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Original article

Thymidylate synthase genetic polymorphism and plasma total homocysteine level in a group of Turkish patients with rheumatoid arthritis: relationship with disease activity and methotrexate toxicity

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ABSTRACT

Background: The polymorphism of thymidylate synthase (TS) gene and homocysteine are reported to have a relationship to methotrexate (MTX) metabolism, with conflicting results. The aim of this study was to determine homocysteine levels and the frequency of TS gene triple repeat (TS3R) and double repeat (TS2R) polymorphisms in a group of Turkish RA patients and evaluate its association with MTX toxicity and disease activity.

Methods: Sixty-four patients with RA and 31 control subjects with a mean age of 48.7 ± 12.5 and 46.2 ± 13.4 years were enrolled for the study. Demographic characteristics were obtained and a number of patients with MTX-related adverse affects were recorded in the patient group. The homocysteine levels and TS2R/TS3R polymorphisms of the TS gene were analyzed and the distribution of genotypes according to MTX toxicity and disease activity was determined.

Results: The demographic properties were similar between the patient and control subjects. Folic acid supplementation with a mean dose of 5 mg folic acid/week was present in all patients. Thirty-six of the 64 patients showed adverse effects to MTX treatment. The respective frequency of TS2R and TS3R polymorphisms was found to be similar in the patient and control groups. TS2R and TS3R gene polymorphisms were found to be similar in patients with and without MTX-related adverse events. The mean homocysteine level was also similar in patients with and without TS gene polymorphism, but was found to be higher (12.45 μmol/L vs 10.7 μmol/L) in patients with MTX-related side effects than in patients without side effects. The mean level of homocysteine was correlated with levels of ESR in the patient group.

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Conclusions: In conclusion, homocysteine levels might affect the disease activity and toxicity of MTX but 2R and 3R polymorphisms in the TS gene were not related with MTX-related toxicity in RA patients receiving folate supplementation. Further studies are needed to illuminate the polymorphisms in other enzymes that might be responsible for the MTX toxicity in patients suffering from RA.

Polimorfismo do gene timidilato sintase e nível plasmático total de homocisteína em um grupo de pacientes turcos com artrite reumatoide: relação com a atividade da doença e toxicidade ao metotrexato

Palavras-chave:
Artrite reumatoide
Timidilato sintase
Homocisteína
Polimorfismo
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RESUMO

Introdução: Relata-se que o polimorfismo do gene timidilato sintase (TS) e a homocisteína têm relação com o metabolismo do metotrexato (MTX), com achados conflitantes. O objetivo deste estudo foi determinar os níveis de homocisteína e a frequência de polimorfismos de repetição tripla (TS3R) e dupla (TS2R) do gene TS em um grupo de pacientes turcos com AR e avaliar sua associação com a toxicidade ao MTX e a atividade da doença.
Métodos: Foram incluídos no estudo 64 pacientes com AR e 31 indivíduos no grupo controle, com média de 48,7 ± 12,5 e 46,2 ± 13,4 anos. Foram obtidas as características demográficas e foi registrado o número de pacientes que relataram efeitos adversos ao MTX no grupo AR. Foram analisados os níveis de homocisteína e os polimorfismos TS2R/TS3R. Foi determinada a distribuição de genótipos de acordo com a toxicidade ao MTX e a atividade da doença.
Resultados: Os dados demográficos foram semelhantes entre os pacientes e controles. Todos faziam suplementação de ácido fólico a uma dose média de 5 mg/semana. Dos 64 pacientes, 36 apresentaram efeitos adversos ao tratamento com MTX. Encontrou-se uma frequência de polimorfismos TS2R e TS3R semelhante nos grupos AR e controle. Encontrou-se que os polimorfismos TS2R e TS3R eram semelhantes em pacientes com e sem eventos adversos relacionados com o MTX. O nível médio de homocisteína também foi similar em pacientes com e sem polimorfismo do gene TS, mas era mais elevado (12,45 μmol/L vs. 10,7 μmol/L) em pacientes com do que sem efeitos adversos relacionados com o MTX. O nível médio de homocisteína se correlacionou com o VHS no grupo AR.
Conclusões: Os níveis de homocisteína podem afetar a atividade da doença e a toxicidade ao MTX, mas os polimorfismos 2 R e 3 R no gene TS não se correlacionaram com a toxicidade ao MTX em pacientes com AR que recebem suplementação de ácido fólico. São necessários mais estudos para esclarecer os polimorfismos em outras enzimas que podem ser responsáveis pela toxicidade ao MTX em pacientes com AR.

Introduction

Methotrexate (MTX) is the cornerstone and the most commonly used disease modifying anti-rheumatic drug (DMARD) for the treatment of rheumatoid arthritis (RA). The beneficial effects of MTX in RA stem primarily from two aspects of the mechanism of action. It directly inhibits several folate dependent enzymes such as dihydrofolate reductase and thymidylate synthase (TS); leading to disrupted purine and pyrimidine synthesis and extracellular release of adenosine, a potent antiinflammatory agent respectively.[1,2] Several studies demonstrated the effect of gene polymorphisms on MTX efficacy and safety but the available evidence is not yet conclusive.[1–13] Thymidylate synthase is an important target for methotrexate and overexpression of TS is linked to resistance of TS-targeted drugs.[14] Polymorphism of TS gene is reported to have a relationship to MTX metabolism with conflicting results.[3–13] Homocysteine is an aminoacid containing the thiol group and is an intermediate product of the metabolism of methionine. Some previous authors concluded that plasma homocysteine may be important in mediating the side effects of MTX.[15–17] Increasing pharmacogenetic studies show that a variety of gene polymorphisms including the TS-5′UTR 3R/2R variant result in hyperhomocysteinaemia, which could be responsible for more common and severe MTX-related side effects.[7,17,18] Besides hyperhomocysteinaemia has been linked to inflammation in RA. Homocysteine levels are also known to be influenced by nutrients such as folic acid and the vitamin B complex, and anti-RA drugs such as MTX.[6,7,16]

The aims of this study were to determine the frequency of TS gene double-triple repeat (2R/3R) polymorphism and homocysteine levels and in a group of patients with RA and

to evaluate their relationship with disease activity and MTX toxicity.

Methods

Sixty-four RA patients who met the ACR revised criteria[19] were recruited from the outpatient rheumatology unit of Clinic of Physical Medicine and Rehabilitation, Ankara Training and Research Hospital between February and July 2009.

Current or past treatment with MTX was the main criterion for the inclusion of RA patients into the study. Patients with a mean BMI >35, aged <18, and >80 years, having B12 and folic acid depletion and patients with these comorbid diseases (chronic renal failure, hepatic insufficiency, thyroid disease, heart failure and uncontrolled diabetes mellitus) were not included to the study. One hundred and four patients with rheumatoid arthritis were assessed for eligibility. Ninety-eight patients had current or past MTX treatment, 5 patients refused to participate in the study and 29 patients were excluded due to age or comorbid diseases. Therefore 64 RA patients were included to the study. Thirty-one age and sex similar (within ±2 years) healthy control subjects who were the members of the hospital staff and volunteered to participate in the study, were comprised. Informed consent was obtained from all the patients and controls, and the study was approved by the ethics committee of the hospital.

Demographic characteristics including age, sex, body mass index of all subjects, and disease duration and drug intake of RA patients were recorded. All the patients underwent a detailed clinical interview and physical examination. The number of patients having MTX-related adverse event was determined from the files and patient interviews.

Clinical measurements

The numbers of tender and swollen joints as well as disease and treatment duration were collected from patient files. DAS 28 was calculated for each patient using well-known calculations which included the number of tender and swollen joints, erythrocyte sedimentation rate (ESR), and patients' global assessment of general health expressed on visual analog scale.[20] The scores of Health Assessment Questionnaire (HAQ) indicating the functional status of RA patients were recorded from the files.[21,22] The ESR, C reactive protein (CRP), Disease Activity Score (DAS) 28 and HAQ were considered as disease activity parameters.

Laboratory measurements

Blood samples of the patients were taken to determine hemoglobin (Hb), ESR using the Westergren method, rheumatoid factor (RF) using nephelometric method and CRP by turbidimetric method in RA group. The blood samples were centrifuged and the plasma samples were frozen at −20 °C until the analysis of homocysteine. Plasma homocysteine was measured by enzyme-linked immunoassay according to the manufacturer's instructions (Axis Biochemicals, ASA Norway). The homocysteine levels among patient and control groups were compared. The relationship between homocysteine level and, disease characteristics and side effects was also analyzed.

Genomic studies

Genomic DNA was prepared from peripheral blood of patients and controls. The 2R and 3R polymorphisms of the TS gene were analyzed by polymerase chain reaction amplification as described previously with slight modifications.[23] The PCR amplification program consisted of an initial denaturation step at 95 °C for 15 min, followed by 30 cycles of denaturation of 94 °C for 1 min, annealing at 64 °C for 1 min, extension at 72 °C for 1 min and a final extension of 72 °C for 10 min. PCR products were separated by 6% denaturing polyacrylamide gel electrophoresis (PAGE) for 3 h at 300 V and visualized by ethidium bromide. The allelic frequencies and genotype distributions among patient and control groups were compared. The distribution of genotypes according to disease characteristics and side effects was also analyzed.

Statistical analysis

Descriptive statistics were performed and indicated as mean ± standard deviation and median (maximum–minimum) for continuous variables. All qualitative data are expressed as frequencies and percentages. The normality of the distribution of continuous variables was analyzed by Shapiro–Wilk test. Differences in genotype distribution were tested with two sided chi-square test. The intergroup comparisons for continuous variables between the two groups (with and without polymorphism) were performed by Student *t* test and Mann–Whitney *U* test. Spearman's correlation test was used to evaluate correlation between continuous variables and Pearson's chi square test or Fisher's exact outcome chi square tests were used to define the categorical variables. The level of significance was set to 0.05. All statistical analyses were done using SPSS for windows version 11.5.

Results

A total of 64 RA patients and 31 control subjects were included to the study. Demographic data of RA patients and control subjects are presented in Table 1. There was no statistical difference between the mean age, sex and BMI of the patient and control groups ($p > 0.05$). Most patients were receiving MTX at the time of recruitment but some had discontinued MTX treatment due to adverse effects. Folic acid supplementation with a mean dose of 5 mg folic acid/weekly was recorded in all patients.

Thirty-four (53%) patients were using MTX and other DMARDs. 38 (59.4%) patients were using oral MTX, while 26 (40.6%) patients were on subcutaneous MTX therapy. The mean duration and dose of the MTX were 2 years (0.2–10) and 15 mg/week (10–20) respectively.

Thirty-six of the 64 patients (56.2%) had experienced adverse effects to MTX treatment, of which gastrointestinal toxicity was the most common. MTX had been discontinued in 12 (18.8%) patients due to adverse side effects. There was no significant difference in the MTX dosage, the demographic

Table 1 – Demographic and clinical characteristics of patients and controls.

	Patients ($n=64$)	Controls ($n=31$)
Age (mean ± SD) (years)	48.7 ± 12.5	46.2 ± 13.4
Sex (female/male)	53/11	26/05
BMI (kg/m^2)	27 ± 5	27.5 ± 4.7
Duration of the disease (years)	6.5 (0.50–34)	
Duration of MTX treatment (years)	2 (0.2–10)	
Concurrent DMARD treatment (n) (%)	34 (53.1%)	
Rheumatoid factor positivity (n) (%)	41 (64.1%)	
Homocysteine (μmol/L)(median)	11.2 (5–146)	10.1 (6–18)

BMI, body mass index; MTX, methotrexate; DMARD, disease-modifying antirheumatic drugs.

and clinical features between the patients with and without adverse effects during MTX treatment ($p>0.05$).

The frequencies of TS2R/TS3R polymorphisms were determined in all subjects and were found to be statistically similar between patient and control groups (Table 2). The distribution of allele polymorphisms in regard to MTX-related adverse events was carried out. TS2R/TS3R gene polymorphisms were found to be similar in patients with and without MTX-related adverse events (Table 3). In order to define the relationship between MTX-related side effects and polymorphisms in TS gene, we have analyzed the patients receiving only MTX treatment. The distribution of TS 2R and 3R polymorphisms in patients with and without adverse effects was again found to be similar ($p>0.05$) (Table 4).

Table 4 – The genotypic distribution according to adverse events in patients receiving only methotrexate treatment.

Variables	Adverse effect (–) ($n=13$)	Adverse effect (+) ($n=17$)	p
TS 2R2R	3 (23.1%)	7 (41.2%)	
TS 2R3R	5 (38.5%)	7 (41.2%)	
TS 3R3R	5 (38.5%)	3 (17.6%)	
2R3R + 3R3R	10 (76.9%)	10 (58.8%)	0.440

TS, thymidylate synthase.

The distribution of disease activity parameters in regard to allele polymorphisms was also determined. No statistical difference among ESR, CRP, HAQ and DAS 28 values in regard to TS 2R and 3R polymorphisms was observed in the patient group (Table 5).

The mean plasma homocysteine levels were statistically similar in patient and control subjects. In the patient group, the mean homocysteine level was correlated with ESR levels ($p<0.05$, $r=30$) and found to be significantly higher in patients with MTX-related side effects than in patients without side effects (Table 6). The homocysteine values were also similar in patients with and without TS gene polymorphism (Table 7).

Discussion

The most studied polymorphisms in the TS gene was described by Horie et al.[24] The TS gene contains a tandem repeat found in the 5′ untranslated region and subjects were observed with two tandem repeats, three tandem repeats or heterozygous genotype. This tandem repeat region acts as an

Table 2 – The distribution of genotypes (TS polymorphisms) in patient and control groups.

	Control ($n=31$) n (%)	RA ($n=64$) n (%)	p	Odds ratio (% CI)
TS 2R2R	6 (19.4%)	14 (21.9%)	–	1.000[a]
TS 2R3R	20 (64.5%)	33 (51.6%)	0.539	0.707 (0.234–2.137)
TS 3R3R	5 (16.1%)	17 (26.6%)	0.593	1.457 (0.366–5.801)
2R3R + 3R3R	25 (80.6%)	50 (78.1%)	0.778	0.857 (0.294–2.499)

TS, thymidylate synthase; CI, confidence interval.
[a] Reference category.

Table 3 – The genotypic distribution of RA patients according to presence of adverse events.

	Adverse events (–) ($n=28$)	Adverse events (+) ($n=36$)	p	Odds ratio (95% CI)
TS 2R2R	4 (14.3%)	10 (27.8%)	–	1.000[a]
TS 2R3R	14 (50.0%)	19 (52.8%)	0.375	0.543 (0.141–2.093)
TS 3R3R	10 (35.7%)	7 (19.4%)	0.098	0.280 (0.062–1.266)
2R3R + 3R3R	24 (85.7%)	26 (72.2%)	0.195	0.433 (0.120–1.567)

CI, confidence interval; TS, thymidylate synthase.
[a] Reference category.

Table 5 – The distribution of disease activity parameters according to the polymorphisms in thymidylate synthase gene.

Variables	2R2R (n = 14)	2R3R (n = 33)	3R3R (n = 17)	p
HAQ (median)	0.8 (0–3)	0.6 (0–3)	0.75 (0–2.5)	0.695
DAS28 (median)	4.0 (2.1–7.5)	4.0 (1.7–7.8)	4.5 (1.9–6.9)	0.909
ESR (median)	29.0 (10.0–120.0)	30.0 (2.0–82.0)	25.0 (7.0–65.0)	0.695
CRP (median)	0.7 (0.3–10.0)	0.7 (0.1–10.5)	0.8 (0.2–4.8)	0.409

HAQ, health assessment questionnaire; DAS, disease activity score; ESR, erythrocyte sedimentation rate; CRP, C-reactive protein.

enhancer to the TS promoter and leads to an increase in TS activity. The promoter enhancer region of TS gene contains a double (2R) or a triple (3R) 28-base-pair (bp) tandem repeat polymorphism where 3R genotype is associated with a higher TS gene expression.[24,25]

Our study showed that the frequency of TS2R and TS3R polymorphisms was similar in Turkish RA patients and healthy control subjects. The rates of polymorphism in the tandem repeat of 5′ UTR region were significantly lower from the previous studies which could be related to ethnic differences.[26]

Previous authors reported that genotyping for TS polymorphism may become a useful indicator for determining the appropriate dosage of MTX and for monitoring MTX toxicity in RA patients.[2,6,9,11] There have been confusing data regarding the impact of TS polymorphism on MTX treatment response in RA patients, as TS is one of the enzymes in the folate metabolic pathway that is directly inhibited by MTX. A lower disease activity and an improved response to low dose MTX were reported in patients having TS polymorphism.[2] Kumagai et al.[9] and Inoue et al.[11] evaluated the relationship between the response to MTX and TS genotype in Japanese patients and reported a better response to MTX in patients with 6 bp alleles in TS 3′ UTR region. But the regarded patients in their study were on low dose (6 mg/week) MTX therapy. On the other hand, the 3R/3R genotype was associated with poor response to MTX therapy in some previous studies.[6,13] Regarding MTX toxicity, polymorphism of TS gene was associated with occurrence of alopecia[18] while no relation between polymorphism of TS gene and MTX toxicity was reported in other previous studies.[6] Contrary to some previous studies, the present study also showed that genetic polymorphisms in the TS gene did not influence the disease activity or toxicity of MTX treatment in RA patients receiving folate supplementation. Grabar et al.[8] studied a group of gene polymorphism including TS and reported no relationship between polymorphisms and disease activity parameters including ESR, CRP and DAS 28 values. Regarding the adverse effects due to MTX treatment, TS2R/3R polymorphisms were found to decrease bone marrow toxicity in their study group.[8] In the presence of risk genotype combinations among variants in TS and other genes, a higher likelihood to exhibit toxicities was reported.[27] Table 8 represents a review of published literature on TS gene polymorphisms affecting MTX treatment and/or toxicity in RA patients.

MTX inhibits the enzyme dihydrofolate reductase, thereby depleting the pool of reduced folates and producing a state of effective folate deficiency.[1] Folic acid improves MTX tolerability rates without compromising efficacy.[1,2] 5 mg/day folic acid has been proposed to be given in all patients receiving MTX therapy.[28,29] In our study all of the patients were on concomitant folic acid supplementation, which might be a limitation of our study. It would be valuable to study these gene expressions according to side effects of MTX, in both patients on folate supplementation and in patients not receiving this supplementation. Our study also included a relatively small number of patients and not all of the polymorphic genes involved in the MTX metabolism were tested. Therefore additional data might be needed to demonstrate more conclusive results. But our study was not biased by genetic heterogeneity, as all the patients were members of an ethnically homogeneous population. We have previously studied polymorphisms in MTHFR A1298C and C677T genes but failed to show any relationship with toxicity of MTX in the same group of RA patients.[30] The discrepancies between previous studies and the present study might be due to the differences in the frequencies of TS2R/3R homozygotic polymorphisms, since our study group has lower frequencies of this polymorphisms (5% and 6%) than in previous studies.[5,18]

Table 6 – Homocystein levels according to the presence of adverse effects of methotrexate in patients with AR.

	Homocystein	p
Adverse effects		0.024
Absent	10.7 (5.0–33.0)	
Present	12.45 (6.8–146.0)	

Table 7 – The levels of homocystein according to the polymorphism of Thymidylate synthase gene.

	Homocystein	p
		0.893[a]
TS 2R2R	11.3 (5.2–146.0)	
TS 2R3R	11.0 (5.2–50.0)	
TS 3R3R	11.0 (5.0–42.0)	
2R3R + 3R3R	11.0 (5.0–50.0)	0.655[b]

TS, thymidylate synthase.
[a] Variance analysis among genotypes.
[b] Comparison between genotypes with risky allele and reference genotype.

Table 8 – Literature review on TS gene polymorphisms affecting MTX treatment and/or toxicity in RA patients.

Author	RA patients (*n*)	Studied polymorphisms	MTX efficacy	MTX toxicity	Receiving folic acid
Dervieux et al.[26]	255	TS 2R/3R (*and other genes*)	ns	Increased toxicity	Not known
Dervieux et al.[2]	108	TS 2R/3R (*and other genes*)	Increased MTX response	Not studied	1 mg/day
Takatori et al.[10]	124	TS 6 bp deletion polymorphism (*and other genes*)	ns	ns	Not known
Derwieux et al.[6]	48	TS 2R/3R (*and other genes*)	ns	ns	+ (95% of patients)
Weisman et al.[18]	214	TS2R/3R 3R (*and other genes*)	ns	Increased alopecia	+ (81% of patients)
Grabar et al.[8]	213	TS 2R/3R (*and other genes*)	ns	Increased bone marrow toxicity	+ (62–91% patients)
Ghodke et al.[5]	34	TS 2R/3R and 6bp deletion	ns	ns	Not known
Inoue et al.[11]	36	TS 6bp deletion and TS 2R/3R	6bp deletion related with CRP and better response to MTX	Not studied	5 mg/week
Kumagai et al.[9]	105	TS 2R/3R and 6bp deletion	Poor response to MTX	ns	Not known
Ranganathan et al.[13]	222	TS 1494 of TTAAAG (*and other genes*)	Not studied	ns	1–3 mg/day
Zeng et al.[7]	85	TS 3R/2R	Not studied	ns	–
Present study	64	TS 2R/3R	ns	ns	5 mg/week

TS, thymidylate synthase; MTX, methotrexate; ns, non-significant.

Plasma homocysteine levels were reported to be related with inflammatory markers in some RA patients[16,17] in accordance with our data indicating a relationship between ESR and homocysteine levels in our study group.

Previous studies on the effect of MTX on homocysteine concentrations in patients with RA showed a substantial rise in homocysteine concentrations during MTX treatment, an effect that was diminished by folate supplementation.[31] Plasma homocysteine levels were measured in 105 RA patients and patients receiving MTX showed a persistent greater increase in plasma homocysteine which was explained by further enhancement of MTHFR polymorphism. These authors concluded that plasma homocysteine may be important in mediating the gastrointestinal side effects of MTX.[15] Some previous authors speculated that impeded homocysteine metabolism introduced by intracellular conversion of folate was the mechanism of the increased frequency of toxicities in patients with the mutant allele at C677T polymorphism.[4,29] In our study the homocysteine levels were similar between patients and healthy control subjects which may be due to the regular receiving of folate supplementation in all patients. Previous studies, in agreement with our study, indicated that MTX doses with adjuvant folic acid did not increase homocysteine levels.[28] But homocysteine levels were found to be higher in patients with adverse effects due to MTX than in patients without adverse effects. This may be explained by the active disease in patients who have to decrease or stop MTX treatment due to side effects.

Methotrexate metabolism is complex and involves the interaction of a number of genes in the folate pathway. In our study, 2R and 3R polymorphisms in the TS gene were not related with MTX efficacy or toxicity in RA patients receiving folate supplementation. We speculate that the effects of other genetic polymorphisms on the sensitivity to MTX toxicity may be stronger than those of TS 2R and 3R gene polymorphisms. However, homocysteine levels were correlated with ESR levels, and found to be higher in patients with adverse effects than in patients without these effects, which might indicate the effect of homocysteine on disease activity and/or MTX toxicity.

In conclusion homocysteine levels may affect the disease activity and toxicity of MTX but 2R and 3R polymorphisms in the TS gene were not related with MTX-related toxicity in RA patients receiving folate supplementation. Further larger studies are needed to illuminate the reasons and

polymorphisms in other enzymes that might be responsible from clinical toxicity of MTX in patients suffering from RA.

Conflicts of interest

The authors declare no conflicts of interest.

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