Common Polymorphisms in the Folate Pathway Predict Efficacy of Combination Regimens Containing Methotrexate and Sulfasalazine in Early Rheumatoid Arthritis

HEATHER M. JAMES, DAVID GILLIS, PRAVIN HISSARIA, SUSAN LESTER, ANDREW A. SOMOGYI, LESLIE G. CLELAND, and SUSANNA M. PROUDMAN

ABSTRACT. ***Objective.*** To study genetic polymorphisms in the folate pathway, a site of action of methotrexate (MTX) and sulfasalazine (SSZ), as predictors of efficacy of combination disease modifying antirheumatic drug (DMARD) regimens containing MTX and SSZ in early rheumatoid arthritis (RA).

Methods. Ninety-eight Caucasian patients with early RA received MTX with SSZ, hydroxychloroquine, and folate according to a standardized protocol. Efficacy was evaluated using the Disease Activity Score (DAS28) and European League Against Rheumatism response criteria at 12 months. Nine polymorphisms in 7 genes of the folate pathway were studied.

Results. Response to therapy was associated with *SLC19A1, MTR,* and *TYMS* polymorphisms. Two favorable allele combinations associated with responder status at 12 months were identified: the *MTR* 2756A allele in combination with either the *SLC19A1* 80A allele or the *TYMS* 3R-del6 haplotype (multivariate analysis, $p = 0.0002$, $p = 0.009$ respectively). Seventy of the 72 patients with these allele combinations responded compared to 12/24 patients without [odds ratio (OR) 35.0, 95% confidence interval (CI) 6.9-176, $p < 0.0001$]. An association with remission (DAS28 < 2.6) was also observed (OR 3.4, 95% CI 1.1-10.0, $p = 0.04$). When analyzed over 3 years, both the change in DAS score from baseline and the final DAS scores were significantly higher and lower, respectively, with the favorable genotype group ($p < 0.0001$, $p < 0.0001$).

Conclusion. Polymorphic variations in the *MTR*, *SLC19A1*, and *TYMS* genes were associated with better clinical response to combination DMARD regimens containing MTX and SSZ. Allele combinations of these genes may predict response to combination DMARD and assist in treatment decisions in patients with early RA. (First Release Mar 1 2008; J Rheumatol 2008;35:562–71)

Key Indexing Terms:
METHOTREXATE RHEUMATOID ARTHRITIS
GENETIC POLYMORPHISM PHARMACOGENETICS FOLATE PATHWAY

Methotrexate (MTX) is the cornerstone of treatment of rheumatoid arthritis (RA) and is the most widely used disease modifying antirheumatic drug (DMARD) in newly diagnosed patients[1-3]. However, the use and efficacy of MTX varies among treated patients[4]. Increasingly, early, intense disease suppression, ideally remission, is considered necessary if joint damage is to be avoided; moreover, remission is unlikely to be achieved with monotherapy[5]. Further, factors predictive of poor prognosis tend to be unreliable in the individual patient, although combinations of adverse factors may increase the power of prediction. With the advent of new, typically expensive therapies, the ability to predict an individual patient's response to MTX, particularly in combination with other DMARD, would permit newer therapies to be targeted to those otherwise less likely to respond and to limit joint damage by avoiding prolonged use of MTX in unhelpful combinations. Certainly in some countries, prescribing criteria for subsidized use of agents such as tumor necrosis factor (TNF) inhibitors require prior failure of certain treatment regimens, often combinations containing optimal doses of MTX. Consequently, use of DMARD combinations that include MTX, particularly in patients

From the Division of Human Immunology, Institute of Medical and Veterinary Science; Arthritis Research Laboratory, Hanson Institute; Discipline of Pharmacology, School of Medical Sciences, University of Adelaide; and Rheumatology Unit, Royal Adelaide Hospital, Adelaide, Australia.

Supported by a Royal Adelaide Hospital Clinical Project Grant, Adelaide, and the National Health and Medical Research Council of Australia.

H.M. James, MSc; D. Gillis, MBBS, FRCPA, FRACP; P. Hissaria, MD, FRCPA, Division of Human Immunology, Institute of Medical and Veterinary Science; S. Lester, BSc (Hons), Arthritis Research Laboratory, Hanson Institute; A.A. Somogyi, PhD, Discipline of Pharmacology, School of Medical Sciences, University of Adelaide and Royal Adelaide Hospital; L.G. Cleland, MD, FRACP; S.M. Proudman, MBBS (Hons), FRACP, Rheumatology Unit, Royal Adelaide Hospital.

Address reprint requests to Dr. H.M. James, Division of Human Immunology, Institute of Medical and Veterinary Science, PO Box 14, Rundle Mall, Adelaide SA 5000 Australia. E-mail: heather.james@imvs.sa.gov.au

Accepted for publication November 20, 2007.

deemed to have a poor prognosis, has become commonplace. The triple therapy regimen of MTX, sulfasalazine (SSZ), and hydroxychloroquine (HCQ) has been demonstrated to be effective with minimal toxicity[6], and most recently in an inception cohort of recent onset RA[7].

MTX has a complex mechanism of action including inhibition of enzymes of folate metabolism with the accumulation of adenosine, a potent antiinflammatory agent, believed to be one mediator of the therapeutic effects of MTX in RA[8-10]. Polymorphisms in the genes of the folate pathway are potentially responsible for interpatient variation in MTX efficacy and toxicity. MTX enters the cell through the solute carrier family 19, folate transporter, SLC19A1 (Figure 1) and is converted to MTX-polyglutamates, which inhibit thymidylate synthase (TYMS), dihydrofolate reductase (DHFR), and 5-aminoimidazole-4-carboxamide ribonucleotide formyltransferase/IMP cyclohydrolase (ATIC)[9]. Other key enzymes in the folate pathway are 5,10-methylenetetrahydrofolate reductase (MTHFR), serine hydroxymethyltransferase 1 (SHMT1) and 5-methyltetrahydrofolate-homocysteine methyltransferase (MTR). *TYMS* polymorphisms in the promoter and 3' untranslated region (3'UTR) have been associated with clinical outcomes in RA patients, and varying results are reported for *SLC19A1* G80A, *ATIC* C347G, *MTHFR* C677T, and *MTHFR* A1298C[11-18]. Altered levels of plasma homocysteine, a biomarker for disruption of the folate cycle, have been observed in patients with the 19bp deletion in intron 1 of *DHFR*, *SHMT1* C1420T, and *MTR* A2756G[19-22]. Elucidation of the pharmacogenetics of MTX treatment in RA is complex and is likely to be multigenic, with complex gene–gene interactions. Composite allele and haplotypic analysis as opposed to individual polymorphisms may be more informative than single allele studies[9].

The mechanism of action of SSZ is not completely understood but it also exhibits an antiinflammatory action

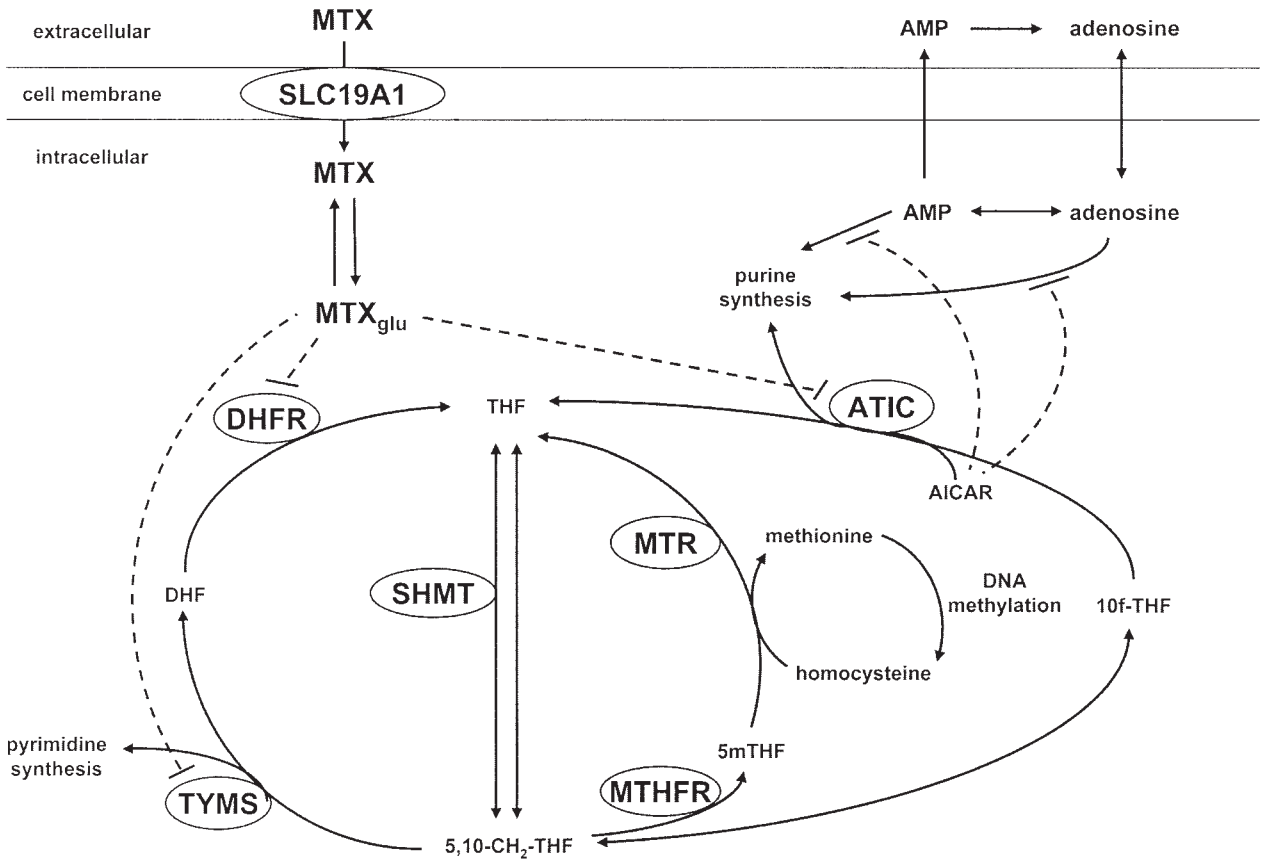

Figure 1. Simplified representation of methotrexate action on the folate pathway. Genes are shown in ovals. ATIC = 5-aminoimidazole-4-carboxamide ribonucleotide formyltransferase/IMP cyclohydrolase; DHFR = dihydrofolate reductase; MTHFR = 5,10-methylenetetrahydrofolate reductase; MTR = 5-methyltetrahydrofolate-homocysteine methyltransferase; SHMT1 = serine hydroxymethyltransferase 1; SLC19A1 = solute carrier family 19, folate transporter (reduced folate carrier); TYMS = thymidylate synthase; AICAR = aminoimidazole-carboxamide ribonucleotide; AMP = adenosine monophosphate; DHF = dihydrofolate; THF = tetrahydrofolate; 5,10-CH_2THF = 5,10-methylene tetrahydrofolate; 5mTHF = 5 methyl-tetrahydrofolate; 10f-THF = 10-formyl-tetrahydrofolate; MTX = methotrexate; MTX_{glu} = methotrexate polyglutamates.

believed to be mediated by adenosine accumulation[23-25]. SSZ has been shown to inhibit enzymes of the folate pathway[26,27], and alter plasma homocysteine levels[28]. However polymorphisms of the folate pathway have not been studied in the context of SSZ efficacy. There is no evidence to date that HCQ has any influence on the folate pathway.

We examined common polymorphisms in a range of candidate genes involved in the folate pathway to assess their role as predictors of clinical improvement, assessed at 12 months, in our inception cohort of patients with early RA receiving MTX in combination with SSZ and HCQ, with pre-defined dosage adjustments according to disease activity.

MATERIALS AND METHODS

RA patient population and treatment. Consecutive patients presenting with recent onset polyarthritis to the Early Arthritis Clinics at the Royal Adelaide and The Queen Elizabeth Hospitals, as well as private practices of participating rheumatologists, were assessed[7]. Included patients were over age 18 years and had a diagnosis of RA according to the 1987 revised American College of Rheumatology criteria[29], symptoms of polyarthritis of more than 6 weeks and less than 24 months, a swollen joint count (SJC) ≥ 3, a tender joint count (TJC) ≥ 6, and erythrocyte sedimentation rate (ESR) > 28 mm/h and/or C-reactive protein (CRP) > 10 mg/l. The protocol stipulated exclusion of patients who had taken other DMARD at any stage or antimalarials for more than 1 month. Patients initially received MTX 10 mg orally weekly, folic acid 500 µg daily, SSZ 500 mg daily orally, increasing over 4 weeks to 1 g twice daily, and HCQ 200 mg twice daily. DMARD doses were adjusted according to a standardized treatment algorithm based on predetermined disease activity criteria[7]. DMARD dose or treatment was adjusted if SJC > 1 (maximum 44 joints) and CRP and/or ESR were elevated or if one of these and 2 or more of the following criteria were fulfilled: TJC > 1 (maximum 53 joints), early morning stiffness > 30 min, patient assessed pain > 30 mm [on a 100 mm visual analog scale (VAS)], and fatigue > 30 mm (on a 100 mm VAS). Briefly, SSZ was increased to 3 g/day, then, if disease was still assessed as active, the dose of MTX was serially increased to a maximum of 25 mg/week. Additional DMARD were then added (leflunomide, gold sodium thiomalate, azathioprine, then cyclosporin A). If at any stage, the Australian prescribing criteria for a TNF inhibitor were fulfilled, one of these agents was added. In the event of drug intolerance or toxicity, dosage adjustments including drug withdrawal if severe, were also made according to pre-defined rules where applicable (e.g., elevated transaminases, reduced neutrophil count) as well as patient preference. Based on the difficulties in defining lack of efficacy of any 1 component of a DMARD combination, DMARD were discontinued on the basis of toxicity only.

This pharmacogenetic study was limited to Caucasian patients. Efficacy was analyzed in the patients who received MTX for at least 3 months after enrolment. A further 6 patients receiving MTX therapy for less than 3 months were included in the analysis of adverse events. The study was approved by the research ethics committees of the Royal Adelaide and Queen Elizabeth hospitals according to the Helsinki Declaration and patients gave informed written consent.

Patients were assessed at baseline for anti-cyclic citrullinated peptide (anti-CCP) and rheumatoid factor (RF) by ELISA and nephelometry, respectively, with positive results defined as anti-CCP ≥ 6 and RF ≥ 20. Shared epitope positivity was based on the amino acid sequence at 70-74 following DNA sequencing of HLA-DRB1. Assessments at baseline and every 6 weeks were made to assess efficacy (SJC, TJC, VAS for patient assessed disease activity, pain, and fatigue, stiffness, CRP, and ESR) and adverse events including elevated transaminases, reduced neutrophil count and patient-reported intolerance[7].

Efficacy evaluation. Efficacy was assessed as improvement between baseline and 12 months based on the DAS28 score[30], with patients classified as responders (good or moderate) or nonresponders according to the European League Against Rheumatism (EULAR) criteria[31]. Clinical remission at 12 months was defined as a DAS28 score < 2.6. Radiographs were scored by readers blinded to patient identity but not chronological order, with data evaluated at baseline, 12, and 36 months using modified Sharp scores (both erosion and total scores)[7,32]. Progression was defined as an increase in score of ≥ 2 (erosions) and ≥ 5 (Sharp score) based on our smallest detectable difference[7].

Genotype determination. Genetic variants of the folate pathway were chosen based on clinical relevance in previous publications[11-14,20,21,33-36], effect on plasma homocysteine levels[19-22] and with the minor allele frequency in Caucasians of ~20% or more. The DHFR C829T polymorphism shown to influence DHFR expression in a Japanese study[37] was not included due to its low incidence in Caucasian populations[20]. *SHMT1* C1420T (leu474phe), *SLC19A1* G80A (Arg27His, rs1232027), *TYMS* 2R/3R (28 bp variable number of tandem repeats, VNTR, in the promoter, and single nucleotide polymorphism, SNP, G116C within this region, 3G/3C) were detected using polymerase chain reaction (PCR) and restriction enzyme digest[38-41]. *DHFR* (19bp deletion in intron 1) was detected by allele specific PCR[36]. *TYMS* ins6/del6 (1494delTTAAAG, 6 bp deletion in 3'UTR) was detected using PCR[42] and separation using 3% Nusieve GTG agarose (Cambrex)/1% agarose gel. Real-time Taqman allelic discrimination was used for the *ATIC* C347G (thr116ser, rs2372536) and *MTR* A2756G (asp919gly, rs1805087) polymorphisms[12,41]. *MTHFR* variations C677T (ala222val, rs1801133) and A1298C (glu429ala, rs1801131) were assayed by primer extension using the ABI PRISM SNaPshot multiplex system (Applied Biosystems) according to the manufacturer's instructions. Control samples for all assays were confirmed by direct DNA sequencing.

Statistical analysis. Univariate associations between responder status at 1 year (responder vs nonresponder) were analyzed by logistic regression for both additive (allele dose) and genotype models. Because of relative sparseness of the genotype tables, significant genotype associations were confirmed by estimating exact p values by permutation (MCMC) using RxC software (Mark Miller, http://www.marksgeneticsoftware.net/). *MTHFR* and *TYMS* haplotypes, and their association with responder status, were analyzed by an additive genetic model, using hapassoc v1.1 (http://stat-db.stat.sfu.ca:8080/statgen/research/hapassoc/) software for R, which implements the EM algorithm for haplotype estimation and incorporates haplotype phase uncertainty in the inference of haplotype trait associations. Genes significant in the univariate analysis were subsequently analyzed in a multivariate genetic additive model with 2 factor interactions, and the best allelic combination predictive of a favorable response was determined using the Akaike Information Criteria (AIC). Associations were expressed as odds ratios (OR) derived by exponentiation of the relevant logistic regression coefficient. To extend our findings relating to the DAS responder status at 12 months, multivariate (i.e., repeated measures type) exponential growth curve models were fitted to the DAS and radiographic scores from baseline to 3 years. Analysis was performed using both BAUW v1.2[43] and WinBUGS v4.1 Bayesian analysis software.

RESULTS

The baseline characteristics of 98 patients are summarized in Table 1. These patients were MTX naive. Eighty-six patients were treated with the triple drug regimen (MTX+HCQ+SSZ), 9 received MTX+HCQ, 2 patients were treated with MTX+SSZ, and 1 received MTX monotherapy. Eighty-nine patients received MTX for the full 12-month period, and the remaining 9 patients took MTX for a mean of 34 weeks (range 31–50 wks). The median MTX dose was

Table 1. Clinical characteristics of 98 early RA patients at time of enrolment. Values are the median (interquartile range) except where indicated otherwise.

Characteristic	Baseline Value
Age, yrs	57 (44–69)
Female/male, %	75/25
Duration of polyarthritis, wks	12 (8–20)
DAS28	5.9 (5.1–6.4)
Baseline erosions present, %	33
Baseline erosion score	0 (0–1)
Total Sharp score	7 (4–11)
CCP antibody positive, %	49
RF positive, %	51
Shared epitope positive, %	60
CRP, mg/l	13 (6–32)
ESR, mm/h	36 (21–51)

RA: rheumatoid arthritis; DAS: Disease Activity Score; CCP: cyclic citrullinated peptide; RF: rheumatoid factor; CRP: C-reactive protein; ESR: erythrocyte sedimentation rate.

15 mg (range 10–25). By 12 months, no patient had progressed to receive a TNF inhibitor.

Using the EULAR response criteria, 49 (50%) patients had a good response, 34 (35%) had a moderate response, and 15 (15%) were nonresponders at 12 months. DMARD efficacy was analyzed further according to the responder (good and moderate) versus nonresponder status. Responder status was not associated with gender, anti-CCP, CRP, RF, shared epitope, baseline erosions, or other baseline characteristics.

The allele frequencies for the 9 folate transport and metabolism polymorphisms were similar to published Caucasian population frequencies (Table 2). There was minor deviation from Hardy-Weinberg equilibrium in the *MTR* locus ($p = 0.04$) due to lower than expected heterozygosity, but all other loci were in Hardy-Weinberg equilibrium.

A logistic regression model of responder status, fitting genotypes of all 9 polymorphisms simultaneously, demonstrated an overall genetic difference between responders and nonresponders ($p = 0.001$, permutation test). Univariate analysis demonstrated that responder status was primarily associated with polymorphisms of the *SLC19A1, MTR*, and *TYMS* genes (Table 3). Patients with the *SLC19A1* 80A variant allele were more likely to be responders [OR = 3.1, 95% confidence interval (CI) 1.3-7.7]. There was a strong association of *MTR* genotype and the response ($p = 0.0003$) with patients carrying the wild type *MTR* 2756A allele being more likely to respond (OR = 2.6, 95% CI 1.1-6.1). The *TYMS* promoter 2R/3R VNTR genotype was also associated with responder status ($p = 0.009$), and all 29 3R/3R homozygote variants were classified as responders. While not statistically significant, a similar trend was observed for the del6 allele of the *TYMS* 3'UTR ins6/del6 polymorphism.

Table 2. Allele frequency of polymorphic variants of the 7 genes studied in 98 early RA patients.

Gene	Polymorphism	Minor Allele	Frequency of Minor Allele
MTHFR	C677T	677T	0.30
MTHFR	A1298C	1298C	0.34
SLC19A1	G80A	80A	0.45
TYMS	promoter VNTR 2R→3R	3R	0.46
TYMS	3'UTR 6 bp deletion	6 bp deletion (del6)	0.29
DHFR	19 bp deletion in intron 1	19 bp deletion	0.39
SHMT1	C1420T	1420T	0.33
MTR	A2756G	2756G	0.19
ATIC	C347G	347G	0.45

The *TYMS* promoter 2R/3R VNTR and 3'UTR 6bp deletion were in strong linkage disequilibrium ($p = 1 \times 10^{-15}$), forming 3 common haplotypes: 2R-ins6, 3R-ins6, and 3R-del6 with frequencies of 0.28, 0.23, and 0.44, respectively. Consistent with the individual polymorphism results (Table 3), the 3R-del6 haplotype was associated with a clinical response (OR 2.9, 95% CI 1.0–9.2). The additional G/C single-nucleotide polymorphism (SNP) within the *TYMS* VNTR subdivided the favorable 3R-del6 haplotype, but was uninformative in terms of the responder status (data not shown). Further analysis utilized the *TYMS* 3R-del6 haplotype rather than the individual polymorphisms.

To further investigate the relationship between clinical response and the 3 genes associated with responder status at 12 months, we analyzed a multivariate allele dosage model of *SLC19A1* 80A allele, *MTR* 2756A allele, and the *TYMS* 3R-del6 haplotype with 2-factor interactions. The best model, selected by AIC criteria, comprised not main effects, but rather 2 interaction terms indicative of epistatic interaction effects between these genes. Patients with either the *SLC19A1* 80A/*MTR* 2756A allele combination ($p = 0.0002$) or the *TYMS* 3R-del6 haplotype/*MTR* 2756A allele combination ($p = 0.009$) are more likely to be responders following combination DMARD therapy. Of the 72 patients with these favorable allele combinations, 70 (positive predictive value 97%) responded to treatment (Table 4), whereas only 12/24 (negative predictive value 50%) of patients without either of these allele combinations were responders (OR 35.0, CI 6.9–176, $p < 0.0001$). Remission (DAS28 < 2.6) was achieved in 34/72 (47%) of patients with a favorable allele combination compared to 5/24 (21%) of patients without (OR 3.4, CI 1.1–10.0, $p = 0.04$).

Adverse events attributed to MTX by the treating clinician occurred in 50 (48%) of the total cohort (n = 104) and were not associated with the genetic polymorphisms studied. The 6 patients who took MTX for less than 3 months had similar allele frequencies for all polymorphisms compared to the 98 patients who continued with MTX therapy.

DAS scores decreased rapidly over the first 6 months with a plateau beyond 1 year. To extend our findings relating to the favorable/unfavorable genotype combination and

Table 3. Responder status and genotypes of *SLC19A1*, *MTR*, *TYMS* *MTHFR, ATIC, SHMT1,* and *DHFR* in 98 early RA patients.

Polymorphism	Genotype Distribution		p_{exact}	Allele	Allele Frequencies		p
	Responders, n = 83 (%)	Nonresponders n = 15 (%)			Responders, n = 83	Nonresponders, n = 15	
SLC19A1 G80A							
AA	20 (24)	1 (7)		A	0.49	0.23	0.009
GA	41 (49)	5 (33)	0.036	G	0.51	0.77	
GG	22 (27)	9 (60)					
MTR A2756G							
AA	58 (70)	10 (67)		A	0.84	0.67	0.06
AG	23 (28)	0 (0)	0.0003	G	0.16	0.33	
GG	2 (2)	5 (33)					
*TYMS 2R/3R**							
3R/3R	29 (35)	0 (0)		3R	0.57	0.39	0.09
2R/3R	35 (43)	11 (79)	0.009	2R	0.43	0.61	
2R/2R	18 (22)	3 (21)					
TYMS 6bp del							
del6/del6	10 (12)	0 (0)		del6	0.31	0.17	0.10
ins6/del6	32 (39)	5 (33)	0.31	ins6	0.69	0.83	
ins6/ins6	41 (49)	10 (67)					
MTHFR C677T							
CC	43 (52)	6 (40)		C	0.71	0.63	0.39
CT	32 (39)	7 (47)	0.64	T	0.29	0.37	
TT	8 (10)	2 (13)					
MTHFR A1298C							
AA	36 (43)	6 (40)		A	0.66	0.67	1.00
AC	38 (46)	8 (53)	0.92	C	0.34	0.33	
CC	9 (11)	1 (7)					
ATIC C347G							
CC	22 (27)	3 (20)		C	0.56	0.50	0.56
CG	49 (59)	9 (60)	0.85	G	0.44	0.50	
GG	12 (14)	3 (20)					
SHMT1 C1420T							
CC	37 (45)	6 (40)		C	0.67	0.67	1.00
CT	37 (45)	8 (53)	0.92	T	0.33	0.33	
TT	9 (11)	1 (7)					
DHFR 19bpdel, intron 1							
w/w	30 (36)	6 (40)		w	0.61	0.60	1.00
w/del	41 (49)	6 (40)	0.80	del	0.39	0.40	
del/del	12 (15)	3 (20)					

* Two patients carrying *TYMS* VNTR alleles with > 3 repeats were excluded from the analysis.

Table 4. Association of clinical outcome at 12 months and favorable allele combination*.

Clinical Outcome	Allele Combination†		Odds Ratio	p
	Favorable, n = 72 (%)	Other, n = 24 (%)		
Responder status				
Responders	70 (97)	12 (50)	35 (6.9–176)	< 0.0001
Nonresponders	2 (3)	12 (50)		
Remission				
Remission	34 (47)	5 (21)	3.4 (1.1–10)	0.04
Nonremission	38 (53)	19 (79)		

* Favorable allele combination consists of *MTR* 2756A allele in combination with either the *SLC19A1* 80A allele or the *TYMS* 3R-del6 haplotype.
† Two patients carrying *TYMS* VNTR alleles with > 3 repeats were excluded from the analysis.

DAS responder status at 12 months, a multivariate exponential growth curve analysis was performed on the DAS scores from baseline to 3 years (Figure 2). The DAS scores (mean ± SE) were not significantly different between the genotype groups at baseline (favorable 5.46 ± 0.18 and unfavorable 5.07 ± 0.31, p = 0.28). However, the plateau of the DAS scores was significantly higher in RA patients with the unfavorable genotype (3.63 ± 0.27 compared with 2.34 ± 0.17, p = 0.00007) and consequently, the overall improvement in DAS score was significantly greater in RA patients with the favorable genotype (–3.12 ± 0.21) compared with the unfavorable genotype (–1.45 ± 0.36), p = 0.00004. Therefore the effects of the favorable/unfavorable genotypic combinations on DAS responder at 12 months reflected sustained differences in the DAS response over 3 years. Further, mean

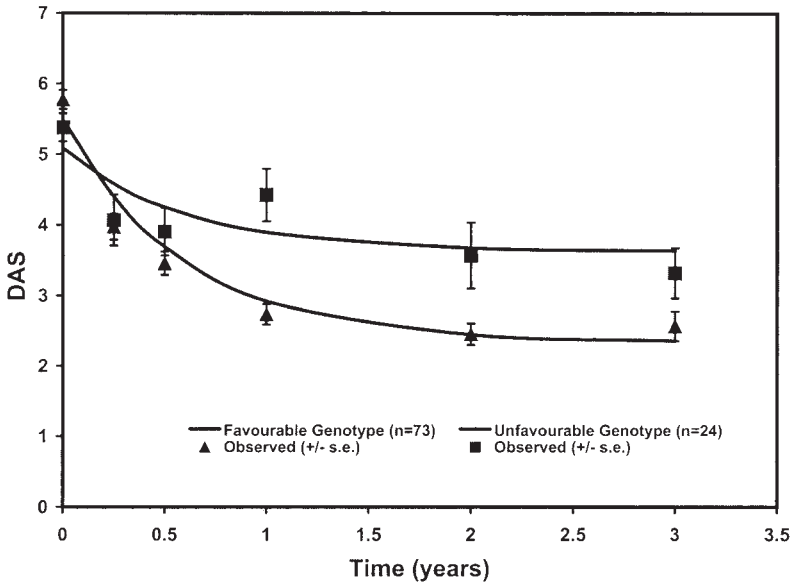

Figure 2. Changes in DAS scores over time by genotype. Analysis was performed by multivariate exponential growth curve analysis. The fitted model was of the form DAS = $ß_{11}$ + $ß_{12} - (ß_{21} + ß_{22})e^{-b*Time}$, where the ß terms are the regression coefficients for each genotype. The observed data points (± standard error) and the fitted regression curves (solid lines) are both shown. The DAS scores decrease over the first year, and then plateau. The baseline DAS scores were not significantly different between the 2 genotype groups (p = 0.28), but the change in DAS scores over time were significantly different (p = 0.00004).

plateau DAS scores in patients with favorable genotype combinations were consistent with ACR remission criteria (2.34 ± 0.17), whereas DAS scores for patients with unfavorable genotypes (3.63 ± 0.27) were not.

Radiographic data were available for 92 patients. Erosion progression between baseline and 12 months was observed in 13/92 (14%) patients, and progression in the modified Sharp score was observed in 22/92 (24%) patients. The total joint scores were higher overall in the RA patients with the unfavorable genotype (probably reflecting the higher DAS scores), but there was no evidence of an increased rate of progression compared with the favorable genotype group at either 12 months, or in the extended analyses over 36 months (data not shown).

DISCUSSION

Ours is the first study in which associations between genetic polymorphisms at multiple loci in the folate pathway and efficacy of MTX and SSZ used in combination DMARD therapy have been studied in patients with early RA. In this cohort, the alleles *SLC19A1* 80A variant, the *TYMS* 3R variant, and the *MTR* 2756A wild-type were associated with better control of disease activity after MTX treatment administered in combination with SSZ and HCQ. We found evidence of gene interaction with the allelic combinations of *MTR* 2756A with either *SLC19A1* 80A or *TYMS* 3R-del6 being predictive of patients who were more likely to respond to treatment. Perhaps more importantly, this enabled us to identify a subset of 25% of patients who were less likely to respond to treatment as assessed by the responder status. Further, the patients with favorable genotype combinations defined using responder status at 12 months maintained a better DAS response up to 3 years compared to patients with the unfavorable genotype combinations.

Earlier publications in this field centered around the utility of variants in single genes in the folate pathway for predicting the efficacy of MTX[35,44]. As MTX has a very complex mechanism of action in RA[10], it is more informative to analyze multiple genes in this pathway[11-15,18,45], but previous studies have been limited by cross-sectional design, have been performed in cohorts of patients with RA in varying stages of disease, or have involved limited followup. In addition, studies to date have been based on MTX monotherapy, and although this minimizes confounding factors, MTX in combination with other DMARD is now commonly used in order to achieve rapid disease suppression.

Previous studies on the role of *TYMS* polymorphisms in the response to MTX therapy in RA have yielded conflicting results. The *TYMS* del6/del6 genotype was associated with a significantly improved response to MTX as assessed by CRP in a Japanese study[11], and this is consistent with our finding that the 3R-del6 haplotype was associated with responder status. In other RA studies *TYMS* alleles have been classified only as 2R or 3R. In a recent 6 month longi-

tudinal study of MTX monotherapy, no association between 2R/3R status and DAS response was seen[18], but in a cross-sectional study[12], 2R/2R homozygotes were associated with a better clinical response. Our haplotype analysis indicates that these 2R homozygotes would be predominantly 2R-ins6 homozygotes, and so the results of this cross-sectional study[12] differ from our results and those previously reported in Japanese patients with RA[11].

TYMS 3R promoter variant has been found to be associated with increased mRNA expression[39,46,47] and the 6bp del in the 3'UTR of *TYMS* is associated with decreased mRNA stability and expression[48]. In cancer association studies, it has been suggested that both the functional variants 2R/3R and ins6/del6 should be studied simultaneously[34]. Further, an additional G/C single-nucleotide polymorphism within the 3R VNTR is associated with greater translation efficiency of the 3G allele with higher *TYMS* expression and is associated with chemotherapy response[40,49,50]. The 3G/3C SNP subdivided the 3R-del6 haplotype identified in this study, but was uninformative in terms of response to therapy. The overall functional significance of the 3R-del6 haplotype, identified in the current study as influencing response to therapy in RA, is not known. We believe it is not yet clear which *TYMS* polymorphism is the better marker of MTX response in RA and *TYMS* haplotypic analysis should be considered in assessing efficacy and toxicity of MTX in RA until the role of these polymorphisms is further elucidated.

Our results for *SLC19A1* were in agreement with those of previous studies in which an association of *SLC19A1* 80AA genotype with efficacy was observed[12,16], although this was not confirmed in other studies[13,15]. The functional effect of the *SLC19A1* G80A variation is not clear, although RA patients with the homozygote variant genotype had a higher level of the active metabolite, MTX-polyglutamate, compared to the G/G or G/A genotypes[9,12,51].

The *MTR* A2756G polymorphism has been studied in the context of DAS response to MTX treatment in RA, although no association was observed[15,45]. In a recent study, an association of the variant *MTR* 2756G allele with RA susceptibility was observed[52]. Further, the *MTR* 2756GG genotype was associated with MTX-induced acceleration of rheumatoid nodulosis. Their cross-sectional study at a hospital outpatient clinic may have been enriched for patients who were more refractory to therapy, which is consistent with our finding that the *MTR* 2756G allele is less favorable[52]. Homozygous *MTR* 2756 GG individuals have lower homocysteine levels compared to individuals with the AA genotype[19,22], suggesting a higher enzyme activity with the GG genotype and relative resistance to MTX. Interestingly, similar to our results, an interaction between *MTR* A2756G and *TYMS* del6 alleles with colorectal adenoma risk was observed in women[33].

Previous pharmacogenetic studies of MTX efficacy in RA have been in the context of MTX monotherapy. We found no association of *MTHFR* polymorphism with disease activity following combination DMARD therapy. Recently, *MTHFR* C677T and A1298C polymorphisms were reported to be associated with efficacy of MTX monotherapy using a study design and patient population similar to our study but with a shorter, 6-month followup[13,15]. This association was not observed in a 12 month study[17]. The functional effects of the *MTHFR* polymorphism may be more evident with MTX monotherapy, or have less impact on clinical outcome at 12 months.

Pharmacogenetic studies involving MTX are likely to require multigenic analysis, but few studies have used this approach for the prediction of response to DMARD in RA. In a cross-sectional study, an additive pharmacogenetic index, based on genotypes of *SLC19A1* G80A, *TYMS* 2R/3R, and *ATIC* C347G, was associated with swollen and tender joint counts as well as disease activity as measured by the VAS[14]. In a recent 6 month longitudinal study of MTX monotherapy, 17 genetic polymorphic sites in 13 genes related to folate and adenosine pathways were examined[18]. Four of these genes, ATIC, methylenetetrahydrofolate dehydrogenase, adenosine monophosphate deaminase, and inosine triphosphate pyrophosphatase, were combined with clinical variables to give a pharmacogenetic score that was used with some success to predict response to MTX. However, these studies did not examine genetic interactions.

We found evidence of epistatic interactions between the *MTR* and *SLC19A1*/*TYMS* genes. The folate pathway includes a number of alternate pathways for regeneration of tetrahydrofolate (THF) from 5,10-methylene-tetrahydrofolate as shown in Figure 1. MTX polyglutamates inhibit the enzymes DHFR, TYMS, and ATIC, thus affecting 2 of the pathways of THF regeneration and resulting in increased production of adenosine, an antiinflammatory agent. Lower enzyme activity in patients with the *MTR* 2756A allele could result in reduced flux through a third pathway. Patients with the *SLC19A1* 80A have increased levels of MTX-polyglutamates so a higher level of inhibition of the MTX target enzymes could be expected. Functional studies of TYMS have focused on the individual polymorphisms rather than a *TYMS* haplotype, but we postulate that the overall functional effect of the *TYMS* 3R-del haplotype is a reduction in TYMS enzyme levels. The favorable allele combinations *MTR* 2756A with either *SLC19A1* 80A or *TYMS* 3R-del would have lower enzyme activity of MTR with either increased inhibition of DHFR, TYMS, and ATIC or decreased amount of TYMS. It is possible that the reduced MTR and TYMS activity play a key role in increased flux through alternate pathways of THF regeneration, with a resultant increased production of adenosine, an antiinflammatory agent. Further studies designed to elucidate the functional role of these polymorphisms and to achieve a better understanding of the complex folate cycle are required for a fuller understanding of these epistatic interactions.

Eighty-eight (90%) of the patients in our study were treated with SSZ in combination with MTX. SSZ is also a folate antagonist, which can interact with the transport protein SLC19A1, and like MTX, is an inhibitor of enzymes of the folate pathway including DHFR, ATIC, MTHFR, and SHMT[26,27,53]. The additional inhibition of these enzymes in patients receiving SSZ would be expected to enhance the flux through pathways leading to increased adenosine production. Differences in our results from studies of MTX monotherapy could be due to the combined effects of MTX and SSZ. As MTX is commonly used in combination with SSZ, our findings have "real life" relevance, which is underlined by the extent to which favorable genotype combinations are associated with therapeutic responses to DMARD regimens involving these agents.

The goal of therapy in RA is suppression of disease activity in order to reduce the cumulative disease burden over time and to minimize irreversible joint damage. We did not observe any associations with radiographic progression and folate pathway polymorphisms, despite the identified folate metabolism polymorphisms being associated with better suppression of disease activity in the first 12 months of treatment. This suggests a degree of disassociation between radiographic damage and joint inflammation[54] and/or a greater sensitivity of disease activity scores to change under conditions of the study.

We followed an inception cohort of patients with early RA, treated with a standardized DMARD regimen, longitudinally for 1 year to assess the influence of folate metabolism polymorphism on response to combination regimens containing MTX and SSZ. Ours is the first study to utilize haplotypic analysis of *TYMS* and to incorporate potential gene–gene interaction in the analysis to identify favorable genetic combinations. At 12 months, patients with the favorable allele combinations of *MTR* 2756A allele and either *SLC19A1* 80A or *TYMS* 3R-del6 haplotype were 35 times more likely to respond to a MTX-containing DMARD combination compared to other patients. Further, these genotypic effects on disease activity were sustained beyond 1 year. We acknowledge that these results may not be applicable to MTX monotherapy. However, it is of particular significance that these polymorphisms predict response to MTX in combination with other drugs (SSZ and HCQ) so that these results are relevant to the contemporary management of RA that is based on "triple therapy" (i.e., MTX, SSZ, and HCQ), which is safe and demonstrably superior to MTX monotherapy[6,7,55,56].

Analysis of common genetic polymorphisms allowed us to identify a small but important proportion of patients with less favorable genetics for whom alternative treatments such as TNF inhibition may be considered earlier. Future, larger prospective pharmacogenetic studies are needed and could ultimately enable selection of appropriate therapy for patients with RA at or soon after the time of diagnosis.

ACKNOWLEDGMENT

We thank Guiseppina Morelli, Matthew Fenech, Aygul Simsek, and Graeme Casey for their technical assistance, and Helen Keen, Lisa Stamp, Anita Lee, Fiona Goldblatt, Oliver Ayres, Maureen Rischmueller, Michael James, Catherine Hill, Gillian Caughey, Sam Whittle, Sally Cox, Eliza Pontifex, Fin Cai, Tim Lu, Debashish Danda, Leah McWilliams, and Cindy Hall for their involvement in patient care and data collection.

REFERENCES

1. Mikuls TR, O'Dell J. The changing face of rheumatoid arthritis therapy: results of serial surveys. Arthritis Rheum 2000;43:464-5.
2. O'Dell JR. Therapeutic strategies for rheumatoid arthritis. N Engl J Med 2004;350:2591-602.
3. Weinblatt ME, Coblyn JS, Fox DA, et al. Efficacy of low-dose methotrexate in rheumatoid arthritis. N Engl J Med 1985;312:818-22.
4. Alarcon GS, Tracy IC, Blackburn WD, Jr. Methotrexate in rheumatoid arthritis. Toxic effects as the major factor in limiting long-term treatment. Arthritis Rheum 1989;32:671-6.
5. Mottonen T, Hannonen P, Korpela M, et al. Delay to institution of therapy and induction of remission using single-drug or combination-disease-modifying antirheumatic drug therapy in early rheumatoid arthritis. Arthritis Rheum 2002;46:894-8.
6. O'Dell JR, Haire CE, Erikson N, et al. Treatment of rheumatoid arthritis with methotrexate alone, sulfasalazine and hydroxychloroquine, or a combination of all three medications. N Engl J Med 1996;334:1287-91.
7. Proudman SM, Keen HI, Stamp LK, et al. Response-driven combination therapy with conventional disease-modifying antirheumatic drugs can achieve high response rates in early rheumatoid arthritis with minimal glucocorticoid and nonsteroidal anti-inflammatory drug use. Semin Arthritis Rheum 2007;37:99-111.
8. Cronstein BN. Low-dose methotrexate: a mainstay in the treatment of rheumatoid arthritis. Pharmacol Rev 2005;57:163-72.
9. Ranganathan P, McLeod HL. Methotrexate pharmacogenetics: the first step toward individualized therapy in rheumatoid arthritis. Arthritis Rheum 2006;54:1366-77.
10. Kremer JM. Toward a better understanding of methotrexate. Arthritis Rheum 2004;50:1370-82.
11. Kumagai K, Hiyama K, Oyama T, Maeda H, Kohno N. Polymorphisms in the thymidylate synthase and methylenetetrahydrofolate reductase genes and sensitivity to the low-dose methotrexate therapy in patients with rheumatoid arthritis. Int J Mol Med 2003;11:593-600.
12. Dervieux T, Furst D, Lein DO, et al. Polyglutamation of methotrexate with common polymorphisms in reduced folate carrier, aminoimidazole carboxamide ribonucleotide transformylase, and thymidylate synthase are associated with methotrexate effects in rheumatoid arthritis. Arthritis Rheum 2004;50:2766-74.
13. Wessels JA, de Vries-Bouwstra JK, Heijmans BT, et al. Efficacy and toxicity of methotrexate in early rheumatoid arthritis are associated with single-nucleotide polymorphisms in genes coding for folate pathway enzymes. Arthritis Rheum 2006;54:1087-95.
14. Dervieux T, Furst D, Lein DO, et al. Pharmacogenetic and metabolite measurements are associated with clinical status in patients with rheumatoid arthritis treated with methotrexate: results of a multicentred cross sectional observational study. Ann Rheum Dis 2005;64:1180-5.
15. Dervieux T, Greenstein N, Kremer J. Pharmacogenomic and metabolic biomarkers in the folate pathway and their association with methotrexate effects during dosage escalation in rheumatoid arthritis. Arthritis Rheum 2006;54:3095-103.

16. Drozdzik M, Rudas T, Pawlik A, Gornik W, Kurzawski M, Herczynska M. Reduced folate carrier-1 80G>A polymorphism affects methotrexate treatment outcome in rheumatoid arthritis. Pharmacogenomics J 2007;7:404-7.
17. Hughes LB, Beasley TM, Patel H, et al. Racial or ethnic differences in allele frequencies of single-nucleotide polymorphisms in the methylenetetrahydrofolate reductase gene and their influence on response to methotrexate in rheumatoid arthritis. Ann Rheum Dis 2006;65:1213-8.
18. Wessels JA, van der Kooij SM, le Cessie S, et al. A clinical pharmacogenetic model to predict the efficacy of methotrexate monotherapy in recent-onset rheumatoid arthritis. Arthritis Rheum 2007;56:1765-75.
19. Chen J, Stampfer MJ, Ma J, et al. Influence of a methionine synthase (D919G) polymorphism on plasma homocysteine and folate levels and relation to risk of myocardial infarction. Atherosclerosis 2001;154:667-72.
20. Gellekink H, Blom HJ, van der Linden IJ, den Heijer M. Molecular genetic analysis of the human dihydrofolate reductase gene: relation with plasma total homocysteine, serum and red blood cell folate levels. Eur J Hum Genet 2007;15:103-9.
21. Heil SG, Van der Put NM, Waas ET, den Heijer M, Trijbels FJ, Blom HJ. Is mutated serine hydroxymethyltransferase (SHMT) involved in the etiology of neural tube defects? Mol Genet Metab 2001;73:164-72.
22. Harmon DL, Shields DC, Woodside JV, et al. Methionine synthase D919G polymorphism is a significant but modest determinant of circulating homocysteine concentrations. Genet Epidemiol 1999;17:298-309.
23. Gadangi P, Longaker M, Naime D, et al. The anti-inflammatory mechanism of sulfasalazine is related to adenosine release at inflamed sites. J Immunol 1996;156:1937-41.
24. Nurmohamed MT, Dijkmans BA. Efficacy, tolerability and cost effectiveness of disease-modifying antirheumatic drugs and biologic agents in rheumatoid arthritis. Drugs 2005;65:661-94.
25. Plosker GL, Croom KF. Sulfasalazine: a review of its use in the management of rheumatoid arthritis. Drugs 2005;65:1825-49.
26. Selhub J, Dhar GJ, Rosenberg IH. Inhibition of folate enzymes by sulfasalazine. J Clin Invest 1978;61:221-4.
27. Baggott JE, Morgan SL, Ha T, Vaughn WH, Hine RJ. Inhibition of folate-dependent enzymes by non-steroidal anti-inflammatory drugs. Biochem J 1992;282:197-202.
28. Haagsma CJ, Blom HJ, van Riel PL, et al. Influence of sulphasalazine, methotrexate, and the combination of both on plasma homocysteine concentrations in patients with rheumatoid arthritis. Ann Rheum Dis 1999;58:79-84.
29. Arnett FC, Edworthy SM, Bloch DA, et al. The American Rheumatism Association 1987 revised criteria for the classification of rheumatoid arthritis. Arthritis Rheum 1988;31:315-24.
30. Prevoo ML, van 't Hof MA, Kuper HH, van Leeuwen MA, van de Putte LB, van Riel PL. Modified disease activity scores that include twenty-eight-joint counts. Development and validation in a prospective longitudinal study of patients with rheumatoid arthritis. Arthritis Rheum 1995;38:44-8.
31. van Gestel AM, Haagsma CJ, van Riel PL. Validation of rheumatoid arthritis improvement criteria that include simplified joint counts. Arthritis Rheum 1998;41:1845-50.
32. van der Heijde D. How to read radiographs according to the Sharp/van der Heijde method. J Rheumatol 2000;27:261-3.
33. Goode EL, Potter JD, Bigler J, Ulrich CM. Methionine synthase D919G polymorphism, folate metabolism, and colorectal adenoma risk. Cancer Epidemiol Biomarkers Prev 2004;13:157-62.
34. Ulrich CM, Curtin K, Potter JD, Bigler J, Caan B, Slattery ML. Polymorphisms in the reduced folate carrier, thymidylate synthase, or methionine synthase and risk of colon cancer. Cancer Epidemiol Biomarkers Prev 2005;14:2509-16.
35. Urano W, Taniguchi A, Yamanaka H, et al. Polymorphisms in the methylenetetrahydrofolate reductase gene were associated with both the efficacy and the toxicity of methotrexate used for the treatment of rheumatoid arthritis, as evidenced by single locus and haplotype analyses. Pharmacogenetics 2002;12:183-90.
36. Johnson WG, Stenroos ES, Spychala JR, Chatkupt S, Ming SX, Buyske S. New 19 bp deletion polymorphism in intron-1 of dihydrofolate reductase (DHFR): a risk factor for spina bifida acting in mothers during pregnancy? Am J Med Genet A 2004;124:339-45.
37. Goto Y, Yue L, Yokoi A, et al. A novel single-nucleotide polymorphism in the 3'-untranslated region of the human dihydrofolate reductase gene with enhanced expression. Clin Cancer Res 2001;7:1952-6.
38. Chango A, Emery-Fillon N, de Courcy GP, et al. A polymorphism (80G->A) in the reduced folate carrier gene and its associations with folate status and homocysteinemia. Mol Genet Metab 2000;70:310-5.
39. Horie N, Aiba H, Oguro K, Hojo H, Takeishi K. Functional analysis and DNA polymorphism of the tandemly repeated sequences in the 5'-terminal regulatory region of the human gene for thymidylate synthase. Cell Struct Funct 1995;20:191-7.
40. Marcuello E, Altes A, del Rio E, Cesar A, Menoyo A, Baiget M. Single nucleotide polymorphism in the 5' tandem repeat sequences of thymidylate synthase gene predicts for response to fluorouracil-based chemotherapy in advanced colorectal cancer patients. Int J Cancer 2004;112:733-7.
41. Skibola CF, Forrest MS, Coppede F, et al. Polymorphisms and haplotypes in folate-metabolizing genes and risk of non-Hodgkin lymphoma. Blood 2004;104:2155-62.
42. Ulrich CM, Bigler J, Velicer CM, Greene EA, Farin FM, Potter JD. Searching expressed sequence tag databases: discovery and confirmation of a common polymorphism in the thymidylate synthase gene. Cancer Epidemiol Biomarkers Prev 2000;9:1381-5.
43. Zhang Z, Wang L. BAUW: Bayesian Analysis using Winbugs, Version 1.0. 2006. Available from: http://bauw.psychstat.org
44. van Ede AE, Laan RF, Blom HJ, et al. The C677T mutation in the methylenetetrahydrofolate reductase gene: a genetic risk factor for methotrexate-related elevation of liver enzymes in rheumatoid arthritis patients. Arthritis Rheum 2001;44:2525-30.
45. Wessels JA, Kooloos WM, De Jonge R, et al. Relationship between genetic variants in the adenosine pathway and outcome of methotrexate treatment in patients with recent-onset rheumatoid arthritis. Arthritis Rheum 2006;54:2830-9.
46. Kawakami K, Salonga D, Park JM, et al. Different lengths of a polymorphic repeat sequence in the thymidylate synthase gene affect translational efficiency but not its gene expression. Clin Cancer Res 2001;7:4096-101.
47. Pullarkat ST, Stoehlmacher J, Ghaderi V, et al. Thymidylate synthase gene polymorphism determines response and toxicity of 5-FU chemotherapy. Pharmacogenomics J 2001;1:65-70.
48. Mandola MV, Stoehlmacher J, Zhang W, et al. A 6 bp polymorphism in the thymidylate synthase gene causes message instability and is associated with decreased intratumoral TS mRNA levels. Pharmacogenetics 2004;14:319-27.
49. Mandola MV, Stoehlmacher J, Muller-Weeks S, et al. A novel single nucleotide polymorphism within the 5' tandem repeat polymorphism of the thymidylate synthase gene abolishes USF-1 binding and alters transcriptional activity. Cancer Res 2003;63:2898-904.
50. Kawakami K, Watanabe G. Identification and functional analysis of single nucleotide polymorphism in the tandem repeat sequence of thymidylate synthase gene. Cancer Res 2003;63:6004-7.
51. Dervieux T, Kremer J, Lein DO, et al. Contribution of common

polymorphisms in reduced folate carrier and gamma-glutamylhydrolase to methotrexate polyglutamate levels in patients with rheumatoid arthritis. Pharmacogenetics 2004;14:733-9.
52. Berkun Y, Abou Atta I, Rubinow A, et al. 2756GG genotype of methionine synthase reductase gene is more prevalent in rheumatoid arthritis patients treated with methotrexate and is associated with methotrexate-induced nodulosis. J Rheumatol 2007;34:1664-9.
53. Jansen G, van der Heijden J, Oerlemans R, et al. Sulfasalazine is a potent inhibitor of the reduced folate carrier: implications for combination therapies with methotrexate in rheumatoid arthritis. Arthritis Rheum 2004;50:2130-9.
54. Cohen G, Gossec L, Dougados M, et al. Radiological damage in patients with rheumatoid arthritis on sustained remission. Ann Rheum Dis 2007;66:358-63.
55. Grigor C, Capell H, Stirling A, et al. Effect of a treatment strategy of tight control for rheumatoid arthritis (the TICORA study): a single-blind randomised controlled trial. Lancet 2004;364:263-9.
56. Mottonen T, Hannonen P, Leirisalo-Repo M, et al. Comparison of combination therapy with single-drug therapy in early rheumatoid arthritis: a randomised trial. FIN-RACo trial group. Lancet 1999;353:1568-73.