The Egyptian Journal of Hospital Medicine (October 2019) Vol. 77 (1), Page 4777-4784

Detection of Some Genetic Polymorphism and Serological Factors Associated with Rheumatoid Arthritis in Egyptian Patients

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ABSTRACT

Background: Rheumatoid arthritis is a chronic inflammatory autoimmune disease thatis characterized by destruction of cartilage and bone. PADI4 gene encoding the peptidyl arginine deiminase 4 citrullinating enzyme that has been found to be over expressed in rheumatoid arthritis patients.

Objective: To investigate relation between PADI4 gene polymorphisms and anti–cyclic citrullinated peptide (Anti-CCP) autoantibody levels in sera of Egyptian rheumatoid arthritis patients, and to find its relevance to the disease activity(DAS28 score) and other inflammatory markers as Rheumatoid factor (RF).

Methods: PADI4-89 andPADI4 -92 single nucleotide polymorphisms (SNPs) were analyzed in thirty RA patients and twenty healthy controls by polymerase chain reaction- restriction fragment length polymorphism (PCR-RFLP) method. Serum anti-CCP levels were measured by enzyme-linked immunosorbent assay (ELISA).

Results: PADI4-89 genotype A/G was present in RA patients at higher frequency compared to controls (P = 0.024). (G) allele frequency was significantly higher in RA patients than in controls (P= 0.047). Level of Anti – CCP was significantly higher in RA patients serum than in controls (P= 0.000).PADI4-89 A/G + G/G genotype and PADI4-92 C/G+GG genotype were associated with higher DAS28 score, Rheumatoid factor (RF), and anti-cyclic citrullinated protein (Anti – CCP). Significant positive correlation was found between serum level of Anti– CCP and DAS28 score (P= 0.023).

Conclusion: The SNPs A/G and G/G of PADI4-89 were associated with RA susceptibility. Relation was found between both PADI4 polymorphism and anti-CCP levels.

Keywords: Rheumatoid arthritis, PADI4, Single nucleotide polymorphism, Anti– CCP.

INTRODUCTION

Rheumatoid arthritis (RA) is the most common inflammatory arthropathy. The majority of evidence from genetics, tissue analysis models and clinical studies showed that an immune-mediated etiology and stromal tissue dysregulation together lead to chronic inflammation and articular destruction. A pre-RA phase lasting months to years may be characterized by the presence of circulating auto antibodies, increasing range and concentration of inflammatory cytokines, chemokines and altered metabolism [(1)]. Different evidences have shown that genetic alterations mainly of the single nucleotide polymorphisms (SNPs) type that located in genes that regulate the innate and adaptive immune response are the main genetic risk factor in RA [(2)]. The PADI4gene located at the 1p36 region was recently found as one in association with RA[(3)]. This gene encoding peptidyl arginine deiminase 4 enzyme that catalyzes the protein conversion of arginine residues into citrulline, producing citrullinated proteins [(4)]. This process can cause loss of immune tolerance and anti-CCP development. Anti-CCP identification provides accurate prognosis and diagnosis of RA [(5,6)]. The protein peptidyl arginine deiminase (PADI4) consists of 663 amino acid residues with a 74 kDa molecular weight [(7)]. A candidate gene study identified several PADI4 SNPs (PADI4-89, PADI4-90, PADI4-92 and PADI4-104) involved with risk for RA [(3)]. The first positive association between PADI4 and RA was shown in a Japanese population [(3)]. A meta-analysis showed that positive association between PADI4 and RA not only in the Japanese population but also in European Caucasian populations. However, a recent genome-wide association studies found a weak association of PADI4 polymorphisms and RA in most Caucasian European studies [(9)].

Objectives: The aim of this study was to investigate the association between PADI4 gene polymorphisms and anti–cyclic citrullinated peptide (Anti-CCP) autoantibody levels in sera of Egyptian patients with rheumatoid arthritis and to find out its relevance to the disease severity (DAS28 score) and other inflammatory markers such as rheumatoid factor (RF), C-reactive protein (CRP) and erythrocyte sedimentation rate (ESR).

SUBJECTS AND METHODS

Study population

The study included thirty Egyptian patients with RA, all fulfilled the American College of Rheumatology (ACR) 2010 criteria for diagnosis of RA [(10)] and ACR criteria for classification of rheumatoid arthritis (1987) [(11)]. They were 26 (86.7%) Female and 4(13.3%) males, with the age between (23-70) years old at the time of diagnosis and they were recruited from Physical Medicine, Rheumatology and Rehabilitation Department at Sayed Galal University Hospital during the time period from January 2018 to May 2018.All patients were subjected to full history taking, musculoskeletal examination including signs of rheumatoid arthritis, plain X-ray of both hands AP view. Disease activity score was performed for each patient

Received:05/07/2019
Accepted:05/08/2019

according to (DAS28 score) [12].Patients with other rheumatic diseases, liver diseases, acute chronic inflammatory diseases and malignancy were excluded from the study. Twenty age and sex matched healthy individuals were included as a control group.

Ethical approval:

This study was approved by the Ethical Committee of Faculty of Pharmacy (Girls),AL-Azhar University and informed consent was obtained from all subjects.

Blood sample collection

Five ml of venous blood were collected aseptically and divided into two portions 2 mL of whole blood that were collected in sterile EDTA-containing tubes and stored at-20^0C for DNA extraction, and the remainder were left for 30 to 60 minutes for clotting at room temperature before centrifugation at 3000 rpm for10 minutes to measure Anti CCP.

Measurement of Anti-CCP autoantibody:

Anti-CCP measurement using a commercial quantitative sandwich enzyme linked immunosorbent assay (ELISA) kit (QUANTA Lite CCP3 IgG ELISA) according to the recommendations of the manufacturer.

Detection of PADI4 polymorphisms:

DNA Extraction

Genomic DNA was extracted from stored whole blood samples using commercially available extraction kit (QIAamp® DNA Blood Mini Kit) (QIAGEN) following the manufacturer instructions. Extracted DNA was stored at -20^0C until genotyping.

Genotyping

The polymerase chain reaction-restriction fragment length polymorphisms (PCR-RFLP) method was used for detection of PADI4-89and PADI4- 92 SNPs. For PADI4-89,the primer sequence is: forward: (5to3): TCT GCT TTC CCATGT GTC TTG, reverse (5to3): AGG ACA GAG TGT GTG TGG CTG. The primer sequence For PADI4-92 is: forward: (5to3): CCC AAC TTT GTC TCC CCA GT, reverse (5to3): TTG TGG TTC ACT GAC TAA GGA T[13].Amplification PCR reaction using 1µg Genomic DNA in a 50 µl reaction mixture containing 25 µl of TopTaq Master Mix (1.25 units TopTaq DNA Polymerase, 1.5 mM MgCl2buffer and 200 µM of each dNTP) (QIAGEN) ,0.5 µl of each primer (Thermo scientific), 9 µl of RNase-free water and 10 µl of CoralLoad Concentrate and 5 µl of sample. The PCR cycling conditions were initial denaturation at 94 ◦C for 5 minutes, followed by 35 cycles of denaturation at 94 ◦C for 30 seconds, annealing at 62 ◦C for PADI4-89, at 60 ◦C for PADI4-92 extension at 72 ◦C for 30 seconds, and final extension at 72 ◦C for 5minutes.The PCR products were (278) bp for PADI4-89 and (363) bp for PADI4-92.They were digested by 1 µl HaeIII and 1 µl MspI restriction enzymes (New England Biolabs), (Recognized Sequence: GGCC and CCGG) respectively. The digests were subjected to electrophoresis on a 2% agarose gel then stained with ethidium bromide and visualized with UV transilluminator and photographed. The results of digestion were (Allele **G** 100, 95, 43, and 40bp and Allele **A**140, 95, and 43bp) for PADI4-89 and (Allele **G** 195, 134, and 34bp and Allele **C** 329 and 34bp) for PADI4-92[13].

Statistical analysis

Data were collected and analyzed using Statistical Program for Social Science (SPSS) version 23. Quantitative data were expressed as mean± standard deviation (SD) and range. Qualitative data were expressed as frequency and percentage. Independent t-test of significance was used when comparing between two independent groups with quantitative data. Chi-square test was used when comparing between two independent groups with qualitative data. Correlation coefficient test was used when comparing between two quantitative parameters in the same group.

Results

The demographic and clinical data of RA patients are shown in tables (1) and (2).Comparison between patients and control groups was shown in table (3). Serum level of Anti – CCP was significantly higher in RA patients than controls (P = 0.000) as shown in table (3).Detection of PADI4 was shown in figures (1 & 2). The frequency of A/G and G/G alleles was presented in figures (3) & (4). Frequency of PADI4-89 genotype A/G in RA patients is higher than controls (P = 0.024) as shown in table (4). There was significant increase in frequency of (G) allele in RA patients than controls (P= 0.047)as shown intable (4).A/G + G/G genotype of PADI4-89 and C/G+GG genotype of PADI4-92were associated with higher DAS 28score, RF, CRP, ESR and Anti– CCP(P = 0.007, 0.008, 0.019 ,0.012 ,0.011) respectively as shown in tables (5) and (6). Significant positive correlation was found between Serum level of Anti – CCP and ESR, CRP and DAS 28score (P= 0.002, 0.036 and 0.023) as shown in table (7) and figures (5), (6) and (7).

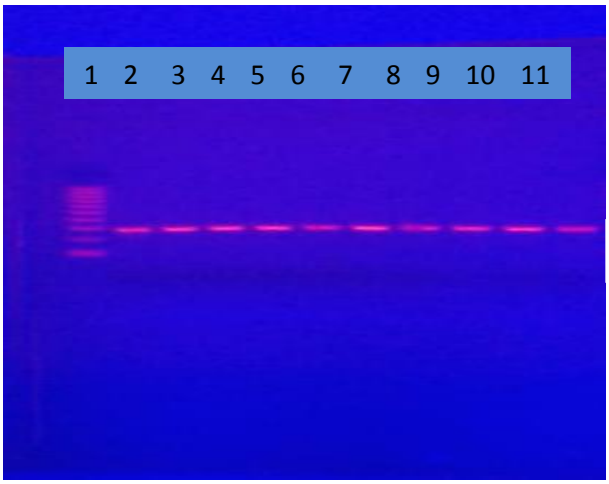

Figure (1): Gel electrophoresis showing PADI4-89 amplification.
Lane 1 represents DNA ladder (100-1000bp).
Lanes 2-11 represent PADI4-89 amplification.

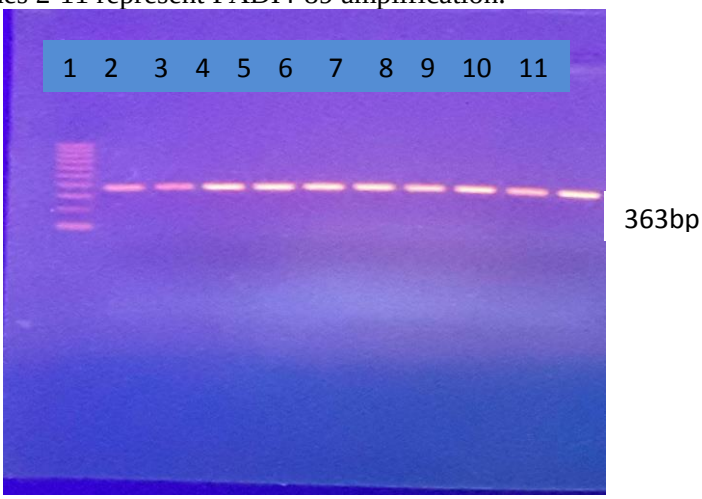

Figure (2): Gel electrophoresis showing PADI4-92 amplification.
Lane 1 represents DNA ladder (100-1000bp).
Lanes 2-11 represent PADI4-92 amplification.

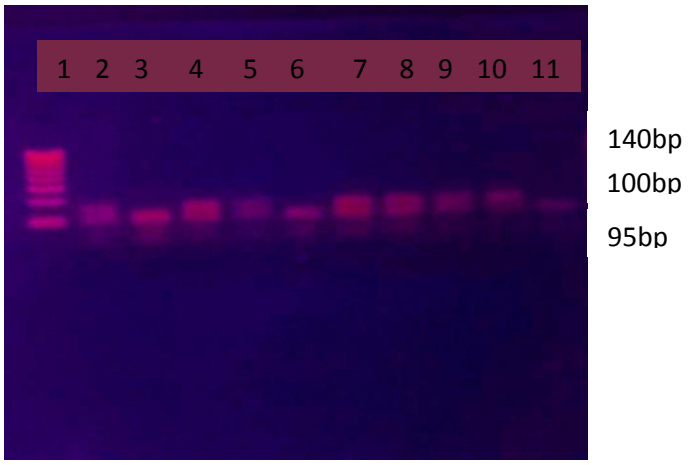

Figure (3): Gel electrophoresis showing PCR-REFLP analysis of PADI4-89using Hae111 restriction enzyme.
Lane 1 represents DNA ladder (100-1000bp).
Lanes 2, 4, 5, 7, 8, 9, 10 represent A/G genotype.
Lanes 3, 6, 11 represent G/G genotype.

Figure (4): Gel electrophoresis showing PCR-REFLP analysis of PADI4-92using Msp1 restriction enzyme.
Lane 1 represent DNA ladder (100-1000bp).
Lanes 2, 3, 4, 6 represent C/G genotype.
Lane 7 represents G/G genotype.
Lane 5 represents C/C genotype.

Table (1): Demographic data of RA patients

		Patients group
		No. = 30
Age	Mean ± SD Range	51.03 ± 11.47 23 – 70
Sex	Female Male	26 (86.7%) 4 (13.3%)
Duration (yrs)	Median (IQR) Range	5 (4 – 13) 0.5 – 23
History	Negative Diabetic Hyper tension Diabetic+ Hypertension	15 (50.0%) 6 (20.0%) 2 (6.7%) 7 (23.3%)

Table (2): Clinical parameters of RA patients

		Patients group
		No. = 30
ESR(mm/h)	Mean ± SD Range	61.63 ± 30.28 30 – 145
RF	Negative Positive	5 (16.7%) 25 (83.3%)
CRP (mg/L)	Mean ± SD Range	31.06 ± 14.98 12 – 86.8
Anti CCP(U/ml)	Median (IQR) Range	128.5 (15.29 – 306.6) 6 – 500
X RAY	Normal Osteopenia	0 (0.0%) 30 (100.0%)
DAS28 Score	Mean ± SD Range	51.70 ± 15.38 17 – 72
SNP Padi489	A/A A/G G/G	5 (16.7%) 15 (50.0%) 10 (33.3%)
SNP Padi492	C/C C/G G/G	9 (30.0%) 14 (46.7%) 7 (23.3%)

Table (3): Comparison between patients and control group regarding demographic and various laboratory parameters

		Control group	**Patients group**	**Test value**	**P-value**	**Sig.**
		No. = 20	**No. = 30**			
ESR(mm/h)	Mean ± SD Range	20.00 ± 3.81 14 – 30	61.63 ± 30.28 30 – 145	-6.096•	0.000	HS
RF	Negative Positive	17 (85.0%) 3 (15.0%)	5 (16.7%) 25 (83.3%)	22.741*	0.000	HS
CRP(mg/L)	Mean ± SD Range	5.22 ± 4.38 2 – 15	31.06 ± 14.98 12 – 86.8	-5.064•	0.000	HS
AntiCCP (U/ml)	Median Range	7 (7 – 8) 6 – 11	128.5 (15.29 – 306.6) 6 – 500	-4.507≠	0.000	HS
Age (years)	Mean ± SD Range	28.25 ± 2.95 23 – 34	51.03 ± 11.47 23 – 70	-8.664•	0.000	HS
sex	Female Male	20 (100.0%) 0 (0.0%)	26 (86.7%)4 (13.3%)	2.899*	0.089	NS

P-value > 0.05: Non significant; P-value < 0.05: Significant; P-value < 0.01: Highly significant
*: Chi-square test; •: Independent t-test.

Table (4): Genotypic and allelic frequencies of PADI4 89and PADI4 92 SNPs of PADI4gene in controls and RA patients

		Control group	Patients group	Test value*	P-value	Sig.
		No. = 20	No. = 30			
SNP Padi489	A/A	14 (70.0%)	5 (16.7%)	7.465	0.024	S
	A/G	3 (15.0%)	15 (50.0%)			
	G/G	3 (15.0%)	10 (33.3%)			
SNP Padi492	C/C	8 (40.0%)	9 (30.0%)	0.756	0.685	NS
	C/G	7 (35.0%)	14 (46.7%)			
	G/G	5 (25.0%)	7 (23.3%)			
Allele						
SNP Padi489	A	31 (77.5%)	35 (58.3%)	3.929	0.047	S
	G	9 (22.5%)	25 (41.7%)			
SNP Padi492	C	23 (57.5%)	32 (53.3%)	0.168	0.682	NS
	G	17 (42.5%)	28 (46.7%)			

Table (5): Comparison between wild genotype and polymorphic genotypes of PADI4 89 regarding various parameters in RA patients

		SNP Padi489		Test value	P-value	Sig.
		A/A	A/G + G/G			
ESR(mm/h)	Mean ± SD Range	47.8 ± 13.73 30 – 70	70.15 ± 32.87 30 – 145	-2.048•	0.050	S
RF	Negative Positive	4 (40.0%) 6 (60.0%)	1 (5.0%) 19 (95.0%)	5.880*	0.015	S
CRP (mg/L)	Mean ± SD Range	21.56 ± 11.08 12 – 36	35.34 ± 14.73 18 – 86	-2.497•	0.019	S
DAS28 score	Mean ± SD Range	38.50 ± 14.10 17 – 67	58.30 ± 11.37 34 – 72	-4.152•	0.000	HS
ANTICCP **(U/ml)**	Median Range	80.37 (8.3 – 100) 7 – 200	225 (67.7 – 373.6) 6 – 500	-2.314ǂ	0.019	S

P-value > 0.05: Non significant; P-value < 0.05: Significant; P-value < 0.01: Highly significant
*:Chi-square test; •: Independent t-test

Table (6): Comparison between wild genotype and polymorphic genotypes of PADI4 92 regarding various parameters in RA patients

		PCR REFLP Padi492		Test value	P-value	Sig.
		C/C	C/G+GG			
ESR (mm/h)	Mean ± SD Range	42.44 ± 12.66 30 – 70	71.38 ± 30.82 30 – 145	-2.699	0.012	S
RF	Negative Positive	4 (44.4%) 5 (55.6%)	1 (4.8%) 20 (95.2%)	7.143	0.008	HS
CRP (mg/L)	Mean ± SD Range	21.56 ± 7.73 12 – 30	35.34 ± 15.61 12 – 86.8	-2.497	0.019	S
ANTICCP **(U/ml)**	Median Range	8.3 (7 – 111.9) 6 – 250	169 (94.24 – 347.2) 7 – 500	-2.539	0.011	HS
DAS28 Score	Mean ± SD Range	40.61 ± 12.55 17 – 59	56.45 ± 14.17 22 – 72	-2.896	0.007	HS

P-value > 0.05: Non significant; P-value < 0.05: Significant; P-value < 0.01: Highly significant
*:Chi-square test; •: Independent t-test.

Table (7): Correlation between Anti-CCP serum level and various clinical parameters

	AntiCCP(U/ml)	
	r	**P-value**
Age (years)	-0.117	0.536
DD (years)	-0.043	0.822
ESR (mm/h)	**0.545****	**0.002**
CRP (mg/L)	**0.392***	**0.036**
DAS28 Score	**0.413***	**0.023**

P-value > 0.05: Non significant; P-value < 0.05: Significant; P-value < 0.01: Highly significant
Spearman correlation coefficient.

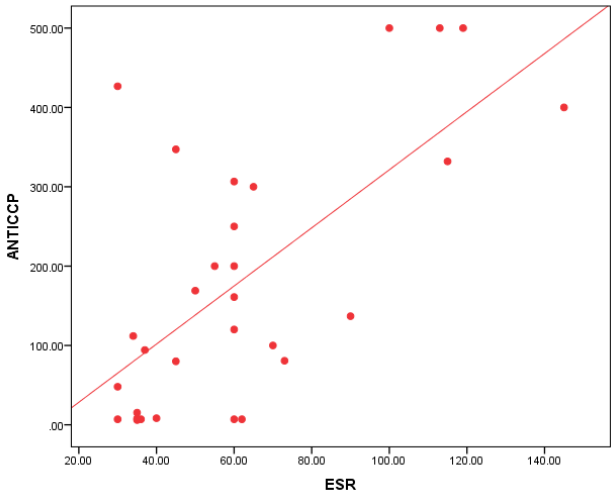

Figure (5): Correlation between Anti- CCP and ESR

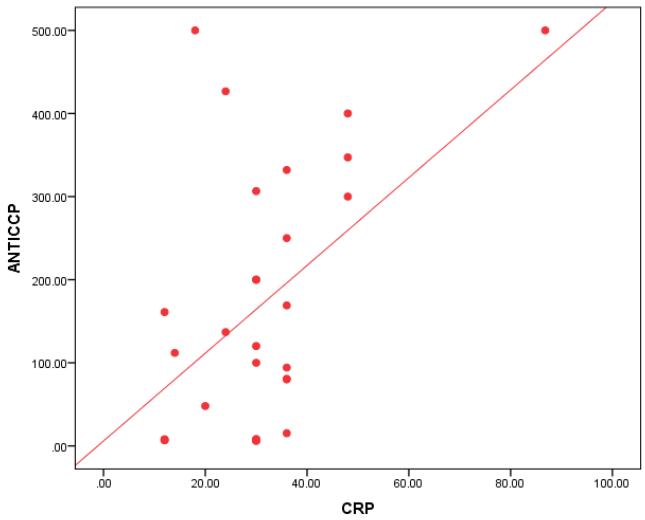

Figure (6): Correlation between Anti- CCP and CRP

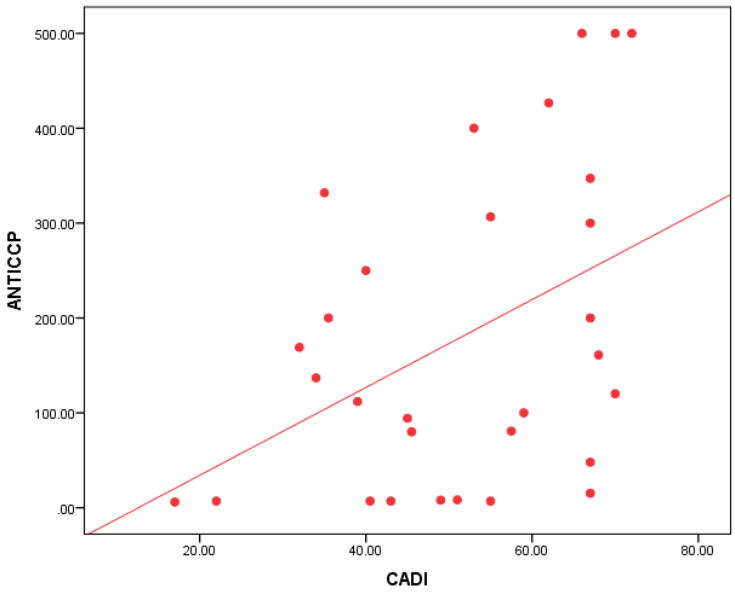

FIGURE (7): CORRELATION BETWEEN ANTI-CCP AND DAS 28

DISCUSSION

Rheumatoid arthritis (RA) is a chronic inflammatory autoimmune disease that initially affects small joints, progressing to larger joints, and eventually the skin, eyes, heart, lungs, and kidneys. The bone and cartilage of joints are destroyed while tendons and ligaments are weakened [14]. It has been found that a high-risk genetic background in combination with environmental exposures leads to a cascade of events inducing synovitis and destructive arthritis. The clinical picture of joint involvement in RA is the result of chronic inflammation of the synovium, which is characterized by interactions of resident cells such as fibroblast-like synoviocytes (FLS) with cells of the innate immune system(e.g. macrophages, dendritic cells, neutrophils and NK cells) and adaptive immune system(e.g. B and T lymphocytes) [15]. Recent genome-wide association studies and Meta-analysis have led to the identification of many alleles that are associated with RA susceptibility [16]. In recent years, particular interest has been given to the contribution of neutrophil extracellular trap (NET) formation (NETosis) to the breaking of immunological tolerance and the maintenance of autoimmunity and inflammation in RA. In RA, neutrophils reveal spontaneous increase in NETosis, associated with elevated reactive oxygen species (ROS) production, nuclear translocation of protein arginine deiminase-4 **(**PADI4**)**, PADI4-mediated citrullination of H3 and altered nuclear morphology [15].

The PADI4 gene has been studied in several RA populations. This gene encodes the PADI4 enzyme, which may contribute in the development of citrullinated proteins through the conversion of peptidyl arginine to peptidyl citrulline residues [17].The association of PADI4 gene with RA susceptibility was first reported in a Japanese population, they observed that 4 exonic PADI4single nucleotide polymorphisms (SNPs), namely PADI4-89, PADI4-90, PADI4-92 and PADI4-104 related to RA [3].And this association has been replicated in several populations including Korean [18, 19], German [20], Chinese [21] and Egyptian[22]. However, studies in other countries such as England [23] and France [24] showed no association between PADI4 and RA. In our study we found significant increase in frequency of PADI4-89 gene SNP A/G in RA patients than controls. We also found that frequency of (G) allele was significantly higher in RA patients than controls. This result agrees with **Baños-Hernández *et al.*** [17]who showed similar result in Southern Mexican population. They found that individual polymorphisms PADI4-89 A/G are considered genetic risk markers for RA. Our results, also are matching with study done by **Guzman-Guzman *et al.*** [25] in western Mexican population. They found a positive relation between A/G + G/G genotype of PADI4-89 and RA susceptibility. Our study, also is similar to result of **Bang *et al.*** [18].They showed thatPADI4-89 SNP are significantly associated with RA. Also our result is matching with **Hoppe *et al.*** [20].They found that the PADI4 haplotype 4 and the SNPs padi4_89A/G were significantly associated with RA in a German population. On the other hand, **Barton *et al.*** [26], **Panati *et al.*** [27] and **Caponi *et al.*** [24]showed no association between SNPs and functional A/G + G/G genotype in PADI4-89with RA susceptibility. In our study, we did not find significant increase in frequency of PADI4-92 gene SNP C/G in RA patients when compared to controls. This result is in agreement with **Fan *et al.*** [21] who reported that PADI4-92 was not associated with RA. Also, **Hoppe *et al.*** [20] showed no allelic association of PADI4- 92 polymorphism with RA. On other hand, **Abd-Allah *et al.*** [22] found that the SNPs padi4_92C/G were significantly associated with RA. The discrepancy in results may be due to genetic heterogeneity in different ethnic groups [18].We also found that A/G + G/G genotype of PADI4-89 and C/G+GG genotype of PADI4-92 were associated with higher DAS 28score, RF and anti–CCP, which is in partial agreement with **Guzman-Guzman *et al.*** [25] since they showed that the higher serum level of anti–CCP was associated with the homozygous susceptibility genotypes (PADI4_89 G/G, and PADI4_92 G/G) than the homozygous non-susceptibility genotypes and heterozygous genotypes, but disease activity showed no relation with PADI4genotypes. We found significant positive correlation between serum level of anti–CCP and DAS28 score. Also, we found significant relation between serum level of anti– CCP and RF. This result is matching with **Panati *etal.*** [27] Who demonstrated that all patients with high DAS28 score were positive for both anti-CCP antibodies and RF antibodies DAS28 score values revealed significant association with the anti-CCP antibody levels in their study.

CONCLUSION

Genotypes A/G and G/G in PADI4 -89 are considered genetic risk factors for RA. SNPs padi4_92C/G was not significantly associated with RA. Significant association of anti-CCP serum levels with both PADI4 polymorphisms. Other studies with large samples are needed to confirm this study.

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