Association of dihydrofolate reductase *(DHFR) -317AA* genotype with poor response to methotrexate in patients with rheumatoid arthritis

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Abstract

Objectives

Identifying genetic predictors of methotrexate (MTX) treatment response in patients with rheumatoid arthritis (RA) may have great importance for optimising drug doses required for clinical benefit without toxicity. In a group of 125 RA patients treated with MTX we investigated whether selected polymorphisms in genes relevant for MTX action (aminoimidazole-4-carboxiamide ribonucleotide transformylase, ATIC, and dihydrofolate reductase, DHFR) modulate disease activity and/or have impact on therapy side effects.

Methods

The efficacy of treatment was estimated both by the disease activity score in 28 joints (DAS28), based on EULAR criteria, and relative DAS28 (rDAS28) score. Adverse drug events (ADEs) were also recorded. RA patients were genotyped using the PCR-RFLP method, followed by an association study between ATIC -129T>G, DHFR -216T>C and DHFR -317A>G polymorphisms and the efficacy and toxicity of MTX.

Results

According to the EULAR response criteria, 96 RA patients (76.8%) were classified as responders (good/moderate response) and 29 (23.2%) as non-responders (poor response). rDAS28 values ranged from -0.01 to 0.80 (mean value 0.31±0.19). Among 125 patients enrolled in this study 39 experienced at least one side effect. The DHFR -317AA genotype was associated with the less favourable response (reduction in rDAS28 score, p=0.05). None of the analysed polymorphisms was associated with MTX toxicity.

Conclusion

RA patients with DHFR-317AA genotype had less favourable response to MTX Further studies in larger patient populations are necessary to confirm the relationship between the analysed polymorphisms and MTX treatment response.

Key words

methotrexate, DHFR, ATIC, polymorphisms, rheumatoid

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Introduction

Methotrexate (MTX) is the most widely used disease modifying antirheumatic drug (DMARD) (1-3). Despite numerous attempts to develop new, more specific therapy for rheumathoid arthritis, MTX remains the first line treatment option (4). Although MTX has a proven efficacy and acceptable safety profile in RA, the clinical response shows great inter-patient variability. Only 50–70% of patients achieve good response and up to one-third discontinues therapy due to adverse drug events (ADEs) (5-7). Currently, it is not possible to predict MTX treatment outcome with any accuracy. Several demographic and clinical risk factors (age, gender, duration of the disease, disease activity, structural joint damage, RA functional class and prior DMARD use) have been studied in relation to the efficacy and safety of MTX treatment, but the results are not yet conclusive (8-10). Gene polymorphisms may as well contribute to the variability in MTX treatment responses in RA, particularly those that are located in genes relevant for MTX action (11-13).

Methotrexate is an analogue and antagonist folate, which affects purine and pyrimidine synthesis and homocysteine-methionine pathway. The folate metabolic pathway is complex (14) and involves a number of enzymes, including dihydrofolate reductase (DHFR) and 5-aminoimidazole-4-carboxiamide ribonucleotide transformylase (ATIC). MTX competitively inhibits DHFR, required for dihydrofolate to tetrahydrofolates (THFs) conversion, resulting in folate depletion, homocysteine accumulation, impairment of DNA methylation and purine and pyrimidine synthesis (15). Inhibition of ATIC by MTX results in intracellular adenosine and adenine accumulation, increased extracellular adenosine concentrations and suppression of pro-inflammatory cytokine production (16). Given the importance of the two enzymes in MTX therapeutic action, several variations in their respective gene have been studied in relation to response to treatment in RA (17-19). We analysed relationship between efficacy and toxicity of MTX treatment in RA with regulatory DHFR and ATIC polymorphisms not assessed in previous studies. The variations are located in the promoter of *DHFR (-216T>C*, *rs6151599* and *-317A>G*, *rs408626*) and 5'UTR of *ATIC* gene (*-129T>G*, *rs4535042*).

Materials and methods

Study design

Patients included in the study fulfilled the American College of Rheumatology (ACR) 1987 revised classification criteria for RA (20). The patients were treated and prospectively followed at the Institute of Rheumatology, School of Medicine, University of Belgrade, Serbia. Patients gave their informed consent to participate in the study. Attending physicians and patients were blinded to the genotypes throughout the entire study, and laboratory personnel were blinded to all clinical information. The study protocol was approved by the Ethics Committee of the Institute of Rheumatology.

Treatment with MTX for at least 6 months was the main criterion for inclusion in the study. Low-dose corticosteroids (≤10mg/day), previous disease-modifying anti-rheumatic drugs other than MTX (DMARDs) and folic acid supplementation were allowed. DMARDs used prior to MTX were aurotherapy, sulphasalazine and chloroquine. Patients receiving intra-articular corticosteroids were not included in the study.

Clinical assessments

Disease activity was prospectively assessed using the Disease Activity Score (DAS28), which is calculated from the 28 tender and 28 swollen joint counts, the erythrocyte sedimentation rate (ESR) and the patient's global assessment of disease-related general health on a visual analogue scale (GH) (21). The DAS28 scores at the start of MTX treatment (DAS28 0) and at 6 months (DAS28 1) were used to estimate the clinical response to MTX therapy, according to the European League Against Rheumatism (EULAR) response criteria (22). Namely, a good response was defined when DAS28 1 was ≤3.2 and an improvement from base-

line (DAS28 0–DAS28 1 or ΔDAS28) was ≥1.2, a moderate response when DAS28 1 value was between 3.2 and 5.1 and ΔDAS28 between 0.6 and 1.2, while DAS28 1 scored higher than 5.1 and a ΔDAS28 lower than 0.6 was considered as a poor response. Patients with a good or moderate response were defined as "responders" and patients with poor response as "non-responders". The efficacy of MTX treatment was also measured through the relative DAS28 (improvement in the DAS28 score relative to the baseline value) which was calculated according to the following formula:

rDAS28 = DAS28 0 -DAS28 1/DAS28 0 (23)

Safety assessments

Safety assessments were based upon patient's reports of all adverse drug events (ADEs), results of routine physical examinations and laboratory analysis. Interview with the patients included questionnaire collected information regarding type, severity, time of onset and duration of the ADEs, as well as therapy used for treatment of the ADEs symptoms. All data were assessed by a single-trained physician. ADEs were gastrointestinal complaints (anorexia, vomitus and nausea), hepatotoxicity (elevation of transaminases), bone marrow toxicity (leucopenia, thrombocytopenia and pancytopenia), dermatological complaints (rashes, vasculitis and hair loss), mucositis and pulmonary toxicity (cough, pneumonitis and dyspnea). According to defined criteria, ADEs were estimated as mild (nausea, alopecia, cough, leucopenia 3–4x10^9cells/L, thrombocytopenia 100–150x10^9cells/L, an elevation of transaminases to <3 times the upper limit of normal), moderate (anorexia, nausea, hair loss, mucositis, dyspnea, leucopenia <3 x10^9cells/L, thrombocytopenia 70–100x10^9cells/L, an elevation of transaminases of >3 times the upper limit of normal) or severe in the case of ADEs which required hospitalisation of the patient.

Genotyping

Genomic DNA was extracted from 5ml Na citrate-anticoagulated peripheral blood, using standard salting out method (24). Genotypes for all analysed polymorphisms were established by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) method. To perform quality control each sample has been genotyped in duplicate.

DHFR gene segment encompassing positions from -317 to -216 was amplified as follows: after 3 minutes of initial denaturation at 95°C, each of 40 cycles consisted of 45 seconds at 94°C, 45 seconds at 56°C and 45 seconds at 72°C. The final extension was 7 minutes at 72°C. The forward and reverse primers sequences were 5'- GCA GCT TTC TAG TCA CCC - 3' and 5'- GTAGGT TCT GTC TGG GAC TGG - 3', respectively (25). The PCR products were 400 bp long. The *-216T>C* genotypes were distinguished by BoxI digestion: TT genotype revealed a single 400bp DNA fragment and TC genotype revealed 3 fragments, 400 bp, 253 bp and 147 bp long. The *-317A>G* genotypes were detected by HinfI digestion: GG genotype was characterised by 266 bp, 83 bp and 51 bp DNA fragments, AA genotype was defined by 266 bp and 134 bp fragments and GA heterozygotes had 266 bp, 134 bp, 83 bp and 51 bp DNA fragments. Genotypes of *ATIC -129T>G* polymorphism were detected by PCR amplification followed by NsbI digestion. Forward and reverse primers sequences were 5'- GAA ACT GAG CAG AGC AGG GC - 3' and 5' - ATG GCT GCA GGC ACT GGG TT - 3', respectively. PCR consisted of a 3-minute initial denaturation at 95°C, followed by 40 cycles, each composed of 30 seconds at 94°C, 30 seconds at 68°C and 30 seconds at 72°C. Final extension was at 72°C for 7 minutes. After digestion of the172 bp long PCR product with NsbI, individuals with the TT genotype presented a DNA fragment of 172 bp, TG genotype revealed 172bp, 114bp and 58bp and GG genotype revealed 114bp and 58bp DNA fragments.

Statistical analysis

Differences in patient, disease and treatment characteristics between responders and non-responders, or those with and without ADEs were analysed by Student's *t*-test for continuous variables (or Mann-Whitney U-test, depending on homogeneity of variable distribution) and chi-square test for dichotomous variables. The chi-square test was also used to examine differences in frequencies between *DHFR* and *ATIC* genotypes in responders and non-responders (as defined by EULAR response criteria). rDAS28 was compared between genotype groups by Mann-Whitney U-test after grouping genotypes as dichotomous variable (based on the presence or absence of a SNP's minor allele). Transformed values (z-scores) were used for analysis of covariance (ANCOVA) when an association of genotype with rDAS in the presence of covariates was assessed. Statistical analyses were performed using SPSS statistical package, version 17.0 (SPSS Inc, Chicago, Illinois, USA).

Table I. Clinical data on 125 RA patients enrolled in the study.

Characteristics	Value
Age (years)	57.9 ± 10.9 (20–84)
Women, n (%)	106 (84.8)
Duration of disease (months)	49.5 ± 41.9 (6–240)
Duration of MTX treatment (months)	22.7 ± 19.9
RF seropositivity, n (%)	103 (82.4)
Administration of low dose corticosteroids, n (%)	84 (67.2)
Weekly MTX dose in mg, mean (range)	10.2 ± 2.8 (7.5–20.0)
Folic acid supplementation, n (%)	62 (49.6)
DAS28 baseline, mean (range)	7.7 ± 0.8 (5.53–9.11)
DAS28 6 months, mean (range)	5,3 ± 1.6 (1.96–8,46)
rDAS28, mean (range)	0.31 ± 0.19 (-0.01–0.80)

RF: rheumatoid factor; MTX: methotrexate; DAS28: Disease Activity Score in 28 joints; rDAS28: relative Disease Activity Score in 28 joints.

Table II. Adverse drug events (ADEs) in 125 genotyped RA patients.

ADEs	6 months of MTX therapy n (%)
Hepatoxicity	13 (10.4)
Vomitus	10 (8.0)
Bone-marrow toxicity	7 (5.6)
Stomatitis	1 (0.8)
Hair loss	7 (5.6)
Cough	1 (0.8)
Overall ADEs	
None	86 (68.8)
Mild to moderate	36 (28.8)
Serious	3 (2.4)

Numbers and frequencies (in brackets) are given separately for each ADS as well as for all ADEs combined.

Table III. Genotype frequencies of analysed SNPs among responders and non-responders according to EULAR criteria.

Gene*	Genotype**	Responders n (%)	Non-responders n (%)	*p*-value
DHFR -216 T>C	TT	89 (93.7)	25 (86.2)	0.2
	TC	6 (6.3)	4 (13.8)	
DHFR -317 A>G	AA	18 (18.7)	7 (24.1)	0.8
	AG	47 (49.0)	14 (48.3)	
	GG	31 (32.3)	8 (27.6)	
ATIC -129T>G	TT	37 (38.5)	15 (51.7)	0.4
	TG	47 (49.0)	11 (38.0)	
	GG	12 (12.5)	3 (10.3)	

*Position of the polymorphisms is given relatively to the first transcription initiation site.
**Genotypes were successfully determined in 125 RA patients for DHFR -317 A>G and ATIC -129T>G polymorphisms and in 124 patients for DHFR -216 A>G polymorphism.

Table IV. DHFR and ATIC genes polymorphisms and MTX-related side effects.

Gene	Genotype	Presence of side effects* n (%)	Absence of side effects n (%)	*p*-value
DHFR -216T>C	TT	36 (92.3)	78 (91.8)	1
	TC	3 (7.7)	7 (8.2)	
DHFR -317G>A	GG	16 (41.0)	23 (26.7)	0.2
	GA	18 (46.2)	43 (50.0)	
	AA	5 (12.8)	20 (23.3)	
ATIC -129T>G	TT	18 (46.2)	34 (39.6)	0.7
	TG	16 (41.0)	42 (48.8)	
	GG	5 (12.8)	10 (11.6)	

*Side effects refer to all ADEs grouped together.

Results

Patients' clinical characteristics

A total of 125 RA patients, 106 (84.8%) women and 19 (15.2%) men, treated with MTX for at least 6 months, were enrolled in a prospective study. Table I summarises patient's detailed clinical data. The mean weekly dose of MTX was 10.2±2.8 mg. Sixty two patients (49.6%) received folic acid supplementation, 24 hours after MTX administration, at a stable weekly dose of 5–10 mg. Eighty four patients (67.2%) concomitantly received low dose corticosteroids (average 6.6 mg/day). At 6-month control, a significant decrease in DAS28 was observed ($p<0.001$). The relative DAS28 values ranged from -0.01 to 0.80 (mean value 0.31±0.19).

According to the EULAR response criteria, 96 RA patients (76.8%) were classified as responders and 29 (23.2%) as non-responders after 6 months of MTX therapy. Among 57 MTX responders, 12 (12.5%) patients were classified as good responders and 84 (87.5%) as moderate responders. The significant difference between the weekly MTX dose received by responders (mean dose 9.7 mg/week) and non-responders (mean dose 12.2 mg/week) was seen ($p<0.001$). There was no correlation between rDAS values and MTX dose.

A higher number of patients who received corticosteroids was noted among non-responders than responders (82.8% *vs*. 62.5%, $p=0.05$). The number of patients taking folic acid supplement and/or have previously received DMARDs other than MTX did not differ between the two groups of patients.

Thirty nine patients (31.2%) experienced at least one ADEs during the treatment with MTX. The frequencies of various types of ADEs are shown in Table II. The majority of the recorded ADEs were mild to moderate. Among patients with serious ADEs, two patients had bone-marrow toxicity and one patient experienced persistent cough. There was no difference between the weekly MTX dose received by patients who experienced ADEs (mean dose 9.7 mg/week) and those without ADEs (10.5 mg/week) ($p=0.1$).

DHFR and ATIC polymorphisms in relation to MTX efficacy and toxicity

Genotype frequencies for the SNPs analysed in this study are in agreement with the results obtained for Caucasian populations (26-28). The distribution of *DHFR* and *ATIC* genotypes in relation to MTX treatment response according to EULAR criteria is presented in Table III. No significant association was found when *DHFR* and *ATIC* genotypes were compared between responders and non-responders. When rDAS28 was used as measure of MTX treatment efficacy, the *DHFR -317 AA* genotype was associated with less favourable response to MTX ($p=0.05$, Fig. 1). This association remains significant if analyses were adjusted for baseline DAS value, or when additionally dose and concomitant use of corticosteroids were added to multivariate model.

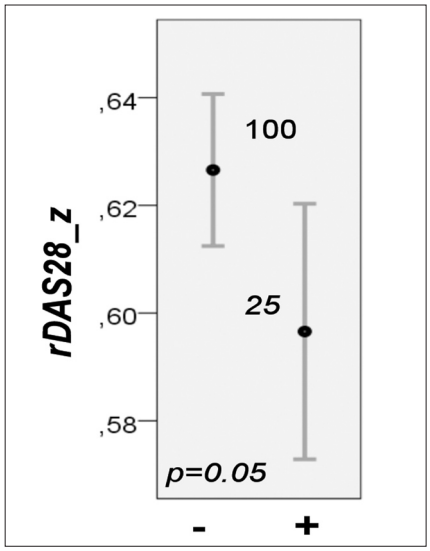

Fig. 1. Association of DHFR *-317AA* polymorphism and MTX efficacy based on rDAS28. Transformed rDAS28 (Z score, mean ±95 confidence interval, CI) according to the presence (+) or absence (-) of *-317AA* genotype. Number of patients presented by each line, as well as *p*-values, obtained by Mann-Whitney U-test (for non-transformed values) are indicated on the plot.

Discussion

Pharmacogenetics can offer additional or alternative modalities to optimise MTX therapy in RA patients. To our knowledge, the influence of *ATIC -129T>G*, *DHFR -216T>C* and *DHFR -317A>G* polymorphisms on MTX efficacy and safety in patients with RA was not previously investigated. Polymorphisms analysed in this study are located in the regulatory gene regions and hence may influence gene expression.

Methotrexate directly binds to DHFR and competitively inhibits its activity (29). Genetic polymorphisms affecting the DHFR level or function can thus affect the response to MTX treatment (30). The relationship between SNPs in *DHFR* gene and reduced MTX efficacy was previously reported in patients with childhood acute lymphoblastic leukemia (ALL) (25). In RA, Wessels *et al*. (17) analysed the influence of two SNPs in the *DHFR* gene, *-473G>A* (*rs1650697*) in 5'UTR and *352896 G>A* (*rs1232027*) on MTX-related toxicity and MTX efficacy, as evaluated by DAS 44 score. No significant association was reported. The study conducted in RA patients from northern India (19) on additional *DHFR A>T* polymorphism in 3'UTR (*rs7387*) revealed its contribution to MTX efficacy only in a multivariate model, when analysed together with other folate-dependent enzymes polymorphisms. We did not find any association between responders and non-responders defined by EULAR criteria for the *-317* and *-216 DHFR* polymorphisms. However, when rDAS28 was used as a measure of MTX efficacy, the *DHFR -317AA* genotype was associated with lower MTX treatment response. rDAS28, as a quantitative variable, may be a more sensitive parameter for the assessment of the efficacy of therapeutic modalities in RA patients, and is usually used in clinical trials (23). Interestingly, the same *-317 AA* genotype has been associated with lower relapse free survival in leukemia patients when compared to the remaining genotypes (25). Haplotype defined by *A-317* allele has been shown to increase promoter activity and correlate with higher mRNA levels (25), possibly explaining lower efficacy of MTX treatment. Our study is the first to demonstrate an association of the *DHFR -317A>G* polymorphism with MTX efficacy in patients with RA. Given the relatively limited number of patients and an association that would not sustain the correction for multiple testing, this finding requires confirmation in additional studies.

There are several studies that analysed *ATIC* gene polymorphisms and MTX treatment response in RA. In a cross-sectional study, the GG genotype of *347 C>G* polymorphism (*rs2372536*) that results in Thr116Ser substitution was associated with better response to MTX in comparison to the remaining genotypes (16). Accordingly, Weisman *et al*. showed that patients with *ATIC 347GG* genotype more frequently experienced gastrointestinal side effects (31). Longitudinal study of the same polymorphism in a group of patients with recent-onset RA that received MTX only showed that the CC genotype was instead associated with good clinical response to MTX (32). A recent study demonstrated a significant association between CC genotype of the intronic *C>T* (*rs4673993*) *ATIC* gene polymorphism and low disease activity in patients with RA (18). The *-129T>G* SNP of *ATIC* gene analysed in the present study did not show correlation with MTX efficacy or toxicity, at least in our group of RA patients.

Conclusion

Our data provide evidence of a significant association of *DHFR -317 AA* genotype with lower efficacy of MTX treatment and identify *DHFR -317A>G* polymorphism as a potential pharmacogenetic marker in patients with RA.

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