The Egyptian Journal of Hospital Medicine (July 2020) Vol. 80 (2), Page 857-864

The Influence of the MDR1 C3435T Polymorphism on Methotrexate Responsiveness in Rheumatoid Arthritis Patients

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ABSTRACT

Background: Methotrexate (MTX) is most widely used for the treatment of rheumatoid arthritis (RA). However, different clinical responses have been observed in patients treated with MTX. **Objective:** The aim of the work was to determine the influence of the MDR1 C3435T polymorphism on Methotrexate responsiveness in RA patients.

Patients and Methods: A cross sectional study included 90 RA patients. Demographics, clinical features, medication history, and the disease activity score (DAS28) were carefully recorded. Genotypes of the C3435T polymorphism were determined by real time polymerase chain reaction for all patients. According to disease activity score, patients were classified into 2 groups: group (1): MTX responders (DAS28 score ≤3.2) and group (2): MTX non-responders (DAS28 score >3.2).

Results: Of the 90 RA patients, 80 were females (88.9%) and 10 were males (11.1%). No statistically significant difference was found in genotype or allele frequencies between MTX responders and non-responders groups. However, patients who had C allele were 1.65 times more likely to be non-responder to MTX treatment when compared to those who had the T allele. The most common MTX adverse effects reported were Gastrointestinal upset in 31.1% followed by undesirable hair loss in 11.1%. The probability of having GIT adverse effects was observed to be higher among the cases with (CT) genotype than the other genotypes but without statistical significance.

Conclusion: It could be concluded that no significant association could be detected between MDR1 gene C3435T polymorphism and responsiveness to methotrexate in the studied rheumatoid arthritis patients.

Keywords: Rheumatoid arthritis**,** MDR1 C3435T SNP, MTX sensitivity.

INTRODUCTION

Rheumatoid arthritis is a systemic inflammatory disease that leads to severe joint damage and affects around 1% of the population worldwide. Its exact cause is unknown, but genetic and environmental factors are contributory [(1)]. Disease-modifying anti-rheumatic drugs (DMARDs) are used in treatment of RA to decrease inflammation, delay bone erosion and disease progression, and improve functional ability in RA patients [(2)].

Methotrexate (MTX) is still the most commonly used DMARD and considered to be the gold standard for treatment of RA. The combination of its perceived efficacy, acceptable safety profile, and low cost, as well as decades of clinical experience, makes MTX the cornerstone of treatment for RA [(2)].

However, in routine clinical practice, different clinical responses have sometimes been observed in patients treated with MTX. This indicates the presence of individual differences in MTX sensitivity. Moreover, 30% of patients discontinue therapy within a year of starting the treatment, usually because of undesirable side effects. Research into the reasons behind patients' unresponsiveness or occurrence of adverse events has generated considerable interest [(3)].

As achieving response early in the disease process is the key to minimizing the joint damage, there is a need to identify patients with a higher risk to experience treatment inefficacy and to predict non-responsiveness to MTX treatment. Pharmacogenetics, the inheritance of drug response, holds the promise not only to explain interindividual variability in drug response, but also to predict efficacy and adverse drug events in individual patients. This so-called personalized medicine or tailored therapy [(4)].

The treatment response is influenced by several factors including female gender, DAS at baseline, rheumatoid factor (RF), smoking and differences in drug metabolism. Many genetic factors may be associated with these differences [(4)].

Translocation of endogenous compounds, as well as drugs, across biological membranes occurs not only via passive diffusion, but also by carrier-mediated processes. Knowledge of cell- and tissue-specific transporter expression, as well as characterization of the substrates of individual transporters leads to a better understanding of the role of these transporters in the treatment efficacy and adverse events of various drugs [(5)].

Received:11 /5 /2020
Accepted:21 /6 /2020

The human multidrug resistance (MDR1) gene is found on chromosome 7, at band q 21-21. It encodes for an integral membrane protein called P-glycoprotein (P-gp) which belongs to ATP-binding cassette (ABC) superfamily of transporters [(6)].

P-glycoprotein functions as the transmembrane efflux pump for various structurally unrelated anticancer agents and toxins. Polymorphisms in the MDR1 gene may have an impact on the expression and function of P-gp, thereby responsiveness to drugs [(7)].

MDR1 was the first ABC transporter to be identified and is the most studied gene in the field of multidrug resistance and it is highly polymorphic; more than 50 SNP have been identified and correlated with efficacy of drug treatment [(6)].

MDR1 3435C>T (rs1045642) is the most widely investigated SNP that is located in exon 26 and represent a synonymous SNP with no effect in amino acid change at position 1145 [(8)]. However, it was found to correlate with P-glycoprotein expression levels and also to alter protein structure and substrate affinity of this protein and thus, the MDR1 polymorphism may have an impact on the response to methotrexate in RA patients [(7)].

The aim of this study was to determine the influence of the MDR1 C3435T polymorphism on Methotrexate responsiveness in rheumatoid arthritis patients.

SUBJECTS AND METHODS

This study is a cross sectional study included 90 RA patients fulfilling the American College of Rheumatology and European League against Rheumatism (ACR / EULAR) 2010 classification criteria [(9)]. The patients were recruited from the outpatient clinic and the inpatient Department of Rheumatology Ain Shams University Hospital after an informed consent.

Ethical approval:

The study was approved by the Local Research Ethical Committee of Ain Shams University and conforms to the provisions of the Declaration of Helsinki in 1995.

Demographics, clinical features, medication history were carefully recorded. Disease activity score (DAS28)[(10)] was assessed and was graded as remission (≤2.6), low (≤3.2), moderate (>3.2-≤5.1) and high (>5.1)

The included 90 subjects were divided into two groups; **Group 1 (**MTX responders**)** consisted of 45 patients, DAS28 score ≤3.2, and Group **2 (**MTX non-responders**)** consisted of 45 patients,
DAS28 score >3.2.

Laboratory investigations:

Six milliliters (ml) of venous blood were collected from all patients under complete aseptic conditions for ESR determination, quantitation of CRP, Deoxyribonucleic acid (DNA) extraction and subsequent genotyping. The allele and genotype frequencies of all the genes were determined in all 90 Patients.

Gene analysis:

Genomic DNA was obtained from peripheral blood by using the QIAamp DNA blood mini kit supplied by QIAGEN, Germany.

- Amplification and Allelic Discrimination:

The extracted DNA was amplified using TaqMan universal master mix II and ready-made TaqMan SNP genotyping assay for rs1045642 supplied by Applied Biosystems, USA.

Two TaqMan probes (one probe labeled with VIC dye to detect the T allele and another probe labeled with FAM dye to detect the C allele sequence).

Probes Sequence:

TGTTGGCCTCCTTTGCTGCCCTCAC[A]ATCTC
TTCCTGTGACACCACCCGGC-VIC
TGTTGGCCTCCTTTGCTGCCCTCAC[G]ATCTC
TTCCTGTGACACCACCCGGC-FAM

- The PCR amplification program consisted of an initial denaturation step at 95 ºC for 10 min, followed by 40 cycles of denaturation at 92 ºC for 15 sec and annealing and extension at 60 ºC for 1 min.
- Real-time PCR was performed on the ***Rotor- Gene Q*** real-time thermal cycler.
- The system software uses the fluorescence measurements from each well made during the plate read, and then plots signal values. The software determines which alleles are in each sample for genotyping analysis.

Statistical analysis

Statistical analysis was done using software version SPSS 20 (Statistical package for the social sciences). Statistical significant differences between the responders and non-responders, and patients with or without adverse drug events and their genotypes were assessed using Chi-Square test, Mann Whitney Test (U test) and the Kruskal-Wallis test. The odds ratios (OR) and their 95% confidence interval (CI) were calculated by using the logistic regression model. The cut-off value for significant differences is 0.05.

RESULTS

Of our 90 RA patients, 80 were females (88.9%) and 10 were males (11.1%). Their ages ranged from 21 to 67 years with the median age 44.5 years. All clinical manifestations, disease activity indices, drugs received and laboratory data were presented in table (1).

Table (1): Descriptive Statistics of clinical, laboratory data and Treatment regimen in all RA Patients (90):

Clinical Characteristics		**N (%)**
Sex	Male, n (%)	10 (11.1%)
	Female, n (%)	80 (88.9%)
Age in years, median (IQR)		44.5 (36 – 51)
Age of disease onset, median (IQR)		38 (29 – 45)
Number of total swollen joints, median (IQR)		0 (0 – 1)
Number of total tender joints, median (IQR)		4 (2 – 16)
DAS 28	Remission, n (%)	13 (14.4%)
	Low, n (%)	32(35.6%)
	Moderate, n (%)	5 (5.6%)
	Severe, n (%)	40 (44.4%)
Parameter	**Median**	**IQR**
ESR (mm/hour)	25	13 – 45
CRP (mg/dl)	0.90	0.5 - 2.4
ALT (U/L)	19.50	15 – 26
AST (U/L)	21	16 – 26
TLC ($x10^3$/ul)	7.40	6.4 - 9.2
Hb (g/dl)	11.60	11 - 12.7
PLT ($x10^3$/ul)	273.50	230 – 324
Treatment regimen		**N (%)**
Patients on MTX and Glucocorticoids		10 (11.11%)
Patients on MTX, Glucocorticoids and Hydroxychloroquine		63 (70%)
Patients on MTX, Glucocorticoids and Leflunomide		7 (7.8%)
Patients on MTX, Glucocorticoids, Hydroxychloroquine and Leflunomide		10 (11.11%)

All patients were treated with MTX for at least 6 months. The most common MTX adverse effects reported (according to clinical and laboratory data) were Gastrointestinal upset (anorexia, nausea, vomiting and heartburn) in 31.1% followed by undesirable hair loss in 11.1%. Figure (1)

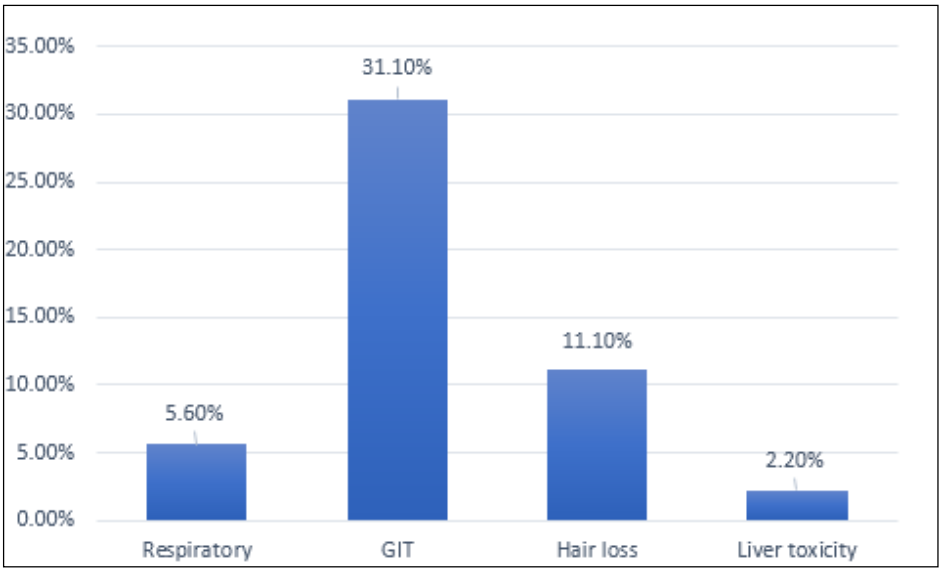

Figure (1): Adverse effects of MTX.

Genotyping of MDR1 gene C3435T polymorphism was done for all 90 patients. The frequency of the wild type CC was 47.8%, while the homozygous TT and the heterozygous CT mutant types were 14.4% and 37.8% respectively. The C allele had a frequency of 66.7% while the T allele had a frequency of 33.3%. Figure (2)

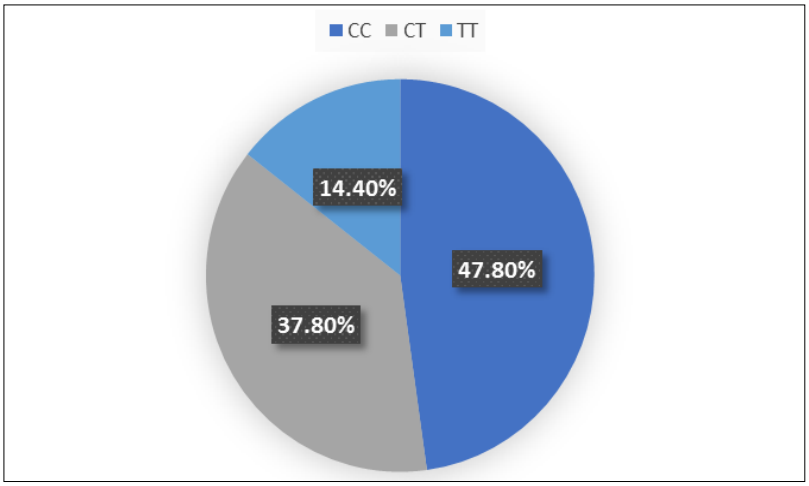

Figure (2): Frequency of MDR1 C3435T genotypes in the whole studied RA patients.

According to disease activity score, patients were classified into 2 groups: group (1): Forty-five patients who were responders to MTX treatment having DAS28 score ≤3.2, group (2): 45 patients who were non-responders to MTX having a DAS28 score >3.2. The comparative statistics of genotype frequencies in the studied MDR1 gene C3435T SNP in both groups (MTX responders and MTX non-responders) revealed no significant difference. Among the non-responders, the MDR1 wild-type (CC) was observed in 25 patients (55.6%), whereas 15 patients (33.3%) were heterozygous (CT) and 5 (11.1%) patients were homozygous (TT) for the mutation. Among the responders, the CC genotype, CT genotype and TT genotype were found in 18 (40.0%), 19 (42.2%) and 8 (17.8%) of the patients respectively. Figure (3)

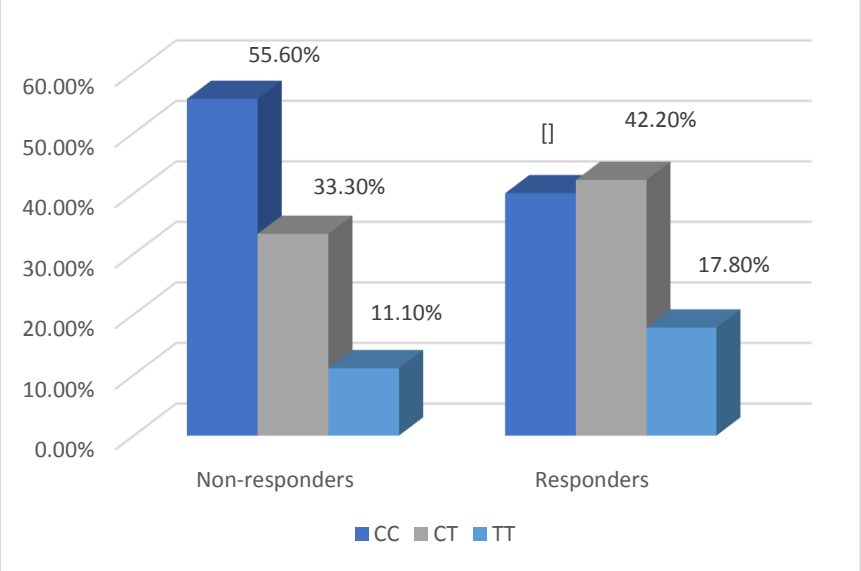

Figure (3)*:* Frequency of MDR1 C3435T SNP genotypes among responders and non-responders groups.

Concerning allelic frequencies of MDR1 C3435T SNP, the C allele was found in 72.2% MTX non-responder patients and 61.6% of MTX responder patients(Figure 4). While the T allele was found in 27.8% and 38.9% of the same groups respectively .Chi square test showed that there was no statistical significant difference between the 2 groups regarding the allele frequency. Odds ratio was 1.65 (95% CI: 0.88- 3.1), patients who had C allele were 1.65 times more likely to be non-responder to MTX treatment when compared to those who had the T allele. Table (2).

Table (2): Comparative Statistics of allele frequencies of the MDR1 C3435T SNP in responders and non-responders groups.

C3435T	Responders N=45 N (%)	Non-responders N=45 N (%)	Chi square test		OR (CI)
C allele	55(61.1%)	65 (72.2%)	**P value**	**Sig.**	1.65 (0.88-3.1)
T allele	35 (38.9%)	25 (27.8%)	**0.114**	NS	

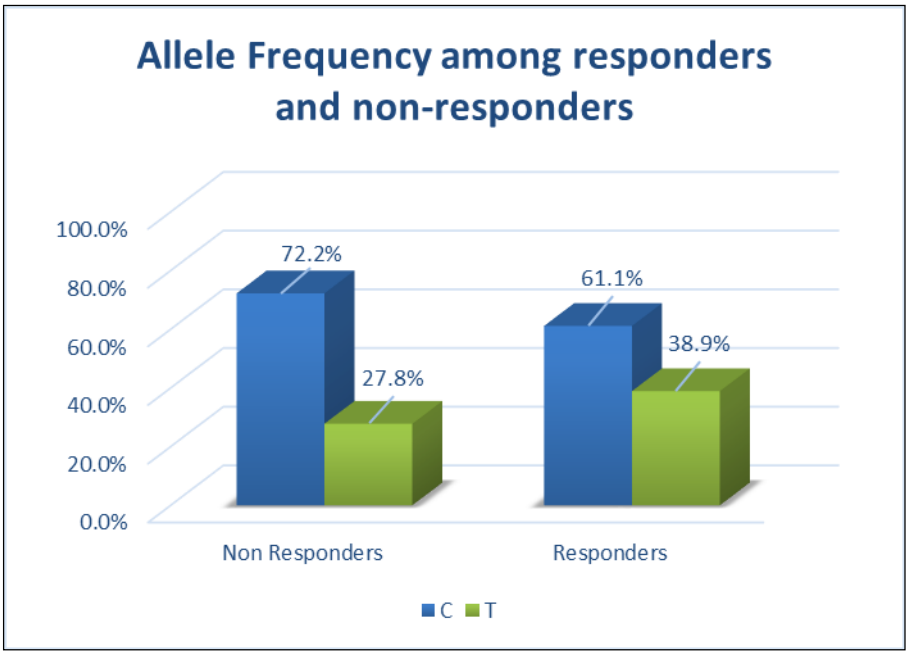

Figure (4): The frequency of C&T alleles of MDR1 C3435T SNP in MTX responder and MTX non- responder RA patients.

Comparative statistics between patients with and without MTX induced adverse effect as regard the MDR1 C3435T polymorphism, revealed no significant difference in MTX toxicity among the different genotypes(Figure 5). However, the probability of having GIT adverse effects was observed to be higher among the cases with (CT) genotype than the other genotypes (Table 3).

Table (3): Comparative statistics of MDR1 C3435T genotypes frequencies as regard MTX induced adverse effects subtypes:

Adverse effect	Genotypes						Fisher exact test	
	CC		CT		TT			
	N	%	N	%	N	%	p value	sig.
Respiratory	5	100.0%	0	0.0%	0	0.0%	0.095	NS
GIT	7	25.0%	13	46.4%	8	28.6%	0.004(C)	S
Hair loss	7	70.0%	2	20.0%	1	10.0%	0.391	NS
Liver toxicity	2	100.0%	0	0.0%	0	0.0%	0.635	NS

C: Chi square test

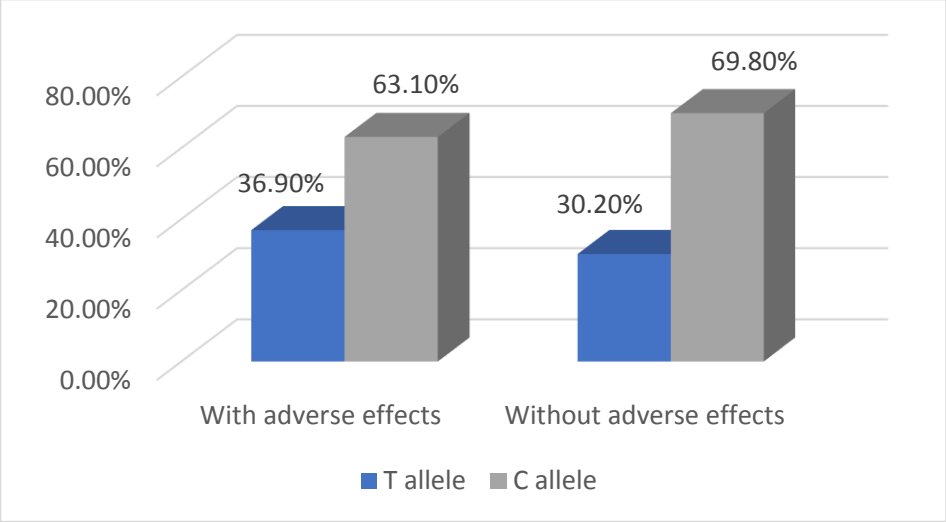

Figure (5): The frequency of C&T alleles of MDR1 C3435T SNP in patients with and without MTX induced adverse effects.

DISCUSSION

Response to MTX treatment is influenced by several factors including factors that affect the drug metabolism. Genetic factors may be responsible for up to 30 % of unresponsiveness to MTX therapy [(12)].

Multidrug resistance protein 1 belongs to the ABC family, and it is a P-glycoprotein expressed on natural killer cells, lymphocytes, and bone marrow progenitor cells. P-glycoprotein functions as a transmembrane efflux pump to decrease the intracellular accumulation of a variety of drugs and toxins [(13)]. The MDR1 gene is highly polymorphic with more than 50 variants residing in the coding region which can influence the expression, efflux, substrate specificity, and mRNA stability of P-gp [(14)].

C3435T (rs1045642) is the most widely investigated SNP of MDR1 that is located in exon 26 and represents a silent SNP with no effect in amino acid sequence but was found to affect P-glycoprotein expression levels and also to alter substrate affinity of P-glycoprotein[(12)].

Several studies have examined the potential contribution of the MDR1 C3435T polymorphism in RA. However, it remains controversial whether the MDR1 C3435T polymorphism may be a marker of efficacy and toxicity of DMARDs including MTX [(15)].

Wang *et al.* [(16)] demonstrated an effect of the MDR1 C3435T polymorphism on P-gp mRNA stability. Also, **Hoffmeyer *et al.*** [(17)] showed that the presence of homozygous T allele resulted in reduced P-gp expression.

As regard the effect of MDR1 C3435T polymorphism on response to MTX therapy in RA, results of the present study showed no significant difference between MTX responders and non-responders patients. This finding was valid for genotype and allele frequencies. However, the C allele frequency was higher among non-responder RA patients, while the T allele was higher among responders but the value didn't reach a statistically significant level (OR 1.65, CI: 0.88-3.1, P > 0.05).

This may be supported by **Drozdzik *et al.*** [(5)] who reported that in a cohort of 174 RA patients receiving MTX and methylprednisolone, the probability of remission of RA symptoms was 4.6-fold higher in patients carrying the MDR1 3435 TT genotype than patients with 3435 CC genotype.

Also, **Pawlik *et al.*** [(18)] found that the risk of having an active form of RA was 2.89-fold greater in MTX treated patients with 3435CC and CT genotypes when compared to patients with 3435 TT genotype.

Moreover, in a Chinese study, **Mo *et al.*** [(11)] evaluated the effects of the interaction of various genes involved in the folate metabolic pathway (such as RFC1, MDR1, GGH, FPGS and MTHFR) on the efficacy and toxicity of MTX. Among the 6 SNPs analysed in the study, only MDR1 C3435T was observed to have an association with efficacy where the TT genotype seemed to confer more possibility of good response among the studied group. The former researchers suggested that the MDR1 3435T allele decreased P-gp expression which results in decreasing of the efflux of MTX. This might lead to a higher rate of remission of the disease attributed to the higher intracellular concentrations of MTX.

In accordance with our study results, **Grabar *et al.*** [(19)] who studied the effect of MDR1 C3435T on efficacy of MTX therapy in 213 patients with RA of Caucasian race reported no significant association between MDR1 C3435T and the efficacy of MTX treatment.

Similarly, in a study that included 159 Jordanian patients with RA, no statistical association was found between MTX response and MDR1 C3435T genotypes [(20)].

The current finding was also supported by a meta-analysis study that included five studies with total 760 RA patients (Caucasian and Asian, 391 responders and 369 non-responders), investigating the response to DMARDs according to subject MDR1 C3435T polymorphism status. The meta-analysis showed no association between the MDR1 C3435T T allele and the response to MTX therapy [(15)].

Surprisingly, **Takatori *et al.*** [(21)] studied 124 Japanese patients with RA and found that MDR1 3435TT genotype frequency was higher among non-responders to MTX compared to responders. Also, a study of 336 Indian patients with RA has shown that the mutant T allele frequency was higher among patients on MTX and having high disease activity than in those with low disease activity.[(12)] The authors suggested that the pumping ability of P-gp was more elevated in the 3435TT patients than in the 3435CC patients.

Concerning the effect of MDRI C3435T polymorphism on MTX toxicity, neither MDRI C3435T genotype nor allele frequencies has shown an association to MTX toxicity. Although the T allele frequency was higher among patients who having MTX induced adverse effects, it didn't reach a statistical significance (OR 1.4, CI: 0.7-2.5, P > 0.05).

Similarly, **Takatori *et al.*** [(21)] **and Mo *et al.*** [(11)] did not find any association between the MDR1 C3435T polymorphism and MTX toxicity in the Japanese and Chinese patients, respectively.

On the other hand, **Samara *et al.*** [(20)] reported a significant association between MDRI C3435T polymorphism and MTX toxicity. Where, Patients with RFC1 80GG genotype were at higher risk for gastrointestinal toxicity (p = 0.036). Patients carrying at least one MDR1 3435T variant allele were at higher risk for MTX overall toxicity (p = 0.04), especially hepatotoxicity (p = 0.028). **Grabar *et al.*** [(19)] also reported similar results. Those studies explained that patients with 3435TT genotype are more liable to develop MTX-adverse events due to a decrease in MTX efflux leading to a prolonged cellular exposure to the drug.

The discrepancy between studies including present study might be explained by the differences in study population, sample size, therapy plan, study design, co-administered drugs, or the confounding effects of other genetic polymorphisms in the folate pathway on MTX efficacy.

In addition, the MDR1 gene is highly polymorphic and this could be one of the factors responsible for the inter-individual variations in the activity of P-gp. One limitation of present study is that not all genetic polymorphisms of the folate pathway were investigated. Also, the study included a relatively small number of patients.

CONCLUSION

It could be concluded that no significant association could be detected between MDR1 gene C3435T polymorphism and responsiveness to methotrexate in the studied rheumatoid arthritis patients.

RECOMMENDATIONS

More studies on larger scale of patients to obtain more accurate results are recommended.

Study of MDR1 polymorphisms in combination with other MTX influencing factors (e.g. enzymes involved in folate metabolic pathway) might unravel complex factors involved in unresponsiveness to methotrexate.

Further genomic studies for the distribution of other common SNPs in MDR1 gene and responsiveness to MTX in Egyptian population are recommended.

Cross genotyping analysis for occurrence of multiple polymorphisms in more than one gene at the same time can reveal the aetiology of MTX unresponsiveness.

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