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Association of C35T polymorphism in dihydrofolate reductase gene with toxicity of methotrexate in rheumatoid arthritis patients

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Abstract

Background: Methotrexate, a folate analogue, is the most commonly used disease-modifying drug in the treatment of rheumatoid arthritis. However, high interindividual differences in drug response are present among RA patients.

Research design and methods*:* In a group of 234 RA patients treated with MTX we investigated whether rs1650697 polymorphism in *DHFR* gene may have impact on MTX efficacy and/or adverse drug effects (ADEs). Relative DAS28 values (rDAS28) were used for MTX therapy estimation and all adverse drug events were recorded. Patients were genotyped for selected polymorphism by real-time PCR method.

Results: According to the EULAR criteria after 6 months of MTX therapy 196 patients (83.8 %) were classified as responders, (25 (10.7 %) were good and 171 (73.1 %) were moderate) and 38 patients (16.2 %) as non-responders. ADEs were observed in 55 patients (23.5 %).

Conclusions: Our results showed that the presence of T allele might be protective against MTX hepatotoxicity measured by transaminaze levels (p=0.05). Furthermore, among patients who also received low-dose corticosteroids we have found a lower rDAS values in patients with CC genotype (p=0.039).

Keywords: DHFR, genetic polymorphism, methotrexate, pharmacogenetics, rheumatoid arthritis, toxicity

1. Introduction

Methotrexate (MTX) is the most commonly used disease-modifying antirheumatic drug (DMARD) for the treatment of rheumatoid arthritis (RA) [1]. Low MTX doses used as either monotherapy or in combination with other drugs have a superior efficacy profile [2]. Despite MTX cost-effectiveness, clinical response to MTX varies widely [3]. Approximately 30-40% patients do not have a good response to MTX despite optimal dosing regimes [4]. Furthermore, some patients develop severe side effects such as cytopenias, gastrointestinal adverse effects (stomatitis, nausea), or abnormal liver function, which may limit its use and may result in additional health care costs [1]. These high interindividual differences in drug response gave rise to the need for prognostic markers in order to individualize and optimize therapy with these antirheumatic agents [5].

The mechanisms of action of methotrexate are complex. Developed as a folic acid analogue, methotrexate inhibits purine and pyrimidine synthesis, which accounts for its efficacy in the therapy of cancer as well as for some of its toxicities [6]. It is considered that the main mechanism of MTX action is based on direct inhibition of dihydrofolatereductase (DHFR) and other key enzymes involved in folate metabolism [7]. DHFR inhibition leads to disruption of DNA replication and thus cell death [8].

DHFR gene contains two alternative promoters: a major and a minor promoter. The major promoter is responsible for 99% transcription of the gene, whereas the minor promoter drives transcription of a non-coding RNA. This RNA harbors sequence of major *DHFR* gene promoter and has been found to fulfill regulatory function through binding to the major promoter in a sequence-specific fashion [9]. Polymorphism

rs1650697 (C35T) resides in a major promoter of *DHFR* gene and is associated with higher *DHFR* expression [10]. We have investigated whether this polymorphism may have impact on MTX efficacy and/or adverse drug effects (ADEs).

2. Patients and methods

234 patients with RA treated and prospectively followed at the Institute of Rheumatology (Faculty of Medicine, University of Belgrade, Serbia) were included in the study. Diagnosis for all patients was set according to the American College of Rheumatology (ACR) 1987 revised classification criteria for RA [11]. Physicians and patients were blinded to the genotypes and lab personnel were blinded to all clinical information throughout the study. Ethics Committee of the Institute of Rheumatology approved the study protocol and signed informed consent form was obtained from each patient involved in the study. Current and past treatment with MTX for at least 6 months was the main criterion for patient inclusion in the study. Patients receiving intraarticular corticosteroids were excluded from the study. Stable dosages of non-steroidal anti-inflammatory drugs (NSAIDs), low-dose corticosteroids ($\leq$10mg/day), previous disease-modifying antirheumatic drugs other than MTX (DMARDs, Aurotherapy, Sulphasalazine, Chloraquine) and folic acid supplementation were allowed.

Safety and clinical assessments were performed as our research group has previously reported [12]. To estimate the clinical response to MTX we have used EULAR (*European League Against Rheumatism*) response criteria and rDAS values [13]. According to EULAR response criteria we have classified patients in groups of

good, moderate and poor responders. Good and moderate responders were considered to be responders and poor responders were considered to be nonresponders. rDAS was calculated for each patient using formula rDAS28 = (DAS280 – DAS281)/DAS280 (DAS280 represents DAS28 score at the beginning of MTX treatment and DAS281 represents DAS28 score after 6 months of therapy). Patient's reports, results of routine laboratory measurements and physical examinations were used for ADEs recording as described in our previous study [14]. In respect to defined criteria ADEs were defined as mild, moderate or severe [14]. Severe ADEs required hospitalization and discontinuation of the MTX therapy.

Molecular-genetic analyses were performed at the Institute of Human Genetics, Faculty of Dentistry, University of Belgrade, Serbia and Institute of Human Genetics, Faculty of Medicine, University of Belgrade. Salting out method was used to extract genomic DNA from peripheral blood leukocytes [15]. C35T polymorphism in *DHFR* gene was analyzed by real-time PCR method. We have used TaqMan probe (TaqMan® SNP Genotyping Assays), C_27863089_10, and each sample was analyzed in duplicate. The reaction mix volume was 25 µl and it included 12,5 µl of *TaqMan Universal Master Mix*, 0,625 µl of 40x *stock*, 10,875 µl of water and 1 µl of DNA. The reaction was conducted in LineGene K real-time PCR apparatus (Bioer Technology) under the following conditions: enzyme activation at 95ºC for 10 minutes; 40 cycles comprised of two steps (15 seconds of denaturation at 92ºC and primer binding, extension and DNA polymerization during 1 minute at 60 ºC) and final extension for 1 minute at 72 ºC. VIC dye corresponded to allele 1 (T) while FAM corresponded to allele 2 (C).

Student's t-test or Mann-Whitney test (depending on homogeneity of variable distribution) for continuous variables and Chi-square test for dichotomous variables were used for analyzing the difference in patients, disease and treatment characteristic between responders and non-responders or those with and without ADEs. Differences in frequencies of genotypes between responders and non-responders and patients with or without ADEs were analyzed by Chi-square test. rDAS28 difference across genotypes was assessed by ANOVA and Student's t-test. When statistically significant results were observed we have used multiple logistic or linear regression analysis. All statistical analyzes were performed by SPSS version 16.0 (SPSS Inc, Chicago, Illinois, USA).

3. Results

All patients included in the study were receiving MTX therapy for at least 6 months. The duration of the MTX treatment was 33.77 ± 34.20 months. 149 patients (63.7%) received low doses of corticosteroides, while 159 patients (67.9%) received folate suplements. Folate suplementation was introduced in the therapy in case of adverse effects appearance in doses recommended by physician. The main demographical and clinical characteristics of the patients are presented in Table 1.

A significant decrease in DAS28 was obsereved at 6-month control ($p < 0.0005$). According to the EULAR response criteria, after 6 months of MTX therapy 196 (83.8%) patients were classfied as responders (good 25 patients (10.7%) and 171 (73.1%) moderate) and 38 (16.2%) patients as non-responders. MTX therapy response by EULAR criteria in regard to genotypes of rs1650697 polymorphism in *DHFR* gene is shown in Table 2. We have observed no association of genotypes with

MTX therapy response. Genotypes frequencies of analyzed polymorphism were not in Hardy-Weinberg equilibrium (HWE; tested using Pearsons's chi-square test, 5% significance threshold). This could be result of higher number of females than males in analyzed RA patients cohort compared with healthy population. Since no genotyping errors were observed and there is no recommendation for exclusion from the analyses of SNPs that are not HWE [16, 17], we did not investigate this further.

Relative DAS28 values (rDAS28) ranged from – 0.02 to 0.85 (0.34 ± 0.18). In the group of 149 patients who received low-dose corticosteroids we have found statistically significant lower rDAS value in patients with CC genotype compared to patients with CT and TT genotypes (p=0.013), as shown in Table 3. To confirm obtained results we have used multiple linear regression analysis using as covariables gender, patients age, duration of the disease, folic acid supplementation, MTX dose and use of DMARDs other than MTX and result remained significant (ß=0.198, p=0.015).

Adverse drug events were observed in 55 patients (23.5%). Most frequent ADEs were nausea (20 patients (8.5%)) and elevetaed transaminaze levels (24 patients (10.3%)), followed by bone marrow toxicity (9 patients (3.8%)), alopecia (7 patients(3%)), stomatitis (4 patients (1.7%)), pneumonitis (2 patients (0.8%)) and caugh (1 patients(0.4%)). Severe ADEs were observed in 11 patients (4.7%) (bone marrow toxicity and pneumonitis) and they were forced to discontinue therapy.

There was statistically significant difference between weekly MTX dose received by patients who have experineced ADEs (10.73 ± 2.92 mg/week) and the one received

by the patients with no ADEs (11.98 ± 3.24 mg/week; p = 0.003). Association between the age of patients when RA simptoms appeared and presence of ADEs has not been observed. Furthermore, we have obsereved no association of rs1650697 polymorphism genotypes with overall ADEs. However, the presence of allele T (CT and TT genotypes) was protective against MTX hepatotoxicity (p=0.05). Distribution of genotypes in relation MTX hepatotoxicity is shown in Table 4. Results remained significant after multiple logistic regression analysis with gender, age of the patients, MTX dose, folic acid suplementation and use of DMARDs other than MTX as covariates (p=0.049, OR=0.356, CI: 0.127-0.993).

4. Discussion

One of the main goals of the MTX therapy in RA is DHFR protein inhibition. *DHFR* gene polymorphisms may influence its product activity and abundance. Hence, polymorphisms in this gene are attractive targets for the search of biomarkers that will allow clinicians to predict responses to MTX therapy. We have already explored several *DHFR* gene polymorphisms and reported influence of two analyzed polymorphisms on efficacy and toxicity of the MTX in RA patients [14, 18].

There is very scarce information regarding rs1650697 polymorphism in RA patients treated with MTX available in the literature. This is a single nucleotide polymorphism located in a major promoter of *DHFR* gene [10]. It was noticed that T allele, which is one of the alleles present in 1b* haplotype may have influence on increased *DHFR* gene expression [10], but it is not clear if this polymorphism alone may influence level of *DHFR* expression. Wessels and associates have investigated a potential influence of several polymorphisms including C35T on efficacy and toxicity of

methotrexate in a group of 205 RA patients, but they have not found any association [19]. No association of this polymorphism with MTX response has been observed in a group of psoriatic arthritis patients [20].

Our study revealed a possible protective role of T allele against MTX hepatotoxicity measured by transaminaze levels in RA patients with elevated levels of transaminase after 6 months of methotrexate therapy in contrast to the results published by Wessels et al. [19]. According to the recent results [21] folates, regardless administration dose, protect against MTX toxicity and do not influence its efficacy. Unfortunately, for some RA patients folate supplementation is not sufficient against MTX toxicity. These patients have to be recognized and included in alternative therapy protocols. In order to accomplish this, identification of genetic polymorphisms specific for those patients is necessary. DHFR protein levels rapidly increase upon exposure to methotrexate due to increase of DHFR translation, provoked by the drug action [22] and there are no evidences that MTX could influence DHFR protein levels through changes of *DHFR* gene expression. Consequently, we did not analyze the expression of the *DFHR* gene before and after MTX treatment. Our findings may contribute to results of other authors [10] and hypothesis that allele T is responsible for higher *DHFR* gene expression. Namely, in a the direct inhibitory action of the drug on the protein, like in case of MTX and DHFR protein, increased activity or level of the protein may result in diminished drug effects in a sense of its reduced efficacy and toxicity. Our results regarding analyzed polymorphism and MTX hepatotoxicity contradict to results of some previously published papers. Although this may be result of different designs of the studies,

total analyzed cohort of the RA patients is small and for the final conclusions additional investigations are necessary. In accordance with previous studies we found no association of C35T polymorphism with methotrexate efficacy according to EULAR response criteria in RA patients, but our results showed lower rDAS value in carriers of CC genotype in a group of patients receiving MTX therapy in combination with low-dose corticosteroids. Lower rDAS values may indicate less favorable response to MTX. There are only several studies that investigated possible interactions between MTX and corticosteroids, but results are controversial. In the study on the C6-glioma cells it has been shown that corticosteroid dexametasone induces high doses MTX resistence [23]. According to study on rats [24] administration of corticosteroids reduce biliary clearance of MTX and consequently contribute to its hepatotoxic effects, while in study on 154 RA patients no interaction between those drugs has been confirmed, although corticosteroid administration significantly delayed the time of MTX ADEs appearance [25]. In a group of 33 RA patients Lafforgue and associates [26] observed an influence of long-term corticosteroids administration on MTX pharmacokinetics. Association of MTX and corticosteroids administration with hepatotoxicity in the children brain tumor therapy has been reported, but again no obvious drug-drug interaction has been proven [27]. It is important to emphasize that rDAS value does not represent common measure of MTX efficacy, but reflects changes in disease activity. Group of patients receiving corticosteroids in cohort analyzed in our study had more active disease at the beginning of the therapy and it is possible that this resulted in less favorable MTX response in CC genotype carriers. Additionally, our patients had more active disease at the beginning of the therapy compared with other published

studies. Obviously, the results so far are not sufficient for final conclusions and considering therapy protocols that often include MTX and corticosteroids and new studies which will finally explain if there is an interaction between these potent antirheumatic drugs are necessary. Further studies in larger patient groups are necessary to confirm the relationship between the analyzed polymorphism and MTX treatment response and reveal its possible role in relation to low-dose corticosteroids treatment in rheumatoid arthritis.

5. Conclusion

In conclusion, our results indicated a possible protective role of T allele against hepatotoxicity in RA patients. No association of C35T polymorphism with methotrexate efficacy in RA patients was found, but our results showed lower rDAS value in carriers of CC genotype in a group of patients receiving MTX therapy in combination with low-dose corticosteroids. Lower rDAS values may indicate less favorable response to MTX.

Further studies in larger patient groups are necessary to confirm the relationship between the analyzed polymorphism and MTX treatment response and reveal its possible role in relation to low-dose corticosteroids treatment in rheumatoid arthritis.

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Declaration of interest

The authors have no relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript. This includes employment, consultancies, honoraria, stock ownership or options, expert testimony, grants or patents received or pending, or royalties.

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Author contributions

The paper was written by DV and BJ, who also designed the experiment and conducted molecular-genetic analysis together with BP. VM, TD, IN and NM participated in the study design. The manuscript was commented and revised by TD, and NM. VM, ND and VB have analyzed clinical assessment data. MK participated in statistical analysis of the collected data.

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Table legends

Table 1. Demographical and clinical characteristics of the RA patients analyzed in the study; DAS28 0 represents DAS28 values at the beginning of therapy, DAS28 represents DAS28 values after six months of therapy.

Table 2. MTX therapy response by EULAR criteria in regard to *DHFR* gene rs1650697 polymorphism genotypes.

Table 3. rDAS28 values in relation to *DHFR* gene rs1650697 polymorphism genotypes of RA patients treated with MTX therapy in combination with low-dose corticosteroids.

Table 4. Hepatotoxicity in relation to *DHFR* gene rs1650697 polymorphism genotypes

Table 1. Demographical and clinical characteristics of the RA patients analyzed in the study; DAS28 0 represents DAS28 values at the beginning of therapy, DAS28 represents DAS28 values after six months of therapy.

characteristic	**value, range (mean)/n (%)**
female/male	184 (78.6)/50 (21.4)
mean age (years)	20-84 (57.49±10.62)
duration of the disease (months)	6-240 (46.75±41.06)
weekly MTX dose (mg)	7.5-20.0 (11.70±3.20)
low dose corticosteroids mg/day	≤10 (6.20±4.81)
DAS28 0	5.10-9.14 (7.50±0.86)
DAS28 1	1.13-8.46 (4.95±1.55)

Table 2. MTX therapy response by EULAR criteria in regards to *DHFR* gene rs1650697 polymorphism genotypes.

Genotypes	**Responders, n (%)**	**Non-responders, n (%)**	**Total, n (%)**	p
CC	113 (48.29)	28 (11.97)	141 (60.26)	0.177
CT	38 (16.24)	5 (2.14)	43 (18.38)	
TT	45 (19.23)	5 (2.14)	50 (21.37)	
Dominant model CC/CT+TT	113/83 (48.29/35.47)	28/10 (11.97/4.28)	141/93 (60.26/39.75)	0.072
Recessive model TT/CT+CC	45/151 (19.23/64.53)	5/33 (2.14/14.11)	50/184 (21.37/78.64)	0.202

Table 3. rDAS28 values in relation to *DHFR* gene rs1650697 polymorphism genotypes of RA patients treated with MTX therapy in combination with low-dose corticosteroids.

Genotype	**Number of patients, (%)**	**rDAS28 values**	**p**
CC	92 (61.7)	0.295±0.166	0.013
CT+TT	57 (38.3)	0.352±0.175	

Table 4. Hepatotoxicity in relation to *DHFR* gene rs1650697 polymorphism genotypes

Genotype	**No hepatotoxicity, n (%)**	**Hepatotoxicity, n (%)**	**p**
CC	122 (52.13)	19 (8.12)	0.05
CT+TT	88 (37.61)	5 (2.14)	