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Genetic polymorphisms and Methotrexate response in patients with rheumatoid arthritis

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ABSTRACT: In the context of rheumatoid arthritis (RA) treatment, Methotrexate (MTX) plays a crucial role in preventing joint damage and bone erosion (BE). RA is characterized by elevated levels of matrix metalloproteinase 2 (MMP2), an enzyme responsible for extracellular matrix degradation, which contributes to joint damage and inflammation. Tissue inhibitor of metalloproteinase 2 (TIMP2) counteracts MMP2, and an imbalance between the two can exacerbate BE. Inosine triphosphatase (ITPA) is an enzyme involved in regulating inosine triphosphate levels, potentially linked to RA susceptibility. Genetic variations in *ITPA*, *MMP2*, and *TIMP2* genes can influence MTX's efficacy. A study of 122 RA patients on MTX monotherapy assessed its effectiveness using Disease Activity Score (DAS28) changes over 6 months following EULAR response criteria. Genotyping, including *MMP2* (rs243866, rs2285053), *TIMP2* (rs2277698), and *ITPA* (rs1127354) polymorphisms, was performed. Among the patients, 87.7% were responders, 63.9% experienced BE, and 24.6% encountered adverse events. Notably, patients with the *MMP2* (rs243866) GG genotype were the only ones reporting nausea (p=0.025). Patients with both the *MMP2* (rs2285053) CC and *TIMP2* (rs2277698) CT genotypes had a lower incidence of BE compared to those lacking this combination (p=0.048). The *TIMP2* (rs2277698) CC genotype was associated with a higher baseline DAS28 score (p=0.035). In summary, this study suggests that specific *MMP2/TIMP2* genotype combinations may serve as predictors for BE development in RA patients undergoing MTX monotherapy.

KEYWORDS: Methotrexate; MMP2; TIMP2; ITPA; rheumatoid arthritis; polymorphism.

1. INTRODUCTION

Rheumatoid arthritis (RA) is a complex and debilitating chronic disease, characterized by persistent inflammation and an autoimmune response that primarily affects the synovial joints, leading to various systemic manifestations [1]. The hallmark of RA is chronic synovitis, which triggers the formation of invasive synovial pannus. This, in turn, causes extensive damage to cartilage, soft tissues, and bone erosions (BE) in patients with RA [2]. The importance of early therapy initiation cannot be overstated, as it is a critical factor in managing the disease, preventing joint damage, and enhancing the overall quality of life for individuals living with RA.

For many decades, Methotrexate (MTX) has stood as the foremost choice for RA therapy due to its proven efficacy, safety profile, affordability, and the flexibility it offers in dose adjustment [3, 4]. However, it is disheartening to note that roughly one-third of RA patients experience inefficacy when treated with MTX [5]. As a consequence, the patients do not attain remission or maintain low disease activity, following the treat-to-target approach [3]. Despite the significant progress in the field, researchers have not yet managed to identify genetic biomarkers that can guide personalized treatment decisions for RA patients before they embark on their therapeutic journey [6].

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The mechanisms underlying the therapeutic effects of MTX in RA are not entirely understood, although it is known that MTX operates primarily by inhibiting the folate pathway and increasing the accumulation of extracellular adenosine [6, 7]. One intriguing aspect of this is the role played by Inosine triphosphate pyrophosphatase (ITPA), an enzyme that governs the adenosine cycle. Polymorphism in the second exon of the ITPA gene, denoted as rs1127354, leads to conformational changes in the enzyme (variant P32T) that result in reduced activity [8].

RA is associated with an overexpression of proinflammatory cytokines, including TNFα, IL1, IL6, and IL17A, which, in turn, induce the transcription and translation of matrix metalloproteinases (MMPs) [2, 9]. MMPs are zinc-dependent endopeptidases that have a crucial role in the expansion of the pannus and the destruction of subchondral bone. These MMPs are regulated by specific proteins, among which tissue inhibitors of metalloproteinases (TIMPs) are significant [2, 10, 11]. It is worth noting that MTX's therapeutic impact in RA may, in part, be attributed to its ability to modulate cytokine levels and subsequently influence the expression of MMPs/TIMPs [6, 11, 12].

The aim of the study was to investigate the association between the rs243866 and rs2285053 polymorphisms in the MMP2 gene, the rs2277698 polymorphism in the TIMP2 gene, and the rs1127354 polymorphism in the ITPA gene. We aimed to shed light on how these genetic variants might influence disease activity and therapeutic outcomes among RA patients who are undergoing MTX monotherapy. This research holds the promise of providing valuable insights into the potential for personalized treatment strategies for RA patients based on their genetic makeup.

2. RESULTS

This study encompassed 122 RA patients who were administered MTX as monotherapy. Table 1 presents demographic and clinical data for the patients. Favorable response to treatment had 107 individuals (87.7%) with 15 patients (12.3%) displaying a good response and 88 patients (72.1%) showing a moderate response. Adverse drug effects were encountered by 30 patients (24.6%), of whom one patient (0.8%) experienced severe, five patients (4.1%) had moderate, and 24 patients (19.7%) mild adverse effects. Over the follow-up period, 78 patients (63.9%) developed bone erosions. Notably, there were no significant differences in genotype and allele frequencies for any of the investigated polymorphisms between responders and nonresponders (Table 2). Furthermore, the presence of different genotypes of these polymorphisms did not appear to influence the overall occurrence of adverse effects or the appearance of bone erosions (data not shown).

Table 1. Demographic and clinical characteristics of patients enrolled in the study

Variable	All patients n=122	Responders n=107	Nonresponders n=15	p-value
Age	56.65±10.93	56.76±10.76	55.87±12.46	0.769
Female, n (%)	90 (73.8)	80 (74.8)	10 (66.7)	0.536
Adverse effects, n (%)	30 (24.6)	24 (22.4)	6 (40)	0.197
Hepatotoxicity (%)	14 (11.5)	11 (10.3)	3 (20)	0.378
Nausea (%)	9 (7.4)	8 (7.5)	1 (6.7)	1.000
Erosions, n (%)	78 (63.9%)	68 (63.6)	10 (66.7)	1.000
MTX [b] dosage (mg)	12.19±3.21	12.29±3.20	11.50±3.25	0.556
Folate use	87 (71.3%)	78 (72.9)	9 (60)	0.362
Corticosteroid use (%)	64 (52.5%)	53 (49.5)	11 (73.3)	0.102
Corticosteroid dosage (mg)	9.53±1.47	9.53±1.47	9.55±1.51	0.972
DAS [a] 28 at baseline	7.41±0.91	7.38±0.88	7.59±1.13	0.297
DAS [a] 28 after 6 months	4.47±1.53	4.16±1.27	6.74±1.27	<0.001

[a] DAS - Disease Activity Score;
[b] MTX – Methotrexate

Table 2. Genotype frequencies for *MMP2*, *TIMP2*, and *ITPA* gene polymorphisms

Genotype	All patients n=122	Responders n=107	Nonresponders n=15	p-value
MMP2 (rs243866)				
GG	71 (64)	62 (63.3)	9 (69.2)	
GA	36 (32.4)	32 (32.7)	4 (30.8)	0.738
AA	4 (3.6)	4 (4.1)	0 (0)	
GG	71 (64)	62 (63.3)	9 (69.2)	0.767
GA+AA	40 (36)	36 (36.7)	4 (60.8)	
MMP2 (rs2285053)				
CC	79 (75.2)	70 (75.3)	9 (75)	
CT	26 (24.8)	23 (24.7)	3 (25)	1.000
TT	0	0 (0)	0 (0)	
TIMP2 (rs2277698)				
CC	88 (80.7)	78 (81.3)	10 (76.9)	
CT	21 (19.3)	18 (18.8)	3 (23.1)	0.713
TT	0	0 (0)	0 (0)	
ITPA (rs1127354)				
CC	110 (90.9)	96 (89.7)	14 (100)	
CA	10 (8.3)	10 (9.3)	0 (0)	0.453
AA	1 (0.8)	1 (0.9)	0 (0)	
CC	110 (90.9)	96 (89.7)	14 (100)	0.208
CC+CA	11 (9,1)	11 (10.3)	0 (0)	

Nausea emerged as a common side effect of therapy, affecting nine patients. Interestingly, all these patients had the *MMP2* rs243866 GG genotype (p=0.025).

Patients with the *TIMP2* gene rs2277698 CC genotype displayed a higher number of tender joints (p=0.026) and greater DAS28 at baseline when compared to patients with the CT genotype (p=0.035). Furthermore, patients with the CT genotype exhibited a more substantial reduction in the number of tender joints after 6 months of MTX treatment (p=0.017).

Additionally, we explored the association between treatment response, the occurrence of adverse effects, or the appearance of bone erosions and genotype combinations. Patients with the *MMP2* rs2285053 CC / *TIMP2* rs2277698 CT genotype combination (n=15) experienced a less frequent occurrence of bone erosions compared to patients with other genotype combinations (40% vs. 66.7%, p=0.048, RR: 0.6, 95% CI 0.317-1.134; OR: 0.333, 95% CI 0.109-1.024). After adjusting for age and gender, logistic regression analysis of this association demonstrated borderline significance (p=0.051).

Next, we analyzed the investigation of *MMP2* haplotypes. We assumed that polymorphisms within the *MMP2* gene are in linkage disequilibrium (LD). Using the Confidence Intervals LD method, a haplotype block was not identified between the *MMP2* gene polymorphisms rs243866 and rs2285053 (r2=0.037, D'=1). The most frequent haplotypes among our patients were GC, AC, and GT (67.6%, 19.8%, and 12.5%, respectively). However, the investigated *MMP2* haplotypes did not appear to be associated with MTX response, the occurrence of bone erosions, or adverse effects.

3. DISCUSSION

Methotrexate is a widely chosen therapy for the treatment of Rheumatoid Arthritis. However, it is concerning that nearly one-third of patients who do not respond favorably to this drug miss the optimal window for initiating more appropriate therapy. As a result, these patients often face more severe manifestations of the disease [3, 4, 5]. A comprehensive understanding of how MTX exerts its therapeutic effects in RA is crucial for identifying such patients promptly and providing them with alternative treatments [6, 7]. Genetic variations in the genes responsible for the metabolic pathways of MTX could serve as valuable biomarkers for predicting treatment responses in RA. However, the results of numerous studies investigating the influence of gene polymorphisms on MTX therapy outcomes in RA remain inconclusive [6].

Most pharmacogenetic studies related to MTX treatment in RA have focused on polymorphisms within genes that control the folate and adenosine cycles [13, 14]. Based on these studies, CPIC (Clinical Pharmacogenetics Implementation Consortium) still wasn't able to incorporate genetic testing into the daily routine for RA patients starting MTX therapy [15-17]. While there have been numerous investigations into the association between MMP gene polymorphisms and RA susceptibility, none have explored the impact of *MMP2/TIMP2* gene polymorphisms on MTX therapy outcomes [18]. It's worth noting that MMPs play a critical role in subchondral bone destruction and the appearance of bone erosions [18, 19]. At the same time, the primary goal of MTX therapy is to prevent bone erosions and subsequent joint damage [20, 21]. This leads to the question of whether polymorphisms altering MMP2/TIMP2 expression could influence the course and outcome of MTX treatment.

Inflammation and tissue destruction in RA are mediated by proinflammatory cytokines [2, 6, 19]. These cytokines stimulate synovial fibroblasts and chondrocytes to secrete enzymes like MMPs, which are responsible for cartilage destruction, angiogenic processes, extracellular matrix remodeling, pannus formation, bone destruction, and joint dysfunction in RA [9, 12, 18]. The activity of these enzymes is regulated by tissue inhibitors of MMPs (TIMPs) [11, 19]. While the synovial fluid of RA patients is characterized by elevated concentrations of various MMPs, the precise role of these enzymes remains incompletely understood [6, 11, 19]. It has been demonstrated that while MMP1, MMP2, and MMP9 contribute to joint impairment in RA, MMP2 appears to play a suppressive role in the disease's development, possibly through the degradation of cytokines, chemokines, and growth factor receptors [2, 9, 22]. A recent study even suggested that MMP3 concentration could serve as a valuable biomarker for estimating therapeutic response in RA patients [23]. MTX indirectly influences MMP and TIMP production by modulating synovial cytokine levels [2, 9, 22].

The activity levels of the MMP2 enzyme may be influenced by variations in the *MMP2* gene, as well as polymorphisms in the gene coding for its inhibitor, the TIMP2 enzyme. While we observed no association between the analyzed *MMP2* and *TIMP2* genotypes and MTX therapy response, we did find that patients with the *MMP2* rs2285053 CC / *TIMP2* rs2277698 CT genotype combination had a significantly lower rate of bone erosion. Additionally, our patients with *TIMP2* rs2277698 CT genotype had fewer tender joints, lower disease activity assessed by the DAS28 score at the beginning of the disease, and a greater reduction in the number of tender joints after six months of MTX monotherapy, suggesting a better response to treatment than patients with the CC genotype. Previous studies have indicated that the *MMP2* promoter polymorphism -375C>T (rs2285053) disrupts the Sp-1 binding site and reduces gene expression [24]. *TIMP2* rs2277698 is a silent polymorphism with an unexplained functional effect. It has been suggested that this polymorphism might be involved in splicing or transcriptional alterations [10]. Kukkonen et al. found that *TIMP2* rs2277698 T-allele represents a risk factor for emphysema and speculated that the presence of the T-allele leads to TIMP2 downregulation [10]. According to these studies, the *MMP2* rs2285053 CC / *TIMP2* rs2277698 CT genotype combination could result in increased MMP2 activity. Since the present study demonstrated that patients with this genotype combination have a lower rate of bone erosion, our results support previous research on MMP2's protective role in RA development [22].

ITPA is one of the enzymes that regulate adenosine [7, 25]. A genetic variation known as polymorphism rs1127354 (p.P32T), located in exon 2, leads to a conformational change in the enzyme, resulting in reduced activity. Heterozygotes with this variation retain only 25% of the normal enzyme activity [8]. Several studies have explored the influence of this polymorphism on MTX therapy response in RA, but results remain inconclusive. Some authors have found an association between the A allele and a poor response to MTX, as measured by changes in DAS scores [18, 26]. However, Lee et al. did not find such an association in their study, which included 120 RA patients on MTX monotherapy, consistent with our results [27]. Dervieux et al. reported an association between the rs1127354 polymorphism and gastrointestinal adverse effects, but we found no association between this polymorphism and any therapy side effects [14]. As the *ITPA* rs1127354 polymorphism is functional and already included in several pharmacogenetic models developed for predicting MTX therapy response in RA, further studies on this polymorphism are necessary [28].

The ability to predict the response of each patient to MTX would be invaluable in everyday clinical practice. However, we are only beginning to uncover MTX pharmacogenetics in RA treatment. Our study has some limitations, such as the number of participants. While we have uncovered valuable insights into genetic factors that may impact bone erosion development in rheumatoid arthritis patients undergoing methotrexate treatment, a larger sample size would be desirable to ensure the reliability of our findings and their applicability to a broader patient population. Further research with a larger cohort of participants could provide additional confirmation and a better understanding of the genetic factors at play in this context,

potentially leading to the development of more tailored and precise approaches to rheumatoid arthritis treatment.

4. CONCLUSION

In summary, our study findings propose that when assessing the risk of bone erosion in rheumatoid arthritis RA patients treated with Methotrexate, it may be more informative to consider the combined genetic profile of MMP2 (rs2285053) CC and TIMP2 (rs2277698) CT genotypes, rather than analyzing these genes separately. This combination of genotypes could offer a more accurate and refined predictor for the development of bone erosion in this specific patient group. However, it's important to note that our results should be validated through further research and larger studies to establish their reliability in clinical practice.

5. MATERIALS AND METHODS

5.1. Patients

We conducted an analysis involving 122 patients with Rheumatoid Arthritis (RA) who received Methotrexate (MTX) as monotherapy for a minimum of six months. MTX was administered orally once a week. No other synthetic or biological Disease-modifying Anti-Rheumatic Drugs (DMARDs) were used concurrently with MTX treatment. Patients were allowed to use low-dose corticosteroids orally (≤ 10 mg/day) and folic acid supplementation (5 mg, taken three days after MTX dosage). However, patients who received intraarticular corticosteroids were not included in the study. All patients received treatment from the same physician at The Institute of Rheumatology, Faculty of Medicine, University of Belgrade, and each patient's diagnosis adhered to the ACR 1987 revised classification criteria [29].

Demographic data were collected when patients were enrolled in the study. The study was conducted in accordance with the principles of the Declaration of Helsinki and was approved by the Ethics Committee of the Institute of Rheumatology, Belgrade. All patients provided informed consent to participate.

To evaluate disease activity, we used the Disease Activity Score (DAS28) at the baseline (DAS28 0) and after six months of treatment (DAS28 1). We employed the EULAR response criteria to assess treatment efficacy [30]. Patients who exhibited a good response achieved a DAS28 1 score of ≤ 3.2 with an improvement from baseline of ≥ 1.2 (DAS28 0 – DAS28 1 or ΔDAS28). A moderate response was defined as a DAS28 1 value between 3.2 and 5.1, with a ΔDAS28 between 0.6 and 1.2. Poor responders had a DAS28 1 higher than 5.1 and a ΔDAS28 lower than 0.6. We classified patients with good and moderate responses as "responders," while those with a poor response were classified as "non-responders."

Adverse effects (AE) were monitored throughout the study period, with data collected from patient reports, routine physical examinations, and laboratory analysis results. Patients reported AE information related to the type, onset time, severity, and duration of the AE during interviews. A single rheumatologist evaluated all the data. AE observed during the six-month period were categorized into different types, including gastrointestinal symptoms (anorexia, nausea, and vomiting), hepatotoxicity (elevated transaminase levels), bone marrow toxicity (leukopenia with < 4x109 cells/l, thrombocytopenia with < 150x109 cells/l, and pancytopenia), dermatological issues (hair loss, rashes, and vasculitis), mucositis, and pulmonary toxicity (dyspnea, cough, and pneumonitis). AE were further classified as severe, moderate, or mild based on established criteria [31]. The presence of bone erosions was assessed after six months of treatment using standard radiography. This comprehensive analysis aimed to provide valuable insights into the effectiveness and safety of MTX monotherapy for RA patients.

5.2. DNA extraction and genotyping

Molecular genetics analyses were performed at The Institute of Human Genetics, Faculty of Medicine, University in Belgrade. Genomic DNA was extracted from blood using standard Salting out procedure [32]. Genotyping tests were done in replicate. *TIMP2* gene rs2277698 polymorphism and *MMP2* gene rs243866 polymorphism were analyzed using TaqMan assays on ABI7500 RealTime PCR system (Applied Biosystems, Foster, CA) according to manufacturer's instructions.

Analysis of *MMP2* -735C/T polymorphism (rs2285053) was carried out by polymerase chain reaction followed by restriction fragment length polymorphism analysis (PCR-RFLP). Polymerase chain reaction (PCR) amplification was performed according to following conditions: 3 minutes of initial denaturation at 94˚C were followed by 35 cycles consisting of denaturation at 94˚C for 30s, hybridization for 45s at 67˚C and extension at 72˚C for 1min. Final extension at 72˚C lasted 7min. The forward and reverse primers sequences

were 5′-GGA TTC TTG GCT TGG CGC AGG 3′ and 5′-GGG GGC TGG GTA AAA TGA GGG TG 3′, respectively. PCR products were digested with Hinf I enzyme. Uncut PCR fragments of 391bp corresponded to C allele, while PCR products cut to 338bp and 53bp long fragments corresponded to T allele.

For genotyping of *ITPA* rs1127354 polymorphism, KASP (Kompetitive Allele Specific PCR) genotyping kit was used, according to manufacturer's instructions and the analysis was performed on Real-time PCR (ABI7500 Real-time system, Applied Biosystems, Foster, CA).

5.3. Statistical analysis

Statistical analyses were performed using SPSS 16 (IBM SPSS Inc, Chicago, IL, USA). Tests were two-sided and p-values under 0.05 were considered statistically significant. The difference in genotype and allele frequencies between patients' groups were analyzed using the $\chi 2$ test or Fisher's exact test. Students t-test, ANOVA, split-plot ANOVA, or corresponding nonparametric tests were performed for quantitative variables. The Hardy–Weinberg equilibrium, linkage disequilibrium and haplotype analysis were performed using the Haploview software (version 4.2.) [33].

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