Good response to methotrexate is associated with a decrease in the gene expression of ABCG2, a drug transporter, in patients with rheumatoid arthritis

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ABSTRACT

Objectives: Methotrexate (MTX) is used as an anchor drug in the treatment of rheumatoid arthritis (RA), although more than a half of the patients with RA require additional treatments. We designed a prospective study involving two medical centers in Japan to examine the association between the expression of MTX-related genes including a drug transporter ATP-binding cassette sub-family G member 2 (ABCG2) gene and the clinical response to MTX in MTX-naive patients with RA.

Methods: The primary endpoint of this study was good response based on the European League Against Rheumatism (EULAR) response criteria by Disease Activity Score using 28-joint count (DAS28). We evaluated the association between the baseline expression of six genes involved in the intracellular pharmacokinetics of MTX, including ABCG2, as well as their temporal changes, and the clinical response at week 12 from the initiation of MTX.

Results: Based on the clinical response at 12 weeks after the initiation of MTX, 24 patients were classified into good responders (n = 9) and non-good responders (n = 15; 10 moderate responders and 5 non-responders) groups. A univariate logistic regression analysis of the baseline gene expression levels to predict the EULAR good response at week 12 showed a significant association with ABCG2 expression alone. Furthermore, the rate of baseline expression of ABCG2 mRNA above the cut-off value determined using a receiver operating characteristic curve was higher in good responders than in non-good responders (p = 0.012). Moreover, ABCG2 expression decreased in almost all good responders, but not in non-good responders, after MTX treatment for 12 weeks (median −76% vs. +41% from baseline, respectively; p = 0.011). The ABCG2 expression level did not correlate with DAS28 at baseline or week 12.

Conclusions: Our study revealed that good response to MTX is associated with a decrease in the expression of ABCG2 in patients with RA.

Introduction

Methotrexate (MTX) is a first-line therapeutic drug for the treatment of rheumatoid arthritis (RA). It is used as an anchor drug for disease-modifying anti-rheumatic drugs (DMARDs) and is globally used for treatment-naive patients with active RA [1–3]. Approximately 30% of patients with early RA achieve clinical remission with MTX monotherapy after 6 months of treatment [4,5]. The remaining patients are classified as partial responders or non-responders, and they are treated with additional DMARDs according to the “treat to target” strategy [6].

The prediction of the clinical and radiographic effectiveness of MTX is an area of research interest. Low disease activity, male sex, lack of seropositivity, low serum tumor necrosis factor (TNF) level, and TNF-producing cell proportion in the peripheral blood have been reported to be associated with the clinical effectiveness of MTX [7–10]. Recently, activities in daily life as measured using the health assessment questionnaire-disability index (HAQ-DI), body mass index, anxiety/depression, and smoking/alcohol have also been demonstrated to be associated with MTX effectiveness [11–13]. In addition, the gene expression levels of biomolecules involved in the intracellular pharmacokinetics of MTX are likely to play a crucial role in the prediction of clinical response [11,14] and the development of dose-dependent adverse events such as liver dysfunction and cytopenia.

In the present study, we aimed to identify a simple method to predict the clinical response to MTX by analyzing the expression of six genes involved in the intracellular pharmacokinetics of MTX. MTX is transported into target cells via a transporter, reduced folate carrier 1 (RFC-1).
[15,16], and converted to polyglutamated MTX (MTX-PG) by folypolyglutamyl synthase (FPGS) [17]. MTX-PG is highly stable in the intracellular compartment, where it inhibits dihydrofolate reductase (DHFR) significantly more potently than non-polyglutamated MTX [18–21], resulting in the inhibition of DNA synthesis. MTX-PG is reconverted to MTX by γ-glutamyl hydrolase (GGH) [22,23] and is excluded through transporters such as ATP-binding cassette sub-family C member 1 (ABCC1)/multidrug-resistance associated protein 1 (MRP-1) [24] and ATP-binding cassette sub-family G member 2 (ABCG2)/breast cancer resistance protein (BCRP) [25,26]. ABCG2 can also exclude MTX-PG, in addition to MTX [27,28].

In our previous cross-sectional study, we found that the expression level of ABCG2 was lower in patients who received MTX monotherapy than patients who received additional therapies [29]. However, reduced ABCG2 expression may be a result of RA control with MTX. Moreover, whether the baseline expression of ABCG2 or other relevant genes has the potential to predict the clinical response of patients with RA to MTX should be elucidated.

Therefore, we designed a prospective study to evaluate the association of the baseline expression of six genes involved in the intracellular pharmacokinetics of MTX and the changes in their expression over time with the clinical response at 12 weeks (after the initiation of MTX) in MTX-naïve patients with RA. Our findings might help interpret our previous observations from a cross-sectional study.

Methods

Patients

MTX-naïve patients with RA who fulfilled the American College of Rheumatology/European League Against Rheumatism 2010 classification criteria [30] and visited Toho University Ohashi Medical Center or Sakura Medical Center were enrolled in this study before starting MTX between April 2017 and September 2018. Patients without an indication of MTX [3] and those with a history of MTX administration were excluded from the study.

Study design and the primary endpoint

This was a pilot prospective study performed in two medical centers in Japan. After obtaining written informed consent from the patients to participate in this study, blood was sampled at baseline (before starting MTX) and again after 3 months. The primary endpoint of this study was good response based on the European League Against Rheumatism (EULAR) response criteria by Disease Activity Score using 28-joint count (DAS28) [31]. The number of patients required for the study, for analyses between two or among three groups, was determined by an estimation using 15 good responders and 35 moderate or non-responders (non-good responders) according to the clinical response to MTX. However, due to delayed patient recruitment, we terminated recruitment halfway through the study and focused on an analysis between good responders and non-good responders. This study was approved by three ethics committees of Toho University (the Faculty of Pharmaceutical Sciences [approval number 2017-001], Ohashi Medical Center [approval number H16096], and Sakura Medical Center [approval number S17009]).

Clinical assessment of RA

The following demographic and clinical data were obtained from patients at baseline and after 12 weeks: sex, age, disease duration, height, weight, body mass index (BMI), anti-cyclic citrullinated peptide (CCP) antibody, rheumatoid factor (RF), Steinbrocker’s radiographic stage and functional class, tender joint count-28 (TJC), swollen joint count-28 (SJC), patient global assessment (PtGA), health assessment questionnaire-disability index (HAQ-DI), erythrocyte sedimentation rate (ESR), C-reactive protein (CRP), DAS28 based on ESR, the Simplified Disease Activity Index (SDAI), and MTX dose.

RNA extraction and real-time polymerase chain reaction (PCR)

EDTA-treated venous blood (10 mL) samples were collected from patients. Peripheral blood mononuclear cells (PBMCs) were isolated from the samples by density gradient centrifugation using Histopaque 1077 (Sigma), washed three times by centrifugation with PBS, and used for RNA extraction. The total RNA was extracted from the PBMCs using ISOGEN reagents (Wako), according to the manufacturer’s instructions. It was then treated with TURBO DNA-free (Ambion) to remove any possible contaminating DNA. cDNA was prepared from the DNase I-treated total RNA using ReverTra Ace-α (Toyobo).

The cDNA isolated was used as a DNA template to assess gene expression. Quantitative analyses of ABCC1, ABCG2, DHFR, FPGS, GGH, and RFC mRNA expression were performed by real-time polymerase chain reaction (PCR) using the ABI 7500 Fast Real-Time PCR System (Applied Biosystems). TaqMan Gene Expression Master Mix (Applied Biosystems) and TaqMan Gene Expression Assays (HS00219905_m1 for ABCC1, HS00184979_m1 for ABCG2, HS00758822_s1 for DHFR, HS00191956_m1 for FPGS, HS00914163_m1 for GGH, HS00953344_m1 for RFC, and HS99999905_m1 for glyceraldehyde-3-phosphate dehydrogenase [GAPDH]) were used for all quantitative real-time PCR assays. The samples were incubated at 50°C for 2 min and 95°C for 10 min, and then amplified for 40 cycles, each cycle at 95°C for 15 s and annealing at 60°C for 1 min. All assays were performed in duplicate in 96-well plates. The expression levels of all genes of interest were normalized against the expression level of GAPDH and expressed as a percentage of GAPDH expression. The entire real-time PCR experiment was repeated two or three times for each patient sample, and the mean expression level of each gene in each patient in all replicate assays was calculated.
Statistical analyses

Statistical analyses were performed using EZR software (Saitama Medical Center, Jichi Medical University, Saitama, Japan, version 1.37) [32], which provides a graphical user interface for R (The R Foundation for Statistical Computing, Vienna, Austria, version 3.4.1), and complemented by JMP Pro (version 14.2.0, SAS Institute Japan Ltd., Tokyo, Japan) for drawing Supplementary Figure 1. Continuous variables are presented as median and inter-quartile range (IQR) and analyzed using Mann–Whitney U test or Kruskal–Wallis rank-sum test, whereas binominal data from the two groups were examined using Fisher’s exact test. The relationships among the continuous variables were assessed using Spearman’s rank correlation coefficient. We conducted logistic regression analyses followed by receiver operating characteristics (ROC) analyses for baseline gene expression levels to predict the EULAR good response. Cut-off values were determined according to the maximal Youden Index, which represents the highest combination of sensitivity and specificity. Results with a p value of < .05 were considered statistically significant.

Results

Characteristics of the patients

Patient characteristics and disease activities before the administration of MTX are shown in Table 1. Based on their response at 12 weeks after the initiation of MTX (according to the EULAR criteria), 24 patients were classified into good responders (n = 9) and non-good responders (n = 15; 10 moderate responders and 5 non-responders) groups. All demographic and clinical characteristics were comparable between the groups. The median dose of MTX was 6 mg/week initially and 10 mg/week at week 12 in both groups. Four patients had received salazosulfapyridine at study entry. All the enrolled patients completed the 12-week treatment without any adverse events or drop-outs. However, seven patients in the moderate responders group received additional anti-tumor necrosis factor (TNF) biologic DMARDs (infliximab, etanercept, and golimumab for two patients each) or prednisolone 10 mg/day before 12 weeks.

Comparison of gene expression levels between the response groups

First, we analyzed the expression levels of the six genes of interest (ABCC1, ABCG2, DHER, FPGS, GGH, and RFC1) by subgroups (good responders vs. non-good responders) before and after the initiation of MTX (Figure 1). The baseline expression of ABCG2 was higher in good responders (closed triangles) than in non-good responders (closed circles), and the elevated expression of ABCG2 in good responders at baseline reduced substantially at week 12 (solid lines). Although a non-parametric analysis using Mann–Whitney U test did not show a significant difference between the groups, the univariate logistic regression analysis of baseline gene expression levels for predicting the EULAR good response at week 12 showed a significant association in ABCG2 expression alone (p = .0064, Supplementary Figure 1). Moreover, the rate of baseline expression of ABCG2 mRNA above the cut-off value determined using the logistic regression analysis was higher in good responders than in non-good responders (p = .012, Table 2). The rate of baseline expression of the remaining five genes was comparable between good responders and non-good responders.

A significant difference was observed in the rate of change in ABCG2 expression between good responders and non-good responders (median –76% vs. +41% from baseline, respectively; p = .011) during MTX treatment for 12 weeks, although no such change was observed for the remaining five genes (Figure 2). These results were further confirmed by a correlation analysis of gene expression between baseline and week 12. The results of the correlation analysis showed distinct subgroups of good and non-good responders in terms of ABCG2 expression (Supplementary Figure 2). Furthermore, a significant association of the reduction of ABCG2 mRNA expression with the EULAR response was also confirmed by a comparison among good-, moderate-, and non-responders (p = .031; Supplementary Figure 3).

Correlation between RA disease activity and ABCG2 expression

Finally, we examined whether the above findings were attributable to an association between RA disease activity (as defined by the DAS28 score) and ABCG2 expression level. The ABCG2 expression level did not correlate with DAS28 at baseline or at week 12 (Figure 3(a,b), respectively). Moreover, no correlation was observed between the rate of change in DAS28 score and the rate of change in ABCG2 expression (Figure 3(c)). Therefore, the significant decrease in ABCG2 expression observed in good responders during treatment with MTX for 12 weeks cannot be simply attributed to decreased RA activity.

Discussion

Our previous cross-sectional study revealed that amongst the six genes involved in the intracellular pharmacokinetics of MTX, the expression level of ABCG2 was significantly lower in MTX responders than in non-responders [29]. To elucidate the cause–effect relationship between clinical response to MTX and ABCG2 expression, we performed a prospective study, in which the expression level of ABCG2 and the other five genes involved in the intracellular pharmacokinetics of MTX was examined at baseline and week 12 after the initial treatment of RA with MTX. As in our previous study, an association between ABCG2 expression and clinical response to MTX in patients with RA was demonstrated in this study. No similar association was observed with the expression of the other five genes. The baseline expression of ABCG2 was noticeably higher in a
Figure 1. Changes in the mRNA expression of MTX-related genes during the 12 weeks of MTX treatment. The mRNA expression level of each gene is provided as a percent of GAPDH expression at baseline and week 12. Closed triangles with a solid line represent individual patients in the EULAR good responders group and closed circles with a dotted line represent individual patients in the EULAR moderate or non-responders (non-good responders) group.

Table 1. Patient characteristics.

|                       | Total (n = 24) | Good (n = 9) | Non-good (n = 15) | p Value |
|-----------------------|---------------|--------------|-------------------|---------|
| Sex, female           |               |              |                   | .15     |
|                       | 18 (73)       | 5 (56)       | 13 (87)           |         |
| Age, years            |               |              |                   | .25     |
|                       | 58 (46–70)    | 69 (51–73)   | 53 (45–67)        |         |
| Disease duration, years|              |              |                   | .57     |
|                       | 0.8 (0.3–1.0) | 0.8 (0.3–1.5)| 0.8 (0.4–2.0)    |         |
| Height, cm            |               |              |                   | .44     |
|                       | 161 (155–167) | 163 (159–167)| 159 (155–167)    |         |
| Weight, kg            |               |              |                   | .999    |
|                       | 59 (50–60)    | 58 (57–61)   | 52 (47–59)        |         |
| BMI                   |               |              |                   |         |
|                       | 21.8 (19.8–22.8) | 22.5 (20.8–24.0) | 21.8 (18.7–22.3) | .12     |
| TJC                   |               |              |                   | .20     |
|                       | 2 (1–4)       | 4 (2–6)      | 2.1 (0–4)         |         |
| SJC                   |               |              |                   | .67     |
|                       | 4 (2–6)       | 4 (3–6)      | 3 (2–7)           |         |
| PtGA                  |               |              |                   | .61     |
|                       | 41 (20–53)    | 32 (24–45)   | 44 (17–74)        |         |
| DAS28-ESR             |               |              |                   | .40     |
|                       | 4.54 (3.52–5.44) | 4.61 (4.07–5.32) | 4.16 (3.09–5.74) |         |
| HAQ-DI                |               |              |                   | .91     |
|                       | 0.6 (0–1.1)   | 0.6 (0.4–1.0)| 0.8 (0–1.5)       |         |
| Steinbrocker’s classification class |           |              |                   |         |
| I                     | 7 (42)        | 3 (33)       | 4 (27)            | .68     |
| II                    | 15 (63)       | 6 (7)        | 9 (60)            | –       |
| III                   | 2 (8)         | 0 (0)        | 2 (13)            | –       |
| Steinbrocker’s radiographic stage |           |              |                   | .42     |
| I                     | 13 (54)       | 6 (67)       | 7 (47)            |         |
| II                    | 11 (46)       | 3 (33)       | 8 (53)            | –       |
| CRP, mg/dL            | 2.21 (0.40–6.53) | 2.31 (0.62–4.87) | 0.79 (0.08–7.36) | .74     |
| ESR, mm/hr            | 52 (22–75)    | 54 (24–73)   | 36 (20–80)        | .95     |
| RF positive           | 11 (46)       | 5 (56)       | 6 (40)            | .68     |
| Anti-CCP positive     | 10 (42)       | 4 (44)       | 6 (40)            | 1.00    |
| MTX, mg/week          |               |              |                   |         |
| baseline (initial dose)|              |              |                   |         |
| CRP                   | 6 (6–8)       | 6 (6–8)      | 6 (6–7)           | .73     |
| at week 12            | 10 (8–12)     | 10 (8–16)    | 10 (8–10)         | .39     |
| Prior csDMARDs        | 4 (17)        | 2 (22)       | 2 (13)            | .62     |
| salazosulfapyridine   |               |              |                   |         |
| aRF positive, > 15 IU/mL |           |              |                   |         |
| Anti-CCP positive, ≥ 4.5 U/mL |       |              |                   |         |

The values are expressed as median (IQR) and number (%). Fisher’s exact test or Mann–Whitney U test was used for group comparisons. BMI: body mass index; TJC: tender joint count; SJC: swollen joint count; PtGA: physician’s global assessment; DAS28-ESR: Disease Activity Score 28-ESR; HAQ-DI: Health Assessment Questionnaire disability index; CRP: serum C-reactive protein; ESR: erythrocyte sedimentation rate; RF: rheumatoid factor; CCP: cyclic citrullinated peptide; MTX, methotrexate.

aRF positive, > 15 IU/mL.

bAnti-CCP positive, ≥ 4.5 U/mL.
portions of good responders, and the reduction in ABCG2 expression was significantly higher in good responders than in non-good responders ($p = .011$). As a result, the mRNA expression of ABCG2 at week 12 was numerically lower in good responders than in non-good responders (median 0.098 vs. 0.23, respectively).

One patient in the good responders group showed a 4.6-fold increase in ABCG2 expression (Figures 1(b) and 2(b), and Supplementary Figures 2 and 3). In the same patient, PtGA (/100 mm) decreased from 92 at baseline to 15 at week 12, although objective findings such as SJC and ESR/CRP remained unchanged. This further confirmed the association between ABCG2 expression and the clinical response of patients with RA to MTX. In addition, six patients in the moderate responders group who subsequently received anti-TNF biologic DMARDs (the mean value of 0.167 at baseline and 0.164 at week 12, $p = .90$) and one patient received prednisolone 10 mg/day (0.241 at baseline and 0.340 at week 12) did not show a reduction in ABCG2 expression. Despite previous reports on the prediction of clinical response of

| mRNA | Gene expression level | Cut-off value | AUC | Number of patients (% > the cut-off value in good responders (n = 9)) | Number of patients (% > the cut-off value in non-good responders (n = 15)) | p Value |
|------|-----------------------|--------------|-----|---------------------------------------------------------------|---------------------------------------------------------------|--------|
| ABCG1 | 0.48 (0.27–1.53)      | 0.44         | 0.59 | 4 (44)                                                        | 10 (67)                                                        | .40    |
| ABCG2 | 0.22 (0.10–0.41)      | 0.64         | 0.67 | 4 (44)                                                        | 0 (0)                                                          | .012   |
| DHFR  | 6.61 (4.06–9.76)      | 1.93         | 0.53 | 8 (89)                                                        | 11 (73)                                                        | .62    |
| FPGS  | 8.28 (5.77–14.1)      | 11.36        | 0.53 | 1 (11)                                                        | 6 (40)                                                         | .19    |
| GGH   | 9.15 (0.36–15.8)      | 0.14         | 0.51 | 8 (89)                                                        | 12 (80)                                                        | 1.0    |
| RFC1  | 2.59 (1.91–4.52)      | 2.61         | 0.59 | 5 (56)                                                        | 6 (40)                                                         | .68    |

The gene expression levels are expressed as median (IQR).
AUC: area under the curve.

Figure 2. Comparison of the rate of change in mRNA expression of MTX-related genes between the EULAR good and non-good (moderate or non-) responders. The values (open circles) are expressed as the percent change in mRNA expression over 12 weeks and the bars represent the median values of each group. A Mann–Whitney U test was used for group comparisons.
patients with RA to MTX [7–14], the “treat-to target” strategy is dependent on a “trials and errors” algorithm [6]. Although the number of patients was limited in our study, the demographic and clinical features of our patients were comparable between the good responders and non-good responders groups. Only 40%–50% of patients were seropositive, and this may be partially associated with the relatively short disease duration (median 0.8 years) [33]. To improve the accuracy of the prediction of clinical response to MTX, an analysis of different cell types, including T cells, B cells, NK cells, monocytes, and fibroblast-like synoviocytes, should be included in future studies [34]. We previously reported such a study on the baseline gene expression signatures to predict the clinical responses of patients with RA to three biologic agents (infliximab, tocilizumab, and abatacept) [35]. It should be noted that the association of ABCG2 expression and clinical response to MTX could not be explained by RA disease activity. To clarify the underlying mechanisms, the effects of cytokines, growth factors and hormones on the expression of ABCG2 should be considered. As it has been previously reported that the activation of NF-$\kappa B$ can upregulate the expression of ABCG2 [36,37], pro-inflammatory cytokines may be responsible for the increased expression of ABCG2 observed in the present study. Moreover, other studies have reported that ABCG2 expression in MCF-7 human breast carcinoma cell lines was upregulated after treatment with IL-1$\beta$ or TNF$\alpha$ [38] and that this upregulation in ABCG2 expression was further enhanced by the presence of estrogen 17$\beta$-estradiol [39]. Conversely, treatment with IL-1$\beta$, IL-6, and TNF$\alpha$ cytokines has been shown to reduce ABCG2 expression in hCMEC/D3 cell lines (a model of the human blood–brain barrier) [40] and in cervical cancer HeLa cell lines [41]. Thus, the effects of pro-inflammatory cytokines on ABCG2 expression seem to be complicated and may be specific to cells and tissues, and ABCG2 expression may be affected by the presence of other factors. The underlining mechanisms of the present results should be clarified in the future researches.

A recent study investigated various single nucleotide polymorphisms in genes related to MTX susceptibility [42]. However, the association of ABCG2 polymorphisms with the effectiveness of MTX has not been observed [11,43]. Therefore, our findings of ABCG2 expression may not be genetically determined, but might be associated with environmental factors such as cytokines, growth factors and hormones, although we could not observe any correlation between ABCG2 expression and DAS28 (Figure 3) or CRP (data not shown). In addition, the expression level of ABCG2 may be inversely associated with the intracellular concentration of MTX-polyglutamates, which has been reported to be related to the safety and/or effectiveness of MTX in Japanese patients with RA [44,45]. In this context, a high expression of ABCG2 in good responders before MTX administration was unexpected, although the decreased ABCG2 expression in them after MTX administration was expected from our previous cross-sectional study [29].

The limitations of this study include the small number of patients and the analysis of expression of only six genes in PBMCs. Nonetheless, the prospective nature of this study has enabled us to clarify the interpretation of our previous study results [29].

In conclusion, we confirmed the association between the gene expression level of the drug transporter ABCG2 and the clinical response of patients with RA to MTX. Our results may justify a dose reduction of MTX in RA patients with sustained remission, and this should be investigated further.

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Conflict of interest

None.

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