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Association of *MTHFR* and *RFC1* gene polymorphisms with methotrexate efficacy and toxicity in Chinese Han patients with rheumatoid arthritis

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Abstract

Objective: The objective was to explore the association of methylene tetrahydrofolate reductase (*MTHFR*) C667T and A1298C and reduced folate carrier 1 (*RFC-1*) A80G single nucleotide polymorphisms (SNP) with rheumatoid arthritis (RA) and efficacy and toxicity of methotrexate (MTX) treatment in Chinese Han patients in Henan, China.

Methods: Two hundred ninety-six patients with RA were enrolled (cases) and 120 healthy individuals served as controls. The genotypes of *MTHFR* C667T and A1298C SNP and *RFC-1* A80G SNP were detected by restriction fragment length polymorphism-PCR and compared between cases and controls. We analyzed correlations of clinical effect, toxicity, and SNPs after 6 months of MTX treatment.

Results: We detected no significant differences in *MTHFR* C677T and A1298C and *RFC-1* A80G SNPs between cases and controls. The *RFC-1* A80G SNP differed between RA patients with good and poor efficacy after 6 months of MTX, and was an independent factor of MTX efficacy. The *MTHFR* C677T SNP was differently distributed in the adverse drug reaction (ADR) and non-ADR groups and was an independent factor of MTX toxicity.

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Conclusions: In Chinese Han patients with RA, the *MTHFR* C667T SNP may correlate with MTX toxicity, whereas the *RFC-1* A80G SNP may correlate with MTX efficacy rather than toxicity.

Keywords
Rheumatoid arthritis, methotrexate, *MTHFR*, *RFC-1*, polymorphism, toxicity, efficacy

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Introduction

Rheumatoid arthritis (RA), a chronic autoimmune disease featuring articular synovitis, causes the destruction of joint cartilage and bony erosion, eventually resulting in joint deformities and incapacitation.[1,2] At present, four types of medications for RA treatment are used: nonsteroidal anti-inflammatory drugs (NSAIDs), disease-modifying antirheumatic drugs (DMARDs), corticosteroids, and biological agents. The DMARD methotrexate (MTX) is the most commonly used drug for treatment of RA. However, individual variation in efficacy and adverse reactions to MTX treatment in RA have been reported, and one-third of patients failed to attain remission (due to toxicity or low efficacy), which seriously limits the clinical application of MTX.[3]

MTX is a folic acid antagonist. In addition to the environmental, physiological, and pathological factors that can influence MTX efficiency and toxicity, genetic polymorphisms in the MTX-metabolizing enzyme and transporter also result in individual differences of MTX efficacy and toxicity. Methylene tetrahydrofolate reductase (MTHFR) and reduced folate carrier 1 (RFC-1) are key proteins in MTX metabolism and transport, respectively, affecting the metabolism and transport of MTX in vivo. Previous studies showed that single nucleotide polymorphisms (SNP) in the *MTHFR* and *RFC-1* genes were associated with susceptibility to RA and the efficacy and toxicity of MTX. However, the results were controversial.[4–7] In this study, we aimed to investigate associations of *MTHFR* SNPs C677T and A1298C and *RFC-1* A80G SNP with susceptibility to RA and efficacy and toxicity of MTX to provide a theoretical basis for the clinical application of MTX.

Materials and methods

Patients

Two hundred ninety-six patients with RA, diagnosed from January 2016 to October 2017 in the Department of Rheumatology of The First Affiliated Hospital of Henan University, were collected as the case group, which included 88 male and 208 female patients, aged 30 to 70 years (average of 54.6 ± 11.6 years), with an average disease course of 5.93 ± 5.48 years. All patients met the RA classification standard stipulated by the American College of Rheumatology/European League Against Rheumatism in 2010.[8] Patients who were treated with MTX or other DMARDs for anti-rheumatic therapy within 3 months prior were excluded. All patients received MTX treatment at least for 6 months; no patients terminated MTX treatment. The DAS28 criteria were used to evaluate RA activity (low: <3.2, moderate: 3.2–5.1, and high: >5.1).[9] One hundred twenty healthy individuals in the same period were randomly collected as a control group, which included 38 men and 82 women from 28 to 71 years old, with an average age

of 35.7 ± 2.6 years old. The two groups did not differ statistically in sex or age. This study was reviewed and approved by the ethical inspection committee of the First Affiliated Hospital of Henan University, and all participants signed informed consent.

Genotype detection by restriction fragment length polymorphism (RFLP)-PCR

Four milliliters of peripheral venous blood was collected from each participant. Genomic DNA was extracted using a DNA extraction kit (Sigma Chemical Co., St. Louis, MO, USA) and stored at −20°C. The genotypes of *MTHFR* C677T and A1298C and *RFC-1* A80G were detected by RFLP-PCR analysis. The specific primers were synthesized by Sangon Biotech (Shanghai, China) and are shown in Table 1. The specific fragments containing the *MTHFR* C677T and A1298C and *RFC-1* A80G were amplified by PCR, and 10 μL of amplification product was digested with restriction endonucleases *Hinf*I, *Mbo*II, and *Ade*I (Takara Biotechnology Co. Ltd., Dalian, China) at 37°C for 3 hours, respectively. The digested fragments were detected by 3% agarose gel electrophoresis.

Treatment regimen

All patients with RA were given folic acid, vitamin D, and gastric mucosal protective agents during treatment. The combination therapy regimen included methotrexate tablets (Shanghai Xinyijinzhu Pharmaceutical Co. Ltd., Shanghai, China; SFDA approval number: H31020644) 10 to 15 mg, oral, once a week, combined with appropriate NSAIDs or hormonal drugs according to the specific condition of each patient. Treatment was continued for 6 months, after which the efficacy of MTX was evaluated.

Evaluation of MTX efficacy

The efficacy of MTX treatment was defined as a 20% improvement according to the American College of Rheumatology (ACR20) criteria, with the following indicators:[10] (1) tender joint count; (2) swollen joint count; (3) degree of pain determined by patient using a visual analogue scale (VAS); (4) patient's comprehensive assessment of RA; (5) physician's comprehensive assessment of RA patient; (6) global health assessment questionnaire to determine the health status of patients; (7) C-reactive protein level and erythrocyte sedimentation rate. If the first two indicators and at least three other indicators were improved by more than 20%, the ACR20 standard was met. According to whether patients reached the ACR20 standard, they were divided into a good efficacy group and a poor efficacy group.

Criteria of adverse drug reactions

MTX can cause adverse reactions in blood, the digestive system, liver, and other systems, and the criteria were as follows:[11] (1)

Table 1. PCR primer sequences (F: forward; R: reverse).

SNP	Primer sequence (5′-3′)	Amplification length
C677T in *MTHFR*	F: 5′-TGAAGGAGAAGGTGTCTGCGGGA-3′ R: 5′-AGGACGGTGCGGTGAGAGTG-3′	198 bp
A1298C in *MTHFR*	F: 5′-CTTTGGGGAGCTGAAGGACTACTAC-3′ R: 5′-CACTTTGTGACCATTCCGGTTTG-3′	163 bp
A80G in *RFC-1*	F: 5′-CTTCCAAGGTGCCCTGACT-3′ R: 5′-GCCATGAAGCCGTAGAAGC-3′	200 bp

MTHFR, methylene tetrahydrofolate reductase; *RFC-1*, reduced folate carrier 1.

gastrointestinal reactions: nausea, vomiting, or bloating after taking the drug; (2) skin and mucosa reactions: amount of hair loss or oral ulcers increased after taking the drug; (3) blood system symptoms: reduction of leukocytes ($<1.5 \times 10^3/\mu L$) or thrombocytopenia ($<70 \times 10^3/\mu L$) after taking drugs; and (4) liver function: increase in serum transaminase or glutamyl transpeptidase to two times higher than normal. The drug reactions were screened according to the National Adverse Drug Reaction Center adverse drug reaction (ADR) correlation.[12] The patients were divided into an ADR group and a non-ADR group, according to the above criteria. If severe ADRs had occurred, patients would stop the MTX treatment or measures would be taken to reduce ADRs.

Statistical analysis

We used SPSS 19.0 software (IBM Corp., Armonk, NY, USA) to analyze the data. Whether the genotype distribution fit Hardy–Weinberg (H-W) equilibrium was tested by the Chi-squared test; $P > 0.05$ indicated the sample was benign. Measurement data are shown as mean $\pm$ standard deviations, and the *t*-test was used to compare results between two groups. The Chi-squared test was conducted to compare enumeration data, genotype frequency, and allele frequency. $P < 0.05$ indicated a significant difference. The factors affecting MTX efficacy and toxicity were analyzed by logistic regression, and odds ratios (OR) and 95% confidence intervals (95% CI) represented relative risk.

Results

Distribution of genotype and allele frequency of MTHFR and RFC-1 polymorphisms in cases and controls

To explore the relationship of *MTHFR* and *RFC-1* polymorphisms with RA susceptibility, we analyzed the distribution of *MTHFR* C677T and A1298C and *RFC-1* A80G genotypes and allele frequencies statistically. The results showed that the genotype distribution of SNP of *MTHFR* and *RFC-1* conformed to H-W equilibrium ($P > 0.05$), indicating that the samples were typically representative. There was no difference in the distribution of *MTHFR* C677T and A1298C and *RFC-1* A80G genotypes and allele frequencies between cases and controls (Table 2), which indicated that the *MTHFR* C677T and A1298C and *RFC-1* A80G SNPs were not associated with RA susceptibility in the Henan Han population.

Association between MTHFR and RFC-1 SNP and MTX efficacy

After 6 months of MTX treatment, 59.5% of the patients were in the good efficacy group and 40.5% were in the poor efficacy group according to ACR20 criteria. There was no difference in sex, age, weight, RA course, or MTX dose between the two efficacy groups. The patients with good efficacy had lower DAS28 scores than patients with poor efficacy, and disease activity in the two groups was significantly different (Table 3). No differences between groups was found for *MTHFR* C677T and A1298C SNPs, whereas the frequency of the RFC-1 *AA* allele was higher in the good efficacy group than in the poor efficacy group. These results indicated no association between MTX efficacy and *MTHFR* C677T and A1298C SNP, whereas the *RFC-1* A80G SNP was associated with MTX efficacy.

Logistic regression analysis of MTHFR and RFC-1 SNP and MTX efficacy

The general data, MTX efficacy as a dependent variable, and *MTHFR* and *RFC-1* SNP were incorporated into a

Table 2. Comparison of the distribution of *MTHFR* and *RFC-1* SNP genotypes and allele frequencies in case and control groups.

	Case group (n = 296)	Control group (n = 120)	χ^2	*P*	Sig.	OR value (95% CI)
MTHFR C677T polymorphism						
Genotype [n (%)]						
CC	160 (54.0)	68 (56.7)	2.640	0.267	0.240	1
CT	110 (37.2)	47 (39.2)			0.489	0.984 (0.154–1.334)
TT	26 (8.8)	5 (4.1)			0.150	1.005 (0.606–1.668)
Alleles [n (%)]						
C	430 (72.6)	183 (76.2)	1.151	0.283	0.282	1
T	162 (27.4)	57 (23.8)				0.827 (0.559–1.224)
H-W	$\chi^2 = 0.180$, $P = 0.914$	$\chi^2 = 0.529$, $P = 0.768$				
MTHFR A1298C polymorphism						
Genotype [n (%)]						
AA	178 (60.1)	70 (58.3)	0.799	0.671	0.670	1
AC	90 (30.4)	41 (34.2)			0.584	1.158 (0.684–1.961)
CC	28 (9.5)	9 (7.5)			0.685	0.817 (0.334–1.998)
Alleles [n (%)]						
A	446(75.3)	181 (75.4)	0.001	0.981	0.983	1
C	146(24.7)	59 (24.6)				0.996 (0.671–1.478)
H-W	$\chi^2 = 1.808$, $P = 0.405$	$\chi^2 = 0.421$, $P = 0.810$				
RFC-1 A80G polymorphism						
Genotype [n (%)]						
AA	60 (20.3)	33 (27.5)	4.489	0.106	0.103	1
AG	154 (52.0)	64 (53.3)			0.334	0.746 (0.412–1.352)
GG	82 (27.7)	23 (19.2)			0.074	0.523 (0.256–1.066)
Alleles [n (%)]						
A	276 (46.6)	130 (54.2)	3.595	0.058	0.056	1
G	316 (53.4)	110 (45.8)				0.729 (0.518–1.026)
H-W	$\chi^2 = 0.249$, $P = 0.883$	$\chi^2 = 0.205$, $P = 0.903$				

MTHFR, methylene tetrahydrofolate reductase; *RFC-1*, reduced folate carrier 1; OR, odds ratio; CI, confidence interval; H-W, Hardy–Weinberg.

logistic regression model to analyze the independent risk factors of MTX efficacy (Table 4). The results showed that disease activity, MTX dose, and *RFC-1* A80G SNP might be independent factors influencing MTX efficacy; patients with the *RFC-1 GG* genotype had a higher risk of poor efficacy, which was 2.819 times higher than that of patients with the *AA* genotype.

Association between MTHFR and RFC-1 SNP and MTX toxicity

One hundred forty patients in the case group had ADR during MTX treatment. A comparison of baseline data and genotype frequency showed a significant difference in disease activity and *MTHFR* C677T genotype frequency in the ADR group and the non-ADR group ($P < 0.05$), whereas *MTHFR*

Table 3. Associations between baseline data, *MTHFR* and *RFC-1* SNP, and MTX efficacy.

Baseline data and genotype	Good efficacy group (n = 176)	Poor efficacy group (n = 120)	χ^2/t	*P*
Sex [n (%)]				
Male	60 (34.1)	36 (30.0)	0.545	0.460
Female	116 (65.9)	84 (70.0)		
Age (years)	52.65 ± 10.62	54.08 ± 10.17	0.649	0.518
Weight (kg)	57.87 ± 10.15	56.44 ± 10.98	0.470	0.639
RA course (years)	7.07 ± 4.61	6.41 ± 4.34	0.879	0.383
MTX dose (mg)	13.89 ± 1.99	14.19 ± 2.03	1.281	0.201
DAS28 score	3.88 ± 1.66	4.42 ± 1.45	2.021	0.045
Disease activity [n (%)]				
Low	28 (31.8)	10 (16.7)	7.069	0.029
Moderate	37 (42.0)	23 (38.3)		
High	23 (26.1)	27 (45.0)		
MTHFR C677T genotype [n (%)]				
CC	102 (58.0)	58 (48.3)	3.569	0.168
CT	62 (35.2)	48 (40.0)		
TT	12 (6.8)	14 (11.7)		
MTHFR A1298C genotype [n (%)]				
AA	112 (63.6)	66 (55.0)	4.192	0.123
AC	52 (29.5)	38 (31.7)		
CC	12 (6.8)	16 (13.3)		
RFC-1 A80G genotype [n (%)]				
AA	56 (31.8)	24 (20.0)	8.828	0.012
AG	78 (44.3)	50 (41.7)		
GG	42 (23.9)	46 (38.3)		

MTHFR, methylene tetrahydrofolate reductase; *RFC-1*, reduced folate carrier 1; MTX, methotrexate.

Table 4. Logistic regression analysis of *RFC-1* SNP and MTX efficacy.

Factors	Regression coefficient	χ^2	*P*	OR (95%CI)
RFC-1 A80G polymorphism				
AA	–	11.209	0.004	1
AG	0.063	0.034	0.853	1.065 (0.547–2.074)
GG	0.986	8.089	0.004	2.819 (1.359–6.997)
Disease activity				
Low	–	8.046	0.018	1
Moderate	0.177	0.399	0.528	1.193 (0.690–2.064)
High	1.350	8.045	0.005	3.158 (1.518–9.805)
MTX dose	0.819	5.229	0.0321	0.441 (0.306–0.864)

MTHFR, methylene tetrahydrofolate reductase; RFC-1, reduced folate carrier 1; MTX, methotrexate.

A1298C and *RFC-1* A80G SNP did not differ between the two groups (Table 5).

The general data and *MTHFR* and *RFC-1* SNP were incorporated into the logistic regression model to analyze the independent factors affecting MTX adverse reactions (Table 6). Disease activity, MTX dose, and *MTHFR* C677T SNP might be

Table 5. Association between baseline data, *MTHFR* and *RFC-1* SNP and MTX toxicity.

Baseline data and genotype	ADR group (n = 140)	Non-ADR group (n = 156)	χ^2/t	*P*
Sex [n (%)]				
Male	44 (23.9)	52 (33.3)	0.122	0.727
Female	96 (76.1)	104 (66.7)		
Age (years)	51.38 ± 10.43	53.49 ± 10.10	1.763	0.079
Weight (kg)	57.49 ± 11.04	57.59 ± 10.91	0.088	0.930
RA course (years)	6.71 ± 4.13	6.88 ± 4.72	0.095	0.891
MTX dose (mg)	14.25 ± 1.96	13.71 ± 2.03	1.625	0.108
DAS28 score	3.98 ± 1.62	4.27 ± 1.55	1.749	0.081
Disease activity [n (%)]				
Low	28 (20.0)	52 (33.3)	6.692	0.035
Moderate	64 (45.7)	58 (37.2)		
High	48 (34.3)	46 (29.5)		
MTHFR C677T genotype [n (%)]				
CC	82 (58.6)	78 (50.0)	6.785	0.034
CT	42 (30.0)	68 (43.6)		
TT	16 (11.4)	10 (6.4)		
MTHFR A1298C genotype [n (%)]				
AA	76 (54.3)	102 (65.4)	4.327	0.115
AC	46 (32.8)	42 (26.9)		
CC	18 (12.9)	12 (7.7)		
RFC-1 A80G genotype [n (%)]				
AA	32 (22.8)	28 (17.9)	2.712	0.258
AG	76 (54.3)	80 (51.3)		
GG	32 (22.8)	48 (30.8)		

MTHFR, methylene tetrahydrofolate reductase; *RFC-1*, reduced folate carrier 1; RA, rheumatoid arthritis; MTX, methotrexate; ADR, adverse drug reaction.

Table 6. Logistic regression analysis of *MTHFR* C677T SNP and MTX adverse reaction.

Factors	Regression coefficient	χ^2	*P*	OR (95% CI)
MTHFR C677T polymorphism				
CC		10.037	0.007	1
CT	0.703	1.244	0.265	0.495 (0.296–0.830)
TT	1.062	9.021	0.003	2.892 (1.446–5.782)
Disease activity				
Low		9.910	0.024	1
Moderate	0.949	9.243	0.360	0.398 (0.195–0.814)
High	0.799	6.189	0.012	2.307 (0.394–4.351)
MTX dose	0.594	5.834	0.016	0.552 (0.341–0.894)

MTHFR, methylene tetrahydrofolate reductase; MTX, methotrexate.

independent factors influencing MTX toxicity, and the adverse reaction risk of patients with *MTHFR TT* genotype at C677T was 2.307 times higher than that of patients with *CC* genotype.

Discussion

MTHFR is a key enzyme and DNA methyl donor in the folate metabolic pathway, catalyzing the irreversible conversion of 5,10-methylene tetrahydrofolate into 5-methyltetrahydrofolate. The SNP in *MTHFR* might cause hypomethylation of genomic DNA and hyperhomocysteinemia, which are related to the pathogenesis of RA, meaning that *MTHFR* might be a susceptible gene in RA. In the *MTHFR* gene, two missense mutations have mostly been studied: C677T and A1298C, but the associations with genetic susceptibility of RA were inconsistent among different studies.[13,14] MTX enters cells mediated by RFC-1, which is the dominant transporter molecule for folinic acid on the cell membrane. The *RFC-1* SNP might affect the reduction of dihydrofolate to tetrahydrofolate and the levels of folate and homocysteine in serum, and thus be involved in the pathogenesis of RA. However, few studies have examined the relationship between *RFC-1* SNP and RA. In the present study, we detected no difference in genotypes and allele frequencies of *MTHFR* C677T and A1298C and RFC-1 A80G between patients with RA (cases) and controls, indicating that these polymorphisms were not associated with RA susceptibility in the Henan Han population, consistent with the results of Gonzalez-Mercado et al.[15] and Hashiguchi et al.[16]

MTX, as a DMARD, is the first-line anchor drug for treating RA. It plays a role in the folate transport pathway by inhibiting the key enzymes. However, associations between the *MTHFR* polymorphism and MTX efficacy or adverse reactions remain controversial.[17] Several studies have reported that the *MTHFR* C677T SNP is not associated with MTX efficacy but is associated with adverse reactions to MTX. The correlations between *MTHFR* A1298C and MTX efficacy or adverse reactions are inconsistent. The meta-analysis of Shao et al.[18] found that *MTHFR* C677T SNP was associated with MTX toxicity, but not efficacy, in RA patients. The meta-analysis of Fan et al.[19] suggested that *MTHFR* A1298C SNP had no significant effect on MTX toxicity or efficacy in RA patients, whereas there was a significant association between *MTHFR* A1298C SNP and MTX efficacy in a South Asian population. Berkani et al.[20] indicated that *MTHFR* C677T and A1298C SNP were associated with MTX toxicity and efficacy, respectively, in RA patients. In the current study, *MTHFR* C677T SNP was not associated with MTX efficacy but was associated with MTX toxicity, and A1298C SNP was not associated with MTX efficacy or toxicity, results that are not fully consistent with previous results. The *MTHFR* C-to-T SNP at position 677, causing an Ala-Val missense mutation at codon 222, might decrease the activity of *MTHFR* and result in hyperhomocysteinemia and an adverse reaction to MTX, whereas the *MTHFR* A-to-C SNP at position 1298, which also resulted in a missense mutation, might influence activity of MTHFR but did not affect the level of serum homocysteine, indicating that it was not related to an adverse reaction to MTX. However, in addition to the MTHFR-folate metabolic pathway, MTX could cause remission of RA through other pathways, such as the ATIC-5-aminoimidazole-4-carboxamide ribonucleotide (AICAR)-adenosine pathway.[17] This might explain why the *MTHFR* polymorphism was not related to MTX efficacy. RFC-1 transports MTX and converts it to polyglutamate, which plays an immunosuppressive role

in cells. Multiple studies have reported that *RFC-1* A80G might be related to MTX efficacy and toxicity, but the results were not consistent. For example, Hayashi et al.[21] and Tazoe et al.[22] reported that RA patients with the G allele had less intracellular MTX and poor efficacy compared with patients with the A allele, suggesting that the *RFC-1* A80G SNP may be associated with MTX efficacy in Japanese RA patients. The meta-analysis of Kung et al.[23] indicated that *RFC-1* A80G was associated with MTX efficacy but not toxicity in RA patients, consistent with the results of Li et al.[24] Samara et al.[25] suggested that patients with the *RFC-1 GG* genotype had higher risk for gastrointestinal toxicity, and that the *RFC-1* A80G SNP affected the toxicity but not the efficacy of MTX. In the present study, *RFC-1* A80G SNP was associated with MTX efficacy and the independent influencing factors of MTX efficacy. Patients with the *GG* genotype had poor efficacy, but this was not related to MTX toxicity. It has been speculated that the A80G SNP is associated with a His-to-Arg missense mutation at codon 27, which interferes with MTX and folate transport into cells, thus affecting the efficacy of MTX.[26]

In conclusion, in Henan Han patients with RA, *MTHFR* C677T and A1298C SNPs were not associated with MTX efficacy, although the C677T SNP was related to MTX toxicity. The *RFC-1* A80G SNP was correlated with MTX efficacy but not with MTX toxicity. However, all of the participants were Han Chinese from Henan Province, so our results have certain limitations. The results were not identical to the previously published reports, for the following possible reasons: (1) the genetic background may have been affected by race, environment, and geographic latitude; (2) metabolism and transport of MTX could be regulated by multiple genes, so a single gene or SNP analysis is inconclusive; (3) the sample size was too small or the inclusion criteria were too restrictive; and (4) the SNP in this study might be in linkage disequilibrium with other SNP loci. Therefore, further studies should be conducted using a larger sample size from a different geographical region and with participants of different ethnicity to reveal gene polymorphisms associated with MTX efficacy and toxicity in RA patients and establish a theoretical basis for the application and individualization of MTX in clinical practice.

Declaration of conflicting interest

The authors declare that there is no conflict of interest.

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