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Research Article

Association of the rs1801133 and rs1801131 polymorphisms in the MTHFR gene and the adverse drug reaction of methotrexate treatment in a sample of Iraqi rheumatoid arthritis patients

Qassim Mahdi Mutlak[1], Ali Abdulhussain Kasim[1]

1 *College of Pharmacy, University of Baghdad, Baghdad, Iraq*

Corresponding author: Qassim Mahdi Mutlak (qasem.mahdi1100p@copharm.uobaghdad.edu.iq)

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Abstract

Background: Methotrexate is one of the mainstays for treating rheumatoid arthritis (RA) with a wide range of adverse drug reactions, however, it's the relationship between adverse drug reactions and genetic polymorphism remains to be highlighted, and there is a lack of studies concerning Arabic Iraqi population regarding this aspect.

Objective: Evaluate the association between genetic mutations in the MTHFR gene in SNPs (rs1801133G>A and rs1801131T>G) on the adverse drug reaction for RA Iraqi patients.

Methods: An observational study, that involved 95 Iraqi RA patients with established RA. Patients were divided according to the occurrence of adverse drug reactions. A polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) was being utilized for *MTHFR* variants (rs1801133 and rs1801131). The Macrogen Company (Korea) provided the forward and reverse primers in lyophilized form. All PCR procedures are carried out using a PCR thermal cycler (Germany).

Results: 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%). No significant association between individual genetic polymorphism with adverse drug reactions. AG haplotype for rs1801133 rs1801131 polymorphism is associated with reducing the risk of overall adverse drug reactions, meanwhile, GT haplotype for rs1801133, and rs1801131 polymorphism were marginally associated with increased risk of adverse drug reactions.

Conclusion: In conclusion, we have successfully found a panel of pharmacogenetic indicators that have the potential to be valuable in predicting the response to methotrexate treatment in patients with rheumatoid arthritis. Haplotypes for rs1801133 rs1801131 polymorphism are associated with reducing or increasing the risk of MTX adverse drug reactions. It is very important to evaluate patients' haplotypes before starting the therapy program so that we can expect the treatment outcome with the most suitable dose and most tolerable one at the same time.

Keywords

methotrexate, rheumatoid arthritis, adverse drug reaction, polymorphism, MTHFR gene

Introduction

Rheumatoid arthritis (RA) is a chronic systemic inflammatory disorder characterized by pain and swelling in the hands and feet on both sides (Faiq et al. 2019b; Kadhim Jwad et al. 2022; Mohammed et al. 2022b). It is characterized by both articular and extra-articular manifestations, and permanent joint deformities can develop in improperly managed cases (Ali et al. 2010; Hadi and Ali 2010; Mikhael and Ibrahim 2017).

Methotrexate (MTX) functions as a folic acid inhibitor, exerting anti-inflammatory and anti-proliferative effects by closely resembling the structural and physiochemical properties of folic acid. Methotrexate (MTX) is extensively employed in the treatment of rheumatoid arthritis (RA) due to its notable efficacy, favorable safety profile, and cost-effectiveness (Faiq et al. 2019a; Abdul-Wahab and Al-Shawi 2020). The enzyme known as methylenetetrahydrofolate reductase (MTHFR) plays a crucial role in the metabolic processes involving folate and homocysteine. The primary purpose of this entity is to enable the transformation of 5,10-methylenetetrahydrofolate into 5-methyltetrahydrofolate. The aforementioned form of folate is the prevailing circulating form and is employed in the process of remethylating homocysteine to methionine (Rosenblatt 1995).

Pharmacogenetics refers to the examination of the correlations between genetic variations among individuals and their impact on the efficacy and adverse effects of specific drugs. Therefore, it has the potential to aid clinicians in tailoring treatment plans for individual patients (Malik and Ranganathan 2013).

The MTHFR gene is influenced by racial and ethnic differences (Hughes et al. 2006), some reported that the frequency of rs1801131 SNP varies racial and ethnicity in which the A allele is associated with more ADR in Caucasians (15.9 fold increase in risk) (Hughes et al. 2006), while rs1801133 reported increase association between TT genotype with Mexican Mestizo and Amerindian populations, while the AA genotype frequency was low in African and Asian ancestry populations (Binia et al. 2014).

Numerous studies have provided evidence of associations between genetic variations in the MTHFR gene and increased susceptibility to methotrexate (MTX) toxicity. The MTHFR 677C>T (rs1801133) genetic variant is associated with elevated hepatic enzyme levels, hyperhomocysteinemia, and increased susceptibility to methotrexate (MTX) toxicity. The single-nucleotide polymorphism (SNP) known as MTHFR 1298A>C (rs1801131) is associated with the combined adverse drug effects (ADEs) on the gastrointestinal, hematologic, and mucosal systems that are linked to the use of methotrexate (MTX) (Haagsma et al. 1999; van Ede et al. 2001; Urano et al. 2002; Berkun et al. 2004).

Numerous studies have been undertaken to examine the genetic polymorphism of enzymes implicated in the mechanisms of methotrexate (MTX) activity. The primary discoveries revolve around the genetic polymorphisms within the methylene tetrahydrofolate reductase (MTHFR) enzyme, which is involved in the activation of folic acid and therefore impacts the functions of MTX. Extensive research has been conducted on the polymorphisms of the gene located on chromosome 1 (1p36.3) due to the enzyme's substantial role in DNA synthesis, repair, and methylation. This study primarily focused on the investigation of two missense mutations: the first mutation, denoted as the C677T substitution (rs1801133), and the second mutation, referred to as the A1298C substitution (rs1801131). The association between these genetic variations and the effectiveness and/or adverse effects of methotrexate (MTX) in the treatment of rheumatoid arthritis (RA) has been documented. However, the results of these investigations have produced contradictory and ambiguous results (Frosst et al. 1995; van der Put et al. 1998; Berkani et al. 2017; Muss et al. 2019).

The current study aims to evaluate the association between MTHFR gene SNPs (rs1801133G>A and rs1801131T>G) and the development of MTX adverse drug reactions in Arab Iraqi RA patients.

Methods

Study design

An observational study, that 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 (Kay and Upchurch 2012). Patients were classified according to the presence or absence of a specific or overall ADR.

Study settings

All the patients enrolled in this study were recruited from the Rheumatology Department of Diwaniya Teaching Hospital. The study was performed between the 1st of June 2022 and to 1st of March 2023.

Inclusion criteria

Adult patients (age ≥18 years), with confirmed RA according to revised 2010 ACR/EULAR RA classification criteria (Kay and Upchurch 2012), and all patients should be on MTX for at least 3 months.

Exclusion criteria

Patients with co-existent diseases other connective tissue diseases, patients who use additional disease-modifying antirheumatic drugs (DMARDs), biological drugs, incomplete data, patients with any chronic infectious diseases, cancer, hepatic or renal dysfunction, endocrinopathy, hematological and cardiac conditions.

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, and p is the prevalence of 2.2% of RA Iraq in 2019 (Al Badran et al. 2022). The Z represents the Z-score at a 95 % confidence interval, and it equals 1.96, *d* represents the marginal error which is accepted according to (Daniel and Cross 2018) to be 0.03. Thus, the minimal sample size was estimated to be approximately 92, and 95 was chosen as the final sample size to account for possible drop cases.

DNA extraction

The genomic DNA was isolated from the peripheral blood of subjects and then it was stored at (-20°C) until its analysis. The extracted DNA is based on Hashim and Al-Shuhaib's study (Hashim and Al-Shuhaib 2020), which depends on non-organic procedures. After the extraction by agarose gel electrophoresis (1% agarose) [Bio-Rad Experion Automated Electrophoresis System (RRID: SCR_019691)], the DNA samples were verified by spectrophotometry using Shimadzu UV-1800 UV/Visible Scanning Spectrophotometer; 115 VAC (Germany) (Tataurov et al. 2008; Hegazi et al. 2018), see Fig. 1.

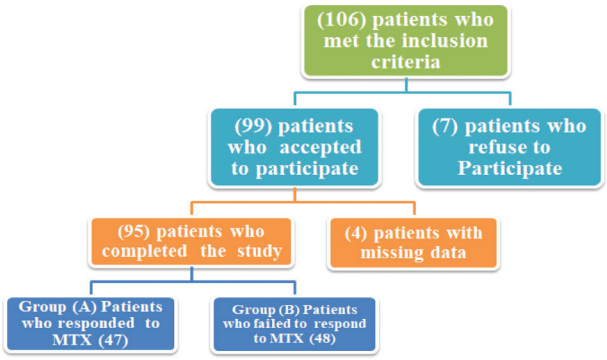

Figure 1. Total number of patients who participated in the current study.

Primer selection

The design of PCR primers for MTHFR variants (rs1801133 and rs1801131) according to the following (see Table 1); briefly as follows The primers were designed with the aid of NCBI-primer BLAST online software [NCBI BLAST (RRID: SCR_004870)], at the same time the produced primers was checked for specificity for their target sequences by performing the BLAST against the human genome, then the primers pair was selected according to the demand criteria such as product length, the similarity of melting temperature, primers length, specificity, etc. The primer ability to form a secondary structure was checked with the aid of Oligo Calc online software [OligoCalc (RRID: SCR_022663)], the primer would be rejected if it had 5 bases or more able to form self-dimerization and/or it had 4 bases able to form hairpin. Each primer pair was checked for dimer formation with the aid of "Multiple Primer Analyzer" online software from Thermo Fisher Scientific Inc.©, the sensitivity of the software was adjusted to the value 2, the primer pair would be rejected if it made any dimers in this degree of sensitivity (Kibbe 2007; Qu et al. 2012; Al-Radeef et al. 2019).

Table 1. Primers sequence, GC%, annealing temperature (Ta), and product size of *MTHFR* genes 1298 (rs1801131) SNP and 677 (rs1801133) SNP (302).

***MTHFR* Gene 1298 (rs1801131) SNP**		
Ta	60°C	
Product size	90 bp	
Primers	**Sequence**	**GC%**
Forward primer	TCCCGAGAGGTAAAGAACGtAGAC	50
Reverse primer	TCCCCCAAGGAGGAGCTGCTGtAGA	60
***MTHFR* Gene 677 (rs1801133) SNP**		
Ta	°C	
Product size	254 bp	
Primers	**Sequence**	**GC%**
Forward primer	CCTGGATGGGAAAGATCCCG	60
Reverse primer	CATCCCTCGCCTTGAACAGG	60

Genotyping of polymorphisms

A polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) was being utilized for *MTHFR* variants (rs1801133 and rs1801131). The Macrogen Company (Korea) provided the forward and reverse primers in lyophilized form. All PCR procedures are carried out using a PCR thermal cycler (Germany). Reactions were adjusted to a final volume of 20 µL including Master mix probes and 10–20 ng/µL of genomic DNA (see Table 2), with the following amplification protocol illustrated in Table 3.

Table 2. The components of PCR for amplification *MTHFR* gene.

Reagents	Concentration	Volume
Genomic template DNA	10–20 ng/µL	2 µL
Master mix	2.5 x	8 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 µL
Nuclease free water	–	7.5 µL
reaction total volume	–	20 µL

Table 3. The components of PCR for amplification *MTHFR* gene.

Stage	Step	Temperature	Interval	Cycles number
1	Initial Denaturation	94 °C	5 minutes	1
2	Denaturation	94 °C	30 seconds	**35**
3	Annealing	58 °C	30 seconds	
4	Elongation	72 °C	30 seconds	
5	Final Elongation	72 °C	5 minutes	**1**

Ethical considerations

The study was approved by College of Pharmacy, Baghdad University (Approval number: RECAUBCP 6620226, date: 6th June 2022), and written informed consent was obtained from all participants in the study, in accordance with the Helsinki Declaration and its later amendments.

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 (Schaid et al. 2006). Both rs1801133 (p-value = 0.018) and rs1801131 (p-value = 0.304) follow HWE (p-value of 0.0057 used for allele frequency less than 100, assuming type I error (α) = 0.01) (Wigginton et al. 2005).

Haplotyping analysis was carried out using SHEsis online software which is based on the partition-ligation-combination-subdivision EM algorithm for haplotype inference with multiallelic markers (Shi and He 2005; Li et al. 2009).

The chi-square test is employed to assess discrete variables. In cases where the chi-square test is not applicable, such as when the sample size is less than 20 or when there are two or more categories with anticipated frequencies less than 5, the Fisher exact test is used as an alternative. The Fisher-Freeman-Halton exact test of independence is employed for n x k tables, which are an extension of the conventional 2x2 Fisher exact test, where the predicted frequency is below 5% (Ghent 1972). Binary logistic regression analysis was used to calculate the odds ratio (OR) and their 95% confidence intervals when the outcome can be categorized into two binary levels, and Wald was used to assessing which parameters had a stronger effect (Wald is t^2 which is Chi-Square distributed with df=1). GraphPad Prism version 10.0.0 for Windows [GraphPad Prism (RRID: SCR_002798)] was used to make the statistical analysis, p-value considered when appropriate to be significant if less than 0.05. The genotypes of the participants were subjected to association testing, logistic regression, and haplotype analysis.

Data availability

Underlying data: Zenodo: polymorphisms in the MTHFR gene and the adverse drug reaction of methotrexate. https://doi.org/10.5281/zenodo.8400501

The project contains the following underlying data: polymorphisms in the MTHFR gene and the adverse drug reaction of methotrexate.

Results

Demographic and clinical data

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%), and about 35.8% were smokers. The distribution of SE per organ system and overall are illustrated in Tables 4, 5.

Table 4. Assessment of demographic, laboratory, and disease characteristics of RA patients.

Variables	Value
Number	95
Age (years), mean ± SD	43.1 ± 10.6
Sex, n (%)	
Female	81 (85.3%)
Male	14 (14.7%)
Smoking, n (%)	34 (35.8%)
ESR (mm/hour), median (IQR)	24.0(16.0–39.0)
RF (U/ml), median (IQR)	23.0(16.4–29.0)
MTX dose (mg), median (IQR)	10.0(7.5–10.0)
MTX duration (month), median (IQR)	19.0(11.0–31.0)
Duration of disease, median (IQR)	23.0(12.0–34.0)

IQR: interquartile range, n: number, ESR: erythrocyte sedimentation rate, RF: rheumatoid factor.

Table 5. Assessment of the adverse drug reactions.

ADR	N (%)
Dermatological	21 (22.1%)
Liver	7 (7.4%)
GIT	43 (45.3%)
Respiratory	8 (8.4%)
Hematological	4 (4.2%)
Renal	1 (1.1%)
Overall	84 (88.4%)

Adverse drug reaction

Liver ADR

No significant association between the hepatological ADR with SNP polymorphism, as illustrated by Table 6.

Table 6. Hepatological ADR and its relationship with genetic polymorphism.

Genotype	Negative ADR	Positive ADR	p-value
RS1801133G>A			
AA	26(86.7%)	4(13.3%)	0.131
AG	29(80.6%)	7(19.4%)	
GG	19(65.5%)	10(34.5%)	
RS1801131T>G			
TT	11(68.8%)	5(31.3%)	0.557
TG	31(77.5%)	9(22.5%)	
GG	32(82.1%)	7(17.9%)	

Skin ADR

No significant association between the dermatological ADR with SNP polymorphism, as illustrated by Table 7.

GIT ADR

No significant association between the GIT ADR with SNP polymorphism, as illustrated by Table 8.

Table 7. Dermatological ADR and its relationship with genetic polymorphism.

Genotype	Negative ADR	Positive ADR	p-value
RS1801133G>A			
AA	26(86.7%)	4(13.3%)	0.062
AG	36(100.0%)	0(0.0%)	
GG	26(89.7%)	3(10.3%)	
RS1801131T>G			
TT	16(100.0%)	0(0.0%)	0.374
TG	35(87.5%)	5(12.5%)	
GG	37(94.9%)	2(5.1%)	

Table 8. GIT ADR and its relationship with genetic polymorphism.

Genotype	Negative ADR	Positive ADR	p-value
RS1801133G>A			
AA	17(56.7%)	13(43.3%)	0.229
AG	16(44.4%)	20(55.6%)	
GG	19(65.5%)	10(34.5%)	
RS1801131T>G			
TT	6(37.5%)	10(62.5%)	0.234
TG	25(62.5%)	15(37.5%)	
GG	21(53.8%)	18(46.2%)	

Respiratory ADR

No significant association between the respiratory ADR with SNP polymorphism, as illustrated by Table 9.

Table 9. Respiratory ADR and its relationship with genetic polymorphism.

Genotype	Negative ADR	Positive ADR	p-value
RS1801133G>A			
AA	28(93.3%)	2(6.7%)	0.908
AG	33(91.7%)	3(8.3%)	
GG	26(89.7%)	3(10.3%)	
RS1801131T>G			
TT	16(100.0%)	0(0.0%)	0.502
TG	36(90.0%)	4(10.0%)	
GG	35(89.7%)	4(10.3%)	

Overall ADR

No significant association between the overall ADR with SNP polymorphism, as illustrated by Table 10.

Table 10. Overall ADR and its relationship with genetic polymorphism.

Genotype	Negative ADR	Positive ADR	p-value
RS1801133G>A			
AA	5(16.7%)	25(83.3%)	0.518
AG	4(11.1%)	32(88.9%)	
GG	2(6.9%)	27(93.1%)	
RS1801131T>G			
TT	0(0.0%)	16(100.0%)	0.130
TG	4(10.0%)	36(90.0%)	
GG	7(17.9%)	32(82.1%)	

Haplotyping analysis

AG haplotype for rs1801133 rs1801131 polymorphism is associated with reduced risk of overall adverse drug reactions, meanwhile GT haplotype for rs1801133 rs1801131 polymorphism was marginally associated with increased risk of adverse drug reactions, as illustrated by Table 11.

Table 11. Haplotype analysis of the 2 SNPs with overall adverse drug reaction.

Haplotyping	With ADR	Without ADR	OR [95%CI]	p-value
$A_{rs1801133}$ $G_{rs1801131}$	52.41(0.312)	11.52(0.524)	0.412 [0.168~1.012]	0.048 [S]
$A_{rs1801133}$ $T_{rs1801131}$	29.59(0.176)	2.48(0.113)	1.684 [0.424~6.694]	0.454
$G_{rs1801133}$ $G_{rs1801131}$	47.59(0.283)	6.48(0.294)	0.947 [0.357~2.514]	0.912
$G_{rs1801133}$ $T_{rs1801131}$	38.41(0.229)	1.52(0.069)	3.989 [0.739~21.522	0.084

Global result: Total no ADR=22.0, total ADR=168.0. Global chi^2 is 5.42 while df=3 (frequency<0.03 in both ADR & no ADR has been dropped.). Fisher's p-value is 0.143744.

Discussion

The genetic influence on the propensity, development, severity, and therapeutic response of rheumatoid arthritis (RA) has been extensively reported in academic literature. The subject of MTX holds significant interest in the field of pharmacogenomics research. This enables the identification of factors that can predict the most effective outcomes while minimizing any negative effects (Owen et al. 2013; Ad'hiah et al. 2018; Al-Radeef et al. 2019; Muss et al. 2019; Al-Saffar and Al-Saadi 2022; Hussain 2022).

The focus of pharmacogenomics research on rheumatoid arthritis (RA) has been on the identification of genetic markers that can potentially predict a patient's clinical response or the occurrence of side effects associated with a specific therapy. Additionally, researchers have investigated the potential interactions between a patient's genetic profile and environmental factors about RA treatment (Mohammed et al. 2022a; Younis et al. 2022; Awni et al. 2023).

In the present study, the genetic association between MTHFR gene rs1801131, and rs1801133 SNP polymorphism and MTX ADR was sought, and there was no association between individual ADR or overall ADR with MTHFR genes. In terms of haplotype analysis, the AG haplotype for rs1801133 rs1801131 polymorphism is associated with a reduced risk of overall adverse drug reactions, meanwhile, the GT haplotype for rs1801133 rs1801131 polymorphism was marginally associated with an increased risk of adverse drug reactions.

This lack of association between individual SNP polymorphism with ADR could be related to the individual's response to a treatment being influenced by various factors, including other genetic, epigenetic, co-morbidities, and environmental factors. The impact of a certain gene is exceedingly minimal. Thus, when we used haplotype analysis, we found that AG haplotype for rs1801133 rs1801131 polymorphism is protective, while GT haplotype increase risk of overall ADR, indicating that the interaction between two SNPs is responsible for variation in ADR outcomes, since the A allele in Rs1801133 is associated with reduced enzyme activity, elevated total homocysteine levels and altered distribution of folate, while mutations in rs1801131 also affect MTHFR enzyme activity and homocysteine levels but to a lesser extent than rs1801133 (De Mattia and Toffoli 2009), T-allele of rs1801133 potentially raise the risk of hypertension and hyperhomocysteinemia by suppressing the expression of PPARG by downregulating folate levels and upregulating homocysteine levels (Liang et al. 2022), G allele of rs1801131 linked to higher risk of neurotoxicity and liver toxicity, which could be linked to higher doses of MTX needed to treat and maintain remission for RA patients (Dervieux et al. 2009; Mena et al. 2011), so variation in haplotyping resulted in unexpected behaviour of ADR, and to the best of our knowledge this work is the first to study haplotyping of these two SNPs on ADRs specifically in Arabic Iraqi sample. In contrast, the study conducted by Cáliz et al. examined the impact of the C677T and A1298C polymorphisms within the MTHFR gene on the toxicity of MTX in a cohort of pa-

tients diagnosed with rheumatoid arthritis (RA). Regarding the MTHFR A1298C polymorphism, it was reported that there was no significant association between the MTHFR A1298C polymorphism and an increased risk of methotrexate (MTX) toxicity (p = .761) (Cáliz et al. 2012). Comparably, Taraborelli and colleagues concluded that there was no statistically significant association between the MTHFR A1298C polymorphism and methotrexate (MTX) medication toxicity. Which aligns with the findings of the present investigation. (Taraborelli et al. 2009). Other researchers reported no association between individual C677T polymorphism (van Ede et al. 2001) and A1298 polymorphism (Wessels et al. 2006; Xiao et al. 2010) with adverse drug reaction.

The study conducted by Sharaki et al. revealed a noteworthy correlation between the MTHFR rs1801131-GG genotype and the occurrence of methotrexate (MTX) medication toxicity. The phenomenon was elucidated through the observation of diminished medication efficacy in individuals possessing the MTHFR rs1801131-GG genotype (Sharaki et al. 2018). In the study conducted by Choe et al., it was observed that there was a significant difference in the occurrence of drug toxicity between rheumatoid patients with the MTHFR rs1801131-GG genotype and those with the rs1801131-TT genotype. Specifically, a higher proportion of patients with the rs1801131-GG genotype experienced at least one instance of drug toxicity compared to patients with the rs1801131-TT genotype (Choe et al. 2012). In contrast to the findings of the present investigation, Davis et al. and Dervieux et al. reported an association between MTHFR rs1801131-TG/GG genotypes with an elevated susceptibility to toxicity (Dervieux et al. 2006; Davis et al. 2014).

The process of replicating results in genetic association research is inherently complex, leading to challenges in making meaningful comparisons across different studies. The most compelling evidence of a correlation persists in the replication of this correlation in an independent group that shares the same genotype, phenotype, and direction of impact (Wessels et al. 2006). The majority of genetic association studies lack adequate statistical power due to the constrained number of patients exhibiting the homozygous mutant genotype (Wessels et al. 2006).

Study limitations

This study suffers from some objective limitations, one is the–sectional nature of the study, second, a single ethnic group was examined – namely Arabic sample, and we could not examine other ethnicities like Caucasian, African, and Kurdish, which will limit the generalizability of our findings and limit them to Arabic ethnicity.

Conclusion

In conclusion, we have successfully found a panel of pharmacogenetic indicators that have the potential to be valuable in predicting the response to methotrexate treatment in patients with rheumatoid arthritis. The results of our study may assist healthcare professionals in identifying patients who are unlikely to derive optimal benefits from methotrexate (MTX) treatment. These individuals may require supplementary drugs or greater therapeutic doses of MTX. This will enhance the quality of therapy decisions for people with rheumatoid arthritis (RA). Haplotypes for rs1801133 rs1801131 polymorphism are associated with reducing or increasing the risk of MTX adverse drug reactions. It is very important to evaluate patients' haplotypes before starting the therapy program, so that we can expect the treatment outcome with the most suitable dose and most tolerable one at the same time.

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