2756GG Genotype of Methionine Synthase Reductase Gene Is More Prevalent in Rheumatoid Arthritis Patients Treated with Methotrexate and Is Associated with Methotrexate-Induced Nodulosis

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ABSTRACT. ***Objective.*** To investigate the distribution of the A2756G polymorphism of the methionine synthase reductase (MTR) gene in patients with rheumatoid arthritis (RA) treated with methotrexate (MTX) compared with a healthy control group; and to examine the relationships among the A2756G polymorphism, plasma total homocysteine (tHcy), serum folate and vitamin B12 levels, disease activity, and MTX toxicity in patients with RA.

Methods. A cross-sectional study was performed on 86 MTX-treated RA patients, consisting of a clinical interview and physical examination to determine disease activity and MTX-related adverse reactions. Genotype analysis of the MTR gene was performed. Fasting plasma tHcy, serum folate, and vitamin B12 levels were measured. Allele and genotype distributions were compared to a healthy control group.

Results. The frequency of the 2756GG genotype (16.3%) in the RA study group was higher than that expected in the general population (3.6%; $p < 0.000001$). This genotype was associated with MTX-induced accelerated rheumatoid nodulosis (MIARN). No association of disease activity variables or plasma homocysteine with MTR A2756G polymorphisms was observed. The MTR 2756GG genotype, low plasma vitamin B12 levels, and the presence of rheumatoid nodules predicted MIARN. No association of nodulosis with any other indicator of disease activity or medical treatment was found.

Conclusion. In our population of MTX-treated RA patients the 2756GG genotype of the MTR gene was more common than expected and was associated with MIARN. (First Release July 1 2007; J Rheumatol 2007;34:1664–9)

Key Indexing Terms:
RHEUMATOID ARTHRITIS
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Pharmacogenetics, the study of genetic differences and their influence on individual therapeutic responses and tolerability to pharmacological agents, holds great promise for therapeutic medicine. Advances in this area have been slow, as the complexity of gene polymorphisms and drug interactions was underestimated. Nevertheless, there has been a steady influx of data that identify gene-drug interactions and suggest their implication in therapeutic effects and/or toxicity[1].

Rheumatoid arthritis (RA) is a chronic inflammatory disease affecting about 1% of the general population[2]. The new biologic agents recently introduced for RA treatment were developed following an understanding of the pathophysiologic mechanisms involved and the manner in which they target specific molecules. In contrast, methotrexate (MTX), the most frequently used disease modifying antirheumatic drug (DMARD) for RA and many other immune diseases, was introduced for this indication without understanding its mechanism of action[3,4]. MTX is a structural analog of folic acid that inhibits dihydrofolate reductase and influences several

other steps in folate metabolism resulting in cellular folate depletion. The mechanisms behind inter-patient variability in MTX efficacy and toxicity are unknown, but there is evidence for the role of genetic factors[5]. The influence of genes encoding folate pathway enzymes in patients with RA treated with MTX has been studied, and the efficacy and toxicity of MTX were associated with the C677T and A1298C polymorphisms of the MTHFR gene[6-11]. Other than the antifolate activity, MTX exerts its antiinflammatory action by inhibition of AICAR transformylase, an enzyme involved in purine synthesis, resulting in enhanced release of adenosine, a potent endogenous antiinflammatory mediator that activates specific receptors on different cells[12,13]. Ingestion of coffee, rich in caffeine, an adenosine receptor antagonist, interferes with the efficacy of MTX in RA patients with a genetic background similar to that of our cohort[14].

Methionine synthase reductase (MTR) is an enzyme that catalyzes remethylation of homocysteine to methionine using methyltetrahydrofolate formed by MTHFR as methyl donor and vitamin B12 as a cofactor[15]. Methionine is a precursor of S-adenosylmethionine, the universal methyl donor for over 100 biochemical reactions, especially important in DNA methylation. A2756G is a common polymorphism of the MTR gene, with allele frequencies ranging between 0.15 and 0.2, and may cause changes in enzymatic activity following DNA hypomethylation[15,16]. This polymorphism has been associated with birth defects[17], cognitive defects in the elderly, various malignancies[18,19], and sudden sensorineural hearing loss[20], and has not been studied in RA.

Our aims were to investigate the MTR A2756G polymorphism distribution in MTX-treated RA patients compared with a healthy control group, and to determine the relationships among this polymorphism, disease activity, and MTX-related adverse effects.

MATERIALS AND METHODS

Patients. Eighty-six consecutive RA patients treated with MTX were recruited from the rheumatology outpatient clinics of 3 university hospitals. All patients met the American College of Rheumatology revised criteria for RA[21]. All participants underwent a clinical interview and physical examination to determine disease activity, MTX toxicity, and dietetic history. MTX-related adverse effects were defined as one or the combination of gastrointestinal symptoms (nausea, abdominal pain, diarrhea) appearing repeatedly in association with MTX consumption, oral ulcers, elevated liver enzyme tests (alanine aminotransferase more than twice the upper limit of normal), MTX-induced or accelerated skin nodules, hematological toxicity (peripheral blood leukocyte count < 3500), unexplained respiratory complaints or pulmonary infiltrates, and megaloblastic anemia. MTX-induced accelerated rheumatoid nodulosis (MIARN) was diagnosed when it developed in a patient taking MTX without previous rheumatoid nodules or in an area previously not affected by rheumatoid nodules, usually multiple nodules and mainly affecting fingers. Side effects were evaluated during patient recruitment, and confirmed by reviewing patients' medical files. Disease activity was evaluated by European League Against Rheumatism criteria for disease activity measurement of RA that include number of tender and swollen joints, pain score, and physician and patient global score on a visual analog scale[22].

Blood was drawn after 10 h fast 3 to 6 days after the weekly MTX dose. Plasma and serum samples were separated promptly and stored until assayed at –80°C. The population control sample was drawn from the national population registry. Individuals in the control group were of similar age and ethnic background to our RA patient group, i.e., the study group comprised Ashkenazi Jews (32%), non-Ashkenazi Jews (52%), and Arabs (16%), and the control group comprised Ashkenazi Jews (41%), non-Ashkenazi Jews (42%), and Arabs (17%). The study was approved by the ethics committee of the hospital, and informed consent was obtained from all participants.

Biochemistry and genetic analysis. Serum folate and vitamin B12 levels were determined using commercial kits (Vitamin B12 Elecsys reagent kit No. 1820753 and Folate Elecsys reagent kit No. 1820761) using an automated electrochemiluminescence immunoassay (ELICIA). Assays were performed on a Roshe Elecsys 2010 immunoassay analyzer.

Plasma total homocysteine, the sum of protein-bound and free homocysteine, was determined by a procedure modified from Araki and Sako[6,23]. Genomic DNA was prepared from peripheral blood. The genotyping protocol for MTR A2756G polymorphism detection was adapted from Matsuo, *et al*[19] with the following primers: 5'-TGT TCC AGA CAG TTA GAT GAA AAT C-3' and 5'-GAT CCA AAG CCT TTT ACA CTC CTC-3'. Polymerase chain reaction (PCR) conditions were modified as follows: 5 min initial at 94°C followed by 35 cycles at 95°C for 60 s, 60°C for 90 s, and 72°C for 60 s, and the PCR product was digested by *Hae* III (Roche Diagnostics, Indianapolis, IN, USA).

Statistical methods. For comparison of quantitative variables between 2 groups (with/without polymorphism), the 2-sample t test and the nonparametric Mann-Whitney test were applied. When 3 groups were compared (2756GG, 2756AG, 2756AA) the ANOVA procedure was used. The chi-square test and Fisher's exact test were used to assess associations between the various polymorphism groups and the different side effects, or any other qualitative variables among the study and control groups. All statistical tests were 2-tailed, and a p value ≤ 5% was considered statistically significant. A logistic regression model was applied to assess which variables simultaneously correlated with the appearance of MIARN.

RESULTS

Patients' characteristics according to genotypes. The baseline demographic and clinical data comparing the various characteristics of patients with RA carrying the different A2756G polymorphisms are shown in Table 1. Patients in all 3 groups received similar doses of MTX (mean 12.03 ± 3.86 mg/wk) for a similar duration of time (mean 3.37 ± 3.13 yrs). In addition, no difference between the groups was observed regarding the number of patients using additional DMARD, prednisone, or folate supplementation. None of the A2756G genotypes (AA, AG, GG) showed an association with individual indicators of disease activity (erythrocyte sedimentation rate, C-reactive protein levels) or clinical characteristics including number of tender and swollen joints, morning stiffness, pain scores, and physician and patient global scores as measured on a visual analog scale[22].

Genotype and allele distribution. We determined the allele and genotype distributions among the RA patients and compared them with a control population of 324 healthy subjects. Genotype distribution among RA patients was significantly different from that in the control population: 16.3% of RA patients carried the 2756GG genotype compared to only 3.6% of the control group ($p < 0.000001$; Table 2). When allele frequency was compared between the 2 groups, the G allele was significantly more frequent among RA patients (39.5%) compared to controls (14%) ($p < 0.001$). This indicates a statisti-

Table 1. Patients' characteristics.

Characteristic	Total, n = 86	MTR 2756 2756GG, n = 14	2756AG, n = 40	2756AA, n = 32
Age, yrs	58.4 ± 13.8	58.4 ± 9.0	57.4 ± 16.5	59.3 ± 12.3
Females, %	82.8	71.4	87.5	81.8
Rheumatoid factor, IgM, %	68.6	71.4	67.5	68.8
Duration of MTX treatment, yrs	3.37 ± 3.13	3.71 ± 3.50	3.63 ± 2.94	2.87 ± 3.25
MTX dose, mg/wk	12.03 ± 3.84	13.04 ± 3.13	11.81 ± 4.15	11.87 ± 3.76
Folate supplementation, mg/wk	5.6 ± 8.3	8.6 ± 12.0	4.6 ± 7.9	5.8 ± 9.0
CRP, mg/100 ml	2.1 ± 2.9	2.7 ± 2.1	1.5 ± 1.8	2.8 ± 4.5
Rheumatoid nodules	20 (23.3)	6 (42.9)	7 (17.5)	7 (21.9)
No. of tender joints	7.1 ± 7.4	6.5 ± 6.3	7.6 ± 9.0	6.9 ± 6.4
No. of swollen joints	2.6 ± 3.5	2.0 ± 3.3	2.5 ± 4.0	2.8 ± 3.3
Pain score	3.7 ± 2.9	4.6 ± 2.9	3.6 ± 2.8	3.5 ± 3.0
Patient global score	3.6 ± 2.8	3.9 ± 2.9	3.6 ± 2.8	3.5 ± 3.0
Physician global score	3.0 ± 2.2	3.8 ± 2.5	2.7 ± 2.1	3.0 ± 2.4
Morning stiffness, h	0.6 ± 0.8	1.1 ± 1.3	0.5 ± 0.7	0.4 ± 0.6

No significant differences between 2756 GG, AG, and AA genotype carriers for various clinical determinants. Number of carriers of each genotype in parentheses. ANOVA: analysis of variance.

Table 2. MTR 2756 polymorphism distribution and allele frequency in patients and controls. Data are percentages.

	Genotypes*			Allele Frequencies**	
	GG	AG	AA	G	A
Study group, n = 86	16.3	46.5	37.2	39.5	60.5
Control group, n = 324	3.6	20.4	76.0	14	86

* MTR 2756 genotype distribution significantly different between RA patients treated with MTX and controls (one-sample chi-square analysis, $p < 0.000001$). ** Allele frequencies different between RA patients treated with MTX and controls (Fisher's exact test, $p < 0.001$).

cally significant association between the study group and distribution of genotypes/alleles.

MTR 2756 genotype polymorphism and MTX-related side effects. A higher rate of 2756GG genotype carriers was found among patients with MTX-induced side effects, but this did not achieve statistical significance. Thirty patients experienced at least one MTX side effect, of whom 23.3% carried the 2756GG genotype, while among the 56 patients who were free of side effects, 12.5% were 2756GG carriers (Table 3). Analyzing the different side effects (see Materials and Methods), the number of MTR 2756GG genotype carriers was significantly higher among RA patients with MIARN than among those without it. Four out of 10 patients with MIARN had 2756GG genotype (40%), while only 10 out of 76 RA patients without this side effect carried this genotype (13.2%; chi-square test for $p = 0.013$).

MIARN and clinical and laboratory characteristics. Using univariate analysis, MTX dosage and duration of treatment, use of other DMARD and prednisone, folic acid supplementation, and serum folate and plasma total homocysteine (tHcy) levels were found not to be significantly different between RA patients with and those without MIARN (Table 4). Previous rheumatoid nodules were much more common in the patients with MIARN (60%) compared to those without nodules (18.4%; $p < 0.01$). In subjects with MIARN, serum vitamin B12 levels were significantly lower than in those without MIARN ($p = 0.03$). Carriage of 2756GG genotype, low plasma vitamin B12 levels, and the presence of rheumatoid nodules predicted MIARN. No correlation of MIARN with any individual measure of disease activity or medical treatment was found.

DISCUSSION

Polymorphisms of MTR gene in patients with RA have not been studied previously. We found significantly higher frequency of the 2756GG genotype and allele frequency of the MTR gene in RA patients treated with MTX compared to controls. The frequency of this genotype in our control group was similar to that in other populations (Asians 2%–3%, Caucasians 1%–5%)[24]. Despite a number of promising candidate genes, a non-MHC gene has not been definitely identified in susceptibility to RA[1].

Our findings suggest that the 2756GG genotype of the MTR gene is associated with increased susceptibility to RA in

Table 3. MTR 2756 genotypes and MTX-related side effects and MTX-induced nodulosis.

	No. of Patients	AA (%)	AG (%)	GG (%)
Patients with MTX-induced nodulosis	10	0	6 (60)	4 (40)
Without MTX-induced nodulosis	76	32 (42.1)	34 (44.7)	10 (13.2)
Patients with any side effect	30	9 (30)	14 (46.7)	7 (23.3)
Without side effects	56	23 (41.1)	26 (46.4)	7 (12.5)
Patients with any GI side effects	11	4 (36.4)	4 (36.4)	3 (27.3)
Without GI side effects	75	28 (37.3)	36 (48)	11 (14.7)
Patients with ulcers	8	4 (50)	3 (37.5)	1 (12.5)
Without ulcers	78	28 (35.9)	37 (47.4)	13 (16.7)
Patients with elevated liver function results	3	0	3	0
Without elevated liver function results	83	32 (38.6)	37 (44.6)	14 (16.9)

* Number of MTR 2756GG carriers significantly higher among RA patients with MTX-induced nodulosis than among those without it (Fisher's exact test p = 0.006).

Table 4. Clinical and laboratory factors and MTX-induced nodulosis.

	MTX-Induced Nodulosis	No. of Patients	Mean ± SD	p
MTX dose, mg/wk	–	76	12.04 ± 3.95	
	+	10	12.00 ± 2.92	NS
MTX duration, yrs	–	76	3.16 ± 3.33	NS
	+	10	4.90 ± 2.93	
Folate supplementation, mg/wk	–	76	5.53 ± 7.95	NS
	+	10	6.00 ± 11.25	
Prednisone, mg/day	–	76	4.19 ± 3.96	NS
	+	10	4.50 ± 4.00	
Other DMARD, %	–	76	36.8	NS
	+	10	60	
Serum folate, ng/ml*	–	63	9.03 ± 5.62	NS
	+	10	9.56 ± 6.77	
Plasma homocysteine, µmol/l**	–	68	13.15 ± 4.36	
	+	10	13.20 ± 5.70	NS
Serum B12, pg/m***	–	61	400.1 ± 211.3	0.03
	+	10	223.3 ± 88.5	
Morning stiffness	–	71	0.51 ± 0.74	NS
	+	10	0.96 ± 1.08	
Rheumatoid nodules, %	–	76	18.4	0.01
	+	10	60	

No significant difference (t test) between patients with and those without MTX-induced nodules in folic acid consumption, serum folic acid level, plasma tHcy level, and MTX dosage. * Data available from only 73 participants. ** Data available from only 78 participants. *** Data available from only 71 participants.

the population we studied. Conceivably, this polymorphism distribution may stem from the cross-sectional design of the study and reflect a selection bias due to therapeutic response to MTX or discontinuation of MTX due to side effects. Evaluation of the A2756G polymorphisms in other RA populations is required in order to reinforce the significance of this polymorphism in RA.

The overall rate of MIARN in our cohort (11.6%) is similar to that reported by others. The prevalence of MIARN varies between 8% and 22% in different series[25-27].

We found a high rate of 2756GG genotype carriers among patients with MIARN (40% as compared to 13.2% in patients without this nodulosis). In addition, among patients with MIARN, 70% carry the G allele, while among patients without MIARN only 35% carry this allele, suggesting a dose response for the G allele with respect to MIARN (p = 0.006, Fisher exact test).

A correlation of MIARN with a genetic factor has been described previously. In a retrospective study of 66 RA patients the frequency of carriers of HLA-DRB1*0401 allele was significantly higher in patients with MIARN compared to other MTX-treated patients. This allele frequency distribution could represent a more aggressive form of rheumatoid disease and may not be a specific marker for MIARN development[28].

In our cohort the 2756GG genotype was not associated with disease activity, suggesting the specificity of this polymorphism for MIARN development.

Most of the side effects associated with MTX (gastrointestinal, hepatic, and myelosuppressive) are thought to be related to its folate antagonism in tissues with high cell turnover requiring purines, thymidine, and methionine[25,29]. An association of these side effects with genotypes of certain enzymes involved in alteration of folate metabolism was found in several studies[6-8], and in addition, folic acid supplementation reduces such toxicity significantly[30]. In our study the appearance of MIARN did not correlate with either folic acid intake or serum folate concentrations. In a previous study we did not find MIARN development to be associated with the MTHFR C677T or A1298C polymorphisms, suggesting a folate-independent mechanism of MTX and nodulosis formation[4].

Upon finding an association between A2756G polymorphisms and the development of MIARN, we investigated the correlation between this type of nodulosis and various clinical and laboratory characteristics in our patients. Sixty percent of our patients with MIARN had had rheumatoid nodules before starting MTX treatment, a rate similar to that reported previously[28], compared to 18.4% in patients without MIARN.

Another biochemical indicator associated with MIARN development was the serum vitamin B12 level. This association suggests a proposed role for MTR using vitamin B12 as a cofactor in nodulosis formation. Vitamin B12 deficiency is a known contributer to decreased MTR activity, and to DNA hypomethylation in rats[31]. In addition, vitamin B12 deficiency and decreased MTR activity may lead to intracellular folate deficiency through depletion of methyl donor tetrahydrofolate[32].

The mechanism for development of MIARN is unknown. A single *in vitro* study showed that adenosine augmented with MTX followed by stimulation of A2 receptors on cultured monocytes resulted in antiinflammatory effects, while stimulation of A1 receptors caused cell migration, fusion to multinuclear giant cells, and spindle-shaped formation of monocytes, cells similar to those seen in nodules[33]. Adenosine A1 antagonists reversed this process. These data suggest that MIARN may develop by stimulation of the adenosine A1 receptor on macrophages, and many other investigations showed that the antiinflammatory effects of MTX are mediated at least in part by increase in extracellular concentrations of adenosine and its binding to the A2 receptors[34,35].

Our data indicate that patients carrying a homozygous variant genotype (2756GG) of the MTR gene may have an increased incidence of MIARN compared with those carrying the AG or AA genotypes. These data may be consistent with the hypothesis that MTX produces its antiinflammatory and nodulosis-inducing effects in part through release of adenosine. One possible explanation is that the 2756GG genotype may alter the enzymatic activity of MTR, resulting in a decrease in methionine and S-adenosylmethionine concentration and activity, and thus more adenosine is available to activate A1 receptors, resulting in nodule formation.

Other studies, including our own, have shown no association between the MTR A2756G polymorphism and homocysteine concentrations[36].

MIARN is treated by discontinuation of MTX or addition of other DMARD. It has been suggested that DMARD might have protective effects against MIARN[26,28]. We found no correlations between MIARN activity and the use of prednisone, hydroxychloroquine, or any DMARD other than the 2756GG genotype and vitamin B12 level. Lack of an influence of DMARD on MIARN in our cohort supports other recent findings[37].

In summary we report a possible significant association between MTR 2756GG genotype carrier state and RA among patients treated with MTX. Further, this genotype may be related to the development of MTX-induced accelerated rheumatoid nodulosis. Since this study was performed among patients with RA taking MTX, it may suffer from selection bias. Conceivably, 2756GG carriers may respond well to MTX treatment with minimal side effects and consequently they remained within the cohort. However, this genotype renders some of the patients susceptible to nodulosis formation. Further studies are needed to evaluate MTR A2756G polymorphism distribution among patients with RA in different populations in order to establish an association that may broaden our understanding of the pathogenesis of RA.

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