Reduced Folate Carrier Gene-1 G80A Polymorphism is Not Related to Methotrexate Response in Egyptian Rheumatoid Arthritis Patients

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Abstract

Background: **Methotrexate (MTX) is considered the prime drug in rheumatoid arthritis (RA) treatment. However, response to MTX varies considerably among patients. Single nucleotide polymorphisms (SNPs) in the genes coding for MTX cellular pathway could be used to predict MTX response which would be clinically beneficial. The reduced folate carrier-1 (RFC-1) is an anion exchanger which plays a significant role in the transport of MTX and folates into the cells. *RFC-1* G80A SNP is reported to affect MTX response in some ethnic populations.** *Aim:* **aimed to examine the relation between *RFC-1* G80A SNP with MTX response in Egyptian RA patients.** *Patients and Methods:* **74 newly diagnosed RA patients within the first month of MTX therapy and 78 healthy controls were included in the present study. Patients' clinical data and laboratory investigations were recorded. The response to MTX in RA patients was evaluated after 3 months of MTX therapy using the European League Against Rheumatism (EULAR) response criteria. RA patients were then classified into MTX responders and MTX non-responders. *RFC-1* G80A SNP was investigated using polymerase chain reaction and restriction fragment length polymorphism (PCR-RFLP).** *Results:* **The results of the present study showed no statistically significant differences between MTX responders and non-responders regarding genotype and allele frequencies of *RFC-1* G80A SNP (p>0.05) in Egyptian RA patients, in addition to recessive, dominant, codominant, homozygotic, and per-allele genetic models (p>0.05).** *Conclusions:* **The current study revealed no association between *RFC-1* G80A SNP and MTX response. Moreover, *RFC-1* A80G SNP was not associated with susceptibility to RA.**

Keywords: Rheumatoid arthritis, Methotrexate, Response, *RFC-1* SNP, Egyptian

Introduction

Rheumatoid arthritis is a chronic systemic inflammatory autoimmune disease that is currently considered primarily as a joint disorder. However, it also involves several other organ systems, which include the pulmonary, cardiovascular, ocular, and cutaneous systems[1]. The incidence of RA has been assessed to be approximately 0.24 percent globally[2]. A similar prevalence (0.29%) was reported in Egypt in

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2015[3]. RA patients usually suffer from joint stiffness and arthralgia leading to disabilities[4]. The disease causes physical impairment and mental stress in addition to an enormous financial burden to patients, their families, and society[5]. MTX is the primary drug prescribed in RA treatment either solely or together with disease-modifying antirheumatic drugs (DMARDs)[6]. However, major drug disadvantages are that only about 50% of patients show a good clinical response, and 30% of patients discontinue therapy due to the unpredictable appearance of side effects[7]. Determining the genetic predictors affecting the treatment response in RA could aid in the early administration of alternated or combined DMARD treatment in patients who are not likely to show a good response to MTX monotherapy to avoid the consequent morbidity and mortality[8]. MTX is transported inside the cells by reduced folate carrier-1[9]. RFC-1 is a constitutively expressed folate transporter that exhibits a high affinity for MTX and plays a major role in the transport of MTX and folate into the cells[10]. The most common single nucleotide polymorphism within the *RFC-1* gene, G80A (rs1051266), has been reported to affect the transporter function[11]. This SNP is characterized by a change of guanine to adenine at position 80 of the transcription start region[12]. The *RFC-1* G80A SNP affects residue 27 of the protein and substitutes arginine with histidine. The conversion of a strongly basic amino acid arginine into a weak base histidine affects folate substrate binding and the uptake rate which may modify human RFC-1 transport properties[8]. Results of the previous studies focused on the association of *RFC-1* G80A SNP with MTX response are inconsistent. Up to our knowledge, no previous studies have investigated the association between *RFC-1* G80A SNP and

MTX response among Egyptian RA patients. Therefore, this study aims to determine whether there is an association between *RFC-1* G80A polymorphism and MTX response in Egyptian RA patients.

Patients and Methods

Study population
The study was conducted on 74 Egyptian RA patients who were recently diagnosed with RA based on the American College of Rheumatology (ACR) 2010 criteria for RA classification and aged over 18 years with both males and females included. The patients were enrolled in the study during the first month of MTX treatment. After 3 months of therapy, patients were classified into 2 subgroups (a: 37 responders and b: 37 nonresponders) according to the response criteria[13] established by European League Against Rheumatism and based on the disease activity score 28 (DAS28) score after 3 months of therapy (DAS28-1) and an improvement from the baseline calculated by the formula $\Delta DAS28 = (DAS28\text{-}1)\text{-}(DAS28\text{-}0)$ (DAS28-0 represents DAS28 at the start of MTX treatment). Namely, good responders declared the following values: DAS28-1≤3.2 and ΔDAS28>1.2, while 3.2<DAS28-1≤5.1 and 0.6<ΔDAS28≤1.2 (or ΔDAS28>1.2 independently of DAS28-1 value) defined moderate responders ("Responders" are those with a good or moderate response) and finally poor responders (classified as non-responders) are those with a poor response value evaluated as ΔDAS28≤0.6 and DAS28-1>5.1 (or ΔDAS28≤0.6 independently of DAS28-1 value)[14]. The intake of biological therapy or another DMARD rather than MTX, intraarticular corticosteroids, and the presence of any other autoimmune disease were exclusion criteria for the study. All groups were subjected to full history taking, entailing age, sex,

smoking status, disease duration, MTX dose (7.5-25 mg weekly based on disease severity), its delivery method as well as other concurrent medications. Laboratory investigations (Rheumatoid Factor (RF), anti-cyclic citrullinated peptide (Anti-CCP), complete blood count (CBC), erythrocyte sedimentation rate (ESR), C-reactive protein (CRP), alanine transaminase (ALT), aspartate transaminase (AST) and creatinine) were documented from the patient's medical records. A total of 78 age and sex-matched healthy individuals, with no clinical history of RA and no family history of autoimmune disease were enrolled as healthy controls. They were selected from healthy blood donors.

RFC-1 G80A rs1051266 genotyping
Three milliliters of peripheral venous blood was collected in EDTA tubes, and genomic DNA was extracted from whole blood using QIAamp DNA Blood Mini Kit (catalog number 51104). *RFC-1* G80A genotyping was performed by PCR-RFLP method. Forward (5'-AGTGTCACCTTCGTCCCCTC-3') and reverse primers (5'- CTCCCGCGTGAAGTTCTT-3') were used[12].

PCR was carried out in a total reaction volume of 25 μl comprising 12.5 μl of PCR Master Mix (2x) (Applied Biotechnology company, Ismailia, Egypt), 10.5 μl of nuclease-free water, 0.5 μl of forward primer, 0.5 μl of reverse primer and 1 μl of template DNA (if the concentration is 35-50ng/ μl). PCR was carried out with the following cycling conditions: Initial denaturation at 95 °C for 5 min, followed by 35 cycles of denaturation at 95 °C for 30 s, annealing at 62 °C for 30 s, extension at 72°C for 1 min and then a final extension at 72°C for 5 min. The 230 bp PCR product (fig.1) was digested with HhaI fast digest enzyme (Thermo Fisher Scientific, Waltham, United States) at 37°C for 5-15 minutes and electrophoresed in 3% agarose gel. Homozygotes for *RFC-1* 80GG genotype were discriminated by 3 fragments (125 bp, 68 bp, and 37 bp), whereas homozygotes for the 80AA genotype were discriminated by 2 fragments (162 bp and 68 bp) while heterozygotes with the 80AG genotype were discriminated by 4 fragments (162 bp, 125 bp, 68 bp, and 37 bp) as shown in Figue 2
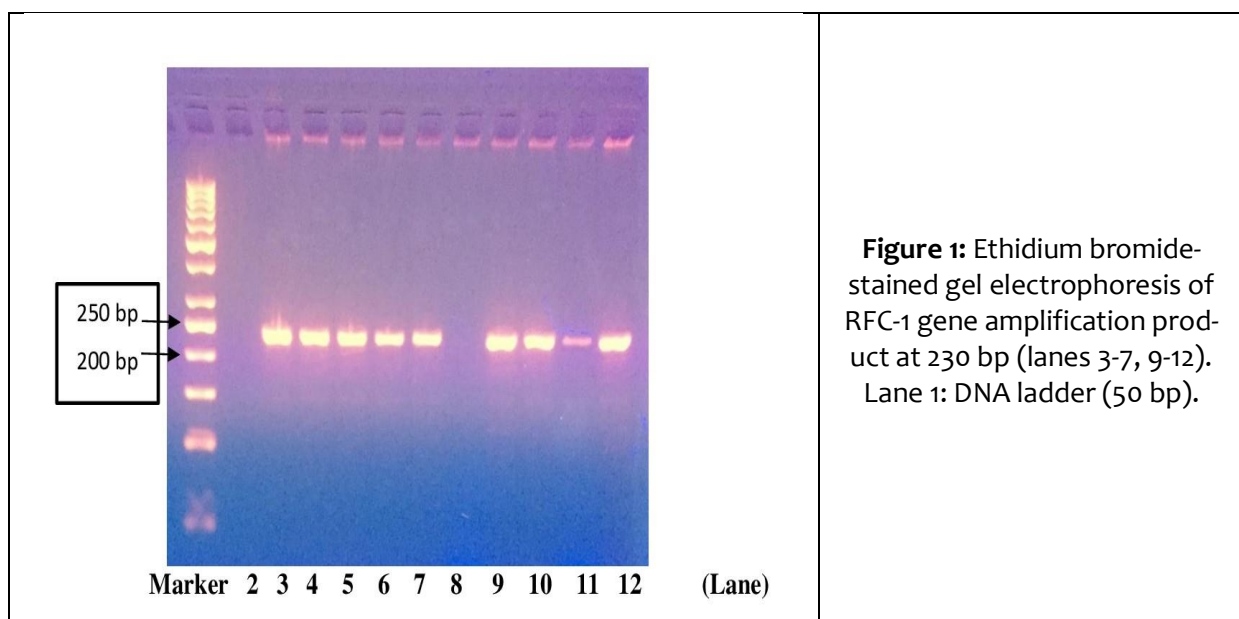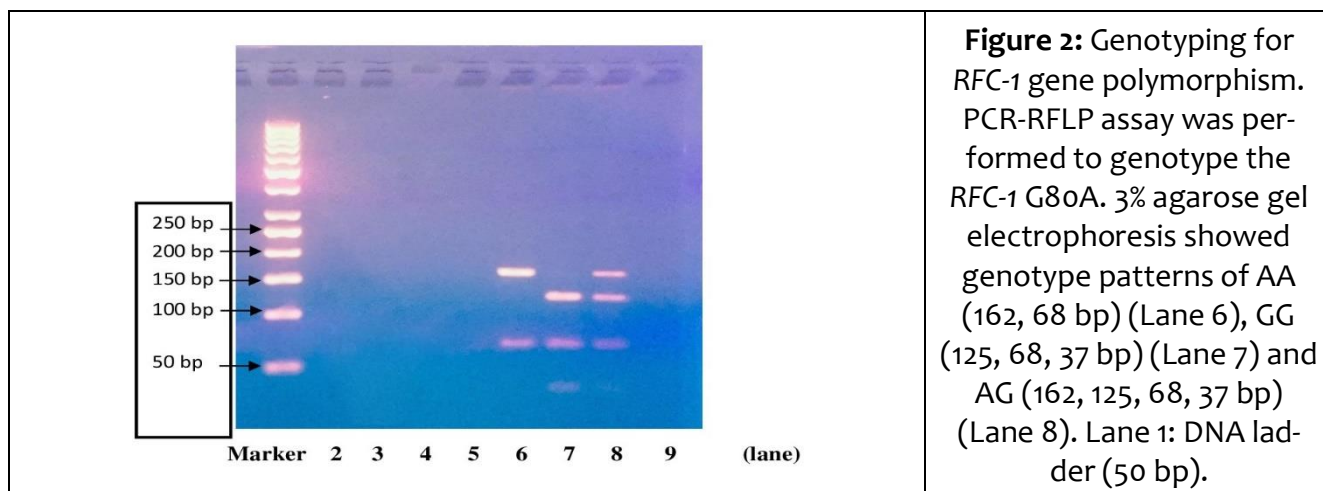

Figure 1: Ethidium bromide-stained gel electrophoresis of RFC-1 gene amplification product at 230 bp (lanes 3-7, 9-12). Lane 1: DNA ladder (50 bp).

Figure 2: Genotyping for *RFC-1* gene polymorphism. PCR-RFLP assay was performed to genotype the *RFC-1* G80A. 3% agarose gel electrophoresis showed genotype patterns of AA (162, 68 bp) (Lane 6), GG (125, 68, 37 bp) (Lane 7) and AG (162, 125, 68, 37 bp) (Lane 8). Lane 1: DNA ladder (50 bp).

Statistical analysis:

Statistical analysis was carried out using IBM SPSS Statistics v 22.0. All numerical data were expressed as the mean ± SD. The Shapiro-Wilk test was used to test normality. Numerical parametric data were compared between the study groups using a t-test for independent samples, while nonparametric data were compared using the Mann–Whitney *U*-test. Allele and genotype frequencies in addition to other categorical variables were compared between the study groups by performing a chi-square test or Fisher's exact. Tests for Hardy–Weinberg equilibrium was carried out using the chi-square test. The odds ratio and 95% confidence interval were calculated for the genetic models. $P<0.05$ was statistically significant.

Results

A. Characteristics of patients
The study included 74 newly diagnosed RA patients treated with MTX for 3 months (66 females and 8 males) and classified into 37 responders and 37 non-responders to MTX treatment. The clinical and laboratory characteristics of all pa-tients are listed in Table 1. The mean age of patients was 44.76 ± 12.16 years, and the percentage of smoking was 1.4%. Of all, 54 (72.9%) patients were RF positive while 58 (78.4%) were Anti-CCP positive. The percentage of Anti-CCP positivity was found to be significantly higher in nonresponders (100%) than in the responders' group (56.8%), $p<0.05$. The mean baseline DAS280 score was 6.9 ± 1.28, whereas the mean DAS281 score after 3 months of MTX therapy was 5.41 ± 1.52 and the mean ΔDAS28 was 1.48 ± 1.22. The mean DAS281 score was significantly lower in respond-ers (4.46 ± 1.22) than nonresponders group (6.35 ± 1.17), $p<0.05$, while the mean ΔDAS28 was significantly higher in responders (2.52 ± 0.9) than nonrespond-ers group (0.45 ± 0.17), $p<0.05$. The mean dose of methotrexate given was 14.73 ± 3.78 mg/week. The mean ESR was 50.85 ± 30.87 mm/hr. while the mean CRP was 18.5 ± 20.91 mg/dl.

B. Relation between RFC-1 G80A SNP and MTX response
No statistically significant differences were detected in genotype and allele fre-quencies between the responder and the nonresponder groups (Table 2).

Table 1: Clinical and laboratory characteristics of patients group.			
Variables	**Total RA (N=74)**	**Responders (N=37)**	**Nonresponders (N=37)**	**p-value**
Age (years)	44.76 ± 12.16	45.75 ± 12.81	43.75 ± 11.57	0.483
Sex (n) (%)	F 66 (89.2%) M 8 (10.8%)	F 31 (83.8 %) M 6 (16.2%)	F 35 (94.6%) M 2 (5.4%)	0.261
Smoking n (%)	1 (1.4%)	0 %	1 (2.7%)	1
MTX dose/week (mg)	14.73 ± 3.78	13.91 ± 2.97	15.54 ± 4.33	0.08
DAS28o	6.9 ± 1.28	6.99 ± 1.41	6.8 ± 1.15	0.331
DAS281	5.41 ± 1.52	4.46 ± 1.22	6.35 ± 1.17	0.000*
ΔDAS28	1.48 ± 1.22	2.52 ± 0.9	0.45 ± 0.17	0.000*
RF (+ve) n (%)	54 (72.9%)	26 (70.3%)	28 (75.7%)	0.6
Anti-CCP (+ve) n (%)	58 (78.4%)	21 (56.8%)	37 (100%)	0.000*
ESR (mm/hr)	50.85 ± 30.87	51.54 ± 32.02	50.16 ± 30.1	0.983
CRP (mg/dl)	18.5 ± 20.91	20.11 ± 21.96	16.89 ± 19.98	0.236

*Data are reported as Means ± Standard Deviation. Comparisons were performed by student t-test for parametric numerical data, Mann Whitney for nonparametric numerical data, chi-square test, and Fisher exact for categorical data. *P < 0.05 is statistically significant.*

The genotypes were in Hardy-Weinberg equilibrium[15]. Additionally, dominant, recessive, codominant, homozygotic, and per-allele genetic models were performed to investigate the association of *RFC-1* G80A SNP with MTX response with no significant differences detected between analyzed groups (Table 3).

C.   Relation between RFC-1 G80A SNP and RA susceptibility
Regarding genotype and allele frequencies between RA patients and the control group, no statistically significant differences were detected (table 4). Dominant, recessive, codominant, homozygotic, and per-allele genetic models were also performed to assess the association between *RFC-1* G80A SNP and RA susceptibility but no significant differences were detected (table 5).

Discussion

RA is one of the most common autoimmune diseases, with increasing incidence worldwide[16]. If proper treatment is not administered early in disease onset, a significant number of patients will develop joint deformities and disability[5]. Currently, MTX is the DMARD of first choice in RA. However, response to MTX, despite being better than most other DMARDs, is not universal[17]. A significant number of patients do not respond well to MTX treatment or cannot tolerate it. Therefore, pre-treatment features that predict response to MTX are of particular interest and potential clinical utility[18]. Previous studies suggest that RA patients' genetic profile may have a substantial role in this variability, especially genes encoding proteins implicated in MTX mechanism of action[19]. RFC-1 is the major cell transporter of MTX[20]. Several studies have reported that *RFC-1* G80A SNP might be related to MTX response. However, it remains controversial whether the *RFC-1* G80A SNP may be a marker of MTX response[21]. To our knowledge, no previous research on the association between this SNP and MTX response in Egyptian RA patients. So, we have the association between *RFC-1* G80A SNP (rs1051266) and MTX response in RA patients attending Suez Canal University Hospital in Ismailia city.

Table 2: Genotypes and allele frequency of *RFC-1* G80A SNP among analyzed groups.			
Genotype	Responders (N=37)	Nonresponders (N=37)	p-value
Genotype (GG)	0 (0%)	3 (8.1%)	
Genotype (AG)	22 (59.5%)	21 (56.8%)	0.353[a]
Genotype (AA)	15 (40.5%)	13 (35.1%)	
Allele			
G	22 (29.7%)	27 (36.5%)	
A	52 (70.3%)	47 (63.5%)	0.382[b]

Fisher's exact [a] and Chi square test [b] test were used.
* Significant when p<0.05.

Table 3: Genetic model analysis of *RFC-1* G80A SNP in responders versus nonresponders.				
Genetic model	Responders N=37 (%)	Non-responders N=37 (%)	P value	OR (95 % CI)
Dominant model AA + GA GG	37 (100%) 0 (0%)	34 (91.9%) 3 (8.1%)	0.2397[a]	----
Recessive model AA GA + GG	15 (40.5%) 22 (59.5%)	13 (35.1%) 24 (64.9%)	0.6316[b]	0.79 (0.31 to 2.04)
Codominant model GA AA + GG	22 (59.5 %) 15 (40.5%)	21 (56.8%) 16 (43.2%)	0.8137[b]	1.12 (0.44 to 2.82)
Homozygotic model AA GG	15 (40.5%) 0 (0%)	13 (35.1%) 3 (8.1%)	0.2492[a]	----
Per-allele model A G	52 (70.3%) 22 (29.7%)	47 (63.5%) 27 (36.5%)	0.3831[b]	0.74 (0.37 to 1.46)

OD: Odds ratio, 95% CI: 95 % Confidence interval
Fisher's exact test [a] and Chi-square [b] were used.* Significant when p < 0.05.

The results of the present study showed no statistically significant differences between MTX responders and nonresponders regarding genotype and allele frequencies (p > 0.05), in addition to recessive, dominant, codominant, homozygous, and per-allele genetic models (p > 0.05). Our results were in agreement with multiple studies that evaluated the association of *RFC-1* G80A with MTX therapeutic outcome and found that *RFC-1* G80A SNP did not affect the response of Tamil Indian, Jordanian, Portuguese, Caucasian, Chinese, Serbian, and Spanish Caucasian RA patients respectively[8, 22- 27]. The present finding of no association between *RFC-1* G80A SNP and MTX response may be explained by the existence of other transport mechanisms, apart from RFC-1 protein, that allow and maintain the entry of folates into the cell. These mechanisms include proton-coupled folate transport-

ers (PCFT) and folate receptors (FRs) which are present in epithelial cell membranes. PCFT is a member of the SLCs superfamily and functions optimally at low pH. It is responsible for the folate transport activity in various tissues, mostly the small intestine [28,29].

Table 4: Genotypes and allele frequency of *RFC-1* G80A SNP among RA patients and controls.			
Genotype	RA patients (N=74)	Controls (N=78)	p-value
Genotype (GG)	3 (4.1%)	6 (7.7%)	
Genotype (AG)	43 (58.1%)	51 (65.4%)	0.319[a]
Genotype (AA)	28 (37.8%)	21 (26.9%)	
Allele			
G	49 (33.1%)	63 (40.4%)	
A	99 (66.9%)	93 (59.6%)	0.188[b]

Fisher's exact [a] and Chi-square test [b] test were used. * Significant when $p < 0.05$.

Previous studies declared that this carrier-mediated transporter that carries its function in a low pH medium possesses a high affinity for MTX as well as folic acid, and reduced folates[30]. It was demonstrated that this low-pH folate transport activity remained intact when RFC-1 function was impaired due to deletion, mutation, or loss of gene expression[31]. In addition, FRs which include α and β isoforms have pharmacological and physiological importance, especially in cells with defective RFC-1 function, because they can greatly allow MTX transport at low MTX blood levels (100–500 nM)[32]. It was obvious that activated mononuclear cells and synovial macrophages in RA patients' synovium give a selective expression to FRβ in which MTX uptake is conveyed through the receptor-mediated endocytosis mechanism[33]. Moreover, MTX resistance due to decreased FR expression was reported[34]. Another factor that may explain our present findings may be the presence of other SNPs in RFC-1 transporter that could counterbalance its functionality[23]. Therefore, this reveals the difficulty in predicting the response to MTX by investigating one SNP. In contrast to our results, multi

ple studies declared a significant association of *RFC-1* G80A SNP with MTX response[7,10,15,20,35-37]. Hayashi *et al.*, 2013 investigated multiple SNPs in the MTX pathway genes in Japanese RA patients treated with MTX alone vs MTX combined with biological DMARD. They declared a higher frequency of *RFC-1* 80A allele in MTX treated patients than in DMARDs treated patients while a higher frequency of 80G allele was recorded in MTX non-responsive patients treated by DMARDs. A low level of intracellular MTX uptake was noted in G allele patients (less potency) in comparison with those carrying A allele[15]. Kung *et al.*, 2014, in a meta-analysis study that tried to identify genetic variant associations with MTX therapeutic outcome, found that *RFC-1* G80A SNP was associated with MTX response[10]. The different results may be due to ethnicity differences in MTX pathway genes in addition to differences in study designs. They may also account for variations in the evaluation criteria of MTX response among studies. Most studies, including our study, assessed MTX response based on the EULAR response criteria[8,27], while some others assessed MTX response according to the ACR20 criteria[21,35].

Table 5: Genetic model analysis of *RFC-1* G80A SNP in RA patients versus controls.				
Genotype	RA Patients N=74 (%)	Controls N=78 (%)	P value	OR (95 % CI)
Dominant model				
AA + GA	71 (95.9%)	72 (92.3%)		0.507 (0.12 to 2.11)
GG	3 (4.1%)	6 (7.7%)	0.5483[a]	
Recessive model				
AA	28 (37.8%)	21 (26.9%)		0.61 (0.30 to 1.20)
GA + GG	46 (62.2%)	57 (73.1%)	0.1501[b]	
Codominant model				
GA	43 (58.1%)	51 (65.4%)		0.73 (0.38 to 1.42)
AA + GG	31 (41.9%)	27 (34.6%)	0.3559[b]	
Homozygotic model				
AA	28 (37.8%)	21 (26.9%)		0.38 (0.08 to 1.68)
GG	3 (4.1%)	6 (7.7%)	0.3413[a]	
Per-allele model				
A	99 (66.9%)	93 (59.6%)		0.73 (0.46 to 1.17)
G	49 (33.1%)	63 (40.4%)	0.1892[b]	

Fisher's exact [a] and Chi square [b] test were used. ***** Significant when $p < 0.05$

The current study used the EULAR response criteria because it is the most common criteria used to evaluate MTX response. Thus, the results of the studies with non-accordant definitions of the MTX response may not be directly comparable. In addition, it seems that only one SNP is insufficient to predict response to MTX therapy[38]. The results showed no statistically significant differences between RA patients and healthy controls regarding genotype and allele frequencies ($p > 0.05$). The same results were shown in the recessive, dominant, codominant, homozygotic, and per-allele genetic models ($p > 0.05$). Our results were in agreement with Hashiguchi *et al.*, 2016[39], Gonzalez-Mercado *et al.*, 2017[40], and Wang *et al.*, 2020[21], on the other hand, Muralidharan *et al.*, 2016[8] found that the heterozygous *RFC-1* AG genotype was associated with less susceptibility to RA. However, regarding the percentage of anti-CCP positivity in the current study patients, it was signifi-

cantly higher in nonresponders (100%) vs responders (61.5%), $p < 0.05$. This was consistent with Lima *et al.*, 2014[41] and Sharaki *et al.*, 2018[42]. In contrast, Saearsdottir *et al.*, 2011 [43] have presented no association between anti-CCP positivity and MTX response in RA patients. The significant association that was found between anti-CCP positivity and MTX unresponsiveness may be explained by the correlation between the anti-CCP antibodies and the consequent progressive disease course and functional disabilities, which were associated with less response to treatment[44]. A major limitation is this study is the investigation of only one SNP in the MTX pathway. However, further prospective multicentric studies with larger sample sizes from different geographical regions are required to support the results of the present study. In addition, genome-wide association studies are needed to reveal novel predictors of MTX response and toxicity by investigating several SNPs

in MTX transporters and metabolic pathways among Egyptian Patients.

Conclusions

The current study revealed no association between *RFC-1* G80A SNP and MTX response in the analyzed population. Moreover, *RFC-1* A80G SNP is not associated with susceptibility to RA. The study could be beneficial to the field of pharmacogenomics and precision medicine by aiding in the identification of SNPs related to MTX response in RA patients. However further studies with larger sample sizes are required to support the results of the present study.

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