Genetic Polymorphism and Serum Levels of RORc2 in Rheumatoid Arthritis

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RORc2 is the master transcription factor of T helper 17 cells. We aimed to evaluate whether RORc2 genetic polymorphism and serum levels have association with the risk and activity of rheumatoid arthritis (RA). RORC genetic polymorphisms were investigated by real time PCR. Serum RORc2 protein levels were determined by enzyme-linked immunosorbent assay. Protective effects of rs370515 CT, rs370515 CT + TT, rs3828057 CT, rs3828057 CT+TT and rs9826 GG genotypes were detected. The genotype-phenotype analysis showed no significant differences in the disease activity score 28 (DAS 28) under the recessive versus dominant genotypes. RORc2 protein serum levels were significantly higher in RA patients than controls (*P*= 0.001) and had a positive correlation with DAS-28. In conclusions, RORC genetic polymorphisms correlate with the risk but not activity of RA, whereas RORc2 serum levels have a positive correlation with both risk and activity of RA.

Approximately, 0.5–1% of the human population are affected with rheumatoid arthritis (RA) making it one of the most common autoimmune disorders that is associated with systemic complications [1]. The disease results from a complex interaction between epigenetic changes and unknown environmental triggers [2]. The genetic pathogenesis of RA involves multiple genes that encode many proteins with significant functions in T cell response regulation [3]. RORc2 is a transcriptional factor that regulates the differentiation of T helper 17 (Th17) cells and could be a genetic risk factor in RA [1]. Expression of chemokine receptors 6 on Th17 cells and production of chemokine ligands 20 by synoviocytes induce recruitment of Th17 cells to inflamed joint [4]. Th17 cells and Th17 cytokines [especially interleukin (IL)17] play important roles in the pathogenesis of RA by induction of osteoclastogenesis, activation of tissue-destructive enzymes, angiogenesis and pannus growth [5]. IL-17 acts on osteoblasts, resulting in cyclooxygenase mediated prostaglandin 2 synthesis and osteoclast differentiation factor (ODF) expression. ODF induces differentiation of osteoclast progenitors into mature osteoclasts by binding to its receptors that present in osteoclast progenitors resulting in pathogenic resorption of bone in RA [4]. Both increased presence of Th17 cells in the synovial fluid and elevated IL-17 levels correlate with increased disease activity [disease activity score (DAS28), erythrocyte sedimentation rate (ESR), and C-reactive protein (CRP) levels] [6]. The master transcription factor for the differentiation of Th17 cells is a nuclear hormone retinoic acid receptor-related orphan receptor variant 2 (RORc2). RORc2-deficient T cells fail to differentiate into Th17 cells, whereas forced expression of RORc2 induces production of IL-17 by naive T cells [7]. Recently, small

molecule inhibitors of RORc2 drew attention as a possible therapy for autoimmune diseases [8]. Generation of Th17 cells is promoted by proinflammatory cytokines like IL-6 and IL-23 while the serum levels of TGF-β and the number of Treg cells that express X-linked forkhead box protein 3 (FOXP3) and have important role in immunological tolerance are decreased leading to imbalance of Th17/Treg in RA patients [9]. The balance between expression of RORc2 and FOXP3 plays a critical role in Treg and Th17 lineage determination [10]. TGFβ induces FOXP3 expression and Treg differentiation shifting the ROR/FOXP3 balance towards FOXP3 and Treg differentiation. Addition of IL-6 or IL-21 inhibits the induction of FOXP3 by TGFβ and activates RORc2 expression, thereby shifting the ROR/FOXP3 balance in favor of Th17 [11].

In the present study, genetic polymorphism of three SNPs in RORC gene, rs3790515 C/T, rs3828057 C/T and rs9826 A/G, were examined. Only rs9826 A/G was found previously to be associated with RA [12] whereas, to the best of our knowledge, no previous study on rs3790515 C/T and rs3828057 C/T in relation to RA was done. Serum RORc2 protein levels were estimated. We aimed in this work to investigate the association of genetic polymorphism and serum protein levels of RORc2 with susceptibility and disease activity in Egyptian RA patients.

Subjects and Methods

Patients Characteristics

This is hospital based descriptive case control study. A total of 55 RA patients diagnosed according to 2010 ACR / EULAR Rheumatoid Arthritis Classification Criteria [13] were recruited from Rheumatology and Clinical Immunology unit, Internal Medicine department, Assiut University Hospitals. Disease activity score 28 (DAS28) was used for each patient to assess the disease activity. Exclusion criteria include patients coaffected with other connective tissue, associated autoimmune diseases, chronic liver or kidney diseases, or genetic diseases. Informed consent was taken from all subjects. A total of 34 age and sex matched healthy controls randomly selected from healthy volunteers were included in the study. This study was approved by ethical committee of Faculty of Medicine, Assiut University according to principles of the Declaration of Helsinki (Approval number 17200212).

Sample collection

Blood samples were obtained from all patients and controls and divided into two tubes; EDTA tube for extraction of genomic DNA (gDNA) and serum-separating tubes for RORc2 serum level determination by enzyme-linked immunosorbant assay (ELISA). Samples were preserved at -70˚C for further use.

DNA extraction and gDNA quantification

Genomic DNA was extracted from whole blood using GeneJET Genomic DNA Purification Kit purchased from ThermoFisher Scientific, USA (according to the manufacturer's instruction). Quantification of gDNA was done using NanoDrop spectrophotometry.

SNP Genotyping

TaqMan SNP Genotyping Assays perform genotyping studies using the TaqMan® assay-based (5′ nuclease) chemistry for amplifying and detecting specific polymorphisms in purified gDNA. The materials used were: (i) the 40✕ SNP Genotyping Assay for each SNP that contains the sequence-specific forward and reverse primers to amplify the polymorphic sequence of interest. For rs3790515: CTTTGCCCTCCAACAAGGTATATCT[C/T]GTTGA GGTGAACAGGACATACAGCT, for rs3828057: TCTCCTCAGAGCAAAGAAGTCCGCT [C/T]ATTCTTCCTGGGCTAGGACAAACCC, and for rs9826: AAGTCGCTCGCACTGGT CAGTCGGA[A/G]GGCAAGATCAGATCCTGGA GGACTT; two TaqMan® MGB probes: One probe labeled with VIC® dye detects the Allele 1 sequence (C allele of rs3790515 and rs3828057 and A allele of rs9826) and one probe labeled with FAM™ dye detects the Allele 2 sequence (T allele of rs3790515 and rs3828057 and G allele of rs9826), (ii) 2X TaqMan® Universal PCR Master Mix (No AmpErase® UNG) contains AmpliTaq Gold® DNA Polymerase, dNTPs with dUTP, passive reference dye (ROX dye) and optimized buffer components,

and (iii) a total of 20 ng of purified gDNA diluted into nuclease-free water.

The reaction was performed in a total volume of 20 ul consisted of; 10 μl Taqman universal PCR master mix, 0.5 ul SNP genotyping assay, and 9.5 ul of 20 ng purified gDNA diluted in nuclease free water on 7500 Real-Time PCR System with the following amplification protocol: denaturation at 95 °C for 10 min, followed by 40 cycles of denaturation at 92 °C for 15 sec, and annealing and extension at 60 °C for 1 min.

To ensure optimal analysis and troubleshooting of the TaqMan SNP Genotyping Assays, an optical reaction plate containing the following for each assay: two NTCs; DNase-free water was used to orient the VIC-dye and/or FAM-dye clusters to an origin and enable the detection of DNA contamination on a given set of plates, and DNA samples with unknown genotype at the SNP of interest.

Post-PCR plate read was performed using the fluorescence measurements made during the plate read then determines which alleles are present in each sample using automatic or manual allelic call. For the automatic allelic call, the real-time PCR instrument software plots the results of the allelic discrimination data as a scatter plot of Allele 1 (VIC® dye) versus Allele 2 (FAM™ dye); while, the manual allelic call depends on determination of the genotype based on the amplification curve and the multicomponent plot. An increase in either FAM or VIC dye fluorescence indicates homozygosity for VIC or FAM -specific alleles (X:X or Y:Y), and an increase in the fluorescence of both dyes indicates heterozygosity (X:Y).

Determination of RORc2 serum Level

Serum RORc2 levels were determined by a sandwich enzyme-linked immunosorbent assay (ELISA) technique according to the manufacturer's instructions (SinoGeneClon Biotech Co., Ltd, China). ELISA plate was read at absorbance of 450nm using ELISA plate reader from BioTek Instruments, Inc, USA. The concentration of RORC2 in the samples is then determined by comparing the optical density of the samples to the standard curve. The detection range is 15 pg/mL-480 pg/mL.

Statistical Analysis

The collected data were verified, coded by the researcher and analyzed using the Statistical Package for Social Sciences (IBM-SPSS/PC/VER 21). Test of significances: Chi square and Fisher Exact tests were used to compare the difference in distribution of frequencies among different groups. For continuous variables; independent t-test analysis was carried out to compare the means of normally distributed data, while Mann-Whitney U test was calculated to test the median differences of the data that don't follow normal distribution. For variables with more than two categories; ANOVA test was calculated to test the mean differences of the data that follow normal distribution, post-hoc test was calculated using Bonferroni corrections. Correlation analysis was used to test the association between variables (Spearman's rank correlation). Multivariate logistic regression analysis was calculated to investigate the predictive power of the genotypes and alleles of the studied cohort on the RA [Odds Ratio (OR), 95% confidence interval (95% CI) and Likelihood Ratio Test (LRT)] [14]. The HaploView program (version 4.2) was applied to estimate the haplotypes and linkage disequilibrium (LD) which uses the expectation maximization (EM) algorithm [15].

Results

Demographic, clinical and laboratory characteristics of RA patients

The mean age±SD of RA patients and control group was 45.13±10.6 years with a median age of 45 years (20-73 years). As for the control group, it was 43.00±11.1 years with a median age of 43 years (20-65 years). RA patients were 50 females and 5 males and controls were 31 females and 3 males with no statistically significant age and sex differences between RA patients and controls (P=0.369 and 0.624, respectively). The main complaint of RA patients was arthralgia (94.5 %). Nearly half of them had extra-articular manifestation and positive family history. Other clinical and laboratory data characteristics of the RA patients are shown in table 1.

Table 1. Clinical and laboratory data of rheumatoid arthritis patients

Clinical data	RA patients (n=55)
Main complaints:	
Arthralgia	52 (94.5%)
Arthritis	32 (58.2%)
Fever	29 (52.7%)
Disease Duration/years	11.45 ± 7.3 (1 - 40)*
Age of Disease Onset/years	33.49 ±9.1 (17 - 53)*
Positive Family History	25 (45.5%)
Associated comorbidities	10 (18.2%)
Extra-articular Manifestations	27 (49.1%)
Morning Stiffness Duration (minutes)	46.45 ± 35.6 (0 - 120)*
Tender joint number	≥ 4 (n=29)
	< 4 (n=26)
Swollen joint number	≥ 1(n=19)
	0 (n=36)
Global Score	36.82 ± 32.4 (0 - 90)*
DAS-28	4.41 ± 1.3 (2 - 7)*
Remission	5 (9.1%)
Low	12 (21.8%)
Moderate	19 (34.5%)
High	19 (34.5%)
Laboratory data	
Erythrocyte sedimentation rate (mm/hr)	60.80 ±28.2 (18 - 150)*
Rheumatoid factor (mg/dl)	48.84 ± 38.4 (1 - 214)*
C reactive protein (mg/dl)	33.44 ±24.1 (0.5 - 124)*

*mean ± SD (range).

RORC gene polymorphisms and risk of RA

Only rs3828057 observed genotypes distribution in controls was comparable to that predicted by Hardy-Weinberg equilibrium (HWE) (P=0.437) but those of rs3790515 and rs9826 show significant deviation from it (P=0.0004 and 0.009, respectively).

rs3790515 (C/T) SNP polymorphism

Significant increase was detected in rs370515 CT and rs370515 CT + TT genotypes versus rs370515 CC wild genotype in controls than patients [P =0.028, OR (95% CI) = 0.152 (0.028-0.818) and P=0.015, OR (95% CI) = 0.293 (0.110-0.785), respectively]. Allelic frequency showed no statistically significant differences between RA patients and controls (table 2).

rs3828057 (C/T) SNP polymorphism

Significant increase was detected in rs3828057 CT and rs3828057 CT + TT genotypes versus rs3828057 CC wild genotype in controls than patients [p=.016, OR (95% CI) = 6.044 (1.405-26.01) and *P*=0.023, OR (95% CI) =5.136 (1.259-20.59), respectively]. Allelic frequency showed no statistically significant differences between RA patients and controls (table 2).

rs9826 (A/G) SNP polymorphism

GG genotype was significantly more frequent in controls than RA patients [*P*=0.012, OR (95% CI) = 0.122 (0.023-0.632)]. Allelic frequency showed no statistically significant differences between RA patients and controls (table 2).

Table 2. Genotypic & Allelic Frequencies of the studied Cohort (Cases vs. Controls)

		Controls (n=34)	RA Cases (n=55)	*P*-value*	OR (95% CI) **
rs3790515 (C/T)					
	CC	21 (61.8%)	46 (83.6%)	-----	1 (Reference)
Genotypes	CT	5 (14.7%)	2 (3.6%)	0.028	0.152 (0.028-0.818)
	TT	8 (23.5%)	7 (12.7%)	NS	0.399 (0.128-1.247)
	CT + TT	13 (37.2%)	9 (16.3%)	0.015	0.293 (0.110-0.785)
Alleles	C-Allele	47 (69.1%)	94 (85.5%)	-----	1 (Reference)
	T-Allele	21 (30.9%)	16 (14.5%)	NS	0.462 (0.521-2.698)
rs3828057 (C/T)					
	CC	8 (23.5%)	3 (5.5%)	-----	1 (Reference)
Genotypes	CT	14 (41.2%)	34 (61.8%)	0.016	6.044 (1.405-26.01)
	TT	12 (35.3%)	18 (32.7%)	NS	.886-18.19)
	CT + TT	26 (76.5%)	52 (94.3%)	0.023	5.136 (1.259-20.59)
Alleles	C-Allele	30 (44.1%)	40 (34.4%)	-----	1 (Reference)
	T-Allele	38 (55.9%)	70 (63.6%)	NS	1.841 (0.721-12.16)
rs9826 (A/G)					
	AA	18 (53%)	37 (67.3%)	-----	1 (Reference)
Genotypes	AG	8 (23.5%)	16 (29.1%)	NS	0.865 (0.321-2.332)
	GG	8 (23.5%)	2 (3.6%)	0.012	0.023-0.632)
	AG + GG	16 (46.0%)	18 (32.7%)	NS	0.515 (0.216-1.229)
Alleles	A-Allele	44 (64.7%)	90 (81.8%)	-----	1 (Reference)
	G-Allele	24 (35.3%)	20 (18.2%)	0.041	0.298 (0.131-9.612)

*P value>0.05 is not significant (NS). **OR: odds ratio, CI: confidence interval. RA= Rheumatoid arthritis

RORC gene polymorphisms and RA activity

The genotype-phenotype analysis showed no significant differences in the disease activity and laboratory parameters in rs3790515, rs3828057 and rs9826 polymorphisms under the recessive genotypes (CT, TT; CT, TT; AG, GG, respectively) versus the dominant genotypes (CC, CC, AA, respectively) with no significant variation in DAS-28 scores in RA patients in relation to the three studied SNPs polymorphisms as shown in table 3 for rs3790515. The mean ± SD of DAS-28 was 4.40± 1.3, 5.76 ± 0.1 and 4.07 ± 1.4 in CC, CT and TT rs3790515 genotypes, respectively (P=.194). Regarding rs3828057, The mean ± SD of DAS-28 level was 5.21 ± 0.7, 4.69 ± 1.3 and 4.19 ± 1.3 in CC, CT and TT genotypes, respectively (P=0.245) and for rs9826, The mean ± SD of DAS-28 level was 4.43 ± 1.4, 3.26 ± 0.8 and 4.51 ± 1.2 in AA, AG and GG genotypes, respectively (P=0.410).

Table 3. Disease activity and laboratory parameters in relations to rs3790515 C/T Model

Parameter	CC (n=46) (Dominant genotype) Median (Range)	CT + TT (n=9) (Recessive genotypes) Median (Range)	P-value*
Disease Duration (years)	10 (1 - 25)	13 (6 - 40)	NS
No. of tender Joints	4 (0 - 22)	0 (0 - 12)	NS
No. of swollen Joints	0 (0 - 4)	0 (0 - 6)	NS
ESR	60 (18 - 150)	58 (30 - 90)	NS
CRP	19.5 (1 - 124)	16 (1 - 68)	NS
Haemoglobin	12 (7 - 14)	11 (6 - 13)	NS
Global Score	35 (0 - 90)	40 (10 - 70)	NS
DAS-28	4.6 (2 - 7)	4.7 (2.5 - 6)	NS
	N (%)	N (%)	P-value**
Sex (Female)	42 (91.3%)	8 (88.9%)	NS
RF (Presence)	30 (65.2%)	5 (55.6%)	NS

*Mann-Whitney U was used to compare the median difference between groups

**Chi-square test was used to compare the Proportion difference between groups

*P value>0.05 is not significant (NS). . *Abbreviations*: CRP=C reactive protein; DAS-28 = Disease activity score of 28 joints; ESR= Erythrocyte sedimentation rate; RF=Rheumatoid factor

Haplotypes analysis of the studied SNPs and their correlation with the risk and activity of RA

Analysis was performed with data derived from chromosome 1 on RORC gene with an average distance between SNP markers of less than 1 kb and revealed that LD between rs9826 and rs3828057 was 37% in control and 7% in cases, while LD between rs9826 and rs3790515 was 5% in control and 56% in cases, LD between rs3828057 and rs3790515 was 51% in control and 53% in cases as demonstrated in figure 1.

rs3790515 - rs3828057 - rs9826 CTA showed highest frequency in both cases and controls (51.1% and 36.2%, respectively)

followed by CCA (17.5%) and CCG (12.8%) haplotypes in cases and CCG (17.1%) and TCA (11%) haplotypes in controls. CTA and CCA haplotypes showed significantly higher frequency (*P*=0.049), while TCG haplotype showed significantly lower frequency in cases when compared to control group (*P*=.003) as shown in Table (4). There were no differences in haplotype frequency between RA patients and control group for the other haplotypes. As regard the haplotypes correlation with the activity of RA, no significant differences were found in rs3790515 - rs3828057 - rs9826 haplotypes between cases with remission and active RA.

Table 4. Comparison of rs3790515 - rs3828057 - rs9826 haplotypes frequencies between cases and control subjects.

rs3790515 -rs3828057-rs9826	Control (n=34)	Cases (n=55)	*P value
CTA	0.362	0.511	0.049
CCG	0.171	0.128	NS
CCA	0.072	0.175	0.049
TTA	0.099	0.076	NS
TCA	0.110	0.056	NS
CTG	0.081	0.040	NS
TCG	0.090	0.004	0.003
TTG	0.016	0.010	NS

*P value>0.05 is not significant (NS).

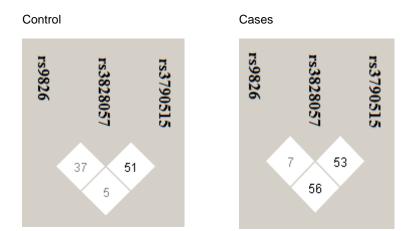Control   Cases

Figure 1. Linkage disequilibrium plot for RORC gene studied SNPs in (A) control and (B) cases.

Relation between RORc2 serum levels and risk and activity of RA

The mean value of RORc2 protein serum levels was higher in RA patients than controls. The mean $\pm$ SD of RORc2 protein serum levels in RA patients was 63.56 ± 9.1 pg/mL, while in controls was 32.04 ± 1.5 pg/mL ($P=0.001$).

RORc2 serum levels were higher in patients with number of tender joints ≥ 4 ($P=0.002$), number of swollen joints ≥ 1 ($P=0.039$), rheumatoid factor (RF) ≥ 32 mg/dl ($P=0.02$), CRP ≥ 18 mg/dl ($P=0.043$) and DAS 28 score ≥ 4.7 ($P=0.012$), as shown in table 5.

Table 5. The disease activity and laboratory parameters (RA phenotype) in relation to RORc2 Protein levels in 55 RA patients

Parameter	Category (n)	RORc2 levels (pg/ml) Median (range)	*P*-value*
Disease Duration/year	≥ 10 (n=32)	38 (21 - 440)	NS
	< 10 (n=23)	37 (21 - 188)	
No of tender Joint	≥ 4 (n=29)	50 (24 - 440)	0.002
	< 4 (n=26)	31 (21 - 111)	
No of swollen Joint	≥ 1 (n=19)	47 (25 - 440)	0.039
	0 (n=36)	33 (21 - 188)	
RF (mg/dl)	≥ 32 (n=31)	44 (24 - 440)	0.020
	< 32 (n=24)	33 (21 - 132)	
ESR (mmHg)	≥ 60 (n=29)	41 (21 - 440)	NS
	< 60 (n=26)	35 (21 - 169)	
CRP (mg/dl)	≥ 18 (n=29)	40 (21 - 440)	0.043
	< 18 (n=26)	32 (21 - 123)	
DAS-28 score	≥ 4.7 (n=25)	55 (27 - 440)	0.012
	< 4.7 (n=30)	33 (21 - 169)	

*Mann-Whitney U was used to compare the median difference between groups, **P* value>0.05 is not significant (NS).
CRP=C reactive protein; DAS-28=Disease activity score of 28 joints; ESR=Erythrocyte sedimentation rate; RA= Rheumatoid arthritis; RF=Rheumatoid factor

RORc2 genetic polymorphism in relation to its serum levels

Serum levels of RORc2 in RA patients with rs3790515 CC, rs3790515 CT, rs3828057 CC, rs3828057 TT and rs9826 GG genotypes were significantly higher than controls with the same RORc2 genotypes ($P=0.003$, 0.02, 0.003, 0.021, and 0.002, respectively), as shown in table 6.

No significant differences were found in rs3790515 - rs3828057 - rs9826 haplotypes between cases with RORc2 serum levels below and above median.

Table 6. Variation in RORc2 expression levels in RA patients and control group in relation to RORc2 gene polymorphisms

		RORc2 levels [Median (Range)]		P-value*
		Control (n = 34)	RA Case (n = 55)	
rs3790515 (C/T)	CC	29 (15 - 45)	37 (21 - 440)	0.003
	CT	34 (21 - 46)	86 (36 - 136)	0.021
	TT	36 (28 - 48)	41 (30 - 144)	NS
rs3828057 (C/T)	CC	29.5 (15 - 45)	37 (20 - 440)	0.003
	CT	36 (28 - 48)	41.5 (30 - 194)	NS
	TT	34 (21 - 47)	86 (36 - 136)	0.021
rs9826 (A/G)	AA	34 (18 - 48)	36 (21 - 440)	NS
	AG	32 (22 - 46)	32.5 (30 - 35)	NS
	GG	29 (15 - 35)	39.5 (24 - 169)	0.002

*Mann-Whitney U was used to compare the median difference between groups

*P value>0.05 is not significant (NS).

Discussion

RORc2 is the master of Th17 cells programing and is required for the IL17 expression so targeting Th17 lineage by RORc2 small molecule inhibitors are currently used for autoimmune inflammatory diseases treatment as RA [15].

In this study, three selected SNPs of RORC gene (rs3790515 C/T, rs3828057 C/T and rs9826 A/G,) were examined for genetic polymorphism and their inheritance was evaluated by analyzing the distribution of haplotypes in RA patients and control group. Also, serum RORc2 levels were estimated in a cohort of Egyptian RA patients and age and sex matched healthy controls to determine their possible association with susceptibility to, clinical phenotype and laboratory data of RA. Only rs3828057 observed genotypes distribution was comparable to that predicted by HWE but those of rs3790515 and rs9826 showed

significant deviation from it. Significant deviations from HWE regarding genotype frequencies among healthy controls occur in approximately 10% of gene-disease association studies [16]. It has been consistently noted that many more SNPs are out of HWE at any given significance threshold than would be expected by chance. SNPs severely out of HWE should therefore not be eliminated from the analysis, but flagged for further analysis after the association analyses are performed [17]. HWE may be violated because of chance, inbreeding, nonrandom mating, differential survival of marker carriers, genetic drifting, population stratification, genotyping error or combinations of these reasons [18]. Genotyping errors were generally small and did not generate sufficient deviations from HWE to be detected in this study. Of note, Hardy–Weinberg disequilibrium that typically results in excess homozygosity, is caused by many biologic phenomena that are

well observed in Upper Egypt like inbreeding, arranged marriages and greater social stratification [19].

As regards the association of the studied SNP polymorphisms with the susceptibility to RA, a protective effect of rs370515 CT, rs370515 CT + TT, rs3828057 CT, rs3828057 CT+TT and rs9826 GG genotypes against the wild genotypes of these SNPs (rs370515 CC, rs3828057 CC and rs9826 AA) was detected. Allelic frequencies of the three SNPs showed no statistically significant differences between RA patients and controls. Moderate LD between rs3790515 and rs3828057 and between rs3828057 and rs9826 but weak LD between rs3790515 and rs9826 in controls were detected. In RA patients, moderate LD was detected between rs3790515 and rs3828057, rs3790515 and rs9826 and between rs3828057 and rs9826.

rs3790515 - rs3828057 - rs9826 CTA haplotype was the most frequent haplotype in both cases and controls. The wild CCA and CTA haplotypes were associated with increased risk of RA, while TCG haplotype showed a protective effect.

The genotype-phenotype analysis of the three studied SNPs showed no significant differences in RA activity and laboratory parameters under the recessive models versus the dominant model also no significant differences were found in rs3790515 - rs3828057 - rs9826 haplotypes distribution between cases with remission and active RA concluding that RORC genetic polymorphisms had no association with the disease activity.

Genetic polymorphisms of RORC gene in RA were analyzed by Paradowska et al. [12]. The rs9017 G/A, rs9826 A/G and rs12045886 T/C showed no significant proportion differences between cases and controls. There was a high LD between rs9826 and rs9017 but weak LD between rs9826 and rs12045886; and rs12045886 and rs9017. The most frequent haplotype was ATA with a frequency of 49.6% in RA patients and 53.8% in control group and demonstrated significantly lower frequencies in RA patients compared to controls, concluding a protective effect. However genotype–phenotype analysis showed a significant association between rs9017 G/A and rs9826 A/G genotypes together with CRP indicating that genetic polymorphism of RORC gene may be associated with the RA activity.

RORC genetic polymorphisms were also analyzed in type 2 diabetes (T2DM) [20], secondary lymphedema [21], celiac disease [22] and BD [23]. The possibility that linkage of T2DM to 1q21-q23 region where RORC maps, suggesting that variation of RORC sequence could be a possible risk factor for T2DM, was studied by Wang et al. [20]. Approximately 21 kb of DNA was screened for sequence variation. From the 11 SNPs detected, only Ala454Gly variant was more frequent in T2DM patients than controls and so RORC sequence variation cannot explain the linkage of T2DM to this region. Statistically significant association was detected by Newman et al. [21] between tagSNP rs11801866 together with tagSNP rs12128071 in RORC gene and the susceptibility to develop secondary lymphedema after breast cancer. In a study by Medrano et al. [22] on the possible role of genes relevant for the Th17 immune response in CD susceptibility including RORC gene showed that, rs1521186 AA+AG genotypes were significantly more frequent in controls than cases indicating their protective role. Liao et al. [23] reported that high copy number variants (CNVs) of RORc2 and low CNVs of Foxp3 confer risk for BD but not for Vogt-Koyanagi-Harada

syndrome (VKH). Contrary to other studies that showed evidence of association between BD and VKH syndrome and RORC genetic polymorphism in Caucasian populations [24], [25] and [26], no association was found for tested SNPs in RORC gene (including the currently studied 3 SNPs) with BD or VKH syndrome. The tested SNPs showed significant differences in the alleles frequencies when comparing Han Chinese with Caucasians indicating a genetic heterogeneity towards disease susceptibility.

In the present study, RORc2 protein serum levels were significantly higher in patients than controls and were higher in patients with number of tender joints ≥ 4, number of swollen joints ≥ 1, RF ≥ 32 mg/dl, CRP ≥ 18 mg/dl and DAS 28 score ≥ 4.7. Serum levels of RORc2 in RA patients with rs3790515 CC, rs3790515 CT, rs3828057 CC, rs3828057 TT and rs9826 GG genotypes were significantly higher than healthy subjects with the same RORC genotypes. No association was detected between rs3790515, rs3828057 and rs9826 haplotypes and RORc2 serum levels in RA patients.

These results match with the results obtained by Paradowska *et al*. [12] who reported that serum RORc2 levels were higher in RA patients than controls and correlated positively with RA activity. Serum RORc2 levels were higher in RA patients with rs9826AA, rs12045886TT and -TC, and rs9017AA genotypes compared to healthy subjects with the same genotypes. Moreover RORc2 protein level was higher in RA patients with number of swollen joints more than 3 confirming the potential important role of Th17 cells in chronic inflammatory progression of RA.

Nistala *et al*. [4] reported that greater expression of RORc2 mRNA in synovial fluid mononuclear cells in juvenile idiopathic arthritis that closely mirroring RORc2 protein levels in Th17 cells from blood and joints. Fulton *et al*. [27] reported that CD4+ T cell expression of RORc2 is important in the pathogenesis of acute graft-versus-host disease (GVHD) evident by infusing donor T cells lacking RORC and as a consequence the isoform RORc2 leading to significantly attenuated acute GVHD and markedly decreased tissue pathology in the colon, liver, and lung. Significant increase in RORc2 and IL-17 genes expression (Th17 markers) that have osteoclast activating effects compared to GATA-3 and IL-4 (Th4 markers) which have an osteoprotective effect was reported by Behfarnia *et al*. [28] in patients with periodontitis compared to controls.

Burgler *et al*. [29] showed that the siRNA-mediated knockdown of RORc2 lead to increased FOXP3 expression and decreased expression of proinflammatory Th17 cells such as IL-1β, IL-6, and IL-17A indicating the essential role of RORc2 in the regulation of inflammatory processes and therefore would represent an innovative therapeutic regiment for treating autoimmune diseases. RORc2/FOXP3 ratios dysregulations were reported in juvenile idiopathic arthritis [30], type 1 diabetes [31], neuro-Behçet's Disease [32], asthma [33] and chronic obstructive pulmonary disease [34]. Increased FOXP3/RORc2 ratio was reported by Jamshidian *et al*. [35] after plasma exchange therapy that may be a mechanism by which this procedure exerts its improving effects in multiple sclerosis disease. Luo *et al*. [36] showed that changes in microbiota of the gut are intimately linked to significant alterations in Th17/Treg balance, mediated by epigenetic mechanisms, and participating in the development of irritable bowel disease, obesity and may be other chronic

inflammatory conditions. Inversely, a significant decrease in the transcript level of RORc2 was reported in the common variable immunodeficiency patients [37], ICOS-deficient [38) and hyper-IgE patients [39].

In conclusion, RORC genetic polymorphisms rs3790515, rs3828057 and rs9826 have a correlation with the risk but not with the activity of RA suggesting that these SNPs may be used as a genetic marker for the predisposition of RA in Egyptians. RORc2 serum levels have a positive correlation with the risk and activity of RA suggesting that RORc2 inhibitors could play a role in controlling the activation of the disease. Small sample size and lack of functional characterization of the currently studied SNPs are limitations of this study. Therefore, further studies of the association in a big population and the function of these SNPs in the expression of RORc2 and T cell activation are warranted.

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