Retrospective Clinical Research Report

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

Date received: 20 May 2019; accepted: 10 September 2019

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. 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.

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. 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. 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). 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 HinfI, MboII, and AdeI (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’-TGAAGGAGAAGGTGTGCTTGCTGGGGA-3’ | 198 bp               |
|                 | R: 5’-AGGACCGTGGTGAGTGCAGGTG-3’       |                     |
| A1298C in MTHFR | F: 5’-CTTTTCGGGGAGCAGGGAGCTACTAC-3’  | 163 bp               |
|                 | R: 5’-CAGCTTTGGACCCCATCCGGTGTTT-3’    |                     |
| A80G in RFC-1   | F: 5’-CTTCCAAGGTTGCCCTGACT-3’         | 200 bp               |
|                 | R: 5’-CCCATGAGCGGCTAAGGC-3’           |                     |

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 × 10^3/μL) or thrombocytopenia (<70 × 10^3/μL) 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.\textsuperscript{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 ± 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 \textit{MTHFR} and \textit{RFC-1} polymorphisms with RA susceptibility, we analyzed the distribution of \textit{MTHFR} C677T and A1298C and \textit{RFC-1} A80G genotypes and allele frequencies statistically. The results showed that the genotype distribution of SNP of \textit{MTHFR} and \textit{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 \textit{MTHFR} C677T and A1298C and \textit{RFC-1} A80G genotypes and allele frequencies between cases and controls (Table 2), which indicated that the \textit{MTHFR} C677T and A1298C and \textit{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 \textit{MTHFR} C677T and A1298C SNPs, whereas the frequency of the \textit{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 \textit{MTHFR} C677T and A1298C SNP, whereas the \textit{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 \textit{MTHFR} and \textit{RFC-1} SNP were incorporated into a
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.

**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) | χ²   | 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 | 0.984| 0.154–1.334      |
| TT                   | 26 (8.8)             | 5 (4.1)                 | 0.150| 1.005 | 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                  | χ² = 0.180,          | χ² = 0.529,             | 0.914| 0.768 |      |                   |
|                      | P = 0.914            | 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 | 1.158| 0.684–1.961      |
| CC                   | 28 (9.5)             | 9 (7.5)                 | 0.685| 0.817 | 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                  | χ² = 1.808,          | χ² = 0.421,             | 0.405| 0.810 |      |                   |
|                      | P = 0.040            | 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 | 0.746| 0.412–1.352      |
| GG                   | 82 (27.7)            | 23 (19.2)               | 0.074| 0.523 | 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                  | χ² = 0.249,          | χ² = 0.205,             | 0.883| 0.903 |      |                   |

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

**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*...
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 \((n = 156)\) | \(\chi^2/lt\) | \(P\) |
|---------------------------|-----------------------------|-----------------------------|---------------|-----|
| 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 | \(\chi^2\) | \(P\) | OR (95% CI) |
|-----------------------------|------------------------|------------|------|-------------|
| \textit{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) |

\textit{MTHFR}, methylene tetrahydrofolate reductase; MTX, methotrexate.
independent factors influencing MTX toxicity, and the adverse reaction risk of patients with \textit{MTHFR} \textit{TT} genotype at C677T was 2.307 times higher than that of patients with \textit{CC} genotype.

**Discussion**

\textit{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 \textit{MTHFR} might cause hypomethylation of genomic DNA and hyperhomocysteinemia, which are related to the pathogenesis of RA, meaning that \textit{MTHFR} might be a susceptible gene in RA. In the \textit{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.\textsuperscript{13,14} \textit{MTX} enters cells mediated by \textit{RFC-1}, which is the dominant transporter molecule for folinic acid on the cell membrane. The \textit{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 \textit{RFC-1} SNP and RA. In the present study, we detected no difference in genotypes and allele frequencies of \textit{MTHFR} C677T and A1298C and \textit{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.\textsuperscript{15} and Hashiguchi et al.\textsuperscript{16}

\textit{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 \textit{MTHFR} polymorphism and \textit{MTX} efficacy or adverse reactions remain controversial.\textsuperscript{17} Several studies have reported that the \textit{MTHFR} C677T SNP is not associated with \textit{MTX} efficacy but is associated with adverse reactions to \textit{MTX}. The correlations between \textit{MTHFR} A1298C and \textit{MTX} efficacy or adverse reactions are inconsistent. The meta-analysis of Shao et al.\textsuperscript{18} found that \textit{MTHFR} C677T SNP was associated with \textit{MTX} toxicity, but not efficacy, in RA patients. The meta-analysis of Fan et al.\textsuperscript{19} suggested that \textit{MTHFR} A1298C SNP had no significant effect on \textit{MTX} toxicity or efficacy in RA patients, whereas there was a significant association between \textit{MTHFR} A1298C SNP and \textit{MTX} efficacy in a South Asian population. Berkani et al.\textsuperscript{20} indicated that \textit{MTHFR} C677T and A1298C SNP were associated with \textit{MTX} toxicity and efficacy, respectively, in RA patients. In the current study, \textit{MTHFR} C677T SNP was not associated with \textit{MTX} efficacy but was associated with \textit{MTX} toxicity, and A1298C SNP was not associated with \textit{MTX} efficacy or toxicity, results that are not fully consistent with previous results. The \textit{MTHFR} C-to-T SNP at position 677, causing an Ala-Val missense mutation at codon 222, might decrease the activity of \textit{MTHFR} and result in hyperhomocysteinemia and an adverse reaction to \textit{MTX}, whereas the \textit{MTHFR} A-to-C SNP at position 1298, which also resulted in a missense mutation, might influence activity of \textit{MTHFR} but did not affect the level of serum homocysteine, indicating that it was not related to an adverse reaction to \textit{MTX}. However, in addition to the \textit{MTHFR}-folate metabolic pathway, \textit{MTX} could cause remission of RA through other pathways, such as the ATIC-5-aminoimidazole-4-carboxamide ribonucleotide (AICAR)-adenosine pathway.\textsuperscript{17} This might explain why the \textit{MTHFR} polymorphism was not related to \textit{MTX} efficacy. \textit{RFC-1} transports \textit{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.\textsuperscript{21} and Tazoe et al.\textsuperscript{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.\textsuperscript{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.\textsuperscript{24} Samara et al.\textsuperscript{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.\textsuperscript{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.

Funding
This study was funded by Natural Science Foundation of China (No. 81471558).

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References
1. Guo Q, Wang Y, Xu D, et al. Rheumatoid arthritis: pathological mechanisms and modern pharmacologic therapies. Bone Res 2018, 6: 5–18.
2. Emery P. Rheumatoid arthritis: an overview. Tarporley: Springer Healthcare Ltd, 2011, p.1–6.
3. Salliot C and van der Heijde D. Long-term safety of methotrexate monotherapy in patients with rheumatoid arthritis: a systematic literature research. Ann Rheum Dis 2009; 68: 1100–1104.
4. He HR, Liu P, He GH, et al. Association between reduced folate carrier G80A polymorphism and methotrexate toxicity in childhood acute lymphoblastic leukemia: a meta-analysis. Leukemia Lymphoma 2014; 55: 2793–2800.
5. Morgan MD, Al-Shaarawy N, Martin S, et al. MTHFR functional genetic variation and methotrexate treatment response in
rheumatoid arthritis: a meta-analysis. *Pharmacogenomics* 2014; 15: 467–475.

6. Owen SA, Lunt M, Bowes J, et al. MTHFR gene polymorphisms and outcome of methotrexate treatment in patients with rheumatoid arthritis: analysis of key polymorphisms and meta-analysis of C677T and A1298C polymorphisms. *Pharmacogenomics J* 2013; 13: 137–147.

7. Cáliz R, Amo JD, Balsa A, et al. The C677T polymorphism in the MTHFR gene is associated with the toxicity of methotrexate in a Spanish rheumatoid arthritis population. *Scand J Rheumatol* 2012; 41: 10.

8. Aletaha D, Neogi T, Silman AJ, et al. The 2010 rheumatoid arthritis classification criteria. An American College of Rheumatology/European League Against Rheumatism Collaborative Initiative. *Arthritis Rheum* 2010; 62: 2569–2581.

9. Makinen H, Kautiainen H, Hannonen P, et al. Is DAS28 an appropriate tool to assess remission in rheumatoid arthritis? *Ann Rheum Dis* 2005; 64: 1410–1413.

10. Neogi T, Aletaha D, Silman AJ, et al. The 2010 American College of Rheumatology/European League Against Rheumatism classification criteria for rheumatoid arthritis: Phase 2 methodological report. *Arthritis Rheum* 2010; 62: 2582–2591.

11. Sun Q, Xie Y, Zhao WH, et al. Adverse effects of high-dose methotrexate therapy. *Chinese J Contemp Pediatr* 2017; 19: 781–785.

12. National Center for ADRs Monitoring, China. The manual of adverse drug reactions reporting and monitoring. 2012. http://www.cdr-adr.org.cn/xzzx/hyzl/hyzl2013nd/201304/t20130426_5436.html

13. Cen H, Huang H, Zhang LN, et al. Associations of methylenetetrahydrofolate reductase (MTHFR) C677T and A1298C polymorphisms with genetic susceptibility to rheumatoid arthritis: a meta-analysis. *Clin Rheumatol* 2017; 36: 287–297.

14. Saleh MM, Irshaid YM and Mustafa KN. Methylene tetrahydrofolate reductase genotypes frequencies: association with toxicity and response to methotrexate in rheumatoid arthritis patients. *Int J Clin Pharmacol Ther* 2015; 53: 154–162.

15. Gonzalez-Mercado MG, Rivas F, Gallegos-Arreola MP, et al. MTRR A66G, RFC1 G80A, and MTHFR C677T and A1298C polymorphisms and disease activity in Mexicans with rheumatoid arthritis treated with methotrexate. *Genet Test Mol Biomarkers* 2017; 21: 698–704.

16. Hashiguchi M, Shimizu M, Hakamata J, et al. Genetic polymorphisms of enzyme proteins and transporters related to methotrexate response and pharmacokinetics in a Japanese population. *J Pharm Health Care Sci* 2016; 2: 35.

17. Blits M, Jansen G, Assaraf YG, et al. Methotrexate normalizes up-regulated folate pathway genes in rheumatoid arthritis. *Arthritis Rheum* 2013; 65: 2791–2802.

18. Shao W, Yuan Y and Li Y. Association between MTHFR C677T polymorphism and methotrexate treatment outcome in rheumatoid arthritis patients: A systematic review and meta-analysis. *Genet Test Mol Biomarkers*. 2017; 21: 275–285.

19. Fan H, Li Y, Zhang L, et al. Lack of association between MTHFR A1298C polymorphism and outcome of methotrexate treatment in rheumatoid arthritis patients: evidence from a systematic review and meta-analysis. *Int J Rheum Dis* 2017; 20: 526–540.

20. Berkani LM, Rahal F, Allam I, et al. Association of MTHFR C677T and A1298C gene polymorphisms with methotrexate efficiency and toxicity in Algerian rheumatoid arthritis patients. *Heliyon* 2017; 3: e00467.

21. Hayashi H, Tazoe Y, Tsuboi S, et al. A single nucleotide polymorphism of reduced folate carrier 1 predicts methotrexate efficacy in Japanese patients with rheumatoid arthritis. *Drug Metab Pharmacokinet* 2013; 28: 164–168.

22. Tazoe Y, Hayashi H, Tsuboi S, et al. AB0036 Analysis of genetic polymorphisms in folate pathway affecting the efficacy of methotrexate in Japanese patients with rheumatoid arthritis. *Ann Rheum Dis* 2013; 71: 639.

23. Kung TN, Dennis J, Ma Y, et al. RFC1 80G>A is a genetic determinant of methotrexate efficacy in rheumatoid arthritis: a
human genome epidemiologic review and meta-analysis of observational studies. *Arthritis Rheum* 2014; 66: 1111–1120.

24. Li X, Hu M, Li W, et al. The association between reduced folate carrier-1 gene 80G/A polymorphism and methotrexate efficacy or methotrexate related-toxicity in rheumatoid arthritis: A meta-analysis. *Int Immunopharmacol* 2016; 38: 8–15.

25. Samara SA, Irshaid YM and Mustafa KN. Association of MDR1 C3435T and RFC1 G80A polymorphisms with methotrexate toxicity and response in Jordanian rheumatoid arthritis patients. *Int J Clin Pharmacol Ther* 2014; 52: 746–755.

26. Chiusolo P, Giammarco S, Bellesi S, et al. The role of MTHFR and RFC1 polymorphisms on toxicity and outcome of adult patients with hematological malignancies treated with high-dose methotrexate followed by leucovorin rescue. *Cancer Chemother Pharmacol* 2012; 69: 691–696.