Methotrexate (MTX) Pathway Gene Polymorphisms and Their Effects on MTX Toxicity in Caucasian and African American Patients with Rheumatoid Arthritis

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ABSTRACT. ***Objective.*** Research has examined the association of folate-dependent gene polymorphisms with methotrexate (MTX) toxicity in racially homogenous patients with rheumatoid arthritis (RA). We examined the influence of MTX transporter gene polymorphisms on MTX toxicity in 2 racial groups of patients with RA.

Methods. Using a retrospective cross-validation approach, the association of polymorphisms in 6 genes in the MTX cellular pathway with MTX toxicity was examined in training and validation cohorts. The genes analyzed were ATP-binding cassette transporter B1 (ABCB1), C1 (ABCC1), C2 (ABCC2), folylpolyglutamyl synthase (FPGS), methylenetetrahydrofolate reductase (MTHFR), and thymidylate synthase (TYMS). Both cohorts included Caucasian Americans and African Americans. Statistical analyses consisted of Fisher exact tests, multivariable logistic regression models, and survival analyses.

Results. Four of 25 variants displayed significant associations with MTX toxicity in the training cohort. The intronic single-nucleotide polymorphism (SNP) ABCC2 IVS 23+56 T → C was associated with alopecia in Caucasians ($p = 0.035$). ABCB1 1236 C → T was associated with overall toxicity ($p = 0.013$); ABCC2 1249 G → A with gastrointestinal toxicity ($p = 0.009$); and ABCC2 1058 G → A with hepatotoxicity ($p = 0.04$) in African Americans. These 4 SNP and the MTHFR 677 C → T variant were assessed in the validation cohort. Of these, only the MTHFR 677 C → T SNP was associated with alopecia, and only in African Americans ($p = 0.032$). The ABCC2 IVS 23+56 T → C genotype influenced toxicity-related time to discontinuation or dose decrease in the Caucasian validation cohort ($p < 0.0001$).

Conclusion. In addition to SNP in folate-dependent genes, MTX transporter gene SNP may be important markers of MTX toxicity in RA. Such pharmacogenetic associations are race-specific. (First Release Mar 15 2008; J Rheumatol 2008;35:572–9)

Key Indexing Terms:
METHOTREXATE TOXICITY POLYMORPHISMS RHEUMATOID ARTHRITIS

Methotrexate (MTX) is the disease modifying antirheumatic drug (DMARD) most widely used in the treatment of rheumatoid arthritis (RA)[1]. MTX is a folate analog that enters the cell after binding to the folate transporter, reduced folate carrier, or solute carrier family 19, member 1 (SLC19A1; Figure 1). MTX can be pumped out of the cell by members of the ATP-binding cassette (ABC) family of transporters. To prevent this and enhance its intracellular retention, MTX is polyglutamated by the enzyme folylpolyglutamyl synthase (FPGS) into MTX polyglutamates (MTXPG) after cellular entry[2]. The beneficial effects of MTX in RA stem primarily from 2 aspects of its mechanism of action. MTXPG directly inhibit several folate-dependent enzymes such as dihydrofolate reductase (DHFR), thymidylate synthase (TYMS), and 5-aminoimidazole-4-carboxamide ribonucleotide formyltransferase (ATIC), leading to disrupted purine and pyrimidine synthesis and extracellular release of adenosine, a potent anti-inflammatory agent, respectively[3]. However, because of its effects on the intracellular folate pool, MTXPG also indirectly influence methylenetetrahydrofolate reductase (MTHFR), a key enzyme required for the generation of reduced tetrahydrofolate, which is the carbon donor for several important cellular biochemical reactions, including the generation of methionine from homocysteine.

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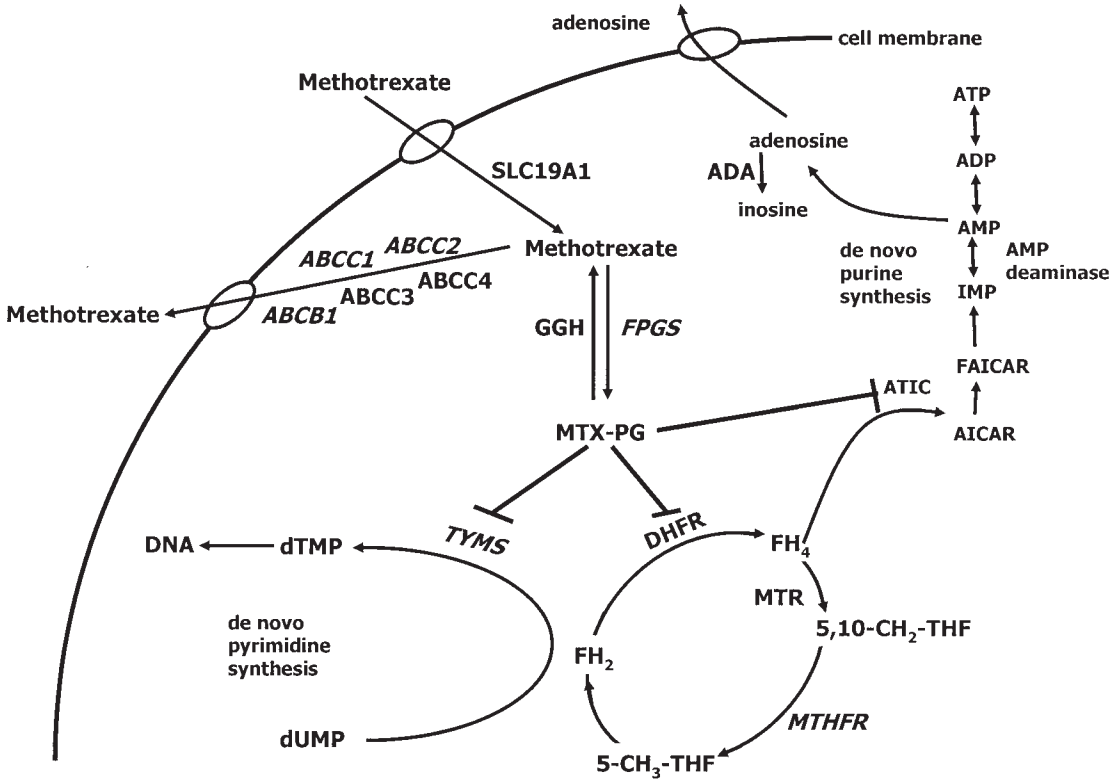

Figure 1. The cellular pathway of methotrexate. Genes denoted in italic type were the targets of pharmacogenetic analyses in this study. SLC19A1: solute carrier family 19, member 1 (also known as reduced folate carrier); ABCB1, ABCC1-4: ATP-binding cassette transporters; GGH: γ-glutamyl hydrolase; FPGS: folylpolyglutamate synthase; MTX-PG: MTX polyglutmate; TYMS: thymidylate synthase; dUMP: deoxyuridine monophosphate; dTMP: deoxythymidine monophosphate; DHFR: dihydrofolate reductase; FH_2: dihydrofolate; 5-CH_3-THF: 5-methyltetrahydrofolate; MTHFR: methylenetetrahydrofolate reductase; 5, 10-CH_2-THF: 5, 10-methylenetetrahydrofolate; MTR: methyltetrahydrofolate reductase; AICAR: aminoimidazole carboxamide ribonucleotide; FAICAR: 10-formyl AICAR; ATIC: AICAR transformylase; IMP: inosine monophosphate; AMP: adenosine monophosphate; ADP: adenosine diphosphate; ATP: adenosine triphosphate; ADA: adenosine deaminase.

It is well known that when MTX is used in RA, a significant number of patients experience toxic side effects[4,5]. The use of folic acid supplements can substantially decrease the occurrence of some MTX toxicities in patients with RA[6]. This supports the hypothesis that folate depletion induced by MTX may be instrumental in mediating MTX toxicity. However, other mechanisms, such as MTX's effects on the adenosine pathway, are also implicated in the occurrence of certain toxicities from MTX[7]. Recent advances in genetics and the availability of high-throughput technologies have led to investigations of variations in genes encoding enzymes in these pathways as possible determinants of MTX response including efficacy and toxicity[8]. The association of a common 677 C → T polymorphism in the MTHFR gene with MTX-related adverse effects in patients with RA has been studied, with conflicting results[9-13]. There are emerging data on the influence of polymorphisms in other genes encoding enzymes in the MTX cellular pathway, such as SLC19A1, DHFR, TYMS, and ATIC, on MTX response including toxicity in RA[11,13-15].

Polymorphisms in the ABC transporter genes have not been studied to date in RA patients for their influence on MTX efficacy or toxicity. By leading to altered function of the encoded proteins, such polymorphisms and those in the FPGS gene may alter levels of MTXPG (the active form of MTX) and thus influence the drug's effects in RA. We previously established that single-nucleotide polymorphisms (SNP) occur frequently in MTX transporter genes in patients with RA[16]. In this study, we investigated the influence of SNP in the MTX transporter genes ABCB1, ABCC1, and ABCC2 and in the FPGS, MTHFR, and TYMS genes on MTX toxicity in a cohort of patients with RA. Also, as all published MTX pharmacogenetic studies in RA to date have been carried out in racially homogenous populations, we examined whether these SNP had differential effects on MTX toxicity in Caucasians and African Americans with RA.

MATERIALS AND METHODS

Study design. This was a retrospective cohort study conducted at Washington University School of Medicine and the Veterans Affairs Medical Center, St. Louis, Missouri. To be eligible, patients (age ≥ 18 yrs) had to meet the American College of Rheumatology revised classification criteria for RA[17] and have received MTX. The dose of MTX and any adjustment in dose (decrease or discontinuation) was determined by the treating rheumatologist based on efficacy and toxicity considerations. Concomitant medications included corticosteroids, sulfasalazine, hydroxychloroquine, leflunomide, and the biological agents etanercept, infliximab, adalimumab and anakinra.

Patients were taking folic acid supplement (1–3 mg/day) at the discretion of the treating physician. In total, 222 patients with RA attending outpatient rheumatology clinics at the 2 centers were enrolled after they provided informed consent. The study was approved by the institutional review boards of both centers. A single sample of blood (10 ml) in EDTA was drawn from each patient for genetic analysis.

The study employed a cross-validation or data-splitting design (Figure 2); 95 consecutive RA patients attending outpatient rheumatology clinics at the 2 centers formed the training cohort and 127 subsequent consecutive RA patients attending these clinics constituted the validation cohort. The model was derived on the first portion of the data (training cohort), and its ability to predict outcome (toxicity) was evaluated on the second (validation cohort) data set. The reasons for using a cross-validation design were 2-fold. The first was to use this as a process of internal validation to validate the prognostic model of genotypes as predictors of MTX toxicity. The second was that the smaller training set served to screen for a relatively large number of SNP with potential associations with MTX toxicity. By recruiting consecutive patients, the training and validation cohorts had groups of RA patients seen in different time periods, ensuring nonrandom data-splitting, a key requirement for cross-validation[18]. Ninety-five patients in the training cohort were recruited over the first 12 months and the subsequent 127 patients in the validation cohort were recruited over the next 18 months of the study period. Ninety-five patients in the training cohort represented a sufficient sample size for the purpose of screening for a large number of candidate SNP, while patients recruited over the remainder of the study period (127 patients over 18 mo) formed the validation cohort. The association of 25 common polymorphisms in 6 genes encoding enzymes in the MTX cellular pathway with MTX toxicity was first established in the training cohort. The genes analyzed were ABCB1, ABCC1, ABCC2, FPGS, MTHFR, and TYMS. Subsequently, SNP that showed significant associations with MTX toxicity in the training cohort were examined in the validation cohort to establish how well the model performed

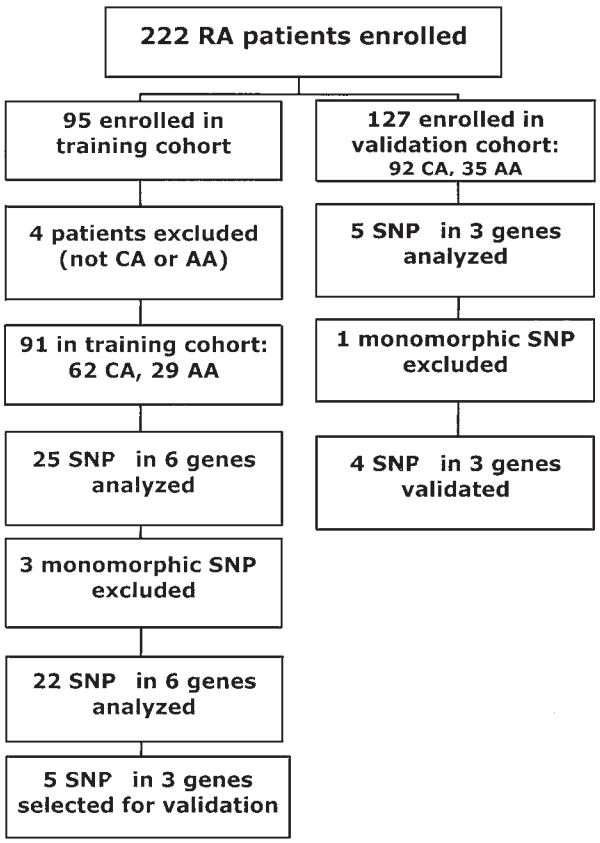

Figure 2. Patient enrollment and outcome.

for other RA patients. A decision was made *a priori* to include the MTHFR 677 C → T SNP in the validation set, to provide a clearer context to the extensive clinical reports in RA with this variant.

Clinical assessments. Patients' clinical, demographic, and MTX toxicity information was collected retrospectively in specifically designed forms. Information was collected on 9 individual MTX toxicities and the omnibus phenotype "overall toxicity," which included patients who had experienced one or more of the individual toxicities. MTX-related adverse events (AE) were defined as alopecia, gastrointestinal (GI) side effects (nausea, vomiting, diarrhea, dyspepsia), hepatotoxicity [elevation of aspartate aminotransferase (AST) or alanine aminotransferase (ALT) above the upper limit of normal (40 units/liter)], leukopenia (white blood cell count < 3500 cells/mm^3), pulmonary toxicity (cough, dyspnea, or pulmonary infiltrate), stomatitis, central nervous system toxicity (headache), post-dosing reactions (fatigue or malaise within 24–48 hours of taking MTX), and rash.

Our definition of hepatotoxicity as elevation of AST or ALT above the upper limit of normal may be considered too sensitive and clinically irrelevant. Our decision to adopt this definition was based findings that any elevation of transaminases into the abnormal range is predictive of a change in hepatic architecture when liver biopsies are obtained annually[19,20]. Non-laboratory and laboratory-based toxicities were recorded as being secondary to MTX if they were ascribed to MTX by the treating rheumatologist upon chart review. The clinical action taken in response to MTX toxicity was also recorded on the data collection forms as "dose maintained," "dose reduced," "dose temporarily interrupted," and "dose permanently discontinued."

Data abstractors were trained by the principal investigator (PR) for the study. Clinical data were abstracted using predefined criteria as described above, under the supervision of the principal investigator. The principal investigator and the data abstractors independently abstracted medical records periodically to compare data entries. Independent abstractions by the principal investigator were performed on roughly every twentieth medical record for this purpose.

Genotyping. All physicians and patients were blinded to the genotypes throughout the study, and the laboratory personnel were blinded to all clinical information. Whole blood (10 ml in an EDTA tube) was drawn from patients. Genomic DNA was extracted using the Gentra Puregene extraction kit, reconstituted in Tris-EDTA buffer, quantitated by fluorometric analysis, and stored at 4°C. Genotyping was performed using Pyrosequencing® technology (Pyrosequencing AB, Uppsala, Sweden). Genotyping procedures for the training cohort were as described[16]. Polymerase chain reaction (PCR) and Pyrosequencing primers and conditions for all variants were as described[16]. Pyrosequencing was performed and genotypes were determined as described[21] using a PSQ hs96A instrument and software (Pyrosequencing AB). Genotypes were automatically assigned by the PSQ software and manually checked by 2 researchers. Negative controls (no DNA) were included in the PCR and Pyrosequencing steps. Failed or ambiguous genotypes were repeated up to 3 times.

Samples were assessed for the presence of 25 polymorphisms in the ABCB1, ABCC1, ABCC2, FPGS, MTHFR, and TYMS genes in the training cohort of 95 RA patients. In the validation cohort of 127 RA patients, samples were assessed for 5 polymorphisms in 3 genes. Polymorphisms in ABCB1 (1236 C → T, a synonymous SNP coding for glycine at position 412), ABCC2 (1058 G → A, a nonsynonymous SNP leading to arginine to histidine substitution at position 353; 1249 G → A, a nonsynonymous SNP leading to valine to isoleucine substitution at position 41; and IVS 23+56 T → C, an intronic SNP in intron 23), and MTHFR (677 C → T, a nonsynonymous SNP leading to alanine to valine substitution at position 222) were analyzed in these patients.

Statistical analysis. The association of the occurrence of each adverse event with the presence of individual SNP in the training and validation cohorts was computed using Fisher exact tests. Statistical associations in the validation cohort were also assessed using a multivariable logistic regression model, where the occurrence of adverse events was the dependent variable and several clinically important variables constituted the independent variables. These independent variables included age, sex, duration of RA, MTX dose

(mg/week), duration (number of months) of treatment with MTX, folic acid use, corticosteroid use, concomitant DMARD use, and NSAID use. Survival analyses were carried out in the validation cohort to determine the effect of genotypes on the time to dose reduction or discontinuation of MTX secondary to toxicity.

As this was a retrospective cohort study in the context of clinical practice at 2 academic institutions, complete clinical and toxicity information was missing in a small number of patients (patients lost to followup because of moving away, loss of insurance, other reasons). However, most missing data were missing at random. The number of patients included in each analysis is indicated in Tables 1 to 4. Given the exploratory design of the research, adjustments for multiple testing in the analysis were not made.

RESULTS

Twenty-five loci in 6 genes in the MTX cellular pathway were analyzed in the first 95 patients with RA (training cohort) for associations with MTX toxicity. Three SNP loci in the ABCC2 gene (2153 A → G, 2677 G → C, and 2934 G → A) were not polymorphic in these patients, hence 22 loci were analyzed. Of the 95 patients, 62 Caucasian Americans and 29 African Americans with RA were included in the analysis and patients of other races were excluded as the numbers in these categories were small (n = 4; 1 Hispanic, 2 Asian, 1 Native American). Neither ethnic subgroup displayed significant deviation from Hardy-Weinberg equilibrium at any of the loci. Since there were significant differences in allele frequencies in the SNP of interest between Caucasians and African Americans with RA in this cohort[16], genotype analyses were stratified by race.

The demographic characteristics of the training and validation cohorts of RA patients are summarized in Table 1 and their toxicity characteristics in Table 2. There were no significant differences between the training and validation cohorts in terms of their demographic or toxicity characteristics. We observed that 58.6% of African Americans and 66.1% of Caucasians with RA in the training cohort had an MTX-related adverse event. Twenty-five SNP in 6 genes in the MTX cellular pathway were analyzed in the training cohort for associations with MTX toxicity. Three SNP in the ABCC2 gene (2153 A → G, 2677 G → C, and 2934 G → A) were not polymorphic in these patients, hence 22 SNP were analyzed, as follows: ABCB1 3435 C → T, 1236 C → T; ABCC1 4002 G → A, IVS 14+115 C → T, IVS 18-30 C → G; ABCC2–24 C → T, 2153 A → G, 2677 G → C, 2934 G → A, 4410 G → A, 4488 C → T, 4544 G → A, 1249 G → A, 1058 G → A, IVS 31+74 C → T, IVS 31-90 A → G, IVS 23+56 T → C; FPGS 1901 T → C; MTHFR 677 C → T, 1298 A → C, 1793 G → A; and TYMS 1494del(TTAAAG). The following SNP did not show any associations with MTX toxicity in this cohort: ABCB1 3435 C → T; ABCC1 4002 G → A, IVS 14+115 C → T, IVS 18-30 C → G; ABCC2–24 C → T, 2153 A → G, 2677 G → C, 2934 G → A, 4410 G → A, 4488 C → T, 4544 G → A, IVS 31+74 C → T, IVS 31-90 A → G; FPGS 1901 T → C; MTHFR 677 C → T, 1298 A → C, 1793 G → A; and TYMS 1494del(TTAAAG) (p-values ≥ 0.05; data not shown).

Fisher exact tests revealed that 4 variants displayed significant associations with toxicity in the training cohort (Table 3). The intronic SNP in ABCC2 (IVS 23+56 T → C) was associated with alopecia in Caucasians (p = 0.035). In African Americans, the ABCB1 1236 C → T SNP was associated with overall toxicity (p = 0.013), ABCC2 1249 G → A SNP with GI toxicity (p = 0.009), and ABCC2 1058 G → A SNP with hepatotoxicity (p = 0.04). Although the MTHFR 677 C → T variant showed only an apparent association with overall toxicity in Caucasians (p = 0.063), this was included with the 4 SNP that showed significant associations with toxicity for validation in the subsequent cohort because of evidence for its clinical relevance[9-13].

In the validation cohort, 55.8% of African American and

Table 1. Demographics of the training (n = 91) and validation cohorts (n = 127).

Characteristics	Training Cohort (AA = 29, CA = 62) Median (IQR) AA	Training Cohort (AA = 29, CA = 62) Median (IQR) CA	Validation Cohort (AA = 35, CA = 92) Median (IQR) AA	Validation Cohort (AA = 35, CA = 92) Median (IQR) CA	p, Differences Between Cohorts AA	p, Differences Between Cohorts CA
Age, yrs	52 (42–58)	61 (51–69)	58 (47–66)	59 (48–67)	0.31	0.30
No. women (%)	25 (86.2)	31 (50)	31 (88.5)	42 (45.6)	0.99	0.62
Duration of RA, yrs	4* (2.5–10.5)	6.5* (3.5–16.5)	6.3 (2–15.3)	7.5 (4–19)	0.41	0.46
MTX dose, mg/wk	15 (7.5–20)	12.5** (0–20)	18.7* (13.7–20)	15* (10–20)	0.11	0.30
Duration of MTX therapy, mo	38# (24–62)	43# (24–60)	27** (15–55)	48** (13.5–84.5)	0.27	0.89
Concurrent DMARD, n (%)	10## (62.5)	39## (76.4)	21*** (61.7)	60*** (72.2)	0.99	0.69
Concurrent NSAID, n (%)	28 (96.5)	60† (98.3)	34# (100)	89# (97.8)	0.46	0.99
Concurrent folic acid, n (%)	21 (72.4)	42†† (71.1)	26## (76.4)	71## (85.2)	0.77	0.06
Concurrent steroids, n (%)	25 (86.2)	51 (82.2)	29 (82.8)	75† (85.2)	0.99	0.65
MTX discontinuation, n (%)	11 (37.9)	30 (48.3)	17 (48.5)	50 (54.3)	0.45	0.51
Duration of MTX therapy until discontinuation, mo	38*** (15–43)	21*** (6–42.5)	43†† (12.5–78.5)	17†† (6.5–32.5)	0.39	0.09

Training cohort: * n = 28AA, 60CA; ** n = 59CA; # n = 28AA, 58CA; ## n = 16AA, 51CA; † n = 61CA; †† n = 59CA; *** n = 7AA, 16CA.
Validation cohort: * n = 24AA, 58CA; ** n = 33AA, 80CA; *** n = 34AA, 83CA; # 34AA, 91CA; ## 34AA, 84CA; † n = 88CA; †† n = 11AA, 44CA.
AA: African American; CA: Caucasian; IQR: interquartile range; DMARD: disease modifying antirheumatic drug; NSAID: nonsteroidal antiinflamamtory drug.

Table 2. Toxicity characteristics of the training (n = 91) and validation cohorts (n = 127).

Toxicity	Training Cohort (n = 91, AA = 29, CA = 62) Frequency (%) African Americans, n = 29	Training Cohort (n = 91, AA = 29, CA = 62) Frequency (%) Caucasians, n = 62	Validation Cohort (n = 127, AA = 35, CA = 92) Frequency (%) African Americans, n = 35	Validation Cohort (n = 127, AA = 35, CA = 92) Frequency (%) Caucasians, n = 92	p, Differences Between Cohorts African Americans	p, Differences Between Cohorts Caucasians
Overall	17 (58.6)	41 (66.1)	19* (55.8)	55 (59.7)	0.99	0.49
Alopecia	6 (20.6)	3* (4.9)	3* (8.6)	8 (8.7)	0.28	0.52
GI side effects (nausea, vomiting, diarrhea)	5 (17.2)	15* (24.5)	6* (17.6)	15 (16.3)	0.99	0.22
Hepatotoxicity (AST or ALT > ULN)	3 (10.3)	18 (29.0)	6* (17.6)	22** (24.1)	0.49	0.58
Leukopenia (WBC ≤ 3500 cells/mm^3)	4 (13.7)	2* (3.2)	4* (11.7)	5** (5.4)	0.99	0.70
Pulmonary (cough, dyspnea, pulmonary infiltrate	3 (10.3)	5* (8.2)	0* (0)	4 (4.3)	0.09	0.49
Stomatitis	3 (10.3)	8* (13.1)	1* (2.9)	16** (17.5)	0.33	0.50
Headache	0 (0)	2* (3.2)	0* (0)	1** (1.1)	NA	0.56
Post-dosing reactions (fatigue, malaise 24–48 hours after taking MTX)	1 (3.4)	3* (4.9)	1* (2.9)	1** (1.1)	0.99	0.30
Rash	0 (0)	0 (0)	1 (2.8)	1 (1.0)	0.99	0.99

* Training cohort: 61 Caucasians. GI: gastrointestinal, ULN: upper limit of normal, WBC: white blood cells, NA: not applicable.
* Validation cohort: 34 African Americans, 61 Caucasians. GI: gastrointestinal, ULN: upper limit of normal, WBC: white blood cells, NA: not applicable.

Table 3. SNPs associated with MTX toxicity in the training cohort.

Toxicity	SNP	No. (%) Patients with Toxicity vs No Toxicity (wild-type)	No. (%) Patients with Toxicity vs No Toxicity (heterozygous)	No. (%) Patients with Toxicity vs No Toxicity (homozygous)	p
Caucasians					
Overall toxicity*	MTHFR C→T	12/12 (50)	16/5 (76.1)	2/5 (28.6)	0.063
Alopecia**	ABCC2 IVS 23+56 T→C	0/17 (0)	1/18 (5.3)	3/2 (60)	0.035
African Americans					
Overall toxicity†	ABCB1 1236 C→T	2/9 (18.2)	6/1 (85.7)	0/0 (0)	0.013
GI side effects††	ABCC2 1249 G→A	2/13 (13.3)	0/9 (0)	2/0 (100)	0.009
Hepatotoxicity***	ABCC2 1058 23+56 G→A	0/24 (0)	1/0 (100)	0/0 (0)	0.04

* 52 Caucasian RA patients with genotype. ** 41 Caucasian RA patients with genotype. † 11 African American RA patients with missing data. †† 3 African American RA patients with missing data. *** 4 African American RA patients with missing data. GI: gastrointestinal.

Table 4. SNPs associated with MTX toxicity in the validation cohort.

Toxicity	SNP	No. Patients with Toxicity (%) vs No Toxicity (wild-type)	No. Patients with Toxicity (%) vs No Toxicity (heterozygous)	No. Patients with Toxicity (%) vs No Toxicity (homozygous)	p
Caucasians, n = 91*					
GI side effects	ABCCS IVS 23+56 T→C	7/24 (22.6)	4/47 (7.8)	3/6 (33.3)	0.0503
GI side effects	MTHFR 677 C→T	7/28 (20)	4/41 (8.9)	4/7 (36.4)	0.0637
African Americans					
Alopecia, n = 34**	ABCB1 1236 C→T	1/18 (5.3)	1/13 (7.1)	1/0 (100)	0.0882
Alopecia, n = 33***	MTHFR 677 C→T	1/28 (3.4)	2/2 (50)	0/0 (0)	0.0326

* Genotyping did not yield SNP information in one Caucasian patient with RA. ** 34 African American RA patients with this toxicity information were available (Table 2). *** Genotyping did not yield SNP information in one African American RA patient. GI: gastrointestinal.

59.7% of Caucasian patients experienced MTX toxicity. Of the 5 SNP analyzed in this cohort, the ABCC2 1058 G → A SNP was not polymorphic in Caucasians with RA, and hence was not examined for toxicity associations. Fisher exact tests in this cohort (Table 4) revealed that the MTHFR 677 C → T SNP displayed a significant association with alopecia in African American patients ($p = 0.032$). None of the other SNP analyzed showed significant associations with toxicity, although the MTHFR 677 C → T and intronic ABCC2 IVS 23+56 T → C SNP showed an apparent association with GI side effects in Caucasian patients ($p \geq 0.05$; Table 4).

Stepwise multivariable logistic regressions were performed with the 9 individual toxicities and the combined phenotype of overall toxicity as the dependent variables in the validation cohort. Analyses were performed separately on the 2 racial groups. Independent variables included in the model were age, gender, duration of RA (months), MTX dose (mg/week), duration of treatment with MTX (months), folic acid use, corticosteroid use, concomitant DMARD use, and NSAID use. None of these variables was a significant predictor of MTX toxicity in either group in this model. Survival analysis revealed a significant effect of the ABCC2 IVS 23+56 T → C genotype on the time to discontinuation or decrease in dose of MTX secondary to toxicity in the Caucasian subset of the validation cohort (Figure 3). Caucasians with RA carrying the T/T genotype (n = 5; homozygotes) had a median survival time of 2 months, while those with the T/C genotype (n = 29; heterozygotes) had a mean survival time of 23 months, and those in the C/C group (n = 10; wild-type) had a mean survival time of 29 months to dose decrease or discontinuation of MTX ($p < 0.0001$). Such an effect of the genotype was not evident in African Americans with RA.

We evaluated whether the SNP-toxicity associations were due to hidden associations between the genotypes and demographic covariates (age, duration of RA, MTX dose, duration of MTX, gender, folic acid use, corticosteroid use, DMARD use, NSAID use). For this, we determined whether the distributions of the genotypes and demographic covariates were independent using Fisher exact tests for the binary covariates and ANOVA for the quantitative covariates. The binary covariates were gender, folic acid use, corticosteroid use, DMARD use, and NSAID use (Fisher exact test). The quantitative covariates were age, duration of RA, MTX dose, and duration of MTX use (ANOVA).We found no genotype-covariate association to account for the p-values reported in Tables 3 and 4. These tests were performed separately for each racial group in the training and validation cohorts. In these analyses, the distribution of p-values conformed to those expected under the null hypothesis.

DISCUSSION

Pharmacogenetic studies to date examining the genetic basis for MTX toxicity and efficacy in RA have all been carried out in racially homogenous RA cohorts. Our study is the first to examine pharmacogenetic associations in Caucasian and African American patients with RA and to suggest that genetic variants may have differential effects in these racial groups. It is also the first to study the influence of SNP in the ABC transporters on MTX toxicity in patients with RA. Studies of MTX pharmacogenetics in RA have been retrospective or cross-sectional, with a few prospective studies. As there are no data from previous studies regarding associations of SNP in the ABC transporter genes and the FPGS gene with MTX toxicity, we adopted a retrospective cross-validation approach. The training cohort was used to screen a relatively large number of candidate SNP in these genes to identify those associated with

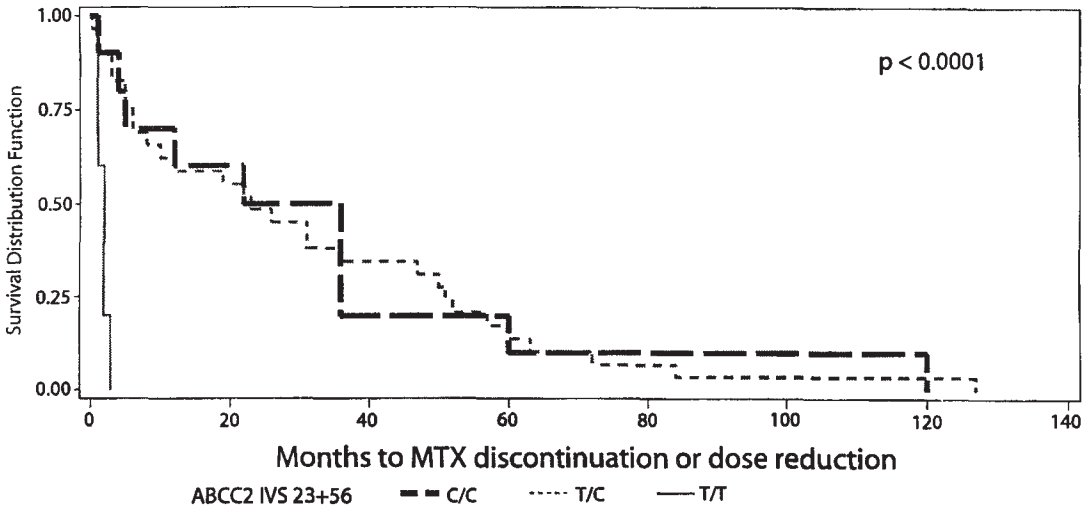

Figure 3. Survival analysis of the effect of the intronic SNP in ABCC2 on MTX dose reduction and discontinuation. Subjects are Caucasians with RA in the validation cohort. ABCC2 23+56 C/C: wild-type genotype (n = 10); T/C: heterozygous genotype (n = 29); T/T: homozygous genotype (n = 5).

MTX toxicity. Subsequently a validation cohort was used to obtain improved estimates of measures of these associations and to gauge the strength of the model in other patients.

The MTHFR 677 C → T SNP is one of the best studied genetic determinants of MTX response in RA. MTHFR is not a direct target of MTX. However, as MTX affects the adequacy of the intracellular folate pools through its inhibition of DHFR, the activity of MTHFR can be influenced by the drug and vice versa. MTHFR is a critical enzyme for the generation of reduced tetrahydrofolate, which is a carbon donor for several biochemical reactions including the remethylation of homocysteine to methionine[22]. The 677 C → T variant causes an alanine to valine substitution at codon 222 of the MTHFR gene. It encodes a thermolabile variant of MTHFR with decreased enzyme activity and subsequent increased plasma homocysteine levels[23].

We used a cross-validation approach to screen for the MTHFR SNP. The training cohort was screened for both 677 C → T and 1298 A → C SNP in MTHFR, and the 677 C → T SNP, which showed a trend toward MTX toxicity in Caucasians with RA, was investigated further in the validation cohort. Interestingly, of all the SNP analyzed, only the MTHFR 677 C → T SNP was associated with MTX toxicity, namely alopecia in the validation cohort of African Americans with RA. Although it showed apparent associations with overall toxicity and GI side effects in the Caucasian training and validation cohorts, respectively, these were not statistically significant. These results suggest that this SNP may be a true marker of MTX toxicity in RA. Studies examining this SNP in relation to MTX toxicity have produced inconsistent results; some studies showed the presence of the SNP was a marker of MTX toxicity[9,10,24], while others did not show such an association[11,12]. It is noteworthy that while most of these studies were carried out in Caucasian populations, some examined Japanese populations[10,11]. The results of these studies, considered along with our results showing the 677 C → T SNP as a marker of MTX toxicity in African Americans with RA, suggest that race may interact with this genetic variant to influence the risk of MTX toxicity.

It has been established that several members of the ABC family of transporters play important roles in the MTX cellular pathway. The ABCB1 (MDR-1) gene encodes a membrane transporter P-glycoprotein (P-gp) whose function is the energy-dependent export of substances from the cell. P-gp has a physiologic role in the protection of cells from toxic metabolites; however, its more important role is in the efflux of several drugs that form its substrates. It remains controversial whether MTX is a substrate of P-gp, for some studies indicate that higher P-gp expression may be a marker of MTX resistance[25], while others contradict this[26,27]. While the role of ABCB1 in contributing to efflux of MTX is controversial, it has been established that ABCC 1–4 are important in mediating the efflux of nonpolyglutamated MTX from the cell[28-31]. ABCC 1–3 can also mediate the efflux of natural folates[32]. Thus genetic variations in the ABCC and possibly the ABCB1 transporters may affect efficacy and toxicity of MTX by influencing intracellular MTX and folate levels.

Our study revealed that the intronic ABCC2 IVS 23+56 T → C SNP was associated with MTX toxicity-related dose decrease or drug discontinuation in Caucasians with RA, but not in African Americans with RA in the validation cohort. This suggests that this intronic SNP may be a true genetic marker of MTX toxicity, or may occur in linkage disequilibrium in Caucasians with a genetic variant that may be the true determinant of MTX toxicity in these patients. Linkage varies between populations, and may explain this race-specific association. A similar hypothesis may explain the association of the synonymous ABCB1 1236 C → T SNP with overall toxicity in African Americans with RA in the training cohort, although this association failed to be validated in the subsequent cohort.

We acknowledge that our study had limitations. An important limitation was the retrospective design necessitating abstraction of all clinical information from medical records. Several uncontrolled variables such as differences in physician documentation, level of experience, and clinical acumen could have influenced the accuracy of data and led to over- or underestimation of MTX toxicity. Our regression analysis failed to show a predictive effect of several clinically important variables (MTX dose, duration of use, concurrent DMARD use, folic acid use) on MTX toxicity. It is likely that such clinical variables do influence and interact with genetic factors to determine the phenotype of MTX toxicity. Although our regression analysis was adequately powered for the phenotype of overall toxicity, it was relatively underpowered for the individual toxicities due to the small number of events and this may have accounted for this result. We would emphasize that this study should be considered a pilot study given the constraints on sample size and power due to stratification by race.

In summary, polymorphisms in several genes in the MTX cellular pathway such as ATIC 347 C → G, serine hydroxymethyl transferase (SHMT) 1420 C → T, TYMS enhancer repeat *2/*3, MTHFR 1298 A → C, methionine synthase 2756 A → G, and methionine synthase reductase (MTRR) 66 A → G have been studied for associations with MTX toxicity[33-35]. However, there are no genetic markers that have shown consistent associations with MTX toxicity. Our findings suggest that besides polymorphisms such as MTHFR 677 C → T, genetic variations in the MTX transporters may also be important in defining an individual's response to MTX, particularly toxicity, and these warrant further study. More importantly, genetic markers for MTX toxicity may be distinct in different racial groups. We acknowledge that the findings of this pilot study are hypothesis-generating and need to be externally validated by replication in a different patient population. Similar to prior research in this area, our findings underscore the need for prospective large-scale pharmacogenetic studies in diverse racial groups to establish race-specific genetic markers for clinically important phenotypes.

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