*, 2010). The differentiation of Th9 and Th2 cells seems to be regulated by different transcription factors depending upon the cytokine environment (Chang *et al.*, 2010). Intriguingly, IL-9 can also induce Th17 cells to differentiate and mediate autoimmune and inflammatory diseases (Li *et al.*, 2010). IL-9 is also produced by Th17 cells, which secrete mainly IL-17A and IL-17F (Noelle *et al.*, 2010). When administered alone or with IL-6 and TGF-β1, IL-9 greatly enhances the production of IL- 17 from Th17 cells *in vitro* (Elyaman *et al.*,2009). In addition to Th9 cells, IL-9 is produced by a variety of other cells, including Th2, Th17 (Elyaman *et al.*, 2009), Treg (Nowak *et al.*, 2009), mast (Wiener *et al.*, 2004), and natural killer cells (Jones *et al.*, 2009). IL-9 has been shown to play a pivotal role in the pathophysiological processes of many autoimmune diseases, including rheumatoid arthritis (Ciccia

et al., 2015), psoriasis (Singh *et al.*, 2013), atopic dermatitis (Ma *et al.*, 2014), colitis (Nalleweg *et al.*, 2015), systemic lupus erythematosus (SLE) (Ouyang *et al.*, 2013), lupus nephritis (Luk *et al.*, 2015), systemic sclerosis (Yanaba *et al.*, 2011), allergic inflammation (Sehra *et al.*, 2015), type 1 diabetes (Ryba-Stanislawowska *et al.*, 2015), and multiple sclerosis (Ruocco *et al.*, 2015).

Discussion of the results of genetic polymorphism first appeared

The present findings lead to suggest that CMV might one of the triggering factors for RA in cooperation with the incidence of anti-CCP antibodies. This was agreed with previous study (Adland *et al.*, 2015).

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