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# Preliminary Study to Identify the Predictive Factors for the Response to Methotrexate Therapy in Patients with Rheumatoid Arthritis

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To identify the major factors predicting the response to Methotrexate (MTX) therapy in rheumatoid arthritis (RA) patients, we evaluated the relationship between the response to MTX and factors such as the concentration of MTX-polyglutamates (MTX-PGs) in erythrocytes (RBCs), genotypes of thymidylate synthase (TYMS) 5′-UTR (2R/3R) and 3′-UTR (−6/+6), 5,10-methylenetetrahydrofolate reductase (MTHFR) C677T and A1298C, and other patient-related factors. Thirty-six Japanese RA patients were enrolled in this cohort study. The concentrations of MTX-PGs in RBCs were measured, and polymorphisms were determined using PCR-RFLP method. As an indicator of the accumulated capacity of MTX-PGs in the RBCs of each patient, the MTX dose/MTX-PGs (AC-MPG, l/week) was calculated. The response to MTX therapy was assessed using the MTX dose for a ≥50% decrease in CRP level (MTX dose for 50% CRP, mg/week), and the relationships between MTX dose for 50%CRP and various other factors were evaluated using multiple linear regression analysis. The MTX dose was 6.9±0.3 mg/week and the MTX-PGs concentration in RBCs was 97.3±8.1 nmol/l ($n$=36, blood samples=95, mean±S.D.). The range of MTX dose for 50%CRP was 2.0–13.0 mg/week. Most individual AC-MPG levels showed no change during the evaluation period (coefficient of variation=5.9%). Based on the results of multiple linear regression analysis, AC-MPG, TYMS 3′-UTR (−6/+6), and ESR at the start of MTX therapy were associated with the MTX dose for 50%CRP. AC-MPG, TYMS 3′-UTR (−6/+6), and ESR might be the major predictive factors for the response to MTX therapy in Japanese RA patients.

**Key words**——rheumatoid arthritis; methotrexate; methotrexate-polyglutamates; polymorphism; clinical trial

## INTRODUCTION

Methotrexate (MTX) is the most widely used disease-modifying antirheumatic drug (DMARD) in the treatment of rheumatoid arthritis (RA) and is regarded as a key drug in the 2002 update of the RA treatment guidelines from the American College of Rheumatology subcommittee.[1] However, it is recognized that there are large individual differences in the optimal dose of MTX in RA patients.[2] In the USA and many European countries, the recommended general target dose of MTX is 15–20 mg/week, but the individual optimal dose is the range of 5–20 mg/week.[3] In Japan, the approved maximum dose of MTX is 8 mg/week, but in practice 2–20 mg/week of MTX is prescribed based on individual sensitivity to and tolerance of MTX.[4] Differences in the optimal MTX dose mainly reflect individual differences in the response to MTX therapy. The reasons for those individual differences in the response to MTX therapy are thought to be different concentrations of intracellular MTX-polyglutamates (MTX-PGs),[5,6] different enzyme activity at MTX-active sites,[5,7–9] and other patient-related factors[10] such as body weight, renal function, disease severity, *etc*.

It was confirmed that MTX exerts its antirheumatic effects by adding up to six glutamates to MTX (MTX-$PG_{1–6}$) *via* folypolyglutamyl synthase (FPGS) in target cells (mononuclear cells, lymphocytes, or synovial cells) which are retained intracellularly over a long period.[11–14] The concentration of MTX-PGs in erythrocytes (red blood cells; RBCs) is reported to reflect that in target cells.[6] Therefore, the concentration of MTX-PGs in RBC is used as a substitute for the MTX-PGs concentration in target cells.[5,6] We measured the concentration of MTX-PGs in RBCs and evaluated the relationship between the response to MTX and the concentration of MTX-PGs in

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RBCs.

Two genetic polymorphisms that influence enzyme activity were reported in both the thymidylate synthase (TYMS) and 5,10-methylenetetrahydrofolate reductase (MTHFR) genes at MTX-active sites.[15–18,22] The two or three 28-bp tandem repeats in the TYMS 5′-untranslated region (UTR) act as an enhancer of the TYMS promoter, and increasing the number of repeats leads to stepwise increases in TYMS mRNA expression.[17] The 6-bp deletion/insertion was located in the TYMS 3′-UTR, 447 bp downstream from the stop codon, and the 6-bp deletion expresses less TYMS mRNA than the 6-bp insertion.[18] The MTHFR C677T polymorphism consists of a C>T change resulting in an alanine to valine substitution that renders the enzyme more thermolabile.[15] In the MTHFR A1298C polymorphism, the A>C change causes a glutamine to alanine substitution and leads to reduced enzyme activity.[16] Several studies evaluated the relation between these genetic polymorphisms and MTX therapeutic effects,[5,7–9] but the indicator of the therapeutic effects and the evaluated genotype site differed in every study. Because the mechanisms of action of MTX are complex, the relation between the above mentioned-factors and the response to MTX therapy should be evaluated not individually but comprehensively. Therefore, the purpose of this preliminary study was to identify the major predictive factors of the individual response to MTX therapy in RA patients by evaluating the concentration of MTX-PGs in RBCs, the four polymorphisms of the TYMS and MTHFR genes, and other patient-related factors.

## PATIENTS AND METHODS

**Study Design** This cohort study was conducted from July 2004 to August 2006 at a single investigational site, Kitasato Institute Hospital (Tokyo, Japan).

**Participants** Patients met the 1987 American College of Rheumatology criteria, and they were receiving MTX or scheduled to start MTX therapy.

The use of low-dose oral corticosteroid (predonisolone, <10 mg/day) and nonsteroidal antiinflammatory drugs (NSAIDs) was allowed, but during the evaluation period the dose was changed as little as possible. For the prevention of adverse effects induced by MTX, all patients were administered folic acid (5 mg/week). This study protocol was approved by the Institutional Review Board of the Kitasato Institute Hospital and written informed consent was given by all patients prior to enrollment.

**Patient Background and Predictive Factors from Patient History** The duration of RA, previous use of DMARDs, age at which MTX administration was initiated, gender, body weight, laboratory data; serum C-reactive protein (CRP) concentration, erythrocyte sedimentation rate (ESR), rheumatoid factor (RF), mean corpuscular volume (MCV), and serum creatinine clearance (Scr) were obtained from the medical records.

**Determination of MTX-PGs Concentration in RBCs** The concentration of MTX-PGs in RBCs was evaluated more than twice per patient at approximately 3-month intervals. For the determination of MTX-PGs in RBCs, heparinized peripheral whole blood (5 ml) from RA patients who had been receiving the same dose of MTX for more than 1 month were collected more than 3 days after MTX administration. All collected blood samples were stored immediately at 4℃ and RBCs and plasma were separated within 48 h in a 10-min centrifugation step.[19] RBCs and plasma were stored at −80℃ until analysis. MTX-PGs concentrations in RBCs were determined using the modified method of Dervieux *et al.*[19] In brief, the MTX-PGs in hemolyzed RBCs were converted to MTX in the presence of plasma $\gamma$-glutamyl hydrolase and mercaptoethanol at 37℃. Then MTX was purified in a perchloric acid deproteinization step, followed by solid-phase extraction. The concentration of MTX was measured by using a TDX analyzer (Abbott Japan, Tokyo). The quantification limit and coefficient of variation (CV) of this analytical method are 30 nmol/l and <12.4%, respectively. Using the values of MTX-PGs concentration in RBCs from each patient, the MTX dose/MTX-PGs (AC-MPG, l/week) was calculated as an indicator of the accumulated capacity (*i.e.*, clearance) of MTX-PGs in the RBCs from each patient. The lower AC-MPG means the higher accumulated capacity of MTX-PGs in RBCs (*i.e.*, lower clearance of MTX-PGs in RBCs).

**Genotype Determination** Peripheral blood samples (5 ml) for genetic analysis were collected in tubes containing EDTA-2Na at the time of the first assessment and stored at −20℃ until DNA extraction. DNA was extracted using the agglutination partition method (Sepa Gene, Sanko Junyaku, Ltd, Tokyo, Japan). The MTHFR C677T polymorphism

was detected using a polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) method, as reported previously.[15] Individuals with the 677CC genotype presented one fragment (198 bp), those with the 677CT genotype presented two fragments (175 and 198 bp), and those with the 677TT genotype presented one fragment (175 bp) when visualized on 3% agarose gels. The MTHFR A1298C polymorphism was detected using a PCR-RFLP method, as reported previously.[16] Individuals with the 1298AA genotype presented five fragments (56, 31, 30, 28, and 18 bp), those with the 1298AC genotype presented six fragments (81, 56, 31, 30, 28, and 18 bp), and those with the 1298CC genotype presented four fragments (81, 31, 30, and 18 bp) when visualized on 10–25% gradient polyacrylamide gels (Daiichi Pure Chemicals Co., Ltd, Tokyo, Japan). The TYMS 5′-UTR polymorphism was detected using a PCR-RFLP method, as reported previously.[20] Individuals with the 2R2R genotype presented one fragment (210 bp), those with the 2R3R genotype presented two fragments (210 and 238 bp), and those with the 3R3R genotype presented one fragment (238 bp) when visualized on 3% agarose gels. The TYMS 3′-UTR polymorphism was also detected using a PCR-RFLP method, as reported previously.[21] Individuals with the +6-bp/+6-bp genotype presented two fragments (88 and 70 bp), those with the +6-bp/−6-bp genotype presented four fragments (152/158, 88, and 70 bp), and those with the −6-bp/−6-bp genotype presented one fragment (152 bp) when visualized on 3% agarose gels.

**Statistical Analysis** The response to MTX therapy in each RA patient was assessed using the MTX dose resulting in a ≥50% decrease in the serum CRP level (MTX dose for 50%CRP, mg/week). The Spearman's rank correlation coeficient was used to investigate the relationship between AC-MPG and MTX dose for 50%CRP. Relationships between genotypes and MTX dose for 50%CRP were analyzed using analysis of variance (ANOVA) or Student's *t*-test. The genetic differences in enzyme activities in MTX-active sites in each patient were evaluated using a pharmacogenetic index, which was the sum of four homozygous variant genotypes (TYMS 5′-UTR 2R/2R, TYMS 3′-UTR −6/−6, MTHFR 677 T/T, and MTHFR 1298 C/C). The relationship between the pharmacogenetic index and MTX dose for 50%CRP was analyzed using ANOVA.

Multiple linear regression analysis was performed to identify the predictive factors of individual response to MTX therapy. The explanatory variables for the dependent variable (*i.e.*, MTX dose for 50% CRP) were AC-MPG, four polymorphisms (TYMS 5′-UTR 2R/3R, TYMS 3′-UTR −6/+6, MTHFR C677T, and MTHFR A1298C), CRP, RF, MCV, and Scr levels and ESR at the start of MTX therapy, duration of disease, previous use of DMARDs, age at which MTX administration was initiated, gender, and body weight. The method of forward-backward stepwise selection was used for the selection of explanatory variables for multiple linear regression analysis. Multicolinearity was evaluated by Pearson correlation coefficient of a correlation matrix.

All statistical analyses were carried out using Statistical Analysis System softwere (14.0J; SPSS, Chicago, IL). A *p*-value of less than 0.05 was considered to represent a statistically significant difference.

## RESULTS

Thirty-six RA patients (31 women and 5 men) were enrolled from July 2004 to August 2006 at the Kitasato Institute Hospital, Tokyo. Twenty-eight patients who were receiving MTX and 8 patients in whom MTX therapy initiation was deemed necessary were included in this study. All patients received folic acid (5 mg/week) from the initiation of MTX therapy. The number of patients with Steinbrocker stage I to Ⅳ were 5, 10, 9, and 12, respectively.

**Factors Predicting Response to MTX Treatment** Data on factors predicting the response to MTX therapy are summarized in Table 1. The median duration of RA was 4.0 years, and 91.7% of patients had used DMARDs previously. The median age at which MTX therapy was initiated was 61.0 years. The median levels of CRP, RF, MCV, and Scr and median ESR at the start of MTX therapy were 1.9 mg/dl, 78.6 IU/ml, 89.0 fl, and 0.6 mg/dl and 51.5 mm/h, respectively. No patient had impaired renal function.

At the start of MTX therapy, 50% of patients were receiving low-dose oral corticosteroids and other DMARDs, and all patients had received NSAIDs. Because the body weight at the start of MTX therapy could not be obtained from the medical records, it could not be assessed. Adverse effects during the evaluation period were observed in only 1 of the 36 patients.

Table 1. Factors Predicting Response to Methotrexate in RA Patients

| | |
|---|---|
| *n* | 36 |
| Women (%) | 86 |
| CRP (mg/dl)* | 1.9 (0.4–12.8) |
| ESR (mm/h)* | 51.5 (13.0–143.0) |
| RF (IU/ml)* | 78.6 (0.0–811.0) |
| MCV (fl)* | 89.0 (75.7–101.1) |
| Scr (mg/dl)* | 0.6 (0.4–1.0) |
| Previous use of DMARDs (%) | 91.7 |
| Duration of disease (yr) | 4.0 (0.0–36.0) |
| Age at MTX initiation (yr) | 61.0 (26.0–86.0) |

Values are expressed as medians; figures in parentheses are ranges (min-max).
* Level and ESR at the initiation of MTX therapy.

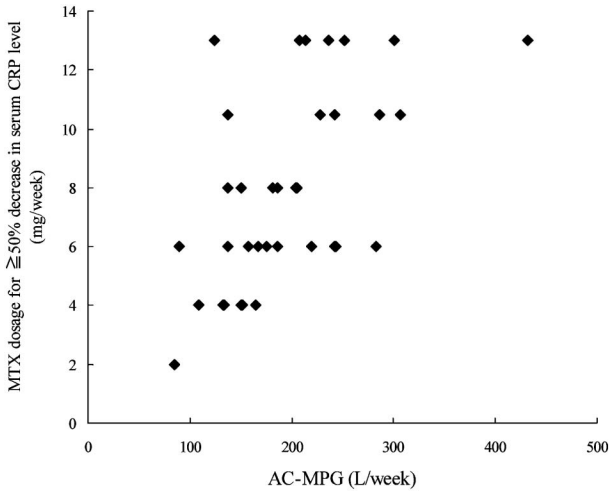


Fig. 1. Linear Regression of AC-MPG and MTX Dose for 50% Decrease in Serum CRP Level in 36 Japanese RA Patients
AC-MPG=MTX dose/RBC MTX-PGs conc. Ratio. A linear regression of $r=0.59$ and $r^2=0.34$ ($p<0.001$) was obtained.

**Relationship between Concentration of MTX-PGs in RBCs and MTX Dose for ≥50% Decrease in Serum CRP Level** Ninety-five blood samples (mean 2.6 times/patient) were collected, and the MTX-PGs concentration in RBCs (mean±S.D.) was 97.3±8.1 nmol/l at the 6.9±0.3 mg/week MTX dose ($n=36$). Mean AC-MPG in the 36 patients ranged from 85.2–432.3 l/week, and there was a difference of about 5.1-fold among them. The mean CV of AC-MPG was 5.9% ($n=36$), and most individual AC-MPG values did not change during the evaluation period. Therefore, AC-MPG was deemed patient-intrinsic value, and it was used as an explanatory variable of the MTX dose for 50%CRP. The relationships between individual AC-MPG and MTX dose for 50% CRP are shown in Fig. 1. The MTX dose for 50% CRP ranged from 2.0 to 13.0 mg/week, and there were differences of approximately 7-fold among patients. The higher the AC-MPG, the higher the MTX dose for 50%CRP became. The analysis revealed a good correlation between AC-MPG and MTX dose for 50%CRP ($p<0.001$). Therefore, AC-MPG was used as an explanatory variable of the MTX dose for 50%CRP. In all samples, the MTX concentrations in plasma were measured using a TDX analyzer, but all MTX concentrations were below the limit of determination.

**Relationships between Genetic Polymorphisms of TYMS or MTHFR and MTX Dose for ≥50% Decrease in Serum CRP Level** The four polymorphisms of TYMS and MTHFR were investigated in the 36 RA patients, and the distribution of TYMS 5′-UTR (2R/3R), TYMS 3′-UTR (−6/+6), MTHFR C677T, and MTHFR A1298C polymorphisms are summarized in Table 2. Allelic frequencies (%) were TYMS 5′-UTR 3R (86) >2R (14), TYMS 3′-UTR 6-bp deletion (65) >insertion (35), MTHFR 677C (61) >T (39), and 1298A (76) >C (24), respectively. No patient had TYMS 5′-UTR 2R/2R.

The relationships between the four polymorphisms of TYMS or MTHFR and MTX dose for 50%CRP were evaluated in three groups (homozygous mutant type, heterozygous mutant type, and homozygous wild type), two groups (without/with homozygous wild type) by every polymorphism site, or three groups classified by the pharmacogenetic index. The MTX dose for 50%CRP (mean±S.D.) in patients without the TYMS 3′-UTR +6 allele was 6.5±2.4 mg/week and in those with the TYMS 3′-UTR +6 allele it was 8.8±3.6 mg/week, and the difference was statistically significant ($p=0.039$). However, no significant difference was found in any other analyses.

**Identification of Major Predictive Factors for Response to MTX Therapy in RA Patients** To identify the predictive factors of MTX dose for ≥50 %CRP, we performed multivariate regression analysis including AC-MPG, the four polymorphisms of TYMS and MTHFR, and other factors. Each genotype was classified into two groups (with/without homozygous wild type), and the pharmacogenetic index was classified into two groups (0 or 1 and 2). The multivariate regression analysis was performed with 15 explanatory variables. No patient had TYMS 5′-

Table 2. Distribution of TYMS and MTHFR Gene Polymorphisms in RA Patients

| | Genotype frequency (%) | | | Allele frequency (%) | |
|---|---|---|---|---|---|
| TYMS 5′-UTR | *2R/2R* | *2R/3R* | *3R/3R* | *2R* | *3R* |
| | 0( 0.0) | 10(27.8) | 26(72.2) | 14 | 86 |
| TYMS 3′–UTR | *+6/+6* | *+6/−6* | *−6/−6* | *+6* | *−6* |
| | 4(11.1) | 17(47.2) | 15(41.7) | 35 | 65 |
| MTHFR 677 | *C/C* | *C/T* | *T/T* | *C* | *T* |
| | 16(44.4) | 12(33.3) | 8(22.2) | 61 | 39 |
| MTHFR 1298 | *A/A* | *A/C* | *C/C* | *A* | *C* |
| | 22(61.1) | 11(30.6) | 3( 8.3) | 76 | 24 |

Values for genotype frequency are expressed as number of patients (%).

Table 3. Multiple Regression Analysis of MTX Dosage for ≥50% Decrease in Serum CRP Level

| | *Dependent variable* MTX dosage for ≧50% decrease in serum CRP level | |
|---|---|---|
| | *β* | *p*-value |
| *Explanatory variable* | | |
| AC-MPG | 0.590 | <0.001 |
| TYMS3′-UTR without+6-bp allele | −0.268 | 0.039 |
| ESR* | 0.293 | 0.025 |
| $R^2$ | 0.51 | |

$R^2$, coefficient of determination. *β*, standard partial regression coefficient.
* ESR at the initiation of MTX therapy.

UTR 2R/2R, and therefore genotypes were divided into 2R/3R and 3R/3R. The results of this analysis are shown in Table 3. In the model, three variables, AC-MPG level, TYMS 3′-UTR (−6/+6), and ESR at the initiation of MTX therapy were major predictive variables of the MTX dose for 50%CRP ($p<0.001$, $p=0.039$, and $p=0.025$, respectively). The AC-MPG level was the most influential explanatory variable for the MTX dose for 50%CRP ($\beta=0.590$). The model-explained variance of the MTX dose for 50%CRP was 51.0%. In this analysis. multicolinearity was recognized between CRP and ESR ($r=0.794$). Therefore, basing on the results of coefficient of determination ($R^2$), ESR was adopted as the explanatory variable.

## DISCUSSION

In this study, we evaluated the relationships between the individual response to MTX and various associated factors in 36 Japanese RA patients, and identified AC-MPG level, TYMS 3′-UTR (−6/+6), and ESR at the initiation of MTX therapy as the major predictive factors for the response to MTX.

The range of MTX dose for 50%CRP which was used as the indicator of the response to MTX therapy[23] was 2.0–13.0 mg/week, with differences of approximately 7-fold among patients. Half of our 36 RA patients were receiving low-dose oral corticosteroids and all were receiving NSAIDs at the start of MTX therapy. However, all continued the same dose of oral corticosteroids and NSAIDs until a ≥50% decrease in the serum CRP level had been achieved, therefore, it was thought that concomitant use of these drugs did not affect in the evaluation of MTX dose for 50%CRP.

The distribution of the allelic frequencies of TYMS 5′-UTR (2R/3R), TYMS 3′-UTR (−6/+6), MTHFR C677T, and MTHFR A1298C in these 36 Japanese RA patients was similar to that in our previous report on 102 healthy Japanese adults.[22] In the evaluation of the relationship between MTX dose for 50%CRP and the genetic polymorphisms of TYMS or MTHFR, there was a significant difference in MTX dose for 50%CRP between patients with and without the TYMS 3′-UTR +6 allele ($p=0.039$). Kumagai *et al.*[8] evaluated the relationship between the response to MTX and TYMS genotype in 105 Japanese RA patients with a history of MTX administration. They reported that a better response to MTX was seen in patients with the TYMS 3′-UTR −6 allele. Our finding supports their result.

In the evaluation of the relationships between the MTX dose for 50%CRP and genetic polymorphisms

of TYMS/MTHFR, the MTX dose for 50%CRP tended to be lower in patients with the homozygous mutant-type TYMS 3′-UTR (−6/+6) or MTHFR A1298C than in patients without. There was a significant difference in the MTX dose for 50%CRP between patients with and without TYMS 3′-UTR (−6/+6) ($p=0.039$), although there was no significant difference in it between patients without and with MTHFR A1298C ($p=0.38$). This result might be explained by the differences in the distribution of TYMS 3′-UTR (−6/+6) and MTHFR A1298C in Japanese. In previous study of ours of healthy Japanese volunteers,[22] the genotype frequency of MTHFR 1298 C/C was 13.7%, whereas that of TYMS 3′-UTR −6/−6 was 35.3%.

As a result of multivariate regression analysis, AC-MPG level, TYMS 3′-UTR (−6/+6), and ESR at the initiation of MTX therapy were major predictive variables for the MTX dose for 50%CRP. The $\beta$ value of AC-MPG and TYMS 3′-UTR (−6/+6) in univariate regression analysis was 0.591 and −0.346, respectively, and these variables are thought to be major independent predictive factors for the response to MTX therapy. To the best of our knowledge, this is the first report on predicting the response to MTX therapy including factors such as the concentration of MTX-PGs in RBCs, genetic polymorphisms of MTX-active sites, and other patient-related factors. The MTX dose for 50%CRP could not be predicted based on ESR and CRP and RF levels alone, although 51% of individual differences in the MTX dose for 50% CRP could be explained by considering the factors of AC-MPG and the genotype of TYMS 3′-UTR (−6/+6). However, their predictive ability was insufficient for clinical use. Therefore, further assessment of the relationships between the response to MTX therapy and other associated factors including body weight, number of tender and swollen joints, and matrix metalloproteinase-3 level reflecting the degree of joint destruction[24] must be made.

Our study had three limitations. First, there was no patient with the TYMS 5′-UTR 2R/2R genotype, and therefore the relationship between TYMS 5′-UTR 2R/2R and MTX dose for 50%CRP could not be assessed. Second, this study was conducted only among Japanese RA patients. Ethnic differences were reported in the distribution of TYMS 5′-UTR (2R/3R), TYMS 3′-UTR (−6/+6), and MTHFR C677T.[22] Therefore, the major predictive factors may differ by ethnic group. Third, this study was a preliminary trial. Therefore, additional data will be needed to demonstrate conclusive results.

The polymorphisms of dihydrofolate reductase (DHFR)[25] and 5-aminoimidazole-4-carboxamide ribonucleotide transformylase (ATIC),[5] which are MTX and MTX-PGs-active sites, $\gamma$-glutamyl hydrolase,[26] which catalyzes the conversion from MTX-polyglutamates to MTX, and reduced folate carrier (RFC)-1,[27] which is responsible for the intracellular transport of MTX, were not analyzed in this study. It has been reported that these genetic polymorphisms influence enzyme activity, and therefore they are also expected to be associated with individual differences in the response to MTX therapy. Dervieux *et al.* reported that the number of homozygous mutant types of TYMS, RFC-1, and ATIC was related to the effect of MTX in RA patients.[5] Therefore, further investigation of these genetic polymorphisms must be performed in the future.

In conclusion, AC-MPG, TYMS 3′-UTR (−6/+6), and ESR at the initiation of MTX therapy might be the major predictive factors of the response to MTX therapy in Japanese RA patients. Genotyping can be carried out before the start of MTX therapy, and the AC-MPG level can also be estimated using one blood sample 1 month after receiving the same dose of MTX. Therefore, the evaluation of AC-MPG and TYMS 3′-UTR (−6/+6) would be useful for individualized MTX therapy in RA patients.

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