—Original—

Polymorphism in Organic Anion-Transporting Polypeptide Gene Related to Methotrexate Response in Rheumatoid Arthritis Treatment

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Background: Methotrexate (MTX) is still the first-choice drug for the treatment of rheumatoid arthritis (RA). In Japan, MTX doses of up to 16 mg/week were approved in 2011. In this study, we aimed to identify the gene polymorphisms that can predict therapeutic effects of MTX in Japanese patients in current clinical settings.

Methods: This study involved 171 patients with RA (all Japanese nationals, age 63.5±10.0 years) who had been administered MTX. The analyzed polymorphisms included 82 single nucleotide polymorphisms (SNPs) involved in the MTX pharmacological pathway or in the pathogenesis of RA. Responders were patients who showed high sustained remission or low disease activity with MTX or conventional disease-modifying anti-rheumatic drugs (DMARDs) treatment beyond 6 months. Non-responders were patients who showed moderate or high disease activity, who were prescribed biological DMARDs. A logistic model was constructed with Responder/Non-responder as the target variable, and minor allele frequency was set as an explanatory variable.

Results: None of the 82 SNPs targeted for analysis met the Bonferroni significance threshold of 6.098×10^{-4}. However, we identified SLCO1B1 rs11045879 as an SNP that might yield significant results if the number of patients were to be increased (P=0.015).

Conclusions: The rs11045879 minor allele in the SLCO1B1 gene is a potential predictor of non-responders to MTX treatment among Japanese RA patients. In future collaborative research, we will investigate whether the association with SLCO1B1 polymorphism is significant by performing statistical analysis with a larger study population. (J Nippon Med Sch 2019; 86: 149–158)

Key words: genetic polymorphism, rheumatoid arthritis, methotrexate, precision medicine, Japanese

Introduction

Rheumatoid arthritis (RA) can be treated with biologic agents. These drugs suppress inflammatory cytokines, which play a key role in RA pathology, and a number have been developed and applied clinically. However, biologics are expensive drugs and cause severe adverse reactions; therefore, conventional synthetic disease-modifying anti-rheumatic drugs (DMARDs), which are cheaper, are currently at the center of therapeutic strategies for RA. The most frequently used DMARD for RA globally is methotrexate (MTX), which was recommended as the drug of first choice in the early treatment phase by the European League against Rheumatic Diseases in 2016[1]. Yet MTX has drawbacks: its therapeutic ef-

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fect differs between clinical cases, with some patients being responders and others being non-responders. MTX also produces a range of unexpected adverse reactions, including gastrointestinal disturbance, pneumonia, hepatic dysfunction, and bone marrow suppression[2,3]. Responders account for 46% to 65% of MTX-treated patients[4], among whom the incidence of adverse drug reactions is around 50%[5]. A range of factors are implicated in such individual differences, including sex, age, body weight, disease duration, and disease activity[6].

MTX reportedly needs to be taken for at least 6 months for its therapeutic effect to be clearly evaluated[2,3]. However, a recent study has demonstrated that longer periods may be needed for this effect to be determined[7]. Nevertheless, non-responders who have a poor prognosis with MTX treatment are recommended to take genetically engineered biologic agents and Janus kinase inhibitors with a stronger pharmacological effect than MTX as the next step[1]. However, these are expensive agents associated with a high incidence of serious adverse drug reactions. Therefore, it is desirable to ascertain the effect of MTX before such biologic agents and Janus kinase inhibitors are used. Recent reports have shown progressive bone destruction in the early phase after RA onset[8,9]. As a consequence, determinations of drug susceptibility would be very useful before MTX treatment rather than over a prolonged period of MTX treatment, with patients screened based on biologic usage and subsequent pharmacotherapy plans decided at the same time.

As mentioned earlier, MTX sensitivity can be influenced by a range of factors. One widely reported factor is gene polymorphism, with reports focusing on genes considered to encode proteins involved in the cellular uptake of MTX, its removal into the extracellular environment, and its onset of action[10]. However, there are differences in MTX sensitivity between human populations.

In Japan, MTX was approved in 1999 with a maximum dose of 8 mg/week. The average weekly dose for Japanese patients subsequent to that approval was around 6 mg, which differed substantially from the dose in other countries. MTX doses of up to 16 mg/week were approved in 2011 after an application was made based on data in the public domain (results of MTX usage outside Japan). Research on the response to MTX treatment in Japanese RA patients was largely conducted before 2011, and involved analysis at a lower dose[11]. Accordingly, it is possible that these evaluations are not an accurate gauge of response.

In this study, we aimed to identify gene polymorphisms that can predict therapeutic effects for MTX in Japanese patients when the drug is administered at doses approved in 2011.

Materials and Methods

Ethics

This study was approved by the Ethics Committee and Ethical Review Board on Human Genome/Gene Analysis Research of Nippon Medical School (No. 25-05); written informed consent was obtained from all patients.

Patients

A total of 180 patients with RA who had been administered MTX were selected from the Rheumatology Outpatient Clinic at the Department of Orthopaedic Surgery, Nippon Medical School Hospital, between October 2013 and September 2014. All patients had been diagnosed based on the 1987 American College of Rheumatology criteria. Eight patients could not continue MTX treatment for 6 months because of adverse events (infection 3, nasal bleeding 1, gastrointestinal symptom 2, depilation 1, eczema 1). One patient was unable to continue MTX treatment due to poor compliance. The remaining 171 patients (all Japanese nationals; 146 women, 25 men, age 63.5±10.0 years) were subject to analysis. All patients with liver dysfunction could continue MTX treatment by dose reduction.

Clinical Information

We collected information on sex, age, serum levels of C-reactive protein, maintenance dose of MTX, and disease activity score 28 (DAS28).

DNA Extraction

A total of 5 mL of venous blood was collected from each patient, transferred to ethylenediaminetetraacetic acid vacuum tubes, and stored at 4℃ before DNA extraction. Genomic DNA was extracted by a contracted clinical laboratory (SRL Inc., Tokyo, Japan) and stored at −20℃. DNA quality and purity were assessed using agarose gel electrophoresis, and optical absorbance was measured at A260/A280.

Single Nucleotide Polymorphism Selection

The candidate polymorphisms in this study included 96 single nucleotide polymorphisms (SNPs) involved in the MTX pharmacological pathway or in the pathogenesis of RA based on the previous reports. A total of 82 SNPs (from the 96 SNPs) met the criteria stated below and were included in the analysis (**Table 1**). The criteria were call rate (CR) ≥0.95, minor allele frequency (MAF) ≥0.01, and a p value ≥0.001 in Hardy-Weinberg equilibrium goodness-of-fit testing. We assumed a trend mode

Table 1 SNPs included in the analysis

Reference SNP ID number	Gene symbol	Possibility of relevance	Ref.
rs10181656	STAT4	Th1 cell differentiation	12
rs1043879	CR 1	Association with anti-TNF treatment efficacy	13
rs1051266	SLC19A1	Association with MTX treatment efficacy	14
rs10760130	TRAF1	Role in RA susceptibility	15
rs10865035	AFF3	Association with anti-TNF treatment efficacy	16
rs10903129	TMEM57	Transmembrane protein 57	13
rs10958713	KBKB	Inhibitor of NF-kB and subsequent TNF induction	17
rs11045879	SLC01B1	Na^+-independent uptake of organic anions such as methotrexate	18
rs11209026	IL23R gene	Rheumatoid arthritis susceptibility	19
rs1143634	IL1B	Possibly associated with IL-1 beta production	20
rs11545078	NFkB2	Role in RA susceptibility	21
rs11586238	CD58	Role in RA susceptibility	22
rs11595324	CHUK	NF-kB induction and subsequent TNF induction	23
rs1160542	AFF3	Role in RA susceptibility	15
rs11935252	TLR2	Role in RA susceptibility	15
rs11986055	IKBKB	Inhibitor of NF-kB and subsequent TNF production	17
rs12081765	Intergenic	Association with anti-TNF treatment efficacy	24
rs13031237	REL	Role in RA susceptibility	25
rs13192841	TNFAIP3	Role in RA susceptibility	26
rs13207033	TNFAIP3	Role in RA susceptibility	26
rs1532269	PDZ2D	Association with anti-TNF treatment efficacy	27
rs1650697	DHFR	Association with MTX treatment efficacy	28
rs1678542	KIF5A	non-HLA rheumatoid arthritis susceptibility loci	15
rs16944	IL1B	Role in RA susceptibility	29
rs17301249	EYA4	Association with anti-TNF treatment efficacy	30
rs17602729	AMPD1	Association with MTX treatment efficacy	31
rs1799724	TNFA	Association with anti-TNF treatment efficacy	32
rs1799964	TNFA	Possible influence on TNF production	33
rs1800471	TGFB1	Influence on TGF-B1 production	34
rs1800610	TNFA	Possible influence on anti-TNF treatment efficacy	35
rs1800629	TNFA	Association with cardiovascular event in RA	36
rs1800896	IL10	Associated with IL-10 production	37
rs1801131	MTHFR	Association with MTX treatment efficacy	31
rs1801133	MTHFR	Association with MTX treatment efficacy	31
rs1801274	FCGR2A	Association with anti-TNF treatment efficacy	38
rs1980422	CD28	Role in RA susceptibility	22
rs2104286	IL2RA	Association with persistence of RA	39
rs2229094	LTA	Possible influence on anti-TNF treatment efficacy	40
rs2230804	CHUK	NF-kB induction and subsequent TNF induction	23
rs2236624	ADORA2A	Association with adverse event in MTX treatment	41
rs2267076	ADORA2A	Association with adverse event in MTX treatment	41
rs2273697	ABCC2	Association with MTX treatment efficacy	42
rs2297480	LTA	Effect on in vitro TNF production	43
rs231775	CTLA4	Role in RA susceptibility	44
rs2372536	ATIC	Association with MTX treatment efficacy	45
rs246240	ABCC1	Association with MTX treatment efficacy	46
rs2476601	PTPN22	Role in RA susceptibility	47
rs2736340	BLK	Role in RA susceptibility	48
rs2812378	CCL21	Role in RA susceptibility	49
rs2900180	TRAF1	Role in RA susceptibility	15
rs3087243	CTLA4	Role in RA susceptibility	15
rs3218253	IL2RB	Role in RA susceptibility	50
rs35592	ABCC1	Association with MTX treatment efficacy	31
rs3758149	GGH	Association with MTX treatment efficacy	51
rs3761422	ADORA2A	Association with adverse event in MTX treatment	42

Table 1 SNPs included in the analysis (Continue)

Reference SNP ID number	Gene symbol	Possibility of relevance	Ref.
rs3761847	TRAF1-C5	Association with anti-TNF treatment efficacy	52
rs394581	TAGAP	Role in RA susceptibility	53
rs396991	FCGR3A	Association with anti-CD20 treatment efficacy	54
rs4149056	SLC01B1	Association with MTX treatment efficacy	42
rs4149081	SLC01B1	Association with MTX treatment efficacy	55
rs419598	IL1RN	Possible influence on IL-1 beta production	56
rs4694890	Intergenic	Involved in TNF-mediated responses	57
rs4750316	PRKCQ	Role in RA susceptibility	58
rs4905865	CCDC85C	Association with MTX treatment efficacy	57
rs5029937	TNFAIP3	Role in RA susceptibility	59
rs540386	TRAF6	Role in RA susceptibility	22
rs548234	PRDM1	Role in RA susceptibility	60
rs5760410	CYTSA	Association with MTX treatment efficacy	57
rs6028945	MAFB	Possible influence on anti-TNF treatment efficacy	57
rs6427528	CD84	Association with anti-TNF treatment efficacy	61
rs6691117	CR1	Association with anti-TNF treatment efficacy	13
rs6822844	IL2/IL21	Regulation of immune responses	62
rs6920220	TNFAIP3	Involved in TNF-mediated responses	63
rs7045953	TLR4	Possible role in RA susceptibility	35
rs7080681	ABCC2	Association with MTX treatment efficacy	64
rs7574865	STAT4	Role in RA susceptibility	65
rs763361	CD226	Role in RA susceptibility	66
rs7744	MyD88	Association with anti-TNF treatment efficacy	23
rs854548	PON1	Association with anti-TNF treatment efficacy	67
rs909253	LTA	Effect on in vitro TNF production	68
rs928655	GBP6	Involved in TNF-mediated responses	69
seq.rs1045642	ABCB1	Association with MTX treatment efficacy	70

for the minor allele analysis and constructed a logistic regression model. We then applied the Wald test to ascertain associations. Strength of association was evaluated based on odds ratio.

Gene Analysis

Genotyping was performed using the Illumina SNP GoldenGate Assay (Illumina, San Diego, CA) according to the manufacturer's specifications. Briefly, 250 ng of genomic DNA was amplified at 37℃ for 20 h, and then the amplified DNA was fragmented and precipitated. The dried pellet was resuspended and hybridized to BeadChips. Hybridized BeadChips were then incubated at 48℃ for 20 h, washed, and a single-base extension step performed. After that, the BeadChips were stained, washed, coated, and dried. Finally, signal intensity data were generated by an Illumina BeadArray Reader. We randomly selected 20% of the samples and genotyped them in duplicate, and 99.8% concordance was observed. Inconsistent data were excluded from the final analysis.

Evaluation of the MTX Response

MTX response was evaluated with Disease Activity Score 28-C-reactive protein in accordance with the European League against Rheumatic Diseases standards for RA activity, as follows: DAS<2.3: remission; 2.3≤DAS<2.7 low disease activity; 2.7≤DAS≤4.1: moderate disease activity; and 4.1<DAS: high disease activity.

MTX and concomitant agent dosages were adjusted monthly and taken as indications of remission or low disease activity.

Responders were those patients who showed sustained remission or low disease activity with MTX alone or MTX with the combination therapy of other conventional DMARDs beyond 6 months. Non-responders were those patients who showed moderate or high disease activity, or those who were prescribed biological DMARDs.

Evaluation of Hepatotoxicity

We assessed MTX-induced hepatotoxicity. We defined the hepatotoxicity as serum levels of alanine aminotransferase (ALT) elevated 1.5 times over the normal range.

Statistical Analysis

A logistic model was constructed with Responder/Non-responder as a target variable, and MAF was set as an explanatory variable, to evaluate associations between the target variable and the candidate SNPs. The signifi-

Table 2 Allele frequency

Gene symbol	Reference SNP ID number	Genotype	Patients in this study (N = 171)	HWE p	HapMap JPT* (N = 172)	P value
SLCO1B1	Rs11045879	TT	57	0.157	70	0.167
		TC	91		74	
		CC	23		28	

*National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov)
HWE p: p value in Hardy-Weinberg equilibrium

Table 3 Characteristics and possible factors for MTX response in 171 patients

Factors	Non-responders	Responders	P value	Crude OR (95% CI)	P value	Adjusted OR (95% CI)	P value
Male/Female	11/87	14/59	0.145	1.88 (0.80-4.42)	0.150		
Age	65.1	62.3	0.133	1.02 (0.99-1.05)	0.135	1.02 (0.99-1.05)	0.135
Mean MTX dose (mg/week)	8.08	8.78	0.070	1.12 (0.99-1.26)	0.076	1.11 (0.98-1.27)	0.107
Hepatotoxicity							
Present/Absent	34/64	13/60	0.014*	0.41 (0.20-0.85)	0.016*	0.47 (0.22-1.00)	0.049*
Adverse event							
Present/Absent	12/86	11/62	0.592	1.14 (0.48-2.80)	0.779		
SLCO1B1							
CC	17	6	0.046*	0.55 (0.34-0.89)	0.015*	0.58 (0.35-0.96)	0.034*
CT	55	36					
TT	26	31					

*P<0.05 was considered statistically significant. Statistical analysis was performed using Student's *t*-test, chi-square test, and multivariate logistic regression.
P value, odds ratio (OR), and 95% confidence intervals (CI) are for multivariate logistic regression adjusted for SLCO1B1 genotype, age, MTX dosage, and presence of hepatotoxicity.

cance level was set using the Bonferroni correction for multiple comparisons. We then performed logistics analysis for the identified SNPs, taking into account clinical information.

Results

In total, 98 patients were non-responders (11 men, 87 women; mean age: 65.1 years), and 73 patients were responders (14 men, 59 women; mean age: 62.3 years). Mean MTX dose did not differ significantly between non-responders and responders, at 8.08 mg/week and 8.78 mg/week, respectively.

None of the 82 SNPs targeted for analysis met the Bonferroni significance threshold of 6.098×10^{-4}. However, we identified SLCO1B1 rs11045879 (complete linkage disequilibrium with rs4149081) as an SNP that might yield significant results if the number of patients were to be increased (OR=0.55, 95% CI=0.34–0.89, P=0.015). The allele frequencies for the SLCO1B1 gene in this study did not significantly differ from those in the Japanese in Tokyo, Japan haplotype map collection (HapMap JPT) (**Table 2**). If the supplementary research included additional 80 (about half of this study) or 180 (same as this study) cases with the same genotype frequencies as the case and control groups and the same proportion of dropouts due to adverse events as the collected sample in this study, the integrated P value by meta-analysis of this research and the supplementary research would be 0.003 and 6.225×10^{-4}, respectively. Additional 180 cases would almost meet the Bonferroni significance threshold.

Hepatic dysfunction was found in 34 non-responders and 13 responders; the incidence was significantly higher in non-responders even in multivariate analysis adjusting for MTX dose (OR=0.41, 95% CI=0.20–0.85, P=0.016). Multivariate analyses adjusting for age, MTX dose, and hepatic dysfunction revealed a significantly greater MAF in non-responders (OR=0.58, 95% CI=0.35–0.96, P=0.034) (**Table 3**). The SLCO1B1 rs11045879 polymorphism showed no association with hepatic dysfunction. Other adverse events that did not stop MTX treatments were seen in 23 patients (gastrointestinal symptom 8, respiratory symptom 8, leukopenia (less than 3,000/μL) 4, depilation 1, itching 2). There was no significant difference between responders and non-responders.

Discussion

In this study, no SNPs met the significance threshold with Bonferroni correction (set to compensate for multiple comparisons). However, we suggest that the rs 11045879 minor allele in the SLCO1B1 gene is a potential predictor of non-response to MTX treatment in Japanese RA patients. SLCO1B1 is one of the genes encoding organic anion-transporting polypeptides; it is specifically expressed in stem cell sinusoids and on the basement membranes of intestinal cells, and plays an important role in absorption of drugs from the digestive tract, subsequent transportation in blood, and uptake in hepatocytes[71]. Drugs that are used clinically recognize the relevant substrate and include β-hydroxy β-methylglutaryl-CoA reductase inhibitors (statins), angiotensin converting enzyme inhibitors, angiotensin II receptor antagonists, hypoglycemic agents, and MTX[72]. The rs11045879 allele in the SLCO1B1 gene is reportedly associated with MTX excretion[73] and has reported utility for the prediction of adverse drug reactions to MTX in childhood acute lymphoblastic leukemia[74]. Individuals homozygous for the rs 11045879 minor allele CC were reported to have prolonged high-MTX plasma levels after MTX treatments due to the low capacity of MTX clearance and to have risk for MTX toxicity. This SNP could meet a Bonferroni significance threshold that was set based on the assumed power of a test for an additional study enrolling 180 patients.

Genes targeted in pharmacogenetics analysis include those encoding drug target proteins such as drug-metabolizing enzymes, drug receptors, and drug transporters. Statistical processing of such genetic information could potentially enable personalized-medicine therapeutic strategies for individual patients. In the case of MTX, associations with therapeutic effect have been reported for a number of polymorphisms. These reports cover solute carrier family 19 (SLC19A1; or the reduced folate carrier-1), which is involved in the cellular uptake of MTX[75]; the ATP binding cassette subfamily C member1 (ABCC1), which affects the expression of P-glycoprotein, a protein involved in MTX excretion[46]; methylenetetrahydrofolate reductase, a folate-metabolizing enzyme[76,77]; thymidylate synthetase (TYMS)[78,79]; and 5-aminoimidazole-4-carboxyamide ribonucleotide formyltransferase (ATIC), an enzyme in the adenosine pathway related to the anti-inflammatory effect of MTX[75]. SNP analyses have revealed large disparities between human populations. In 2011, the Japanese authorities increased the approved dosage from the previous limit of 8 mg/week to 16 mg/week, in consideration of the results seen at the higher dosage levels outside of Japan. This study was the first research on associations between SNPs and the therapeutic effect of MTX in Japan since the raising of the MTX upper dose limit; the mean MTX dose in responders is around 9 mg. The results of our analysis reflect the current treatment of RA in actual clinical settings.

The results of this study showed a low possibility for an MTX therapeutic effect against RA in carriers of the minor allele of the SLCOB1 gene. We consider that MTX treatment can be started while predicting responders and non-responders based on determinations of SLCOB1 polymorphism carried out prior to treatment. We also consider that carriers of the SLCOB1 minor allele should be monitored for therapeutic effect in the early stage of treatment and may require treatment centered on biologics that are completely uninfluenced by the SLCO1B1 gene.

The incidence of hepatic dysfunction was significantly higher in non-responders in the present study. All patients with liver dysfunction could continue MTX treatment by dose reduction, and mean MTX in non-responders was lower than in responders. Therefore, we conducted multivariate analysis for the predictive factor of MTX response, adjusting for liver dysfunction, mean MTX dose, and SLCO1B1genotype, and liver dysfunction was still significant. Another new finding in this research is that hepatotoxicity significantly and independently influenced the response to MTX treatment.

In conclusion, the rs11045879 minor allele in the SLCO 1B1 gene is a potential predictor of non-responders to MTX treatment in Japanese RA patients. In future collaborative research, we will investigate whether the association with SLCO1B1 polymorphism is significant by performing statistical analysis with a larger study population.

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Conflict of Interest: None.

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