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
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. Responders account for 46% to 65% of MTX-treated patients, among whom the incidence of adverse drug reactions is around 50%. A range of factors are implicated in such individual differences, including sex, age, body weight, disease duration, and disease activity.

MTX reportedly needs to be taken for at least 6 months for its therapeutic effect to be clearly evaluated. However, a recent study has demonstrated that longer periods may be needed for this effect to be determined. 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. 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. 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. 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. 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 subjected 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°C before DNA extraction. Genomic DNA was extracted by a contracted clinical laboratory (SRL Inc., Tokyo, Japan) and stored at −20°C. 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
SLCO1B1 SNP and MTX Response in RA

| Reference SNP ID number | Gene symbol | Possibility of relevance | Ref. |
|-------------------------|-------------|--------------------------|-----|
| rs10181656               | STAT4       | Th1 cell differentiation  | 12  |
| rs1043879                | CR1         | Association with anti-TNF treatment efficacy | 13  |
| rs1051266                | SLC19A1     | Association with MTX treatment efficacy | 14  |
| rs10760130               | Traf1       | Role in RA susceptibility | 15  |
| rs10865035               | Afp3        | 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                | Afp3        | 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                | Kiif5a      | 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                 | Ctl4        | 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                | Ctl4        | 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                | FCR3A       | Association with anti-CD20 treatment efficacy | 54   |
| rs4149056               | SLC01B1     | Association with MTX treatment efficacy | 42   |
| rs4149081               | SLC01B1     | Association with MTX treatment efficacy | 55   |
| rs4195998               | 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  | 59   |
| 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   |
| rs7636361               | 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°C 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°C 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 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-
SLCO1B1 SNP and MTX Response in RA

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 Adverse event   | 34/64          | 13/60      | 0.014*  | 0.41 (0.20-0.85) | 0.016*  | 0.47 (0.22-1.00)     | 0.049*  |
| Present/Absent SLCO1B1 CC      | 12/86          | 11/62      | 0.592   | 1.14 (0.48-2.80) | 0.779   |                      |         |
| CT                             | 17             | 6          | 0.046*  | 0.55 (0.34-0.89) | 0.015*  | 0.58 (0.35-0.96)     | 0.034*  |
| TT                             | 55             | 36         |         |                  |         |                      |         |

*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.

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Discussion

In this study, no SNPs met the significance threshold with Bonferroni correction (set to compensate for multiple comparisons). However, we suggest that the rs11045879 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. 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. The rs11045879 allele in the SLCO1B1 gene is reportedly associated with MTX excretion and has reported utility for the prediction of adverse drug reactions to MTX in childhood acute lymphoblastic leukemia. Individuals homozygous for the rs11045879 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; the ATP binding cassette subfamily C member 1 (ABCC1), which affects the expression of P-glycoprotein, a protein involved in MTX excretion; methylenetetrahydrofolate reductase, a folate-metabolizing enzyme; thymidylate synthetase (TYMS); and 5-aminomimidazole-4-carboxamide ribonucleotide formyltransferase (ATIC), an enzyme in the adenosine pathway related to the anti-inflammatory effect of MTX. 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 biology 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 SLCO1B1 genotype, 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 SLCO1B1 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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