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Frequency of ABCB1 C3435T Polymorphism in Rheumatoid Arthritis Patients; Relation to Methotrexate Responsiveness and Methotrexate Adverse Effects

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ABSTRACT

Background: Rheumatoid arthritis (RA) is an inflammatory disease requiring treatment by disease-modifying agents like methotrexate. C3435T is a single nucleotide polymorphism in the ABCB1 gene that affects the expression of the P-glycoprotein responsible for the efflux of several drugs from cells increasing both its action and toxicity.

Objective: Examine the frequency of C3435T in RA patients and assess its influence on responsiveness to methotrexate treatment and occurrence of methotrexate adverse effects.

Patients and Methods: Genotyping C3435T polymorphism was done by real-time polymerase chain reaction and the frequency of the polymorphism was assessed in a sample of 90 RA patients who have received methotrexate treatment and 90 healthy controls. The prevalence of the polymorphism was also assessed in responders to methotrexate compared to non-responders, and also in patients who suffered from methotrexate side effects compared to those who did not.

Results: The CC was the most frequent genotype in patients (47.8%) while the CT was the most frequent in controls (43.3%), however, no association was found between the ABCB1 C3435T genotype and susceptibility to RA under the different genetic models ($p>0.05$). Even though the patients who responded to methotrexate had a higher frequency of the T allele (38.9%) compared to non-responders (27.8%), the genotype and allele frequency were not associated with response to methotrexate ($p>0.05$). Also, no association was observed between the frequency of the C3435T polymorphism and experiencing methotrexate-associated adverse effects ($p>0.05$).

Conclusion: The C3435T polymorphism of the ABCB1 gene may not be a genetic risk factor for RA and does not affect responsiveness to methotrexate or affect the occurrence of adverse effects from the drug.

Keywords: Rheumatoid arthritis, Methotrexate, ABCB1, C3435T.

INTRODUCTION

Rheumatoid arthritis (RA) is an activity-hindering disease that affects females more than males. The hallmark of the disease is the inflammation of joints that leads to local destruction of both the cartilage and bone in the affected joints [1]. In 2021, the worldwide prevalence of RA was around 0.46% [2].

Treatment of RA involves medications in addition to patient education, and physiotherapy. Medical treatment of RA generally involves non-steroidal anti-inflammatory drugs, glucocorticoids and disease-modifying antirheumatic drugs (DMARDs). The latter are immunomodulatory therapeutic agents that aim to target the immune-inflammatory process occurring in the joints. There exists 3 types of DMARDs: 1- conventional e.g. methotrexate, hydroxychloroquine, leflunomide, sulfasalazine and gold compounds; 2- biologic e.g. CTLA4-Ig, TNF antagonists, IL-1 receptor antagonist, anti-human IL-6 receptor antibody and anti-CD20 agents; and 3- targeted synthetic e.g. inhibitors of Janus kinase [3].

The most widely prescribed drug among DMARDs is methotrexate and it remains the gold standard for the treatment of RA. Current treatment guidelines for RA is that patients are empirically initiated on treatment with methotrexate. Unfortunately, around 30% of RA patients do not respond to treatment with methotrexate. Genetic mutations have been implicated in resistance to methotrexate through the effect on either methotrexate metabolism pathway or methotrexate transport carriers resulting in its decreased cellular accumulation hindering its pharmacological action [4, 5].

Methotrexate enters the cell mainly via the solute carrier transporter and exits the cell via several channels among which are the ATP binding cassette (ABC) transporters [6].

ABC transporter family includes seven subfamilies; A – G. The ATP binding cassette subfamily B member 1 (ABCB1) gene [also called multidrug resistance protein 1 (MDR1 gene)] is found on chromosome 7q21.12 and contains 29 exons. The gene encodes the transporter P-glycoprotein which transports various compounds to the outside of cells including several drugs. The gene is highly polymorphic with over 50 reported polymorphisms. The C3435T single nucleotide polymorphism in exon 26 is a synonymous polymorphism that results in exchange of the cytosine nucleotide by thymine.

The mutation causes no alteration in the amino acid constitution but has a significant effect on the P-glycoprotein expression and function by altering mRNA splicing and stability or affecting the efficiency of translation with an end result of decrease surface p-glycoprotein level and a decrease in its efflux function [7].

The C3435T polymorphism has been linked to susceptibility to several diseases including Parkinson's disease [8], inflammatory bowel disease [9], ischemic heart disease [10], breast cancer [11], colorectal cancer [12], and leukemia [13]. The CC genotype of C3435T was also reported to be linked to higher risk of

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pharmacoresistance to a wide range of drugs including antiepileptics [14], antipsychotics, antidepressants [15], glucocorticoids [16] and methotrexate [17].

In the current study, we examined the frequency of C3435T (rs1045642) in rheumatoid arthritis patients and assessed its influence on responsiveness to treatment with methotrexate and its influence on the occurrence of methotrexate adverse effects.

SUBJECTS AND METHODS

Study participants

The study included 90 patients with rheumatoid arthritis, who have been receiving methotrexate as a treatment for 6 months, and 90 age and sex matched healthy controls. Patients were diagnosed to have RA according to the American College of Rheumatology and European League Against Rheumatism (ACR/EULAR) 2010 classification criteria [18], and were enrolled from the rheumatological diseases outpatient clinic. Patients recruited in the study must have received methotrexate for 6 months. Patients with other connective tissue diseases or autoimmune diseases, patients who have been treated with methotrexate for less than 6 months or who have stopped methotrexate therapy due to any reason e.g. recent pregnancy or pregnancy plans were excluded from the study.

The activity of RA was evaluated via the disease activity score 28 (DAS28) [19]. Rheumatoid arthritis patients included 45 patients who had a DAS28 ≤3.2 (these are patients in remission or with low disease activity and were considered responsive to methotrexate therapy) and 45 patients who had a DAS28 >3.2 (these are patients with moderate or high disease activity and were considered to be non-responsive to methotrexate therapy).

Sampling

Six millilitres of venous blood were collected into 3 tubes: 1) 2 ml of blood were added to an EDTA vacutainer and stored at -20ºC till used for deoxyribonucleic acid (DNA) extraction and subsequent genotyping. 2) 1.6 ml of blood were added to a 3.2% sodium citrate tube for erythrocyte sedimentation rate (ESR) determination. 3) 2 ml of blood were added to a gel activated vacutainer for quantitation of c-reactive protein (CRP).

Assessment of ESR: The sedimentation rate was estimated using the Westergren method.

Assessment of CRP: It was measured on Cobas c6000 analyzer supplied by Roche diagnostics, Switzerland.

Genotyping of ABCB1 gene C3435T polymorphism

Extraction of Genomic DNA

DNA was extracted from whole blood using GeneJET™ Whole Blood Genomic DNA Purification Mini Kit supplied by Thermo Fisher Scientific, USA. In brief, 200 µL of whole blood were mixed with 20 µL of Proteinase K Solution by vortexing followed by the addition of 400 µL of Lysis Solution and incubating at 56°C for 10 minutes, after which 200 µL of 96% ethanol were added and the mixture was pipetted into a spin column and centrifuged at 6000g for 1 minute. The column was then washed by 500 µL of Wash Buffer I then washed again using 500 µL of Wash Buffer II. Elution was then done using 200 µL of elution buffer and centrifugation at 8000g for 1 minute.

Amplification and Allelic Discrimination

Real-time PCR was used for amplification and allelic discrimination using TaqMan™ Genotyping Master Mix and TaqMan™ SNP Genotyping Assay for rs1045642 supplied by Applied Biosystems, USA on Rotor-Gene Q.

Amplification was carried out in a total volume of 25 µL containing 12.5 µL of TaqMan genotyping master mix, 1.25 µL TaqMan SNP genotyping assay mix and 5ng of extracted DNA in 11.25 of DNase-free, RNase-free water. Probe sequences and amplification protocol are shown in table 1.

Table (1): Probe sequence and thermal profile

Probe sequence			
Probe 1	TGTTGGCCTCCTTTGCTGCCCTC AC[G]ATCTCTTCCTGTGACACCA CCCGGC-FAM		
Probe 2	TGTTGGCCTCCTTTGCTGCCCTC AC[A]ATCTCTTCCTGTGACACCA CCCGGC-VIC		
Thermal profile			
Step	**Temp (°C)**	**Duration**	**Cycles**
Initial denaturation	95	10 min	HOLD
Denature	95	15 sec	40
Anneal/Extend	60	1 min	

Ethical consent:

The study protocol received ethical approval from the Faculty of Medicine, Ain Shams University. All study subjects signed an informed written consent upon participation in the study. The Declaration of Helsinki for human beings, which is the international medical association's code of ethics, was followed during the conduct of this study.

Statistical analysis

Prism 8 software (GraphPad, La Jolla, CA) was used for data analysis. Number and percentage are used for presenting categorical data. Mean, standard deviation (SD), median and interquartile range (IQR) were used for quantitative parameters. Difference between groups was assessed using Student's t-test and Mann-Whitney test. Differences in genotypic and allelic distribution were examined using Chi-square test and Fisher's exact test. Association of the polymorphism was estimated by odds ratios (OR) with a 95% confidence interval (CI). $\chi 2$ goodness-of-fit test was used for the assessment of Hardy–Weinberg equilibrium. A p-value of <0.05 was considered statistically significant.

RESULTS

Clinical data

A total of 90 RA patients with mean age 43.8±10.6 years and 90 healthy controls with mean age 42.8±11.3 years were enrolled in the study.

Females represented 88.9% of the patients and 84.4% of the controls (Table 2). Patients showed significantly higher levels of ESR and CRP compared to controls (p<0.001 for both parameters). Patients enrolled in the study were chosen so that half of them (45 patients) had a DAS28≤3.2 after 6 months of treatment with methotrexate (responders to methotrexate therapy) while the other half had DAS28>3.2 after 6 months of treatment with methotrexate (non-responders to methotrexate therapy).

All patients were evaluated for adverse effects of methotrexate and 42 were found to show adverse effects.

The adverse effects evaluated were: gastrointestinal in the form of anorexia, nausea, vomiting and heartburn; dermatological in the form of hair loss or skin rash; respiratory in the form of dyspnea and interstitial lung fibrosis. 4) liver toxicity as evidenced by high serum transaminases above reference range. Among the studied patients, 5.6% showed respiratory side effects, 31.1% showed gastrointestinal side effects, 11.1% showed dermatological side effects and 2.2% showed liver toxicity.

Table (2): Clinical data of study participants

	Patients	**Controls**	**p-value**
Male, n (%) **Female, n (%)**	10 (11.1%) 80 (88.9%)	14 (15.6%) 76 (84.4%)	0.381
Age, (mean±SD)	43.8±10.6 years	42.8±11.3 years	0.572
Age at disease onset, median (IQR)	38 (29 – 45) years	-	
ESR, median (IQR)	25 (13-45) mm/h	9 (5-13) mm/h	<0.001
CRP, median (IQR)	9.4 (5.6-24.3) mg/L	1.7 (1.0-3.1) mg/L	<0.001
DAS 28, n (%) **DAS 28 ≤3.2 (responders)** **DAS 28 >3.2 (non-responders)**	 45 (50%) 45 (50%)	-	
Adverse effects of methotrexate **Respiratory** **Gastrointestinal** **Dermatological** **Liver toxicity**	 5 (5.6%) 28 (31.1%) 10 (11.1%) 2 (2.2%)	-	

C3435T polymorphism in RA patients

Genotyping for the C3435T polymorphism was done using real time PCR and frequencies of different genotypes were compared between RA patients and controls (Table 3). The most frequent genotype in patients was the CC genotype (47.8%) followed by the CT genotype (37.8%) and the TT genotype (14.4%) while the most frequent genotype in the healthy controls was the CT genotype (43.3%) followed by the CC genotype (37.8%) then the TT genotype (18.9%). The genotype frequency in patients was not statistically different from the frequency in the controls in the codominant model (p=0.335), dominant model (p=0.175), recessive model (p=0.424), and overdominant model (p=0.448). The frequency of genotypes, in both the patient and control groups, held the Hardy-Weinberg equilibrium (p> 0.05). Also, there was no difference regarding allele frequency, the C allele had a frequency of 66.7% in patients and 59.4% in controls while the T allele had a frequency of 33.3% in patients and 40.6% in controls (p=0.156).

Table (3): Genotype and allele frequency of the C3435T polymorphism

	Patients	Controls	p-value	OR (95%CI)
Codominant Model				
CC, n (%)	43 (47.8%)	34 (37.8%)		Reference
CT, n (%)	34 (37.8%)	39 (43.3%)	0.335	0.701 (0.370-1.345)
TT, n (%)	13 (14.4%)	17 (18.9%)		0.605 (0.258-1.416)
Dominant Model				
CC, n (%)	43 (47.8%)	34 (37.8%)		Reference
CT+TT, n (%)	47 (52.2%)	56 (62.2%)	0.175	0.663 (0.366-1.202)
Recessive Model				
CC+CT, n (%)	77 (85.6%)	73 (81.1%)		Reference
TT, n (%)	13 (14.4%)	17 (18.9%)	0.424	0.725 (0.329-1.598)
Overdominant Model				
CC+TT, n (%)	56 (62.2%)	51 (56.7%)		Reference
CT, n (%)	34 (37.8%)	39 (43.3%)	0.448	0.794 (0.437-1.441)
C allele, n (%)	120 (66.7%)	107 (59.4%)		Reference
T allele, n (%)	60 (33.3%)	73 (40.6%)	0.156	0.733 (0.477-1.126)

C3435T polymorphism and methotrexate therapy

Forty-five RA patients who responded to methotrexate therapy and had a DAS28 of less than or equal to 3.2 were enrolled in the study. Responders had a mean age of 45.4±9.2 years and 84.4% of them were females. The study also included 45 patients that did not respond to treatment with methotrexate and showed a DAS28 of more than 3.2. Non-responders had a mean age of 42.1±11.7 years and 93.3% of them were females. ESR levels and CRP levels were higher in non- responders than responders (p<0.01 for both parameters) (Table 4).

Table (4): Clinical data of responders versus non-responders

	Responders	Non-Responders	p-value
Male, n (%)	7 (15.6%)	3 (6.7%)	0.315
Female, n (%)	38 (84.4%)	42 (93.3%)	
Age, (mean±SD)	45.4±9.2 years	42.1±11.7 years	0.140
ESR, median (IQR)	13 (8-15) mm/h	45 (30-63) mm/h	<0.001
CRP, median (IQR)	5 (4-7) mg/L	24 (14.5-50.1) mg/L	<0.001

C3435T genotype and allele frequencies were compared between responders and non-responders (Table 5). No significant difference was observed regarding genotype frequency under the codominant model (p=0.316), dominant model (p=0.140), recessive model (p=0.368) and overdominant model (p=0.384). Regarding allele frequency, the C allele had a frequency of 61.1% in responders and 72.2% in non-responders while the T allele had a frequency of 38.9% in responders and 27.8% in non-responders. No statistical difference was found between both groups regarding allele frequency (p=0.114).

Table (5): Genotype and allele frequency in responders versus non-responders

	Responders	Non-Responders	p-value	OR (95%CI)
Codominant Model				
CC, n (%)	18 (40%)	25 (55.6%)		Reference
CT, n (%)	19 (42.2%)	15 (33.3%)	0.316	0.633 (0.255-1.571)
TT, n (%)	8 (17.8%)	5 (11.1%)		2.222 (0.623-7.924)
Dominant Model				
CC, n (%)	18 (40%)	25 (55.6%)		Reference
CT+TT, n (%)	27 (60%)	20 (44.4%)	0.140	1.875 (0.811-4.333)
Recessive Model				
CC+CT, n (%)	37 (82.2%)	40 (88.9%)		Reference
TT, n (%)	8 (17.8%)	5 (11.1%)	0.368	1.730 (0.519-5.765)
Overdominant Model				
CC+TT, n (%)	26 (57.8%)	30 (66.7%)		Reference
CT, n (%)	19 (42.2%)	15 (33.3%)	0.384	1.462 (0.620-3.443)
C allele, n (%)	55 (61.1%)	65 (72.2%)		Reference
T allele, n (%)	35 (38.9%)	25 (27.8%)	0.114	1.655 (0.884-3.096)

Patients were assessed for methotrexate adverse effects and 42 patients showed adverse effects (Table 6). These patients were compared to the patients who did not experience methotrexate side effects (n=48) regarding C3435T genotype and allele frequency. No significant difference was observed regarding genotype frequency under the codominant model (p=0.509), dominant model (p=0.652), recessive model (p=0.368) and overdominant model (p=0.384). Regarding allele frequency, the C allele had a frequency of 63.1% in patients who experienced adverse effects and 69.8% in patients who did not, while the T allele had a frequency of 36.9% in patients who experienced adverse effects and 30.2% in patients who did not. No statistical difference was found between both groups (p=0.341).

Table (6): Genotypes and allele frequency as regards methotrexate induced adverse effects

	Patients with adverse effects (n=42)	Patients without adverse effects (n=48)	p-value	OR (95%CI)
Codominant Model				
CC, n (%)	19 (45.2%)	24 (50.0%)		Reference
CT, n (%)	15 (35.7%)	19 (39.6%)	0.509	0.997 (0.403-2.468)
TT, n (%)	8 (19.1%)	5 (10.4%)		2.021 (0.567-7.193)
Dominant Model				
CC, n (%)	19 (45.2%)	24 (50.0%)		Reference
CT+TT, n (%)	23 (54.8%)	24 (50.0%)	0.652	1.211 (0.527-2.777)
Recessive Model				
CC+CT, n (%)	34 (80.9%)	43 (89.6%)		Reference
TT, n (%)	8 (19.1%)	5 (10.4%)	0.368	2.024 (0.606-6.751)
Overdominant Model				
CC+TT, n (%)	27 (64.3%)	29 (60.4%)		Reference
CT, n (%)	15 (35.7%)	19 (39.6%)	0.384	0.848 (0.360-1.997)
C allele, n (%)	53 (63.1%)	67 (69.8%)		Reference
T allele, n (%)	31 (36.9%)	29 (30.2%)	0.341	1.351 (0.726-2.516)

DISCUSSION

Rheumatoid arthritis severely affects the patient's life quality either due to the physical dysfunction that is a result of fatigue, pain, joint deformity and functional disability or due to extraarticular involvement of various systems. RA is a multifactorial disease that is triggered by a combination of genetic predisposition in addition to environmental factors [20]. The C3435T polymorphism has been linked to susceptibility to several diseases including Parkinson's disease [8], inflammatory bowel disease [9], ischemic disease [10], breast cancer [11], cancer colon [12], and leukemia [13].

In this study, we assessed the presence of the C3435T polymorphism of ABCB1 gene in a sample of 90 RA patients and 90 controls. Results of the current study revealed no difference between RA patients and controls regarding the frequency of C3435T polymorphism. Our results indicate that the C3435T polymorphism of the ABCB1 gene may not act as a risk factor for RA susceptibility. **Abdurakhmanova *et al.*** [21] compared the C3435T genotypes between 120 patients with RA and 30 healthy volunteers in Uzbekistan and concluded that no significant difference was found in genotype or allele distribution between RA patients and healthy volunteers. Similarly, in China, **Chen *et al.*** [22] determined that the C3435T genotype does not predispose patients to RA, as the polymorphism distribution in 108 RA patients was similar to that in 103 healthy controls. Moreover, in another study done in India, the authors concluded that the genotype and allele frequencies for the 3435C>T polymorphism were not different between RA cases and controls [23].

Proper treatment of RA effectively slows the progression of joint damage and prevents irreversible disability. The most widely used drug in RA treatment is methotrexate [20]. Predicting unresponsiveness to methotrexate would spare the patient both the time that could be wasted and the adverse effects of high methotrexate doses. Thus, the assessment of possible markers that can predict successful response to treatment or drug toxicity is being the current focus of some research studies [22-24].

The ABCB1 gene encodes the P-glycoprotein that is responsible for export of substances from the cell including different drugs and toxic metabolites. Polymorphisms affecting P-glycoprotein function or expression can alter drug effect and toxicity. Possible identification of allelic variants that modulate the pharmacokinetics of a drug, allows better therapy adjustment and increases its effectiveness and safety [6]. The CC genotype of C3435T has also been linked to higher risk of pharmacoresistance to a wide range of drugs including antiepileptics [14], antipsychotics, antidepressants [15], glucocorticoids [16] and methotrexate [17].

In the current study, the C3435T genotype and allele frequency was compared between responders to methotrexate and non-responders, and was also compared between patients who suffered from methotrexate side effects and those who did not. Our results revealed that the C3435T genotype and allele variants did not affect response to methotrexate in the studied patients. However, data in literature regarding the association between C3435T polymorphism and methotrexate response in RA patients is controversial. Similar results were reported by **Boughrara *et al.*** [25] who reported no difference in polymorphism distribution between the studied 65 RA patients who responded to methotrexate therapy and the 45 patients who did not respond to methotrexate. **Muralidharan *et al.*** [23] also reported the absence of significant differences regarding genotype and allele frequencies of 3435C>T between responders to methotrexate therapy and nonresponders. Moreover, in a study done in Jordan and included a total of 159 RA patients, **Samara *et al.*** [26] found that there was no association between C3435T polymorphism and methotrexate response.

Conversely, other studies did report an association between the C3435T polymorphism and response to methotrexate therapy. **Chen *et al.*** [22] found that patients with the CC genotype had a 2.44 fold greater risk of having refractory RA compared to patients with the TT genotype (OR: 2.44, 95 % CI: 1.13–5.28). **Mo *et al.*** [27] reported that in a cohort of 113 patients with RA, the probability of remission was significantly higher in patients carrying the TT genotype. **Abdurakhmanova *et al.*** [21] found that the TT genotype of the ABCB1 gene is associated with significant clinical response to methotrexate therapy. Data from these studies can be explained by the fact that P-glycoprotein is involved in methotrexate transport and increases methotrexate efflux from the cells. The exchange of the C nucleotide by T nucleotide in the ABCB1 gene results in reduced P-glycoprotein expression on the surface of cells with subsequent increase in cellular levels of P-glycoprotein substrates, which can result in not only greater activity of the drug, but also increased risk of susceptibility to adverse effects [28].

The fact that the results of our study in addition to other studies [23, 25, 26] did not detect this association between the polymorphism and response to methotrexate can be linked to two factors. The first factor is the ethnic variability regarding the C3435T polymorphism that has been documented in a study that assessed the C3435T genotypes in a total of 1280 individuals from 10 different ethnic groups and concluded that the frequency of the polymorphism is significantly influenced by ethnicity [29]. Furthermore, in a meta-

analysis that evaluated the studies regarding the effect of this polymorphism on response to methotrexate in RA patients, **He *et al.* [30]** found that in Asian populations there is an increased efficacy for methotrexate associated with the polymorphism in the codominant model (TT versus CC had an OR of 2.54 (95% CI:1.51-4.28) and the recessive model (OR:2.09; 95%CI:1.37-3.19) and that the T allele versus the C allele is associated with responsiveness with an OR of 1.64 (95% CI:1.09-2.48). However, the authors of the meta-analysis reported that this association is not found in Caucasian RA patients. The second factor explaining the lack of association in our study is that there are other molecular mechanisms that affect methotrexate resistance including impaired cellular uptake as a result of mutations in the reduced folate carrier, decrease in polyglutamation of methotrexate inside the cell that results in rapid efflux of the drug and mutations in the dihydrofolate reductase (DHFR) gene that produce an altered form of DHFR enzyme which has low affinity for methotrexate. The coexistence of one of these polymorphisms with the C3435T polymorphism may potentiate its effect on responsiveness to the drug. In other words, polymorphisms may have a somewhat minor influence on methotrexate response when they exist individually, however in combination, these polymorphisms can genetically alter response to methotrexate. Other factors may also affect response to methotrexate including complex gene-gene interactions, nutritional factors, inhibitions by co-medications as well as influence of co-morbidities [31].

A similar discrepancy is observed regarding the association of the C3435T polymorphism and the occurrence of methotrexate adverse effects and is mostly attributed to the same aforementioned factors. While our study and other studies[25, 32] revealed no association between C3435T genotype and methotrexate adverse effects, other studies did report such association. **Muralidharan *et al.* [23]** reported that the heterozygous CT genotype of MDR1 3435C>T was more frequent in patients with adverse events than in patients without adverse events and had an OR of 2.01 (95 % CI: 1.15–3.52) compared to CC genotype. **Samara *et al.* [26]** also found that the C allele is associated with a lower risk of adverse effects compared to the T allele (OR:0.386; 95%CI:0.152-0.992).

To conclude, our study showed no association between the ABCB1 C3435T polymorphism and susceptibility to RA, indicating that the ABCB1 C3435T polymorphism may not act as a genetic risk factor for RA. Our results also revealed that the ABCB1 C3435T polymorphism is not associated with response to methotrexate or methotrexate associated adverse effects.

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