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Impact of *TYMS* gene polymorphism on the outcome of methotrexate treatment in a sample of Iraqi rheumatoid arthritis patients – identification of novel single nucleotide polymorphism

Cross-sectional study

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Abstract

The current work aims to evaluate the association between genetic mutations in thymidylate synthetase (*TYMS* gene in exon1 and partial regions of promotor and intron 1 [877 bp, 657,220–658,096 bp]) and the therapeutic outcomes for rheumatoid arthritis (RA) Iraqi patients. An observational cross-sectional study involving 95 RA patients with established RA patients based on their methotrexate treatment responsiveness. Genetic sequencing of the TYMS gene was performed for all patients according to the instruction manuals of the sequencing company (Macrogen Inc. Geumchen, South Korea). Four polymorphisms were identified by sequencing 95 randomly selected patients in the noncoding region of TYMS. Three of these polymorphisms were found in the NCBI database's dbSNP (rs2853741, rs2606241, and rs2853742 SNPs), and one SNP polymorphism is novel (657334). The CTAT (657334, rs2853741, rs2606241, and rs2853742 SNPs) haplotype was significantly associated with responder with odd ratio, 95% confidence interval: 0.506, 0.281–0.912 (*P* value = .022). In contrast, the other haplotypes were not associated with MTX responsiveness. In the multivariate analysis, after adjusting to the effect of age, sex, smoking, and disease duration, the $TC_{rs2853741}$ genotype was associated with non-responders (*P* value = .030). In contrast, the $AC_{rs260641}$ genotype, after adjusting to the effect of age, sex, and smoking, was associated with non-responders (*P* value = .035). Genetic polymorphism of the TYMS gene, especially in $TC_{rs2853741}$ and $AC_{rs260641}$, predicts non-responder to MTX treatment in RA, while the presence of the CTAT haplotype predicts a good response to MTX treatment.

Abbreviations: CCSD = Congenital Cardiac Septal Defects, ESR = erythrocyte sedimentation rate, HFS = hand-foot syndrome, MTX = methotrexate, NSCLP = Nonsyndromic Cleft Lip and Palate, RA = rheumatoid arthritis, SNPs = single nucleotide polymorphisms, TYMS = thymidylate synthase, VNTR = variable number tandem repeat.

Keywords: methotrexate, polymorphism, rheumatoid arthritis, treatment response, TYMS gene

1. Introduction

Rheumatoid arthritis (RA) is a chronic systemic inflammatory disorder characterized by pain and swelling in the hands and feet on both sides.[1–3] Over time, if persistent inflammation isn't appropriately managed, it might cause permanent damage to the joint structures.[4–6] Methotrexate (MTX) is the first-line medication that has been used for the treatment of RA since the 1980s. It is an antifolate with significant anti-inflammatory and antiproliferative effects. The mechanism of MTX efficacy in RA is related, at least in part, to the intracellular inhibition of thymidylate synthase (TYMS), which is a crucial enzyme for thymidine biosynthesis, an essential nucleotide for DNA replication and repair. Because of its effectiveness, safety profile, and low cost, MTX is widely used to treat RA.[7,8] TYMS is a folate-dependent enzyme that catalyzes the reductive methylation of deoxyuridine monophosphate into deoxythymidine monophosphate; the methyl group donor in this reaction is N5, N10 methylenetetrahydrofolate, which is converted to dihydrofolate.[9]

Written informed consent obtained.

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The datasets generated during and/or analyzed during the current study are publicly available.

While preparing this work, the authors used [Grammarly Inc.] to [improve grammatical errors]. After using this tool, the authors reviewed and edited the content as needed and took full responsibility for the publication's content.

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Roughly 30% to 50% of patients have a poor response to MTX therapy or relapse with inadequate response to re-treatment, resulting in drug resistance.[10] Variations in gene expression or activity of enzymes along the folic acid-MTX pathway have been proposed to underlie the variability in medication pharmacokinetics and responsiveness to MTX seen across individuals.[11] Previous research showed that TYMS levels predict MTX therapy in RA patients.[12,13] Genetic polymorphisms in the TYMS gene have also been linked to fluctuations in TYMS levels,[14,15] which indicates that genetic polymorphism in the TYMS gene is the possible cause of the variability of MTX response seen in RA patients. To address this issue, pharmacogenomics studies focused on alterations in several genes involved in the genetic regulation of MTX activity and the impact of genetic variations on the clinical response to MTX in patients with RA.[16]

Few polymorphisms concerning TYMS have been reported in RA patients, rs2853542 polymorphisms which involve a change from cytosine to guanine at the twelfth nucleotide position inside the second repeat of the VNTR (Variable number tandem repeat) 3R allele, the presence of cytosine (3RC) disrupts the E-box, decreasing transcriptional activation compared to 3RG, thus reducing TYMS levels.[14] Multiple studies have investigated the relationship between this SNP and the TYMS 28 bp VNTR polymorphism by integrating data from both TYMS enhancer region (TSER) polymorphisms.[17,18] Even though the TYMS gene is important for MTX activation and biological activity, no studies examined this gene in RA patients and established its importance in predicting MTX response during RA treatment; we have undertaken this study to shed light on this subject.

This study aimed to investigate the clinical significance of previously unexplored regions of the *TYMS* gene (in exon1 and partial regions of promotor and intron 1 [877 bp, 657,220–658,096 bp]) in the context of MTX treatment outcomes for Arab-Iraqi RA patients.

2. Materials and methods

2.1. Study design

An observational study involved 95 Iraqi RA patients with established RA according to the revised 2010 American College of Rheumatology (ACR)/European League Against Rheumatism (EULAR) Classification Criteria for RA.[19]

Patients were divided into 2 groups, in which the responder group was defined as (disease activity score) DAS28 ≤ 3.2, and non-responder DAS28 > 3.2 after at least 3 months of MTX treatment (tablet or parenteral formulations), one reading of DAS28 used to calculate treatment response.[20–22] Responder groups included 47 patients and 48 patients as non-responders.

2.2. Study settings

All the patients in this study were recruited from the Rheumatology Department of Diwaniya Teaching Hospital. The study was performed between June 01, 2022 and March 01, 2023.

2.3. Inclusion criteria

Adult patients (age ≥ 18 years) with confirmed RA according to revised 2010 ACR/EULAR RA classification criteria,[23] and all patients should be on MTX for at least 3 months.

2.4. Exclusion criteria

Patients with co-existent diseases, other ethnic groups aside from Arabic ethnicity, other connective tissue diseases, patients who use additional disease-modifying antirheumatic drugs, biological drugs, incomplete data, patients with any chronic infectious diseases, cancer, hepatic or renal dysfunction, endocrinopathy, hematological and cardiac conditions or multiple sclerosis.

2.5. Variables

2.5.1. Disease activity (DAS28-ESR). In the present study, DAS28-ESR [erythrocyte sedimentation rate] (4 variables) was used to examine the disease activity of RA patients.[24] This score is a validated method for such purpose and has been validated in many studies and recognized by ACR/EULAR,[25–28] and it was calculated based on the following equation[24]:

$$DAS28-4 = 0.56 * \sqrt{(\text{Tender Joint Count} - 28)} + 0.28 * \sqrt{(\text{Swollen Joint Count} - 28)} + 0.70 * ln(ESR) + 0.014$$

Patients were classified as remission (<2.6), low (≥2.6–≤3.2), moderate (>3.2–≤5.1), and high (>5.1).[28]

2.5.2. Laboratory analysis. Sufficient venous blood samples were obtained from all patients (5 mL); the samples were taken between 08:00 AM and 10:00 AM during their routine visit to the Rheumatology Department. Measurement of erythrocyte sedimentation rate (ESR) was conducted using the modified Westergren method, which involves the use of whole blood that has been anticoagulated with EDTA (VISION Pro ESR Analyzer; Shenzhen YHLO Biotech Co., Ltd, China),[29–31] while rheumatoid factor (RF) measured using Chemistry Analyzer, Smart – 150; Geno Lab-TEK Corporation, Canada.

2.6. Sample size

Sample size estimation was based on the following equation:

$$minimum\ sample\ size\ \ (n) = p\frac{(1-p)\,Z^2_{0.95}}{d^2}$$

Where n is the minimal sample size, p is the prevalence of 2.34% of RA Iraq in 2023.[32] The Z represents the Z-score at a 95 % confidence interval, which equals 1.96; d represents the marginal error (0.03).[33] Thus, the minimal sample size was estimated to be approximately 97.

2.7. Primer optimization and PCR amplifications

The DNA template was amplified using the primer pair (Forward) and (Reverse) at annealing temperatures of 55, 58, 60, 63, and 65°C. The best annealing temperature for the primer was 60°C, as seen in Table 1. The temperature, time, and number of cycles for the PCR program are listed in Table 2. Table 3 summarizes the calculations used in this reaction.

Table 1

Primers sequence, GC%, annealing temperature (Ta), and product size of TYMS-gene.

TYMS gene		
Ta	60°C	
Product size	877 bp	
Primers	Sequence	GC%
Forward primer	GCCCTGGCGGTTTTTAATCA	50
Reverse primer	CATCGTCCTCCTCGCCTTAC	60

TYMS = thymidylate synthase.

2.8. SNP identification and genotyping

Genomic DNA was isolated from venous blood using a solid-phase DNA extraction method by agarose gel electrophoresis (1% agarose) [Bio-Rad Experion Automated Electrophoresis System (RRID: SCR_019691)],[34,35] the DNA samples were verified by spectrophotometry using Shimadzu UV-1800 UV/Visible Scanning Spectrophotometer; 115 VAC (Germany).[23,36]

The TYMS gene region [exon1 and partial regions of promotor and intron 1 of TYMS gene] (877 bp, chr18: 657,220–658,096 bp, NC_000018.10, GI: 568815580) was amplified by PCR (TProfessional TRIO combi; Biometra GmbH, Germany) from 95 RA patients (as listed in Figure S1, Supplemental Digital Content, http://links.lww.com/MD/M728, and Figure S2, Supplemental Digital Content, http://links.lww.com/MD/M729, and Table S1, Supplemental Digital Content, http://links.lww.com/MD/M732).

The PCR amplicons were commercially sequenced according to the sequencing company's instruction manuals (Macrogen Inc. Geumchen, Seoul, South Korea). The PCR product was sent for Sanger sequencing using ABI3730XL, an automated DNA sequencer, by Macrogen Corporation. The results were received by email and then analyzed using Geneious Prime software (V2021.1.1) (Biomatters Ltd., Auckland, New Zealand; www.geneious.com). Only clear chromatographs obtained from ABI (Applied Biosystems, Inc.) sequence files were further analyzed, ensuring that the annotation and variations were not because of PCR or sequencing artifacts.

The positions and other details of the retrieved PCR amplicons were identified by comparing the observed DNA sequences of the investigated samples with the retrieved correspondence DNA sequences of the NCBI Blastn engine. The retrieved PCR fragments' virtual positions and other details were identified (see Figure S3, Supplemental Digital Content, http://links.lww.com/MD/M730). The sequencing results of the PCR products of different samples were edited, aligned, and analyzed as long as with the respective sequences in the reference database using BioEdit Sequence Alignment Editor Software Version 7.1 (DNASTAR, Madison). The observed variations in each sequenced sample were numbered in PCR amplicons and their corresponding position within the referring genome. The highlighted SNPs (see Figure S3, Supplemental Digital Content, http://links.lww.com/MD/M730) were visualized in the dbSNP database to check their originality. Each particular SNP was positioned according to its place in the reference genome. The previous deposition of the observed SNP was determined by viewing its corresponding dbSNP position. Then, the dbSNP position for the detected SNP was documented. The observed SNPs were submitted to the dbSNP database to check their novelty. Each particular SNP position was checked in its corresponding reference genome to assess whether it was previously deposited in the dbSNP server (see Figure S4, Supplemental Digital Content, http://links.lww.com/MD/M731).

2.9. Ethical considerations

The study was approved by the College of Pharmacy, Baghdad University (Approval number: RECAUBCP 6620226, date: June 06, 2022), and written informed consent was obtained from all participants in the study, in accordance with the Helsinki Declaration and its later amendments.

2.10. Statistical analysis

The genotyping results were analyzed, and frequencies of alleles and genotypes were calculated. Hardy-Weinberg Equilibrium (HWE) Calculator for 2 Alleles using online calculator using the difference in distribution between the actual frequency of genotype compared (observed) to the expected frequency of genotype.[37]

Departure from HWE was defined as p-HWE < 10^{-5} and tested by χ^2 test of goodness of fit between observed and expected genotypes according to the binomial distribution.[38] rs2853741T > C and rs2606241A > C did not follow HWE, while 657334C > A and rs2853742T > C follow HWE.

Haplotyping analysis was done using SHEsis online software based on the partition-ligation-combination-subdivision EM algorithm for haplotype inference with multiallelic markers.[39,40]

The Kolmogorov–Smirnov test is used to test the adherence of variables to normality. The discrete variables analyzed by the chi-square test are used to analyze the discrete variable (or Fisher exact test).[41] Two sample *t* tests were used to analyze the differences in means between the 2 groups (if both follow a normal distribution with no significant outlier). Mann–Whitney *U* test was used to analyze the differences in the median of the 2 groups (if they do not follow a normal distribution).[42] Binary logistic regression analysis was used to calculate the odds ratio (OR) and their 95% confidence intervals when the outcome can be categorized into 2 binary levels, and Wald was used to assess which parameters had a stronger effect (Wald is t^2 which is Chi-Square distributed with df = 1). For multivariate binary logistic regression analysis, an unconditional model was used to address the different patients (model 1), disease (model 2), and treatment (model 3) variables, and the effect of possible interaction between different SNPs, where accounted for by the multivariate analysis. GraphPad Prism version 10.0.0 for Windows [GraphPad Prism, Boston (RRID: SCR_002798)] was used to make the statistical analysis, with the *P* value considered when appropriate to be significant if ≤ .05.

3. Results

By sequencing 95 randomly selected patients in the noncoding region of *TYMS*, 4 polymorphisms were identified (all of them are SNPs), 3 of these polymorphisms were found in dbSNP of the NCBI database, and one SNP polymorphism is novel. The study included 95 patients with RA, with a mean age of 43.1 ± 10.6 years; most of the patients were female (85.3%), about 35.8% were smokers, most of the patients had disease low activity (45.2%), followed by moderate (41.1%), high (9.5%), and remission (4.2%). Overall, 49.5% were responders.

Table 2

PCR Program temperature, duration, and number of cycles.

Stage	Step	Temperature	Interval	Cycles number
1	Initial Denaturation	94°C	5 min	1
2	Denaturation	94°C	30 s	35
3	Annealing	60°C	30 s	
4	Elongation	72°C	90 s	
5	Final Elongation	72°C	5 min	1

Table 3

The components of PCR for amplification of TYMS-gene.

Reagents	Concentration	Volume
Genomic template DNA	10–20 ng/μL	2 μL
Master mix	2.5×	10 μL
Forward primer (100 pmol/μL)	10 pmol/μL	1 μL
Reverse primer (100 pmol/μL)	10 pmol/μL	1 μL
$MgCl_2$	25 Mm/0.5 mL	0.5
Nuclease free water		10.5μL
Reaction total volume		25 μL

TYMS = thymidylate synthase.

There was no significant difference in age, sex, and smoking; meanwhile, ESR, RF, DAS28, MTX dose and duration, duration of disease, and side effect (SE) were significantly lower in responders compared to non-responders, as illustrated by Table 4.

Four single nucleotide polymorphisms (SNPs) were identified; 3 of these polymorphisms were previously documented in the dbSNP of the NCBI database (rs2853741T > C, rs2606241A > C, and rs2853742T > C), and one of the documented SNPs is novel (657334C > A), as illustrated in Figures S3, S4, and Table S2, Supplemental Digital Content, http://links.lww.com/MD/M733.

Three genetic models were taken into consideration during the analyses to determine the relationship between the genetic polymorphism and RA disease activity (since the novel SNP [657334C > A] has only 2 genotypes, CA and CC): a co-dominant (3 distinct genotypes for rs2853741T > C: CC, TC, and TT; rs2606241A > C: AA, AC, and CC; and rs2853742T > C: TT, TC, and CC). A dominant (For the minor allele, heterozygotes and homozygotes were paired together for rs2853741T > C: CC plus TC vs TT; rs2606241A > C: AA plus AC vs CC; and rs2853742T > C: TC plus CC vs TT). And recessive model (rs2853741T > C: CC vs TC plus TT; rs2606241A > C: AA vs AC plus CC; and rs2853742T > C: CC vs TC plus TT).

The distribution of genotypes for the 657334C > A, rs2853741T > C, rs2606241A > C, and rs2853742T > C SNPs were not significant according to responsiveness to MTX therapy, while for allele distribution, the rs2606241A > C SNP showed significant difference for single allele distribution. In contrast, the other SNPs did not show significant differences, as illustrated by Table 5.

For the rs2853741T > C genotype in the co-dominant model, the TC genotype predicted non-responder significantly compared to the TT genotype. At the same time, CC did not increase the risk of non-responders compared to TT in the co-dominant model. Meanwhile, the rs2853741 did not follow either the dominant or recessive models. For the rs2606241A > C genotype, the SNP followed dominant models in which AA was associated with the responder. In contrast, CC and CA were associated with non-responder (the bootstrapping technique was used to account for the small sample size since the unadjusted *P* value was .053 and after 1000 resampling *P* values = .045954, beta = 0.811, bias = –0.008). Finally, rs2853742T > C SNP did not follow any genetic model, as illustrated by Table 6.

The CTAT (657334C > A, rs2853741T > C, rs2606241A > C, and rs2853742T > C SNPs) haplotype was significantly associated with responder with OR: 0.506 (*P* value = .022), while the other haplotypes did not associate with MTX responsiveness, as illustrated by Table 7.

In the final multivariate analysis, $TC_{rs2853741}$ genotypes were independently associated with non-responders after adjustment for patients, disease, and treatment characteristics, as illustrated by Table 8.

4. Discussion

The genetic role in predisposition, development, severity, and response for therapeutic intervention of RA is well documented. MTX is an intriguing subject for pharmacogenomics research.[43–50] Pharmacogenomics studies concerning RA have emphasized the identification of genetic markers that may predict a patient's clinical response or the development of side effects for a given therapy, as well as assessing potential interactions between a patient's genetic profile and environmental factors.[51–53]

This field holds promise for advancing personalized medicine and enhancing RA treatment approaches; this is the first research in the current literature that examined the association between several *TYMS* genes (657334C > A, rs2853741T > C, rs2606241A > C, and rs2853742T > C SNPs) with MTX treatment response.

In the present study, 2 SNPs deviation from HWE (rs2853741T > C, and rs2606241A > C), examine these 2 SNPs in detail; both had a very low frequency of heterogeneous, according to a recent study by Chen et al that examined the departure from WHE in a large sample of short variants from the 1000 Genomes Project, high frequency of heterozygous were mostly associated with the genotyping error, meanwhile, low frequency of heterozygous was associated with a variety of other causes, including purifying selection, copy number variation, inbreeding or population substructure,[38] since the low frequency of heterozygous found in the current study suggests causes other than genotyping error is the cause of deviation; other studies support this[54–58] The current sample came from the same ethnic group, which has a high rate of consanguinity and inbreeding, which is the most likely cause of the deviation from HWE.

Regarding genetic models, for the rs2853741T > C genotype in the co-dominant model, the TC genotype predicted non-responder significantly compared to the TT genotype. At the same time, CC did not increase the risk of non-responders compared to TT in the co-dominant model. Meanwhile, the rs2853741 did not follow either the dominant or recessive models. For the rs2606241A > C genotype, the SNP followed dominant models, in which AA was associated with the responder while CC and CA were associated with the non-responder. And finally, rs2853742T > C SNP did not follow any genetic model. We performed haplotype analysis to assess the tetraploid of *TYMS* genes with MTC treatment response; the CTAT (657334C > A, rs2853741T > C, rs2606241A > C, and rs2853742T > C SNPs) haplotype was significantly associated with responder with OR: 0.506 (*P* value = .022), while the other haplotypes did not associate with MTX responsiveness.

In the present study, the genetic association between *TYMS* genes in different SNPs was significant only for the allele distribution of the rs2606241A > C SNP (C carrier was significantly higher in non-responders), while the distribution of genotypes for the 657334C > A, rs2853741T > C, rs2606241A > C, and rs2853742T > C SNPs were not significantly according to responsiveness to MTX therapy.

Multivariate analysis (sensitivity analysis) was used to assess the relationship between *TYMS* genes with treatment responses, and we ran 3 models; in each step, we excluded the effect of age, sex, and smoking, disease duration, MTX dose, MTX duration, and NSAID. In the final multivariate analysis, $TC_{rs2853741}$ genotypes were independently associated with non-responders after adjustment for patients, disease, and treatment characteristics.

Table 4

assessment of demographic, laboratory, and disease characteristics of RA patients classified by response.

Variables	Responder	Non-responder	*P* value
Number	47	48	–
Age (yr), mean ± SD	42.7 ± 10.2	43.4 ± 11.1	.759
Sex, n (%)			
Female	40 (85.1)	41 (85.4)	.966
Male	7 (14.9)	7 (14.6)	
Smoking, n (%)	20 (42.6)	14 (29.2)	.174
ESR (mm/h), median (IQR)	18 (14–22)	38.5 (25–53.8)	<.001
RF (U/mL), median (IQR)	18 (14.9–23)	28.5 (23.5–37.4)	<.001
DAS28 score, median (IQR)	2.8 (2.7–3)	3.9 (3.5–5.0)	<.001
MTX dose (mg), median (IQR)	7.5 (7.5–10)	10 (7.5–10)	.005
MTX duration (mo), median (IQR)	12 (9–24)	28 (11–41.5)	.003
Duration of disease, median (IQR)	19 (9–29)	30 (16.3–46.8)	.004

DAS = disease activity score, ESR = erythrocyte sedimentation rate, IQR = interquartile range, MTX = methotrexate, n = number, RA = rheumatoid arthritis, RF = rheumatoid factor, SE = side effect.

Table 5

Assessment of genotype of *TYMS* genes according to disease activity.

	Responder	Non-responder	*P* value
Number	47	48	–
657334C > A genotype			
CA	6 (12.8%)	11 (22.9%)	.197
CC	41 (87.2%)	37 (77.1%)	
rs657334 allele			
A	6 (6.4%)	11 (11.5%)	.220
C (wild)	88 (93.6%)	85 (88.5%)	
rs2853741T > C genotype [rs2853741T > C]			
CC	13 (27.7%)	17 (35.4%)	.452*
TC	0 (0.0%)	1 (2.1%)	
TT	34 (72.3%)	30 (62.5%)	
rs2853741 allele			
C	26 (27.7%)	35 (36.5%)	.194
T (wild)	68 (72.3%)	61 (63.5%)	
rs2606241A > C genotype [rs2606241A > C]			
CC	16 (34.0%)	22 (45.8%)	.119
CA	3 (6.4%)	7 (14.6%)	
AA	28 (59.6%)	19 (39.6%)	
rs2606241 allele			
C	35 (37.2%)	51 (53.1%)	.028
A (wild)	59 (62.8%)	45 (46.9%)	
rs2853742T > C genotype [rs2853742T > C]			
CC	2 (4.3%)	3 (6.3%)	.633
TC	4 (8.5%)	7 (14.6%)	
TT	41 (87.2%)	38 (79.2%)	
rs2853742 allele			
C	8 (8.5%)	13 (13.5%)	.269
T (wild)	86 (91.5%)	83 (86.5%)	

The Chi-square test was used.

TYMS = thymidylate synthase.

*Bootstrap was performed based on 1000 resampling (*P* value 99% CI: 0.432–0.458).

Table 6

Assessment of the association between *TYMS* genes SNP polymorphism and disease activity using the 3 genetic models to predict non-responders.

Genotype	Responder	Non-responder	OR (95% CI)	*P* value
rs2853741T > C genotype				
Co-dominant model				
CC	13 (43.3%)	17 (56.7%)	1.482 (0.619–3.439)	.384
TC	0 (0.0%)	1 (100.0%)	1.8×10^9	.002
TT	34 (53.1%)	30 (46.9%)	–	–
Dominant genetic model				
CC + TC	13 (41.9%)	18 (58.1%)	1.569 (0.660–3.731)	.308
TT	34 (53.1%)	30 (46.9%)	–	–
Recessive genetic model				
TT + TC	34 (52.3%)	31 (47.7%)	0.697 (0.292–1.666)	.417
CC	13 (43.3%)	17 (56.7%)	–	–
rs2606241A > C genotype				
Co-dominant model				
AA	17 (56.7%)	13 (43.3%)	0.402 (0.140–1.153)	.090
AC	20 (55.6%)	16 (44.4%)	0.421 (0.153–1.155)	.093
CC	10 (34.5%)	19 (65.5%)	Reference	–
Dominant genetic model				
AC + AA	37 (56.1%)	29 (43.9%)	0.413(0.167–1.022)	.058
CC	10 (34.5%)	19 (65.5%)	–	–
Recessive genetic model				
CC + AC	30 (46.2%)	35 (53.8%)	1.526(0.638–3.647)	.342
AA	17 (56.7%)	13 (43.3%)	–	–
rs2853742T > C genotype				
Co-dominant model				
CC	2 (40.0%)	3 (60.0%)	1.618 (0.256–10.219)	.609
TC	4 (36.4%)	7 (63.6%)	1.888 (0.512–6.965)	.340
TT	41 (51.9%)	38 (48.1%)	–	–
Dominant genetic model				
CC + TC	6 (37.5%)	10 (62.5%)	1.798 (0.596–5.425)	.298
TT	41 (51.9%)	38 (48.1%)	–	–
Recessive genetic model				
TT + TC	45 (50.0%)	45 (50.0%)	1.500 (0.239–9.410)	.665
CC	2 (40.0%)	3 (60.0%)	–	–

Table 7

haplotype analysis of the *TYMS* genes SNPs (657334C > A, rs2853741T > C, rs2606241A > C, rs2853742T > C) with the overall response rate.

Haplotyping	Non-responder	Responder	OR [95% CI]	*P* value
ACCC	1.85 (0.019)	2.37 (0.025)	–	–
ACCT	9.15 (0.095)	3.63 (0.039)	2.607 [0.746–9.111]	.121
CCCC	8.15 (0.085)	4.63 (0.049)	1.780 [0.548–5.783]	.332
CCCC	15.85 (0.165)	15.37 (0.163)	1.004 [0.465–2.168]	.990
CTAT	45.00 (0.469)	59.00 (0.628)	0.506 [0.281–0.912]	.022
CTCC	3.00 (0.031)	1.00 (0.011)	2.983 [0.431–20.647]	.380
CTCT	13.00 (0.135)	8.00 (0.085)	1.675 [0.659–4.254]	.274

All those frequencies < 0.03 will be ignored in the analysis.

SNPs = single nucleotide polymorphisms, TYMS = thymidylate synthase.

A previous study that examined the genetic polymorphism of the *TYMS* gene effect in MTX treatment response in RA patients involved 233 adult RA Portages Caucasian patients on continued MTX treatment, diagnosed with similar criteria to the current study; most of the patients were female (84%), mean age of 51 years, median RA duration of 7 years, and median MTX dose 15 mg/wk, which is similar to the current study. They reported several polymorphisms in the *TYMS* gene, VNTR (rs34743033), TSER (rs2853542 and rs34743033), and 1494del6 (rs34489327), which are associated with treatment responsiveness [25]. However, they did not report the polymorphisms found in the current study.

Others, like Lin et al, in their study, examined the *TYMS* gene polymorphisms in breast cancer women in terms of capecitabine-induced hand-foot syndrome (HFS). Their study genotyped 22 SNPs for *TYMS* genes in 342 Chinese breast cancer women. The authors found that rs2606241 and rs2853741 polymorphisms were associated with HFS (*P* value = .022, .029, respectively). For rs2606241, it followed co-dominant and recessive models and G carrier genotypes associated with HFS, while rs2853741 followed co-dominant, dominant models and C carrier genotypes associated with HFS.[59]

Zhao et al examined *TYMS* gene polymorphisms in the Chinese population and its association with Congenital Cardiac Septal Defects (CCSD); the study involved 270 unrelated individuals with CCSD and compared them to 552 healthy controls, later 32 unrelated individuals consisting of 16 CCSD patients and 16 controls. The authors identified 15 SNP polymorphisms; neither rs2853741 nor rs2606241 was associated with CCSD.[60]

The Blanton et al study examined the association between the Folate Pathway and Nonsyndromic Cleft Lip and Palate (NSCLP). The study involved 120 multiplex NSCLP families, 14 genes identified, and 97 SNPs identified, 7 of them identified from the *TYMS* gene. For rs2853741 polymorphism, no significant association with NSCLP was reported.[61] Pellicer et al study, the risk of chemotherapy-induced toxicity by capecitabine treatment was assessed in 301 cancer patients, rs2853741 polymorphism (CT/TT vs CC) associated with diarrhea (OR = 0.314, 95% CI: 0.133–0.740) in multivariate analysis.[62] In the Lautner-Csorba et al study, the authors examined the genetic polymorphism of folate pathways in Childhood Acute Lymphoblastic Leukemia; rs2853741 polymorphism did not show a significant association.[63] Liu et al study the one-carbon metabolizing genes and lung cancer risk, rs2853742 polymorphism decrease the risk of lung cancer (OR = 0.44, 95% CI: 0.19–0.99).[64]

Table 8

Multivariate analysis of different genotypes of *TYMS* genes to predict non-responders.

	Unadjusted*		Model 1 (patients)*		Model 2 (patients and disease)*		Model 3 (patients and disease and treatment)*	
	OR (95% CI)	*P* value	OR (95% CI)	*P* value	OR (95% CI)	*P* value	OR (95% CI)	*P* value
657334C > A								
CA	2.205 (0.496–9.794)	.320	2.615 (0.563–12.156)	.226	3.302 (0.607–17.959)	.269	2.281 (0.362–14.367)	.510
CC	Ref		Ref					
rs2853741T > C								
CC	0.629 (0.097–4.067)	.568	0.699 (0.101–4.853)	.666	0.512 (0.065–4.034)	.507	0.587 (0.056–6.106)	.574
TC	1.4×10^{9}	.027	1.2×10**9**	.026	5.1×10**8**	.030	4.2×10^{8}	.097
TT	Ref		Red					
rs2606241A > C								
CC	1.621 (0.294–8.942)	.518	1.403 (0.237–8.311)	.682	1.610 (0.245–10.590)	.557	1.587 (0.182–13.880)	.540
AC	3.439 (0.789–14.995)	.057	4.025 (0.867–18.674)	.035	3.483 (0.681–17.798)	.087	2.619 (0.499–13.740)	.215
AA	Ref		Red					
rs2853742T > C								
CC	1.458 (0.194–10.978)	.568	1.493 (0.199–11.175)	.558	2.124 (0.260–17.340)	.412	1.725 (0.197–15.064)	.555
TC	1.649 (0.360–7.566)	.533	1.552 (0.323–7.444)	.629	1.687 (0.303–9.393)	.581	1.950 (0.289–13.140)	.513
TT	Ref		Red					

Model 1 excludes the effect of age, sex, and smoking.

Model 2, in addition to variables in Model 1, excludes disease duration.

Model 3, in addition to model 1 and 2 variables, excludes MTX dose, duration, and NSAID.

MTX = methotrexate, NSCLP = Nonsyndromic Cleft Lip and Palate, TYMS = thymidylate synthase.

*Bootstrap was performed based on 1000 resampling.

Thymidylate synthase is an essential enzyme in DNA synthesis and repair.[65,66] It is known to be inhibited by methotrexate polyglutamate (MTXPGs), which in turn contributes to the antiproliferative and anti-inflammatory effects of methotrexate.[67] Indeed, previous studies have demonstrated that TYMS levels can be a reliable predictor of the therapeutic response to MTX treatment.[12,13] Previous studies have established a correlation between genetic variations in the TYMS gene and levels of TYMS.[17]

Replicating findings in genetic association research is typically sophisticated, and establishing comparability between investigations poses challenges. The most compelling evidence of a correlation persists in replicating this correlation in an independent group that shares the same genotype-phenotype and exhibits the same direction of impact. Most genetic association studies lack adequate statistical power due to the constrained number of patients exhibiting the homozygous mutant genotype.[68]

One of the challenges encountered in assessing the effectiveness of treatment in RA is the requirement for agreement on the measures used to determine efficacy. A divergence of viewpoints exists regarding issues such as the duration and experimental approach required.[69–73] In an ideal scenario, it is desirable to establish definitive threshold values for therapeutic purposes, enabling the classification of patients as either responders or non-responders and facilitating the formulation of treatment guidelines. Nevertheless, clinicians are compelled to modify the treatment when there is any alteration in the effectiveness metrics within clinical practice.[74,75]

4.1. Study limitations

As mentioned earlier, the precise processes that underlie the impact of the genotypes on the efficacy of MTX treatment have yet to be fully proven. Furthermore, it is imperative to acknowledge that these findings must be regarded as preliminary and necessitate further validation. It is recommended that more prospective studies be conducted on a larger cohort of patients to validate and quantify the observed relationships in additional datasets. These studies would also contribute to a better understanding of the underlying mechanisms responsible for MTX responsiveness in patients with RA. Moreover, it is also pertinent to continue making extra endeavors to uncover other polymorphisms and unusual variations linked to MTX responsiveness in individuals with RA. Additionally, the restricted sample size of patients included in the present investigation constrains the extent to which the findings, as mentioned earlier, can be generalized.

5. Conclusion

Genetic polymorphism of the TYMS gene, especially in $TC_{rs2853741}$ and $AC_{rs260641}$, predicts non-responder to MTX treatment in RA, while the presence of the CTAT haplotype predicts a good response to MTX treatment.

Author contributions

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