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ANALYSIS OF ASSOCIATION BETWEEN POLYMORPHISMS OF *MTHFR, MTHFD1* AND *RFC1* GENES AND EFFICACY AND TOXICITY OF METHOTREXATE IN RHEUMATOID ARTHRITIS PATIENTS

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A folate analogue methotrexate (MTX) is the most commonly used disease-modifying drug in the treatment of rheumatoid arthritis. However, the clinical response of RA patients treated with MTX shows interindividual differences and 30% of patients discontinue therapy due to the side effects. In a group of 184 RA patients treated with MTX we have investigated whether polymorphisms in MTHFR (rs1801133, rs1801131), MTHFD1 (rs2236225) and RFC1 (rs144320551) genes may have impact on MTX efficacy and/or adverse drugs effects (ADEs).

The efficacy of the MTX therapy has been estimated using the disease activity score in 28 joints (DAS28-ESR) based on EULAR criteria and relative DAS28 values (rDAS28) and all adverse drug events were recorded. Patients were genotyped for selected polymorphism by PCR-RFLP method. According to the EULAR response criteria after 6

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months of MTX therapy 146 (79.3%) patients were classified as responders, (17 patients (11.6%) were good and 129 patients (88.4%) were moderate responders) and 38 patients (20.7%) as non-responders. ADEs were observed in 53 (28.8%) patients. The majority of ADEs were mild (36 (19.56%) patients) to moderate (12 (6.25%) patients). Five patients (2.7%) had serious ADEs. Association studies have been conducted between obtained genotypes and the efficacy and toxicity of MTX. We have observed no association between polymorphisms and efficacy or toxicity of MTX in RA patients.

Key words: genetic polymorphism, methotrexate, MTHFR, MTHFD1, RFC1, rheumatoid arthritis

INTRODUCTION

Over the last two decades a folate analogue methotrexate (MTX) is the most commonly used disease modifying drug in the treatment of rheumatoid arthritis (GOODMAN *et al*., 2015). It is considered that the main mechanism of MTX action is based on direct inhibition of dihydrofolate reductase (DHFR) and other key enzymes involved in folate metabolism, thereby interfering with the synthesis of pyrimidines and purines (WESSELS *et al*., 2008). The clinical treatment of inflammatory and autoimmune diseases such as rheumatoid arthritis as well as different malignant diseases rely on this mechanism. The clinical response of rheumatoid arthritis (RA) patients treated with MTX is different. About 50% of patients show good treatment outcome, while 30% discontinue therapy due to the side effects (SAAG *et al*., 2008). These interindividual differences can not be predicted and markers such as gene polymorphisms may be useful in the individualization and optimization of the drug treatment.

Absorption of MTX and its uptake into target cells are mainly controlled by the reduced folate carrier protein, RFC-1 (ANDO *et al*., 2013; RANAGANATHAN, 2008). There is a well known *G80A* polymorphism (rs144320551) in *RFC-1* gene, that refers to G-to-A transition at nucleotide 80 and replaces an arginine with a histidine in the protein (CHANGO *et al*., 2000). Although it is not clear whether this change alters folate transport, the 80AA variant has been associated with higher plasma folate levels (DROZDZIK *et al*., 2007).

Methylenetetrahydrofolate dehydrogenase 1 (MTHFD1) is a trifunctional enzyme involved in folate metabolism that mediates the interconversion of 5,10- methylenetetrahydrofolate, 5,10-methenyltetrahydrofolate and 10-formyltetrahydrofolate. The last two act as donors during *de novo* purine and pyrimidine biosynthesis and thus the byosinthesis of DNA (KRAJINOVIC, 2008; WESSELS *et al*., 2008). A common *MTHFD11958G>A* polymorphism (rs2236225) causes an alanine to glycine substitution at codon 653 located within the 10-formyl-THF synthetase enzyme domain. Result of this replacement is reduced enzyme activity and stability (KRAJINOVIC, 2008). Since MTHFD1 is a key player in folate metabolism, the difference in its activity could modulate therapeutic response to antifolate agents such is MTX.

Methylenetetrahydrofolatereductase (MTHFR) catalyzes the conversion of 5,10-methylenetetrahydrofolate (5,10-methylene-THF) into 5-methyltetrahydrofolate (5-methyl-THF), which provides a methyl group for remetilation of homocysteine to methionine (WESSELS *et al*., 2008). Two common polymorphisms, a *C677T* (rs1801133) and *A1298C* (rs1801131) affect enzyme activity and plasma homocysteine level (WEISBERG *et al*., 1998), and consequently might influence the therapeutic response.

Here we analyzed whether *RFC1*, *MTHFD* and *MTHFR* variants influence MTX efficacy and toxicity in RA patients from Serbia.

MATERIALS AND METHODS

Study design

We have used the same patient's cohort as in our previous study (JEKIC *et al*., 2013). The study included 184 patients treated and prospectively followed at the Institute of Rheumatology, Faculty of Medicine, University of Belgrade, Serbia. For each patient diagnosis was established according to American College of Rheumatology (ACR) 1987 revised classification criteria for RA (ARNETT *et al*., 1998). Laboratory personnel were blinded to all clinical information and attending physicians and patients were blinded to the genotypes throughout the study. The Ethics Committee of the Institute of Rheumatology approved the study protocol and each patient gave informed consent to participate in the study. Current or past treatment with MTX for at least 6 months was the main criterion for patient inclusion in the study, while patients receiving intra-articular corticosteroids were excluded from the study. Stable dosages of non-steroidal anti-inflammatory drugs (NSAIDs), low-dose corticosteroids (≤10mg/day), previous disease-modifying antirheumatic drugs other than MTX (DMARDs, Aurotherapy, Sulphasalazine, Chloroquine) and folic acid supplementation were allowed.

Clinical assessments

Clinical and safety assessments were performed as we previously reported (JEKIC *et al*., 2013). In short, clinical response to the MTX therapy was estimated according to the EULAR response criteria through the DAS28 score (PREVOO *et al.*, 1995) improvement from the baseline after 6 months of the therapy, calculated by the formula ΔDAS28=DAS281-DAS280 (DAS280 represents DAS28 at the start of MTX treatment and DAS281 represents DAS28 after 6 months of the therapy).

For the estimation of the clinical response to MTX we have also used the relative DAS28 (rDAS28). rDAS refers to improvement in the DAS28 score relative to the baseline value and is calculated according to the formula: rDAS28 = (DAS28 0 – DAS28 1)/DAS28 0

Safety assessments

Adverse drug events (ADEs) were recorded according to patient's reports, results of routine laboratory measurements and physical examinations, as described in our previous study (MILIC et al., 2012). ADEs were estimated in respect to defined criteria as mild, moderate and severe (MILIC et al., 2012). Severe ADEs were those that required hospitalization of the patient and discontinuation of the MTX treatment.

Detection of genotypes

All molecular-genetic analyses were conducted at the Institute of Human Genetics, Faculty of Medicine, University of Belgrade, Serbia. Genomic DNA was extracted from peripheral blood leukocytes by salting out method (MILLER *et al*., 1988).

MTHFR C677T and *A1298C, RFC-1 G80A* and *MTHFD-1 G1958A* polymorphisms genotypes were detected by the PCR-RFLP method (DERVIEUX *et al*., 2004; FROSST *et al*., 1995; HOL *et al*., 1998; VAN DER PUT *et al*., 1998). After enzyme digestion of PCR products, fragments were analyzed on 8% polyacrylamide gels stained with ethidium bromide.

Statistical analysis

Differences in patients, disease and treatment characteristics between responders and non-responders or those with and without ADEs were analyzed by Student's t-test (or Mann-Whitney depending on homogeneity of variable distribution) for continuous variables and by Chi-square test for dichotomous variables. Differences in frequencies of *RFC1*, *MTHFD1* and *MTHFR* genotypes between responders and non-responders were analyzed by Chi square test. rDAS28 difference across genotypes was assessed by ANOVA or, when applicable, by non-parametric test. All statistical analyzes were performed by SPSS version 17.0 (SPSS Inc, Chicago, Illinois, USA).

RESULTS

Patients

Among 184 patients, 150 (81.5%) were women. Mean age was 58.04±10.20 years (20-84). Duration of the disease and of the MTX treatment (in months), was 48.95±39.95 (6-240) and 36.72±33.13, respectively. Weekly doses of MTX were 10.72±2.83mg (7.5-20.0), 99 (53%) patients received folic acid (5-10 mg/week) and 121 (65.8%) low doses of corticosteroids (6.5±4.9 mg/day). Clinical data, including ADEs are presented in Table 1.

Table 1. Clinical data, including adverse drug events, for 184 RA patients analyzed in the study.

Clinical data	***Value***
RF seropositivity, n (%)	154 (83.7)
DAS28 baseline	7.69±0.82 (5.52-9.14)
DAS28 at 6 months	5.25±1.57 (1.64-8,46)
rDAS28 (range)	0.32±0.18 (-0.02-0.80)
ADEs	***6 months of MTX therapy, n (%)***
Hepatoxicity	18 (9.8)
Vomitus	21 (11.4)
Bone-marrow toxicity	8 (4.3)
Stomatitis	3 (1.6)
Hair loss	7 (3.8)
Cough	1 (0.5)

RF – rheumatoid factor; DAS28 - Disease Activity Score in 28 joints; rDAS28 - relative Disease Activity Score in 28 joints; data are expressed as numbers (frequencies) or mean values± SD with the range in brackets. ADEs – adverse drug events; date are expressed in numbers (number of patients) and percentages in brackets.

A significant decrease in DAS28 was observed at 6-month control ($p < 0.0005$). According to the EULAR response criteria, after 6 months of MTX therapy, 146 (79.3%) patients were classified as responders (17 patients (11.6%) were good and 129 patients (88.4%) were moderate responders) and 38 patients (20.7%) as non-responders. Statistically significant difference ($p<0.0005$) has been observed between average weekly dose received by responders (10.3

mg/week) and non-responders (12.3 mg/week). A higher frequency of patients who received corticosteroids was noted among non-responders than responders (p=0.0005). Further, no significant difference was observed between number of patients, responders and non-responders taking folic acid supplements or DMARDs other than MTX. No significant correlation was observed between rDAS values and MTX dose. During the treatment with MTX, 53 patients (28.8%) experienced ADEs. The majority of the recorded ADEs were mild (36 (19.56%) patients) to moderate (12 (6.52%) patients). Among five (2.7%) patients with serious ADEs, four had bone-marrow toxicity and one experienced persistent cough. Weekly MTX doses received by patients who experienced drug side effects (mean dose 10.8 mg/week) and those who did not (mean dose 10.4 mg/week) were not significantly different.

Relation of MTX efficacy and toxicity with selected polymorphisms in RFC1, MTHFD1 and MTHFR genes

Detected frequencies of genotypes of analyzed *RFC1*, *MTHFD1* and *MTHFR* genes polymorphisms are in agreement with the frequencies previously reported for Caucasians (Database: NCBI). The genotypes distribution in relation to MTX efficacy, defined by EULAR criteria, is presented in Table 2A. Whether frequency of 677TT genotype was higher in the group of non-responders (23.7%) compared with responders (11.6%), this difference was not significant (p=0.07) (Table 2B). We found no association between rs144320551 (*RFC-1* gene) rs2236225 (*MTHFD-1* gene) and rs1801131 (*MTHFR* gene) polymorphisms and efficacy measured by DAS28 score (ΔDAS28) or relative DAS28 (rDAS28).

Table 2A. Genotype frequencies of analyzed polymorphisms among responders and non-responders, according to EULAR criteria.

	Genotype	Responders n (%)	Nonresponders n (%)	p
MTHFR C677T	CC	50 (34.2%)	14 (36.8%)	0.110
	CT	79 (54.1%)	15 (39.5%)	
	TT	17 (11.6%)	9 (23.7%)	
MTHFR A1298T	AA	71 (48.6%)	22 (57.9%)	0.553
	AC	64 (43.8%)	13 (34.2%)	
	CC	11 (7.5%)	3 (7.9%)	
MTHFD1	GG	48 (32.9%)	8 (21.1%)	0.256
	GA	64 (43.8%)	22 (57.9%)	
	AA	34 (23.3%)	8 (21.1%)	
RFC-1	GG	30 (20.5%)	11 (28.9%)	0.536
	GA	67 (45.9%)	16 (42.1%)	
	AA	49 (33.6%)	11 (28.9%)	

Genotypes distribution in relation with MTX adverse effects is shown in Table 3. There was no significant correlation between the observed ADEs and analyzed polymorphisms.

Table 2B. Frequencies of responders among individuals with 677TT genotype compared with individuals with 677CC and 677CT genotypes

	677CC, 677CT	**677TT**	**p**
Responders, n (%)	129 (88.4%)	17 (11.6%)	0.07
Nonresponders, n (%)	29 (76.3%)	9 (23.7%)	0.07

Table 3. MTX side effects in different genotypes of analyzed genes polymorphisms

	Genotype	**Presence of side effects* n (%)**	**Absence of side effects n (%)**
MTHFR C677T	CC	17 (32.1%)	47 (35.9%)
	CT	29 (54.7%)	65 (49.6%)
	TT	7 (13.2%)	19 (14.5%)
MTHFR A1298T	AA	25 (47%)	68 (51.9%)
	AC	22 (41.5%)	55 (42.0%)
	CC	6 (11.3)	8 (6.1%)
MTHFD1	GG	16 (30.2%)	40 (30.5%)
	GA	25 (47.2%)	61 (46.6%)
	AA	12 (22.6%)	30 (22.6%)
RFC1	GG	15 (28.3%)	26 (19.8%)
	GA	24 (45.3%)	59 (45.1%)
	AA	14 (26.4%)	46 (35.1%)

DISCUSSION

MTX is the drug of choice for therapy of RA, because of the low costs and availability, acceptable efficacy and toxicity, despite the significant percent of patients without adequate response to therapy (GOODMAN *et al*., 2015). It is well known that early response to treatment of newly diagnosed RA gives much better prognosis for patients (GOEKOOP-RUITERMAN *et al*., 2007; KORPELA *et al*., 2004). Unfortunately, at this point, it is not possible to predict patient's response to drug administration. Combined strategies that include different DMARDs and anti-TNF agents are, compared with MTX monotherapy, more effective in sense of number of responders, but are associated with risks such as infections and osteoporosis (MURDACA *et al*., 2015; NAM *et al*., 2014). For these reasons, it is important to determine biomarkers and to develop pharmacogenetic model that will enable clinicians to predict patient's response to therapy and apply adequate strategy at the very first moment of disease diagnosis.

There are only two pharmacogenetic models currently developed in order to predict the efficacy of methotrexate monotherapy in rheumatoid arthritis (FRANSEN *et al*., 2012; WESSELS *et al*., 2007). Both models, among other parameters, use rs2236225 polymorphism of the *MTHFD1* gene for estimation of therapy response. It is not clear yet whether different MTHFD1 genotypes of rs2236225 alone could be attributed to different response to MTX therapy. In patients with RA,

RBC folate concentrations and gastrointestinal adverse effects were associated with rs2236225 polymorphism (STAMP et al., 2010), but not with an efficacy of MTX (OWEN *et al*., 2013a). Only Wessels et al. (2007) have reported moderate association of *MTHFD1 G* allele with poor response. We did not find an association of this polymorphism with efficacy and toxicity of MTX in RA.

According to the literature data, two polymorphisms of the *MTHFR* gene, C667T and A1298C, are associated with the lower level of MTHFR enzyme activity (FROSST *et al*., 1995; VAN DER PUT *et al*., 1998) and hence may contribute to different efficacy or toxicity of MTX. Number of studies investigated whether different genotypes of these two polymorphisms are associated with therapeutic effects of MTX in RA, but results still remain contradictory (Table 4). This may result from different designs of studies or interpretation of the results. For these reasons, serious efforts should be made on standardization of these types of investigations.

Table 4. Review of relevant studies of association of MTHFR polymorphisms C667T and A1298C with the toxicity and efficacy of methotrexate in rheumatoid arthritis.

Author	**No. of patients**	**SNPs**	**Response**	**Toxicity**
Soukup et al. 2015	120	C677T A1298C	677CT-1298AC haplotype and inefficacy	/
Świerkot et al. 2015	273	C677T A1298C	677CC - more rapid response	CC / CT reduction of no. of AE 677T - aminotransferase elevation
Saleh et al. 2015	159	C677T A1298C	no	C677T and overall toxicity, some haplotypes with specific AE
Salazar et al. 2014	124	C677T A1298C	no	no
Lima et al. 2014	233 Portuguese	C677T	677TT -nonresponse	/
Song et al. 2014	meta-analysis, 12 studies, 2288 patients	C677T A1298C	/	677TT with overall AE 677TT with overall AE in East Asians, 677TT with overall AE in patients taking folate, 1298CC with overall AE
Morgan et al. 2014	meta-analysis, C677T-4 studies, 812 patients, A1298C-3 studies, 694 patients	C677T A1298C	no	/
Morgan et al. 2014	C677T-302 patients, A1298C-300 patients	C677T A1298C	no	/
Davis et al.	319	C677T	/	1298C with sig. AE

2014		A1298C		
de Rotte et al. 2013	285	C677T	no	no
Owen et al. 2013b	309	C677T A1298C	no	no
Owen et al. 2013b	meta-analysis: efficacy-C677T 10 studies, A1298C 8 studies; toxicity-C677T 13 studies, A1298C 8 studies	C677T A1298C	no	no
Plaza Plaza et al. 2012	67 Spanish	C677T A1298C	/	677TT with overall AE
Kato et al. 2012	55 Japanese	C677T A1298C	1298AA lower mean DAS28	/
Cáliz et al. 2012	468 Spanish	C677T A1298C	/	677TT with overall toxicity (OR=1.95)
Choe et al. 2012	167 Korean	C677T A1298C	/	1298CC and 677C/1298A more often experienced at least one AE than 1298AA and 677C/1298C, respectively
Tasbas et al. 2011	64 Turkish	C677T A1298C	/	no
Mena et al. 2011	70 Mexican	C677T A1298C	/	A1298C with elevated transaminase levels
Xiao et al. 2010	110	C677T A1298C	1298 CC/AC greater clinical response than AA	677 CT/TT higher risk for AE
Inoue et al. 2009	36	C677T A1298C	no	/
Fisher et al. 2009	meta-analysis, 8 studies, 1441 patients	C677T A1298C	/	677TT – with toxicity
Lee et al. 2009	262	C677T	no	/
Taraborelli et al. 2009	79 Northern Italian	C677T A1298C	no	no
Bohanec Grabar et al. 2008	213	C677T A1298C	no	1298CC lower risk for toxicity
Ghodke et al. 2008	34 Indian	C677T A1298C	no	no
Kurzawiski	174	C677T	677T- higher frequency of	/

et al. 2007		A1298C	remission, 1298C- higher remission rate	
Fukino et al. 2007	100 Japanese	C677T A1298C	no	/
Aggarwal et al. 2006	150	C677T	no	no
Weisman et al. 2006	214	C677T	/	677TT – AE in the central nervous system
Kumagai et al. 2003	115 Japanese	C677T A1298C	no	no
van Ede et al. 2001	236	C677T	no	CT/TT - risk for elevated liver enzyme levels

According to our study, the increase of *677TT* genotype frequency in poor responders was of borderline significance and no association was found with *MTHFR A1298C* polymorphism.

Majority of analyzes have found association between *RFC80* polymorphism and toxicity of MTX in RA (BOHANEC GRABAR *et al*., 2008; SAMARA *et al*., 2014; ŚWEIRKOT *et al*., 2015). One meta-analysis available so far confirmed association of this polymorphism with efficacy of the drug, but not with adverse effects (KUNG *et al*., 2014). This can be due to different results obtained in regard to the allele or genotype associated. For example, *RFC80AA* genotype was associated with hepatotoxicity (ŚWEIRKOT *et al*., 2015), *RFC80GG* correlated with gastrointestinal toxicity (SAMARASA *et al*., 2014), *RFC80G* allele was associated with reduced efficacy of the drug (HAYASHI *et al*., 2013), while, in the present study, no association with drug effects was observed (TAKATORI *et al*., 2006).

CONCLUSION

In conclusion, we assume that *MTHFR 677TT* genotype may be important for prediction of poor response of MTX in patients with RA. However, weak association observed suggests that more appropriate genetic markers for pharmacogenetic model are needed for allowing prediction of MTX responses in RA patients.

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Database NCBI links:

1. http://www.ncbi.nlm.nih.gov/projects/SNP/snp_ref.cgi?rs=144320551
2. http://www.ncbi.nlm.nih.gov/projects/SNP/snp_ref.cgi?rs=2236225
3. http://www.ncbi.nlm.nih.gov/projects/SNP/snp_ref.cgi?rs=1801133
4. http://www.ncbi.nlm.nih.gov/projects/SNP/snp_ref.cgi?rs=1801131

ANALIZA POVEZANOSTI IZABRANIH POLIMORFIZAMA *MTHFR, MTHFD1* I *RFC1* GENA SA EFIKASNOŠĆU I TOKSIČNOŠĆU METOTREKSATA KOD PACIJENATA SA REUMATOIDNIM ARTRITISOM

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Izvod

Folatni analog metotreksat (MTX) je najčešće korišćen bolest-modifikujući lek koji se koristi u terapiji reumatoidnog artritisa (RA). Međutim, klinički odgovor pacijenata sa reumatoidnim artritisom na terapiju metotreksatom pokazuje interindividualne razlike i 30% pacijentata je prinuđeno da prekine terapiju usled neželjenih efekata leka. U grupi od 184 RA pacijenta lečenih metotreksatom ispitali smo da li polimorfizmi u genima *MTHFR* (*rs1801133*, *rs1801131*), *MTHFD1* (*rs2236225*) i *RFC1* (*rs144320551*) imaju uticaj na efikasnost MTX i/ili neželjene efekte leka. Efikasnost terapije metotreksatom procenjena je upotrebom parametra koji pokazuje aktivnost bolesti (disease activity score) u 28 zglobova (DAS28-ESR) baziranom na EULAR kriterijumu i relativnim DAS28 vrednostima (rDAS28) i svi neželjeni efekti su beleženi. Genotipizacija pacijenata za izabrane polimorfizme izvedena je PCR-RFLP metodom. Prema EULAR kriterijumima za praćenje odgovora na terapiju nakon 6 meseci terapije 146 (79.3%) pacijenata klasifikovano je u grupu koja pokazuje odgovor na terapiju. Od toga 17 pacijenata (11.6%) pokazalo je dobar odgovor na terapiju, dok je 129 pacijenata (88.4%) pokazalo umeren odgovor. 38 pacijenata (20.7%) nije pokazalo odgovor na terapiju. Neželjeni efekti leka zabeleženi su kod 53 pacijenta (28.8%). Većina neželjenih efekata bila je blaga (36 (20.7%) pacijenata) i umerena (12 (6.25%) pacijenata). Kod 5 pacijenata (2.7%) primećeni su ozbiljni neželjeni efekti. Sprovedene su asocijacione studije između određenih genotipova i efikasnosti i toksičnosti MTX. Nismo primetili asocijaciju između analiziranih polimorfizama i efikasnosti i toksičnosti metoteksata kod pacijenata sa reumatoidnim artritisom.

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