Pharmacogenomics and Personalized Medicine

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ORIGINAL RESEARCH

Associations Between Genetic Polymorphisms Within Transporter Genes and Clinical Response to Methotrexate in Chinese Rheumatoid Arthritis Patients: A Pilot Study

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Purpose: To investigate the associations between genetic polymorphisms within transporter genes and clinical response to methotrexate (MTX) in Chinese rheumatoid arthritis (RA) patients.

Patients and Methods: A total of 100 RA patients receiving MTX were prospectively followed up for approximately 3 months to determine the clinical response based on several criteria, including European League Against Rheumatism (EULAR) good and moderate response, disease activity score in 28 joint counts – erythrocyte sedimentation rate (DAS28-ESR) low disease activity (LDA), change in DAS28-ESR (ΔDAS28-ESR) and ΔDAS28-ESR >0.6. Fifty-four single nucleotide polymorphisms (SNPs) within seven transporter genes, including *SLC19A1, ABCB1, ABCC1~4* and *ABCG2*, were genotyped.

Results: Multivariable analysis revealed that *SLC19A1* rs12659 and rs3788200, *ABCC2* rs3740066, rs4148396 and rs717620 were significantly associated with EULAR good and moderate response, and *ABCC2* rs3740066 and rs717620 were significantly associated with DAS28-ESR LDA, and *ABCB1* rs1128503, rs4148737 and *ABCC3* rs2277624, rs4148416 were significantly associated with ΔDAS28-ESR. Moreover, 12 genetic polymorphisms were found to be significantly associated with ΔDAS28-ESR >0.6. With adjustment for corresponding confounders, *SLC19A1* TGAA haplotype consisting of rs1051266, rs1131596, rs12659 and rs3788200 was significantly associated with EULAR good and moderate response and ΔDAS28-ESR >0.6 compared with the most common haplotype CAGG. The *ABCC2* haplotype TTT composed of rs717620, rs4148396 and rs3740066 was significantly associated with EULAR good and moderate response and ΔDAS28-ESR >0.6 compared with the most common haplotype CCC.

Conclusion: Our results highlight the potential of genetic polymorphisms within transporter genes, particularly *SLC19A1* and *ABCC2*, as predictors of clinical response to MTX in Chinese RA patients.

Keywords: rheumatoid arthritis, methotrexate, transporter, single-nucleotide polymorphism, clinical response

Introduction

Rheumatoid arthritis (RA) is a debilitating systemic autoimmune disease affecting about 5 per 1000 adults worldwide, and irreversible joint damage and disability would ensue if without timely diagnosis and treatment.[1] Over the past decades, the RA treatment landscape has dramatically changed, attributed to the application of treat-to-target approach and the increasing availability of biological disease-modifying anti-rheumatic drugs (bDMARDs).[2] In spite of this, methotrexate (MTX) is still recommended as the initial therapeutic drug for most newly diagnosed RA patients owing to its good efficacy, long-term safety and low price.[3–5] A considerable proportion of RA patients, however, do not respond satisfactorily to MTX,[6,7] often necessitating a switch to or addition of another DMARD

Received: 26 November 2021
Accepted: 28 March 2022
Published: 12 April 2022

according to the treat-to-target principle. Of note, since early sufficient response to MTX determines the long-term prognosis of RA,[8,9] it is of great clinical significance to investigate predictors of clinical response to MTX in RA patients.

Currently, although the precise mechanism of action of MTX has not been fully elucidated, accumulating evidence indicates that MTX entering cells by transporters undergoes serial polyglutamation to form MTX-polyglutamates (MTX-PGs), which then exert anti-inflammatory and immunosuppressive response through multiple mechanisms such as the inhibition of purine and pyrimidine synthesis, transmethylation reactions and the promotion of adenosine release. Finally, the long-chain MTX-PGs are converted into short-chain MTX and ultimately to MTX, which could be transported out of cells via ATP binding cassette (ABC) transporters.[10] Notably, available evidence indicates that solute carrier family 19 member 1 (SLC19A1) is the main transporter mediating the influx of MTX, while the efflux of MTX mainly involves ABCB1, ABCC1~4 and ABCG2.[11,12] These transporters are expressed in multiple tissues and remarkably determine the pharmacokinetics of MTX, including absorption, distribution and elimination. Over the past decades, there is growing interest in pharmacogenetic studies owing to the success of high throughput genotyping technology development and the stable quality of genetic polymorphisms, and much more efforts have been made to dissect the role of single-nucleotide polymorphisms (SNPs) within genes encoding proteins implicated in pharmacokinetics and pharmacodynamics in the inter-individual variability of therapeutic drug response.[13] In the context of pharmacogenetic studies of MTX in RA, based on the considerable effects of transporters on pharmacokinetics, there is indeed a great deal of studies investigating the associations of genetic polymorphisms within aforementioned transporter genes with clinical response to MTX in RA patients with inconsistent results.[11,14] However, the majority of these studies focused on a few SNPs within some transporters, and most were performed in Caucasians. In view of the genetic heterogeneity among different ethnic populations, we determined to systematically examine the associations of genetic polymorphisms within transporter genes (*SLC19A1, ABCB1, ABCC1~4* and *ABCG2*) with clinical response to MTX in Chinese RA patients.

Methods

Study Design

A total of 100 adult RA patients (age ≥18 years) with active disease, who received MTX monotherapy and were prospectively followed up for approximately 3 months, were recruited from the Department of Rheumatology, Ningbo First Hospital between August 2015 and January 2021. All these RA patients fulfilled the 1987 American College of Rheumatology revised criteria[15] or the 2010 American College of Rheumatology/European League Against Rheumatism (EULAR) criteria for the classification of RA.[16] Only DMARD-naive RA patients or RA patients who had discontinued the use of DMARD for more than six months were eligible. The dose of MTX was increased to the maximum based on efficacy and toxicity considerations. Folic acid was given to all patients with RA. Other concomitant DMARDs were not allowed, while the low-dose glucocorticoid (GC) and nonsteroidal anti-inflammatory drugs (NSAIDs) were permitted. This study was conducted in accordance with the Declaration of Helsinki and approved by the Ethics Committee of Ningbo University (Ethics ID: SX2019139). Informed consent was obtained from all participants.

Data Collection

The baseline information of the following variables was collected: age, gender, smoking and drinking status, disease duration, rheumatoid factor (RF) and anti-cyclic citrullinated peptide (CCP) antibody status, tender joint count (TJC) and swollen joint count (SJC) in 28 joints, erythrocyte sedimentation rate (ESR), MTX dosage, concomitant GC use and prednisolone equivalent dosage, and concomitant NSAIDs use. Moreover, the values of the components of disease activity score in 28 joint counts – ESR (DAS28-ESR),[17] namely TJC, SJC and ESR, were collected again at the end of approximate 3-month follow-up.

Clinical Response Assessment

The clinical response to MTX was evaluated based on multiple response criteria, including European League Against Rheumatism (EULAR) response criteria (RA patients who achieved good and moderate response were classified as responders and the remainders achieving poor response were classified as non-responders),[18] DAS28-ESR low disease activity (LDA) (DAS28-ESR $\leq$ 3.2), change in DAS28-ESR (ΔDAS28-ESR) (the difference in DAS28-ESR from baseline to 3 months) and ΔDAS28-ESR > 0.6. When patients received additional DMARDs during the follow-up period, those patients were also classified as non-responders.

SNP Selection

Genetic polymorphisms within genes encoding transporters (SLC19A1, ABCB1, ABCC1~4 and ABCG2) were systematically selected based on literature report and genomic locations. In brief, the list of SNPs reported in the literature and the list of SNPs located in potentially functional regions, including exons, 5'-untranslated regions (5'-UTRs) and 3'-UTRs, with minor allele frequency (MAF) of more than 0.1 in Han Chinese, Beijing were merged. Finally, a total of 57 SNPs were chosen, and details about the selected SNPs are summarized in Table S1. Among these selected SNPs, three (*SLC19A1* rs1051296, rs1051298 and rs7499) were not included for the final genotyping experiment due to technical issues; eventually, a total of 54 genetic polymorphisms were genotyped.

DNA Extraction and Genotyping

About 2 mL EDTA anti-coagulated venous blood sample was collected from each participant, and genomic DNA was extracted from peripheral blood lymphocytes using a full automatic nucleotide acid extraction instrument based on the standard procedures of the corresponding commercial DNA extraction kit (NP968-S system, Tianlong, China). The concentration and purity of DNA samples were determined utilizing a NanoDrop 2000 UV spectrophotometer. The genetic polymorphisms were detected by Shanghai Biowing Applied Biotechnology Co., Ltd. (www.biowing.com.cn) applying the method based on multiplex PCR with next-generation sequencing.[19] The SNPs of seven randomly selected patients were genotyped twice, and the genotyping results are 100% concordant.

Statistical Analysis

Mean (standard deviation, SD) or median (interquartile range, IQR) was employed to describe quantitative variable based on its distribution, and the normality of the distribution was assessed by histograms and Q-Q plots. Qualitative variable was expressed as absolute number and percentage (%). For normally distributed data, two groups were compared using unpaired *t* test, while Mann–Whitney *U*-test was applied when data were non-normally distributed. Chi-square test or Fisher's exact test was applied for categorical variables. The chi-square goodness-of-fit test was applied to test Hardy–Weinberg equilibrium (HWE). For patients who withdrew during the follow-up, the last observation carried forward (LOCF) method was applied Multivariable log binomial regression model was applied to estimate the relative risk (RR) and 95% confidence interval (CI) for the association between transporter gene polymorphism and clinical response to MTX assessed according to dichotomous classification criteria (EULAR response, DAS28-ESR LDA and REM, and ΔDAS28-ESR >0.6) with adjustment for potential confounding factors. Multivariable linear regression model was applied to estimate the beta (β) coefficient and 95% CI for the association between transporter gene polymorphism and ΔDAS28-ESR with adjustment for potential confounding factors. Haplotype analysis was performed for genetic polymorphisms within the same transporter gene shown to be significantly associated with clinical response to MTX based on the SNPStats software.[20] Utilizing the most frequent haplotype as a reference, only relatively common haplotype (frequency greater than 5%) was examined for its association with clinical response to MTX with adjustment for potential confounders. All the above statistical analyses were performed using Stata version 15.0 for Windows (StataCorp, College Station, TX, USA), and a two-tailed *P* value less than 0.05 was considered statistically significant.

Results

The baseline characteristics of RA patients included in our present study are shown in Table 1. In total, 100 RA patients aged 22~78 years (mean age of 52.23 ± 12.71 years) were enrolled, and the majority were female (81.00%) and

Table 1 Baseline Characteristics of Rheumatoid Arthritis Patients Included in the Present Study

Characteristics	RA Patients (n = 100)
Age, years	52.23 ± 12.71
Female, n (%)	81 (81.00)
Disease duration, years, median (IQR)	0.50 (0.25–2.00)
Smoking[a], n (%)	12 (12.77)
Drinking[a], n (%)	12 (12.77)
RF-positive[b], n (%)	82 (84.54)
CCP-positive[c], n (%)	91 (92.86)
Tender joint count (per 28 joints), median (IQR)	5.0 (2.5–10.0)
Swollen joint count (per 28 joints), median (IQR)	4.0 (2.0–8.5)
ESR, mm/h, median (IQR)	37.0 (20.0–59.5)
DAS28-ESR	4.87 ± 1.15
Methotrexate dose, mg/week, median (IQR)	10 (10–10)
Concomitant GC use, n (%)	82 (82.00)
GC dose[d], mg/d, median (IQR)	5.0 (2.5–10.0)
Concomitant NSAIDs use, n (%)	23 (23.00)

Notes: Values are mean ± standard deviation unless otherwise stated. [a]6 Patients' data was missing. [b]3 Patients' data was missing. [c]2 Patients' data was missing. [d]Prednisolone equivalent.
Abbreviations: CCP, cyclic citrullinated peptide; DAS28, disease activity score in 28 joints; ESR, erythrocyte sedimentation rate; GC, glucocorticoids; IQR, interquartile range; NSAIDs, non-steroidal anti-inflammatory drugs; RF, rheumatoid factor.

DMARD-naïve (98.00%). A total of seven genetic polymorphisms were excluded from further data analysis due to the following reasons: 5 (*SLC19A1* rs8199; *ABCC1* rs129081, rs212090 and rs3784862; *ABCC3* rs9895420) with genotyping call rate less than 95%, 1 with genotype distribution deviating from HWE (*ABCG2* rs2231137) (Table S2), and one being triallelic SNP (*ABCB1* rs2032582). Therefore, a total of 47 genetic polymorphisms within transporter genes were analyzed for their associations with clinical response to MTX.

Associations of Genetic Polymorphisms Within Transporter Genes with EULAR Good and Moderate Response

When the baseline characteristics were compared between responders and non-responders classified according to whether achieving ELUAR good and moderate response, significant difference was found for the variables including smoking, drinking, TJC, ESR, DAS28-ESR and concomitant NSAIDs use (Table S3). Since TJC and ESR are components of DAS28-ESR, only DAS28-ESR in addition to smoking, drinking and concomitant NSAIDs use were adjusted in the multivariable analysis, and the detailed results could be found in Table S4. As shown in Table 2, after multivariable analysis, the major alleles of *SLC19A1* rs12659 (G/G + A/G versus A/A, RR = 1.42, 95% CI = 1.02–1.97, $P = 0.04$), rs3788200 (G/G + A/G versus A/A, RR = 1.45, 95% CI = 1.04–2.01, $P = 0.03$) were significantly associated with EULAR good and moderate response under dominant models, and the major alleles of *ABCC2* rs4148396 (C/C versus C/T + T/T, RR = 0.79, 95% CI = 0.65–0.96, $P = 0.02$), rs717620 (C/C versus C/T + T/T, RR = 0.77, 95% CI = 0.63–0.94, $P = 0.01$) were significantly associated with EULAR good and moderate response under recessive models. Moreover, a significant association of the major allele of *ABCC2* rs3740066 with EULAR good and moderate response was found under both the dominant model (C/C + C/T versus T/T, RR = 0.88, 95% CI = 0.80–0.98, $P = 0.02$) and the recessive model (C/C versus C/T + T/T, RR = 0.80, 95% CI = 0.65–0.98, $P = 0.03$).

Associations of Genetic Polymorphisms Within Transporter Genes with DAS28-ESR LDA

When the baseline characteristics were compared between responders and non-responders classified according to whether achieving DAS28-ESR LDA, only ESR and DAS28-ESR were found to be significantly different (Table S5). Since ESR

Table 2 Significant Associations of Genetic Polymorphisms Within Transporter Genes with Clinical Response to Methotrexate Assessed According to European League Against Rheumatism (EULAR) Good and Moderate Response in Rheumatoid Arthritis Patients*

Gene	SNP	Allele[a]	Dominant Model[b]		Recessive Model[c]	
			RR (95% CI)	*p*	RR (95% CI)	*p*
SLC19A1	rs12659	A/G	**1.42 (1.02–1.97)**	**0.04**	1.09 (0.89–1.34)	0.39
SLC19A1	rs3788200	A/G	**1.45 (1.04–2.01)**	**0.03**	1.13 (0.93–1.37)	0.22
ABCC2	rs3740066	T/C	**0.88 (0.80–0.98)**	**0.02**	**0.80 (0.65–0.98)**	**0.03**
ABCC2	rs4148396	T/C	1.03 (0.67–1.58)	0.90	**0.79 (0.65–0.96)**	**0.02**
ABCC2	rs717620	T/C	1.17 (0.65–2.09)	0.60	**0.77 (0.63–0.94)**	**0.01**

Notes: Bold values indicate statistical significance at $\alpha = 0.05$. *Adjusted for smoking, drinking, disease activity score in 28 joints- erythrocyte sedimentation rate (DAS28-ESR) and concomitant non-steroidal anti-inflammatory drugs (NSAIDs) use. [a]Minor allele/major allele. [b]Dominant model: major allele carriers versus minor allele homozygotes. [c]Recessive model: major allele homozygotes versus minor allele carriers.
Abbreviations: CI, confidence interval; RR, relative risk.

is one component of DAS28-ESR, only baseline DAS28-ESR was adjusted in the multivariable analysis, with the detailed results shown in Table S6. As displayed in Table 3, only the major alleles of *ABCC2* rs3740066 (C/C versus C/T + T/T, RR = 0.67, 95% CI = 0.47–0.94, $P = 0.02$) and rs717620 (C/C versus C/T + T/T, RR = 0.66, 95% CI = 0.48–0.92, $P = 0.02$) were found to be significantly associated with DAS28-ESR LDA under recessive models after multivariable analysis.

Associations of Genetic Polymorphisms Within Transporter Genes with ΔDAS28-ESR

When the ΔDAS28-ESR was compared across different groups of baseline categorical variables and the correlation of ΔDAS28-ESR with baseline continuous variables was checked, significant evidence was found for the variables including TJC, SJC, ESR and DAS28-ESR (Table S7). As explained previously, only baseline DAS28-ESR was adjusted in the multivariable analysis, with the detailed results presented in Table S8. As shown in Table 4, RA patients carrying major alleles of *ABCB1* rs1128503 ($\beta = 0.89$, 95% CI = 0.28–1.50, $P = 0.005$), *ABCB1* rs4148737 ($\beta = 0.90$, 95% CI = 0.32–1.49, $P = 0.003$) and *ABCC3* rs2277624 ($\beta = 0.75$, 95% CI = 0.04–1.46, $P = 0.04$) achieved more ΔDAS28-ESR compared with minor allele homozygotes, while the major allele homozygotes of *ABCC3* rs4148416 ($\beta = 0.52$, 95% CI = 0.03–1.01, $P = 0.04$) achieved more ΔDAS28-ESR compared with minor allele carriers.

Associations of Genetic Polymorphisms Within Transporter Genes with ΔDAS28-ESR > 0.6

In a bivariate association analysis between baseline characteristics and clinical response to methotrexate assessed according to ΔDAS28-ESR > 0.6, significant evidence was found for the following variables: disease duration, drinking, TJC, ESR, DAS28-ESR and concomitant NSAIDs use (Table S9). As mentioned previously, DAS28-ESR in addition to disease duration, drinking and concomitant NSAIDs use were adjusted in the multivariable analysis, with the detailed results shown in Table S10. As shown in Table 5, 12 genetic polymorphisms within transporter genes were found to be

Table 3 Significant Associations of Genetic Polymorphisms Within Transporter Genes with Clinical Response to Methotrexate Assessed According to Disease Activity Score in 28 Joints – Erythrocyte Sedimentation Rate (DAS28-ESR) Low Disease Activity in Rheumatoid Arthritis Patients*

Gene	SNP	Allele[a]	Dominant Model[b]		Recessive Model[c]	
			RR (95% CI)	*P*	RR (95% CI)	*P*
ABCC2	rs3740066	T/C	1.31 (0.49–3.54)	0.59	**0.67 (0.47–0.94)**	**0.02**
ABCC2	rs717620	T/C	1.71 (0.58–5.06)	0.33	**0.66 (0.48–0.92)**	**0.02**

Notes: Bold values indicate statistical significance at $\alpha = 0.05$. *Adjusted for disease activity score in 28 joints - erythrocyte sedimentation rate (DAS28-ESR). [a]Minor allele/Major allele. [b]Dominant model: major allele carriers versus minor allele homozygotes. [c]Recessive model: major allele homozygotes versus minor allele carriers.
Abbreviations: CI, confidence interval, RR, relative risk.

Table 4 Significant Associations of Genetic Polymorphisms Within Transporter Genes with Clinical Response to Methotrexate Assessed According to Change in Disease Activity Score in 28 Joints – Erythrocyte Sedimentation Rate (DAS28-ESR) in Rheumatoid Arthritis Patients*

Gene	SNP	Allele[a]	Dominant Model[b]		Recessive Model[c]	
			β (95% CI)	*P*	β (95% CI)	*P*
ABCB1	rs1128503	G/A	**0.89 (0.28–1.50)**	**0.005**	0.07 (−0.38–0.51)	0.77
ABCB1	rs4148737	C/T	**0.90 (0.32–1.49)**	**0.003**	0.06 (−0.37–0.49)	0.78
ABCC3	rs2277624	T/C	**0.75 (0.04–1.46)**	**0.04**	0.00 (−0.42–0.42)	0.99
ABCC3	rs4148416	T/C	−0.39 (−1.87–1.09)	0.60	**0.52 (0.03–1.01)**	**0.04**

Notes: Bold values indicate statistical significance at α = 0.05. *Adjusted for disease activity score in 28 joints - erythrocyte sedimentation rate (DAS28-ESR). [a]Minor allele/major allele. [b]Dominant model: major allele carriers versus minor allele homozygotes. [c]Recessive model: major allele homozygotes versus minor allele carriers.

Table 5 Significant Associations of Genetic Polymorphisms Within Transporter Genes with Clinical Response to Methotrexate Assessed According to Change in Disease Activity Score in 28 Joints – Erythrocyte Sedimentation Rate (DAS28-ESR) > 0.6 in Rheumatoid Arthritis Patients*

Gene	SNP	Allele[a]	Dominant Model[b]		Recessive Model[c]	
			RR (95% CI)	*P*	RR (95% CI)	*P*
SLC19A1	rs1051266	T/C	**1.45 (1.04–2.02)**	**0.03**	**1.23 (1.02–1.48)**	**0.03**
SLC19A1	rs1131596	G/A	**1.45 (1.04–2.02)**	**0.03**	**1.23 (1.02–1.48)**	**0.03**
SLC19A1	rs12659	A/G	**1.44 (1.07–1.96)**	**0.02**	1.20 (0.99–1.44)	0.06
SLC19A1	rs2838956	G/A	**1.39 (1.01–1.90)**	**0.04**	**1.23 (1.03–1.48)**	**0.02**
SLC19A1	rs3788200	A/G	**1.47 (1.08–1.99)**	**0.01**	1.20 (1.00–1.45)	0.05
SLC19A1	rs79091853	T/C	–	–	**0.81 (0.68–0.95)**	**0.01**
ABCB1	rs3842	C/T	0.90 (0.68–1.19)	0.47	**1.23 (1.01–1.49)**	**0.04**
ABCC1	rs3743527	T/C	0.94 (0.76–1.16)	0.56	**1.22 (1.02–1.46)**	**0.03**
ABCC2	rs3740066	T/C	0.77 (0.55–1.07)	0.11	**0.81 (0.67–0.99)**	**0.04**
ABCC2	rs4148396	T/C	0.97 (0.59–1.60)	0.91	**0.81 (0.68–0.98)**	**0.03**
ABCC2	rs717620	T/C	1.08 (0.53–2.17)	0.84	**0.79 (0.65–0.95)**	**0.01**
ABCG2	rs4367138	G/A	**0.74 (0.58–0.95)**	**0.02**	0.88 (0.73–1.06)	0.18

Notes: Bold values indicate statistical significance at α = 0.05. *Adjusted for disease duration, drinking, disease activity score in 28 joints - erythrocyte sedimentation rate (DAS28-ESR) and concomitant NSAIDs use. [a]Minor allele/major allele. [b]Dominant model: major allele carriers versus minor allele homozygotes. [c]Recessive model: major allele homozygotes versus minor allele carriers.

Abbreviations: CI, confidence interval; RR, relative risk.

significantly associated with ΔDAS28-ESR > 0.6. The major alleles of *SLC19A1* rs12659 (G/G + A/G versus A/A, RR = 1.44, 95% CI = 1.07–1.96, *P* = 0.02), rs3788200 (G/G + A/G versus A/A, RR = 1.47, 95% CI = 1.08–1.99, *P* = 0.01) and *ABCG2* rs4367138 (A/A + A/G versus G/G, RR = 0.74, 95% CI = 0.58–0.95, *P* = 0.02) were found to be significantly associated with ΔDAS28-ESR > 0.6 under dominant models, while the major alleles of *SLC19A1* rs79091853 (C/C versus C/T + T/T, RR = 0.81, 95% CI = 0.68–0.95, *P* = 0.01), *ABCB1* rs3842 (T/T versus C/T + C/C, RR = 1.23, 95% CI = 1.01–1.49, *P* = 0.04), *ABCC1* rs3743527 (C/C versus C/T + T/T, RR = 1.22, 95% CI = 1.02–1.46, *P* = 0.03), *ABCC2* rs3740066 (C/C versus C/T + T/T, RR = 0.81, 95% CI = 0.67–0.99, *P* = 0.04), rs4148396 (C/C versus C/T + T/T, RR = 0.81, 95% CI = 0.68–0.98, *P* = 0.03) and rs717620 (C/C versus C/T + T/T, RR = 0.79, 95% CI = 0.65–0.95, *P* = 0.01) were found to be significantly associated with ΔDAS28-ESR > 0.6 under recessive models. In addition, the major alleles of *SLC19A1* rs1051266 (C/C + C/T versus T/T, RR = 1.45, 95% CI = 1.04–2.02, *P* = 0.03; C/C versus C/T + T/T, RR = 1.23, 95% CI = 1.02–1.48, *P* = 0.03), rs1131596 (A/A +A/G versus G/G, RR = 1.45, 95% CI = 1.04–2.02, *P* = 0.03; A/A versus A/G + G/G, RR = 1.23, 95% CI = 1.02–1.48, *P* = 0.03) and rs2838956 (A/A +A/G versus G/G, RR = 1.39, 95% CI = 1.01–1.90, *P* = 0.04; A/A versus A/G + G/G, RR = 1.23, 95% CI = 1.03–1.48, *P* = 0.02) were found to be significantly associated with ΔDAS28-ESR > 0.6 under either dominant or recessive models.

Associations of Haplotypes with Clinical Response to MTX

Based on the findings that all genetic polymorphisms within *SLC19A1* and three genetic polymorphisms within *ABCC2* (rs3740066, rs4148396 and rs717620) were significantly associated with clinical response to MTX, we also determined whether these genetic polymorphisms were in high linkage disequilibrium (LD) and then performed haplotype association analysis. As shown in Figure S1, two LD blocks were defined within *SLC19A1*, with rs1051266, rs1131596, rs12659 and rs3788200 constituting one block and the rest genetic polymorphisms (rs2838956, rs7867 and rs79091853) forming another block. Moreover, genetic polymorphisms within *ABCC2* were found to be significantly associated with clinical response to MTX in high LD (Figure S2). With adjustment for corresponding confounders, TGAA haplotype consisting of *SLC19A1* rs1051266, rs1131596, rs12659 and rs3788200 was found to be significantly associated with EULAR good and moderate response (OR = 0.37, 95% CI = 0.14–0.98, $P = 0.048$) and ΔDAS28-ESR > 0.6 (OR = 0.23, 95% CI = 0.07–0.71, $P = 0.01$) compared with the most common haplotype CAGG (Table 6). With regard to *ABCC2* haplotype, the haplotype TTT composed of rs3740066, rs4148396 and rs717620 was found to be significantly associated with EULAR good and moderate response (OR = 8.83, 95% CI = 1.62–48.15, $P = 0.01$) and ΔDAS28-ESR > 0.6 (OR = 14.96, 95% CI = 1.79–125.24, $P = 0.01$) compared with the most common haplotype CCC (Table 6).

Discussion

In view of the significant effects of transporters on pharmacokinetics of MTX, this study was carried out to comprehensively evaluate the associations of genetic polymorphisms within transporter genes with clinical response to MTX based on multiple response criteria in Chinese RA patients. By adjusting for potential confounding factors, multiple significant signals were found in single marker analysis as well as haplotype analysis, indicating that genetic polymorphisms within transporter genes, especially *SLC19A1* and *ABCC2*, should be incorporated into the future prediction model for clinical response to MTX in Chinese RA patients.

The SLC transporter superfamily consists of more than 300 membrane-bound proteins that mediate the translocation of substrates across biological membranes.[21] Among them, SLC19A1, also known as reduced folate carrier 1 (RFC1), is mainly implicated in the uptake of MTX into target cells, and the roles of genetic polymorphisms within *SLC19A1* in clinical response to MTX have been extensively examined with inconsistent results. A recent meta-analysis found that *SLC19A1* rs1051266[22] and rs2838956[23] were significantly associated with MTX efficacy in RA patients. To date, the *SLC19A1* rs1051266 is the most extensively studied variant, since the allele substitution of this SNP results in amino acid change and substantial impact on the red blood cell (RBC) MTX-PGs levels.[24] While *SLC19A1* rs2838956 is an intronic SNP, it has been postulated that this SNP might potentially influence RNA splicing and thereby affect the structure and function of SLC19A1.[25] In our present study, the major alleles of *SLC19A1* rs1051266 and rs2838956 were found to be significantly associated with ΔDAS28-ESR > 0.6 under either dominant model or recessive model, and borderline significant evidence was found for the association of the major allele of *SLC19A1* rs1051266 with EULAR good and moderate response under dominant model (Table S4). Apart from the two aforementioned SNPs, significant evidence was also detected for two synonymous variants, namely rs12659 and rs79091853, one intronic variant, rs3788200, and one 5'-UTR variant, rs1131596. Specifically, RA patients carrying the major alleles of *SLC19A1* rs12659 and rs3788200 were more likely to achieve EULAR good and moderate response and ΔDAS28-ESR > 0.6 compared with patients homozygous to the minor alleles, yet no studies on the relationships between these two SNPs and clinical response to MTX have been reported. It is worth mentioning that the genotype distribution of *SLC19A1* rs12659 was found to be different between MTX-sensitive and MTX-resistant human cancer cell lines,[26] possibly accounting for the observed association of *SLC19A1* rs12659 with clinical response to MTX. Intriguingly, *SLC19A1* rs1131596 was predicted to be located in the putative activator protein-1 (AP1) transcription factor recognition region, and G allele of this variant was found to be associated with reduced protein expression in contrast to A allele.[27] Consistently, we found that RA patients carrying the major allele A allele of *SLC19A1* rs1131596 were more likely to obtain ΔDAS28-ESR > 0.6. Additionally, the major allele A was associated with increased likelihood of achieving EULAR good and moderate response under the dominant model with borderline significance (Table S4). As for *SLC19A1* rs3788200 and rs79091853, although the biological significance of these two variants remains unknown, the intronic SNP might be involved in alternative splicing, while the

Table 6 Associations Between Haplotypes Consisting of Genetic Polymorphisms Within Transporter Genes and Clinical Response to Methotrexate Assessed According to Different Response Criteria with Adjustment for Potential Confounding Factors

Haplotype				Frequency	OR (95% CI)	*P*
EULAR good and moderate response[a]						
SLC19A1	*SLC19A1*	*SLC19A1*	*SLC19A1*			
rs1051266	rs1131596	rs12659	rs3788200			
C	A	G	G	0.57	Reference	
T	G	A	A	0.42	**0.37 (0.14–0.98)**	**0.048**
SLC19A1	*SLC19A1*	*SLC19A1*				
rs2838956	rs7867	rs79091853				
A	G	C		0.43	Reference	
G	A	C		0.42	0.47 (0.17–1.27)	0.14
A	G	T		0.12	1.67 (0.30–9.36)	0.56
ABCC2	*ABCC2*	*ABCC2*				
rs3740066	rs4148396	rs717620				
C	C	C		0.73	Reference	
T	T	T		0.20	**8.83 (1.62–48.15)**	**0.01**
DAS28-ESR low disease activity[b]						
SLC19A1	*SLC19A1*	*SLC19A1*	*SLC19A1*			
rs1051266	rs1131596	rs12659	rs3788200			
C	A	G	G	0.57	Reference	
T	G	A	A	0.42	0.80 (0.43–1.47)	0.47
SLC19A1	*SLC19A1*	*SLC19A1*				
rs2838956	rs7867	rs79091853				
A	G	C		0.42	Reference	
G	A	C		0.42	0.83 (0.42–1.63)	0.60
A	G	T		0.12	0.70 (0.26–1.88)	0.48
ABCC2	*ABCC2*	*ABCC2*				
rs3740066	rs4148396	rs717620				
C	C	C		0.72	Reference	
T	T	T		0.21	1.87 (0.83–4.20)	0.13
Change in DAS28-ESR[c]						
SLC19A1	*SLC19A1*	*SLC19A1*	*SLC19A1*			
rs1051266	rs1131596	rs12659	rs3788200			
C	A	G	G	0.58	Reference	
T	G	A	A	0.41	−0.08 (−0.39–0.22)[d]	0.59
SLC19A1	*SLC19A1*	*SLC19A1*				
rs2838956	rs7867	rs79091853				
A	G	C		0.42	Reference	
G	A	C		0.40	−0.07 (−0.34–0.20)[d]	0.60
A	G	T		0.12	−0.06 (−0.45–0.33)[d]	0.76
ABCC2	*ABCC2*	*ABCC2*				
rs3740066	rs4148396	rs717620				
C	C	C		0.72	Reference	
T	T	T		0.22	0.16 (−0.20–0.52)[d]	0.39

(*Continued*)

Table 6 (Continued).

Haplotype				Frequency	OR (95% CI)	*P*
Change in DAS28-ESR > 0.6[e]						
SLC19A1	*SLC19A1*	*SLC19A1*	*SLC19A1*			
rs1051266	rs1131596	rs12659	rs3788200			
C	A	G	G	0.57	Reference	
T	G	A	A	0.42	**0.23 (0.07–0.71)**	**0.01**
SLC19A1	*SLC19A1*	*SLC19A1*				
rs2838956	rs7867	rs79091853				
A	G	C		0.43	Reference	
G	A	C		0.42	0.35 (0.12–1.03)	0.06
A	G	T		0.12	3.13 (0.34–29.27)	0.32
ABCC2	*ABCC2*	*ABCC2*				
rs3740066	rs4148396	rs717620				
C	C	C		0.73	Reference	
T	T	T		0.20	**14.96 (1.79–125.24)**	**0.01**

Notes: Bold values indicate statistical significance at $\alpha = 0.05$. [a]Adjusted for smoking, drinking, disease activity score in 28 joints- erythrocyte sedimentation rate (DAS28-ESR) and concomitant non-steroidal anti-inflammatory drugs (NSAIDs) use. [b]Adjusted for disease activity score in 28 joints - erythrocyte sedimentation rate (DAS28-ESR). [c]Adjusted for disease activity score in 28 joints - erythrocyte sedimentation rate (DAS28-ESR). [d]Values denoting beta and 95% CI. [e]Adjusted for disease duration, drinking, disease activity score in 28 joints - erythrocyte sedimentation rate (DAS28-ESR) and concomitant NSAIDs use.

Abbreviations: CI, confidence interval; DAS28-ESR, disease activity score in 28 joints - erythrocyte sedimentation rate; EULAR, European League Against Rheumatism; OR, odds ratio.

synonymous SNP might influence the secondary structure of the mRNA and further regulate protein expression. However, it is also possible that these observed associations might result from their strong LD with other variants possessing significant biological effects. In addition to the single marker analysis, the haplotype analysis was also carried out, and patients carrying the haplotype TGAA comprising rs1051266, rs1131596, rs12659 and rs3788200 were found to be more likely to achieve EULAR good and moderate response and ΔDAS28-ESR > 0.6 in contrast to the most common haplotype CAGG, suggesting that alleles in the form of combination might be more predictive. Collectively, these findings highlighted the impact of *SLC19A1* genetic polymorphisms and haplotype on clinical response to MTX in Chinese RA patients.

As the largest transmembrane transporter family, the ABC transporter superfamily consists of 48 members classified into seven subfamilies, from ABC-A to G.[28] These transporters are ubiquitously expressed in multiple tissues and organs such as small intestinal, liver and kidney, thus playing an essential role in absorption, distribution, metabolism and excretion of oral therapeutic drugs. Of note, the major ABC transporters mediating the efflux of MTX include ABCB1, ABCC1~4 and ABCG2,[11,12] and the impact of genetic polymorphisms within genes encoding aforementioned ABC transporters on clinical response to MTX has also become a focus of increasing interest during the past decade. As for *ABCB1*, rs1045642 (C3435T) is the most widely investigated variant. Consistent with one recent meta-analysis showing that *ABCB1* rs1045642 was associated with MTX efficacy in Caucasians but not in non-Caucasians,[29] we did not find significant evidence for this SNP. Similarly, one recent study performed in Chinese patients with RA applying the same response criterion (ΔDAS28-ESR > 0.6) also did not find significant association between *ABCB1* rs1045642 and clinical response to MTX.[30] Apart from rs1045642, a few studies have assessed the effect of *ABCB1* rs1128503 on MTX efficacy with non-significant results.[11,31,32] Nevertheless, we found that RA patients carrying the major allele A of *ABCB1* rs1128503 were more likely to achieve more DAS28-ESR reduction compared with minor allele homozygotes. The discrepancy might be due to difference in response criteria, duration of MTX therapy and consideration of confounding factors. Intriguingly, our finding was consistent with the results in patients with osteosarcoma, in which the major allele A of *ABCB1* rs1128503 was significantly associated with better response to chemotherapy including MTX.[33,34] As one synonymous variant in exon 13, the allele substitution of *ABCB1* rs1128503 does not lead to an amino

acid alteration but might exert effect on ABCB1 expression via codon usage.[33] Moreover, we found that *ABCB1* rs3842 was significantly associated with ΔDAS28-ESR > 0.6 and rs4148737 was significantly associated with ΔDAS28-ESR, although on studies on these associations have been reported. Of note, *ABCB1* rs3842 was previously reported to be associated with active anti-retroviral treatment response in human immunodeficiency syndrome virus (HIV)/acquired immunodeficiency syndrome (AIDS) patients.[35] As one 3'-UTR SNP, the allele substitution of *ABCB1* rs3842 might disrupt or create microRNA (miRNA) binding site, therefore influencing protein expression. As for *ABCB1* rs4148737, consistent with our finding that RA patients carrying the major allele A were more likely to achieve more reduction in DAS28-ESR, the major allele A of this variant was previously documented to be linked to a better treatment outcome in patients with osteosarcoma[33] and Ewing Sarcoma.[36] Although the biological function of *ABCB1* rs4148737 remains unknown, as one intronic variant, it might lead to alternative splicing and the altered protein expression. Regarding *ABCC1*, only one study performed in Caucasian patients with RA reported that rs246240 and rs3784864 were significantly associated clinical response to MTX.[11] However, we did not successfully replicate these findings, and this might be due to the difference in clinical characteristics, length of follow-up and genetic heterogeneity. Indeed, a total of 15 variants were selected including the two above-mentioned variants. While three variants were excluded due to genotyping call rate less than 95%, 12 variants were eligible for analysis. In our present study, one 3'-UTR variant, namely rs3743527, was detected to be associated with ΔDAS28-ESR > 0.6, and RA patients homozygous for the major allele C were more likely to achieve reduction in DAS28-ESR greater than 0.6 compared with minor allele carries. One previous study found that this SNP was significantly associated with chemotherapy-related toxicities in Chinese acute myeloid leukemia (AML) patients.[37] The possible underlying mechanism responsible for the observed association originated from *ABCC1* rs3743527 might be the alteration of binding ability with miRNA due to its location in 3'-UTR. To date, the impact of genetic polymorphisms within *ABCC2* on clinical response to MTX has only been evaluated in a few studies.[11,31,38,39] One cross-sectional study performed in New Zealand did not find significant associations of *ABCC2* rs2273697 and rs4148396 with DAS28 LDA in RA patients who had been treated by MTX for a median of 3 years before study entry.[38] One retrospective study carried out in Japanese revealed that there was no significant difference in mean DAS28 after MTX monotherapy treatment among RA patients with different genotypes of rs2273697 and rs3740066.[31] Besides, another retrospective cohort study performed in Portuguese did not detect significant evidence for associations of *ABCC2* rs4148396 and rs717620 with clinical response to DAS28 LDA in RA patients treated by for 6 months.[11] However, we found that three out of four selected variants within *ABCC2*, namely rs3740066, rs4148396 and rs717620, were significantly associated with clinical response to MTX. Factors possible accounting for the controversy include different study designs, durations, and regimens of MTX treatment and response criteria. Remarkably, one promoter variant in 5'-UTR, *ABCC2* rs717620, has been recently elucidated to be significantly associated with clinical response to low-dose MTX in patients with psoriasis. In accordance with our finding here, patients carrying the minor allele T were more likely to be responders,[39] and this might be explained by this allele associated with reduced activity and an increased bioavailability of methotrexate.[40,41] As for *ABCC2* rs3740066, one study conducted in Han Chinese epilepsy patients with generalized seizure found that patients carrying the major allele C of this variant were more likely to be resistant to valproic acid (VPA), the first-line antiepileptic drug used to control seizure in epilepsy patients.[42] Moreover, advanced nonsmall cell lung cancer (NSCLC) patients homozygous for the minor allele T of this SNP were more likely to achieve higher response rates and progression-free survival.[43] In agreement with these studies, we also found that patients carrying the minor allele T were more likely to achieve better response to MTX. Regarding *ABCC2* rs4148396, the minor allele T was found to confer increased risk of developing intolerance to antiretroviral therapy in Brazilian HIV-1 positive individuals,[44] possibly due to T allele leading to reduced expression or activity of ABCC2 and increased intracellular drug concentrations. Consistently, we found that patients carrying the minor allele T of this variant were more likely to obtain EULAR good and moderate response and ΔDAS28-ESR > 0.6. Of note, similar to *SLC19A1*, the haplotype TTT consisting of the minor allele of rs3740066, rs4148396 and rs717620 were found to be more likely to achieve EULAR good and moderate response and ΔDAS28-ESR > 0.6 in contrast to the most common haplotype CCC, suggesting that alleles in the form of combination might be more predictive of clinical response. With regard to *ABCC3*, we found that two synonymous variants, rs2277624 and rs4148416, were significantly associated with change in DAS28-ESR, yet no studies on their relationships have been

reported. The minor allele T of *ABCC3* rs2277624 was previously revealed to be associated improved survival of small-cell lung cancer (SCLC) patients with borderline significance.[45] We found that the major alleles carriers of *ABCC3* rs2277624 were more likely to achieve reduction in DAS28-ESR compared with minor allele homozygous. Consistent with previous studies demonstrating individuals homozygous for the major allele C of *ABCC3* rs4148416 having improved survival rate and better response to chemotherapy (including MTX) of osteosarcoma patients,[33,34,46] we found that patients with RA homozygous for the major allele were more likely to achieve reduction in DAS28-ESR. Although the biological significance of these two missense variants remains unknown, the allele substitutions might exert effect on ABCC3 expression via codon usage.[33] To date, there has been no studies on the relationships between genetic polymorphisms within *ABCC4* and clinical response to MTX. In our present study, a total of 14 variants with MAF greater than 0.1 located in potentially functional regions were systematically selected. However, non-significant evidence was found for any variant even though multiple response criteria were employed, suggesting that ABCC4 genetic polymorphisms might not contribute to inter-individual variability in MTX efficacy. Regarding *ABCG2*, to date, only two studies have examined the relationship between one missense SNP, namely rs2231142, and MTX efficacy with non-significant results.[31,38] In accordance with these studies, we did not find significant evidence for this variant. Among the remaining three SNPs, only one intronic variant, rs4367138, was found to be associated with ΔDAS28-ESR > 0.6, and RA patients carrying the major allele A of this variant were less likely to achieve ΔDAS28-ESR > 0.6. Although this intronic variant has not reported in literature, we postulated that the allele substitution may influence alternative splicing and further regulate the expression of encoded protein.

The main strength of our study lies in the comprehensively selected SNPs and the multiple response criteria applied. Nonetheless, several potential limitations should be noted. First, the sample size of our present study was relatively small, thus statistical power was relatively limited to detect variants with weak effect sizes. However, we should also bear in mind that only genetic polymorphisms with relatively stronger effect sizes hold the potential to be applied to future clinical practice. Second, our findings for several polymorphisms showing significant evidence might have limited clinical implication presently. Moreover, most of these significant associations would become non-significant after multiple corrections, whereas this could be due to our relatively small sample size. If our results could be replicated in independent studies with larger numbers of RA patients, these genetic polymorphisms within transporter genes should be incorporated into the future prediction model of clinical response to MTX in Chinese RA patients, thereby guiding individualized treatment of MTX in daily clinical practice. Finally, the biological significance of several SNPs found to be associated with clinical response to MTX needs further clarification.

Conclusion

Our findings herein highlight the potential of genetic polymorphisms within transporter genes, especially *SLC19A1* and *ABCC2*, as predictors of clinical response to MTX in Chinese RA patients.

Acknowledgments

This work was supported by Zhejiang Provincial Natural Science Foundation of China (Grant No. LY20H260001), Medical and Health Planned Science and Technology Project of Zhejiang Province (Grant No. 2019KY570), Nature Science Foundation of Ningbo city (Grant No. 202003N4112).

Disclosure

The authors declare no conflicts of interest in this work.

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