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Forkhead Box P3 (Foxp3) serum level and Foxp3 Gene-Promoter polymorphisms in Egyptian rheumatoid arthritis patients: A case-control study

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Abstract

Regulatory T (Treg) cells are the chief player in induction of autotolerance and the transcription factor, Forkhead Box P3 (Foxp3), is the master regulator of their development and function. Polymorphisms in Foxp3 locus affect Foxp3 expression and can influence Treg cell function. This study aimed to determine the frequency of -3279C/A and -924A/G polymorphisms in the promoter region of the Foxp3 gene in Egyptian rheumatoid arthritis (RA) patients in comparison to apparently healthy controls, to test their association with Foxp3 serum levels as well as with patients' clinical and laboratory features. Also, to evaluate Foxp3 serum level as a putative measure of Foxp3+ Treg cells-mediated immune regulation and disease activity. A total of 136 subjects (68 RA patients and 68 controls) were studied for determining the frequency of both -3279 C/A and -924 A/G polymorphisms in the Foxp3 promoter region by PCR-RFLP and measuring their Foxp3 protein serum levels by ELISA. Our results indicated that; -3279 Foxp3 CA and AA genotypes were significantly higher in patients than controls (OR (95% CI) = 2.86 (1.31-6.26) and 2.79 (1.11-7.07), $P=0.008$ and $p=0.03$, respectively). Similarly, -924 AG genotype was significantly higher in patients than controls (OR (95% CI) = 2.92 (1.35-6.34); $P=0.006$). A significantly higher risk of RA was associated with the Foxp3 polymorphic variants -3279 A and -924 G. There was a statistically significant elevation in Foxp3 serum levels among patients, which was positively correlated to disease activity score and disease grade. In conclusion, Foxp3 polymorphisms influenced the risk of developing RA, but did not influence disease severity or activity. Serum level of Foxp3 is not a reliable indicator of Treg-mediated immune regulation in RA patients.

Keywords: Treg, Foxp3, RA

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Introduction

Rheumatoid Arthritis (RA) is a typical common chronic inflammatory autoimmune disease characterized by joint, bone and cartilage destruction and T cell infiltration of the synovium. RA occupies the forty-second place among diseases highly attributed to global disability, with a two-fold higher morbidity among women compared to men.¹ It is a disease of multifactorial origin; the pathogenesis of which represents a contribution of genetic factors, epigenetic dysregulation in addition to hormonal and environmental influences. The genetic component involves the combined effect of multiple gene variants, not just mere mutations of a specific gene. Although certain HLA-DR alleles play a significant role in genetically determined susceptibility to RA, they are not sufficient for disease induction.²

Foxp3+ regulatory T (Treg) cells are the chief player in induction of autotolerance and immune homeostasis and Foxp3 is their lineage-determining transcription factor and the master regulator of their development and function. Foxp3 gene is located on chromosome Xp11.23 which is an autoimmune diseases susceptibility locus. Consequently, it has been nominated as a candidate gene in pathogenesis of RA.^{3,4}

Different mutations in Foxp3 gene have been described; mutations involving Foxp3 functional domains markedly alter Foxp3 nuclear transport, DNA binding, complex formation with other transcription factors and ultimately its transcriptional activator and repressor functions that result in the heterogeneous biological abnormalities of "Immunodysregulation Polyendocrinopathy Enteropathy X-linked (IPEX)" syndrome. On the other side, polymorphisms in Foxp3 promoter region, mostly affect Foxp3 expression level and consequently alter Treg cell number and function as reported in chronic inflammatory conditions as RA, systemic lupus erythematosus, psoriasis, autoimmune thyroiditis and allergic rhinitis.^{3, 5-10}

Five functionally relevant single-nucleotide polymorphisms (SNPs) have been described in the Foxp3 promoter region; of which, the significance of -3279C/A and -924A/G in immune dysregulation have been appreciated in multiple studies.¹¹⁻¹⁵ The - 3279C/A and - 924A/G polymorphisms alters the Foxp3

transcription by abrogating E47, c-Myb and GATA3 transcription factors attachment to their binding sites respectively, and subsequent autoimmune disease development.^{6, 12}

Currently, it became certain that ethnicity greatly influences polymorphisms in different genes implicated in the pathogenesis of several immune diseases including RA. Consequently, the aim of this study was to determine the frequency of 2 single nucleotide polymorphisms in the promoter region of the Foxp3 gene; - 3279C/A (rs3761548) and -924A/G (rs2232365) in Egyptian RA patients in comparison to apparently healthy controls, to test their association with Foxp3 serum levels as well as with patients' clinical and laboratory features. Also, to evaluate serum Foxp3 protein level as a putative measure of Foxp3+ Treg cells-mediated immune regulation and disease activity.

Subjects and Methods

Subjects and Study design

This case control study was conducted in the Medical Microbiology and Immunology Department, Faculty of Medicine, Zagazig University during the period from December 2018 to October 2019. It included 68 Rheumatoid arthritis patients (62 females and 6 males) attending the outpatient clinic of the Rheumatology and Rehabilitation Department, Zagazig University Hospitals. Patients fulfilled ≥ 4 of criteria of the American College of Rheumatology (ACR 1987).¹⁶

Sixty-eight apparently healthy subjects (60 females and 8 males) with no evidence or history of immunological disorder were included as the control group. The study protocol was reviewed and approved by the IRB of the Faculty of Medicine, Zagazig University (approval number 3717, June 2017). An informed consent was obtained from all participants before enrolment.

Patients were subjected to full clinical examination and laboratory data relevant to their medical condition including; C-reactive protein (CRP), rheumatoid factor (RF), erythrocyte sedimentation rate (ESR) and anticyclic citrullinated peptide antibodies (ACCP) were taken from their medical records.

Sample collection

Blood samples (3ml) were collected from all study subjects, and aliquoted in plain tubes for serum separation (for detection of Foxp3 level) and in EDTA tubes (for DNA extraction). Sera and anticoagulated blood tubes were stored at -20°C until examined.

DNA extraction

EDTA anticoagulated blood samples were subjected to genomic DNA extraction using Gene JET Whole Blood Genomic DNA Purification Mini Kit (Thermo SCIENTIFIC, China), according to the manufacturer's instructions.

Genotyping by Polymerase Chain Reaction-Restriction Fragment Length Polymorphism (PCR-RFLP)

-Amplification of promoter segments containing the polymorphisms

This was performed in a 25- μ l PCR reaction containing: 12.5 μ l of (2X) PCR Master Mix (Thermo SCIENTIFIC, china) that contains DNA polymerase (Phire Hot Start II), nucleotides and optimized reaction buffer including $MgCl_2$, two microliters of the extracted genomic DNA and 10 pmol of each primer (Thermo SCIENTIFIC, China) either forward 5'-CTTAACCAGACAGCGT AGAAGG-3' and reverse 5'-CATCATCACCACGCTCTGG-3', for *Foxp3* -3279 C/A polymorphism detection, or forward 5'-AGGAGAAGGAGTGGGCATT-3' and reverse: 5'-TGTGAGTGGAGGAGCTGAGG-3', for *Foxp3* -924 A/G polymorphism detection. Amplifications were performed in a PCR thermal cycler (Biometra, Germany) under the following conditions: initial denaturing at 95°C for 5 min, followed by 35 cycles at 95°C for 20 s; 55°C for 20s; and 72°C for 30s with a final extension at 72°C for 5 min.¹⁷

-Restriction enzyme digestion of the amplified segments and genotype characterization

Foxp3 -3279 C/A polymorphism: Ten microliters of the 399 base pairs (bp) PCR product were digested with 1 μ l of the restriction enzyme PstI (Thermo SCIENTIFIC, China) for 30 min at 37°C, then separated on ethidium bromide-stained

agarose gel (2.5 %). PstI digestion yielded a 399 bp product for the polymorphic allele A (i.e. undigested), and 188 and 211 bp products for the digested wild type allele (C). (Figure 1)

-Foxp3 -924 A/G polymorphism

Ten microliters of 249 bp PCR product were digested with 1 μ l of the restriction enzyme BsmBI (Thermo SCIENTIFIC, China) for 15 min at 37°C, then separated on ethidium bromide-stained agarose gel (3 %). BsmBI digestion yielded a 249 bp for the wild type allele A (i.e. undigested), and 117 and 132 bp products for the digested polymorphic allele G. (Figure 2)

Determination of FOXP3 protein serum levels by quantitative ELISA

Serum levels of Foxp3 protein were determined for patients and control subjects using FOXP3 (Human) ELISA Kit (Biovision, USA) with a detection range of 0.313 – 20 ng/ml. The assay was performed according to the manufacturer's instructions and results were read on a microplate reader (TECAN, Columbus plus, Australia) at 450 nm within 20 minutes.

Statistical analysis

The collected data were computerized and statistically analyzed using SPSS program version 18.0. Qualitative data were represented as frequencies and relative percentages. While quantitative data were expressed as mean \pm SD (Standard deviation). Chi square test was used to calculate differences between qualitative variables. Independent T test was used to calculate differences between quantitative variables in 2 groups in normally distributed data while Mann Whitney U test was used to calculate differences between quantitative variables in 2 groups in non-normally distributed data. Kruskal Wallis test was used to calculate differences between quantitative variables in more than 2 groups in non-normally distributed data. Spearman's correlation coefficient was used to calculate correlation between quantitative variables. P value of <0.05 indicates significant results.

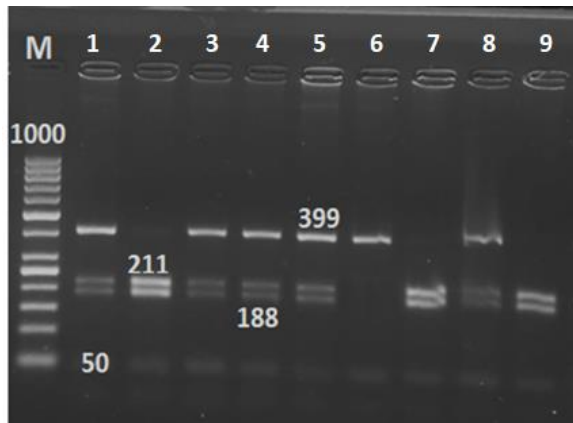


Figure 1. Agarose gel electrophoresis for detection of -3279 C/A *Foxp3* polymorphism. A single band of 399 bps indicates AA genotype (lane 6), two bands at 188 and 211bps indicates CC genotype (lanes 2,7,9) and 3 bands of 399, 211 and 188bps indicate CA genotype (lanes 1,3,4,5,8); (M): molecular weight marker (50, 100, 150, 200, 250, 300, 400, 500, 600, 700, 800,900 and 1000bps).

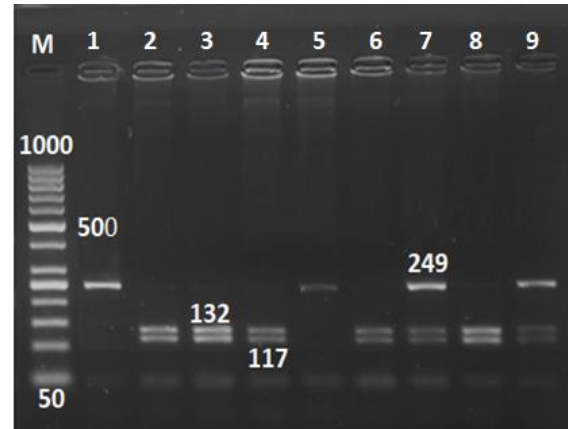


Figure 2. Agarose gel electrophoresis for detection of -924 A/G *Foxp3* polymorphism. A single band of 249 bps indicates AA genotype (lanes 1,5), two bands of 117 and 132 bps indicates GG genotype (lanes 2,3,4,6,8) and 3 bands of 249, 117 and 132 bps indicate AG genotype (lanes 7,9). (M) is the molecular weight marker (50, 100, 150, 200, 250, 300, 400, 500, 600, 700, 800, 900 and 1000bps).

Results

Characteristics of the study population

The studied population mean ages and range were 45.2, 24 – 60 years and 43.8, 25 – 62 years, for patients and control subjects, respectively. Clinical and laboratory data of

patients showed that all of them had an active disease as indicated by Disease Activity Score for 28 joints (DAS28) of more than 5.1 (range=5.8-6.4, mean=6.1), ESR was elevated in all patients and CRP was positive in 60%. (Table 1).

Table 1. Clinical and laboratory features of patients

Variable	Group I (n=68)	
Duration (years)		
Mean ± SD	4.83 ± 4.74	
Median(Range)	3 (0.83 – 20)	
Age of onset (years)		
Mean ± SD	40.34 ± 7.91	
Median(Range)	43 (21– 57)	
Number of swelling joints		
Mean ± SD	5.38 ± 2.91	
Median(Range)	5 (0 – 14)	
Number of tender joints		
Mean ± SD	17.26 ± 9.51	
Median(Range)	20 (2 – 28)	
DAS28		
Mean ± SD	6.12 ± 0.18	
Range	5.8 – 6.4	
Morning stiffness		
30 min	6	8.8
1 h	60	88.2
2 h	2	2.9

Table 1. (Continued)

Variable	Group I (n=68)	
Dry mouth		
Yes	52	76.5
No	16	23.5
Dry eye		
Yes	59	86.8
No	9	13.2
Grade:		
I	48	70.6
II	12	17.6
III	8	11.8
ESR		
Normal	0	0
High	68	100
CRP		
-ve	27	39.7
+ve	41	60.3
RF		
-ve	16	23.5
+ve	52	76.5
Anti-CCP		
-ve	44	64.7
+ve	24	35.3

Abbreviations: DAS28: Disease Activity Score for 28 joints; CRP: C-reactive protein; ESR: erythrocyte sedimentation rate; RF: rheumatoid factor (> 34IU / ml); anti-CCP: anticyclic citrullinated peptide antibodies (> 17 U/ ml), -ve: negative and +ve: positive.

Foxp3 serum levels

Foxp3 serum levels were significantly elevated among RA group compared to the control group (mean \pm SD = 5.52 ± 3.31 and 4.03 ± 1.19 , respectively, $P=0.01$) (Figure 3).

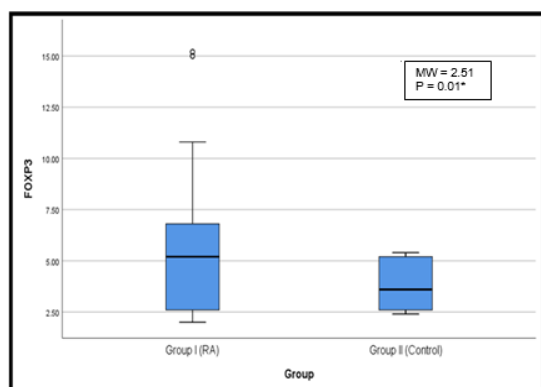


Figure 3. Foxp3 serum level in the studied groups. MW= Mann Whitney U test * = significant

Foxp3 serum level was significantly, directly correlated to disease activity as presented by DAS28 score and diseases grade (Table 2).

Also, it was significantly positively correlated to both disease duration and number of swelling joints (Table 2).

There was a statistically significant increase in Foxp3 levels among cases who tested positive for CRP, RF and ACCP (Table 3).

Foxp3 promotor polymorphisms

Foxp3 -3279 C/A polymorphism: The analysis of -2379 C/A Foxp3 gene polymorphism showed significant differences in distribution between cases and controls under different models of inheritance; RA patients showed a significantly higher frequency of CA and AA genotypes than the control subjects under the codominant model (i.e., both alleles are expressed) ($P=0.008$ and 0.03 , respectively).

Similarly, a significant association was observed with CA and AA genotypes under the

dominant model [OR 2.84; CI (1.40–5.72); $P=0.003$]. Under the over dominant model (i.e., heterozygote advantage; heterozygous individuals have a higher fitness than homozygous ones), CA genotype frequency was significantly higher in patients with RA compared to the control subjects (45.2% vs 28.3%, respectively $P=0.001$).

Also, there was a statistically significant increase in the frequency of A allele (OR=2.06, $P=0.001$) among RA group compared to the control group (Table 4).

Foxp3 -924 A/G polymorphism: The analysis of -924 A/G Foxp3 gene polymorphism revealed statistically significant increase in frequency of AG genotype and G allele (OR=1.65) among patients compared to the control group. Under the codominant and the over dominant models, AG genotype frequency was significantly higher in patients [OR 2.92; CI (1.35–6.34); $P=0.006$] and [OR 2.4; CI (1.19–4.86); $P=0.01$], respectively. A significant association was also observed with AG and GG genotypes under the dominant model, [OR 2.87; CI (1.34–6.12); $P=0.006$] (Table 5).

Table 2. Correlation of serum FOXP3 level with age and clinical data of RA patients

Variable	Foxp3 Group I (RA) (n=68)	
	r	*P value
Age	0.08	NS
Duration	0.24	0.02
Age of onset	0.10	NS
Number of swelling joints	0.21	0.03
Number of tender joints	0.15	NS
DAS28	0.50	<0.001
Morning stiffness	0.06	NS
Grade	0.42	<0.001

DAS28: Disease Activity Score for 28 joints. r: Spearman correlation coefficient test. *: $P>0.05$ is not significant (NS).

Table 3. Relation between Foxp3 serum level and laboratory findings among RA group

Variable		No	group I (RA) Foxp3		MW	*P value
			Mean±SD	Median		
CRP:	-ve	27	3.6±1.92	2.7	4.07	<0.001
	+ve	41	6.78±3.44	6.8		
RF:	-ve	16	4.25±2.55	3.75	2.03	0.04
	+ve	52	5.72±3.51	6		
Anti-CCP:	-ve	44	3.8±1.28	5	2.58	0.03
	+ve	24	5.66±3.39	7		
Hb:	Normal	45	4.80±3.65	5	1.87	NS
	Low	23	5.89±3.1	6		
Creatinine	Normal	63	5.52±3.16	5.2	1.24	NS
	High	5	5.51±3.64	5		
Platelets:	Low	4	4.15±1.31	4	KW 4.06	NS
	Normal	61	5.77±3.38	5.5		
	High	3	4.53±1.44	4.5		

MW: Mann Whitney U test. KW: Kruskal Wallis test. *: $P>0.05$ is not significant (NS), -ve: negative and +ve: positive.

Table 4. Foxp3 3279 C/A genotype frequency in female subjects and allele frequency among all subjects in the studied groups

Variable		Group I (RA)		Group II (Control)		χ^2	* <i>P</i> value	OR (95% CI)
		Females (<i>n</i> =62)		Females (<i>n</i> =60)				
		No	%	No	%			
Codominant	CC	19	30.6	33	55	Ref	NS	--
	CA	28	45.2	17	28.3	7.06	0.008	2.86(1.31-6.26)
	AA	15	24.2	10	16.7	4.87	0.03	2.79(1.11-7.07)
Dominant	CC	19	30.6	33	55.9	Ref	NS	--
	CA+AA	43	69.4	27	44.1	8.65	0.003	2.84 (1.40-5.72)
Recessive	CC+CA	47	75.8	50	83.3	Ref	NS	--
	AA	15	24.2	10	16.7	1.62	NS	1.73 (0.74-4.03)
Over dominant	CC+AA	34	54.8	43	71.7	Ref	NS	--
	CA	28	45.2	17	28.3	3.86	0.04	2.04 (1-4.16)
Allele:		(n=130) ^a		(n=128) ^a				
	C	69	52.9	91	69.9	8.21	0.001	2.06(1.25-3.39)
	A	61	47.1	37	30.1			

χ^2 : Chai square test. OR: Odds ratio CI: Confidence interval. *: P>0.05 is not significant (NS)

^a: The small number of hemizygous males (6 patients & 8 controls) are included in allele frequency analysis.

Table 5. Foxp3 -924 A/G genotype frequency in female subjects and allele frequency among all subjects in the studied groups.

Variable		Group I (RA)		Group II (Control)		χ^2	*P value	OR (95% CI)
		Females (n=62)		Females (n=60)				
		No	%	No	%			
Codominant	AA	13	21	23	38.3	Ref	NS	--
	AG	46	74.2	35	58.3	7.62	0.006	2.92(1.35-6.34)
	GG	3	4.8	2	3.3	1.82	NS	2.49(0.65-9.56)
Dominant	AA	13	21	23	38.3	Ref	NS	--
	AG+GG	49	79	37	61.7	7.65	0.006	2.87 (1.34-6.12)
Recessive	AA+AG	59	95.2	58	96.7	Ref	NS	--
	GG	3	4,8	2	3.3	0.10	NS	1.22 (0.35-4.20)
Over dominant	AA+GG	16	25.8	25	41.7	Ref	NS	--
	AG	46	74.2	35	58,3	6.02	0.01	2.4 (1.19-4.86)
Allele:		(n=130) ^a		(n=128) ^a				
	A	73	56.2	88	67.6	3.99	0.04	1.65(1.01-2.70)
	G	57	43.8	40	33.4			

χ^2 : Chai square test. OR: Odds ratio CI: Confidence interval *: P>0.05 is not significant (NS)

^a: The small number of hemizygous males (6 patients & 8 controls) are included in allele frequency analysis.

Genotype association with clinical and laboratory features, and Foxp3 level

There were no statistically significant differences between -3279 C/A Foxp3 genotypes regarding patients' clinical and laboratory findings except for an increase in

disease duration (KW= 6.89, P=0.03) and morning stiffness duration ($\chi^2=10.18$, P=0.04) among patients with genotype AA. There were no differences between -924A/G Foxp3 genotypes regarding clinical or laboratory findings. There was a significant decrease in

Foxp3 level among patients with -3279 AA genotype ($P=0.03$), but there was no difference among -924 GG genotype (Table 6).

Table 6. Relation between serum Foxp3 level and -3279 C/A and -924 A/G Foxp3 genotypes among RA group.

Variable		No	Group I (RA) Foxp3		KW	*P value
			Mean± SD	Median		
-3279 C/A	CC	22	7.18±3.81	7	7.09	0.03
	AC	28	5.51±3.83	5		
	AA	18	4.24±2.22	3.75		
-924 A/G	AA	14	5.49±3.42	5	0.46	NS
	AG	46	6.25±4.13	6		
	GG	8	4.79±2.23	4.5		

KW: Kruskal Wallis test *: $P>0.05$ is not significant (NS) SD: standard deviation.

Discussion

Sixty-eight RA patients were included in our study. Unsurprisingly, the highest percentage of our patients were females (91.2%). Sex bias in autoimmune diseases is attributed to X chromosome-linked immune related genes, their differential expression in both sexes (i.e. sexual dimorphism) and sex-specific epigenetic regulation including the influence of sex hormones.¹⁸

The mean duration of RA disease was 4.8 years. All patients had an active disease as indicated by a mean DAS28 score of 6.1 (i.e., more than 5.1) and elevated ESR. Positive CRP, rheumatoid factor and anti-CCP were detected in 60%, 76% and 35% of the patients, respectively; more or less similar results were detected in other studies; Alzifzaf *et al.*¹⁹ and Khalil *et al.* in Egypt² and Hashemi *et al.* in Iran.³

Foxp3 expression is subjected to two main types of regulation, transcriptional and posttranslational, both of which are responsive to positive and negative regulation by factors in the tissue environment, including cytokines, metabolic mediators and inflammatory factors.²¹ In this perspective, serum level of Foxp3 was postulated to be a reflection of the overall expression process as well as the activity state of Treg cells that takes part in the inflammation rather than a mere reflection of the individual genotype.

In the present study, the mean Foxp3 serum level was significantly higher in patients than control subjects, this was in accordance with findings of a study by Paradowska-Gorycka *et al.*

who reported an elevated serum Foxp3 more in patients than control group (51% vs 18%, respectively).¹⁷ Higher and lower mean Foxp3 levels were reported by Alzifzaf *et al.*¹⁹ and Gaafar *et al.*²² (16.9 and 1.01 ng/ml in patients with rheumatoid arthritis vs 26.4 and 1.72 ng/ml in control subjects, respectively).

High Foxp3 serum levels in patients compared to controls were explained as attempts of immune system to tame the ongoing inflammation; justified by the observation that elevated Foxp3 serum levels were associated with reduced disease activity in some studies.¹⁷ Nevertheless, elevated Foxp3 serum level in our study was associated with higher disease activity and elevated inflammatory and autoimmune markers.

Treg cell lineage and functional stability depends on the efficient expression of Foxp3 and several mechanisms act to ensure this; T cell receptor signaling, cytokine signaling (IL2 via STAT5 & TGFB via SMAD 2,3,4) and methylation pattern of the Treg cell specific demethylated region (TSDR) that determines efficacy of transcription factors binding.² However, Treg cells, like other immune cells exhibit some degree of plasticity that allows them to adapt to the changing environmental cues. In this context, Foxp3+ Treg can acquire some features of the immune cells they control, which is beneficial for the immune response, but in some autoimmune conditions dysfunctional plasticity of Treg cells is observed with acquisition of a helper function, secretion of pro-inflammatory cytokines and loss of suppressive function, but maintaining Foxp3

expression. They are thus termed "Helper T cell-like Treg cells". Here they fail to maintain peripheral tolerance but mediate immunopathology.²¹

Supporting this idea, in case of rheumatoid arthritis, IL17 secreting Foxp3+ Treg cells (T_H17-like Tregs) were detected in the synovial fluid samples of patients with active RA. Moreover, in a collagen-induced arthritis mouse model, the conversion of Foxp3+ Treg cells into T_H17 cells mediated by IL-6 and IL-1 β had an important role in the pathogenesis of autoimmune arthritis.^{23, 24, 25} Meanwhile, Studies had proved that Foxp3 expression is not an exclusive character of Treg cells and promiscuous Foxp3 expression occurs transiently in human conventional T cells (Tcons) during initial rounds of activation-induced proliferation.²⁶

In addition to the above-mentioned facts that would explain the presence of an active disease despite elevated Foxp3 level, it has been reported that even with normal Treg cell function, the susceptibility of certain effector T cell (Teff) types and states to the suppressive action of Treg cells may differ;²⁷ Teff cells can overcome the inhibitory effect of Treg cells in autoimmune diseases.²⁸ Therefore, Treg cells alone may not be able to control robust T cell and B cell activation.^{29, 30} Consequently, Treg/T_H17 cell balance was proposed as a new paradigm for autoimmunity rather than the sole Treg state.^{31,32}

The present study revealed a significant increase in the frequency of AC and AA genotypes, and A allele among RA group compared to the control group. This was consistent with the results of studies on Iranian and Polish RA patients^{3, 17} as well as on Turkish osteoarthritis (OA) patients,¹³ where Foxp3 - 3279 A allele was associated with a higher risk of disease than the wild type C allele.

The association of -3279 C/A polymorphism with other immune diseases have been reported; Gao *et al.* (2010) found an increased risk of psoriasis associated with the AC and the combined AC + AA genotypes compared with the wild CC genotype in a Han Chinese population.⁸ This polymorphism was also associated with allergic rhinitis and unexplained recurrent spontaneous abortion (URSA) in the same population.^{14, 15} The -3279 C/A polymorphism in the Foxp3 gene was also

associated with the development and recalcitrant nature of Graves' disease⁹ and with a higher risk of systemic lupus erythematosus with lower anti-dsDNA levels in female patients in Taiwan.⁷

Foxp3 -924A/G genotype and allele frequency study showed an increase in frequency of AG genotype and G allele among RA group compared to the control group. Such finding agrees with that of Paradowska-Gorycka *et al.*¹⁷ while Cekin *et al.*¹³ in their study on knee OA detected no association between Foxp3 - 924A/G polymorphism and disease development. Foxp3 - 924A/G polymorphism was also associated with an increased risk of developing psoriasis.³³ and vitiligo.³⁴

The present study demonstrated a decrease in Foxp3 level among cases with Foxp3 - 3279 AA genotype and with Foxp3 - 924 GG genotype. Similarly, Hashemi *et al.*³ who investigated Treg cell frequency in PBMC of Foxp3 -3279 genotypes, demonstrated a significant decrease in Treg cell frequency among patients and control subjects with AA genotype compared to the other genotypes of the same group.

Our results showed that the - 3279 A and - 924 G alleles are RA risk alleles in the Egyptian population as they were significantly higher in patients than control subjects. Due to female predominance in our study population, the small number of hemizygous males (possessing a single copy of X chromosome) was included in allele frequency analysis. Females on the other hand, have two copies of X chromosome but normally one of them is randomly inactivated to balance the dosage of X chromosome-linked genes in both sexes, except for 15% of X-linked genes that escape inactivation. Indeed, Foxp3 gene is subjected to inactivation and one of the two Foxp3 alleles undergoes inactivation so that heterozygous females with a Foxp3 mutation have ~ 50% of their cells with active mutant allele and ~50% with active wild-type allele. However, as exceptions of this rule, skewed X chromosome inactivation (nonrandom silencing of the X chromosome) and imprinting expression of X chromosome genes (allelic expression depends on the sex of the parent from whom the allele was inherited) can occur, which should be taken into consideration.^{35, 36} Therefore, studying significant numbers of male

patients may contribute to a better understanding of the influence of these polymorphisms in the development, severity and progression of the disease.

Our results denied the presence of associations between the studied Foxp3 genotypes with any of the patients' clinical or laboratory findings indicative of disease activity or severity. Controversial results were reported for such associations; Hashemi *et al.*³ reported a more severe disease phenotype with statistically significant increase in disease duration, activity, deformity, anti-CCP and RF in patients with -3279 Foxp3 CA and AA genotypes compared to CC genotype. On the other hand, Paradowska-Gorycka *et al.*¹⁷ detected no association between the Foxp3 -3279 C/A or -924 A/G variants and disease phenotype concerning disease activity, laboratory variables and extra-articular manifestations among patients with RA. Discrepant results are possibly due to the polyetiologic nature of the disease with involvement of other factors, apart from Foxp3 genotype, in determining disease severity, progression and response to treatment.

In conclusion, the findings of our study indicated that Foxp3 polymorphisms influenced the risk of developing RA, but such polymorphisms did not influence disease severity or activity probably due to involvement of other interacting variables. Although Foxp3 represents a chief marker of Treg cell development and function, Foxp3 serum level is not a reliable indicator of Treg-mediated immune regulation in RA patients. Its exact significance in allergic and autoimmune disorders needs further investigations.

Author Contributions

RR conceived and designed the study, RR and EI reviewed the relevant literature and formulated the hypothesis. SA performed patient evaluation and participant selection. EI performed patient sample and data collection and provision of study material and reagents. RR and EI performed the ELISA and genetic molecular laboratory studies. RR and EI participated in statistical analysis and formulation of results. RR drafted the manuscript. AA and TM supervised all steps of the study. All authors reviewed and approved the final manuscript.

Declaration of Conflicting Interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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Ethical approval

The study protocol was reviewed and approved by the IRB of the Faculty of Medicine, Zagazig University (approval number 3717, June 2017).

Informed consent

An informed consent was obtained from all participants before enrolment

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