EXTENDED REPORT

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

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Background: The anti-folate drug methotrexate (MTX) is commonly used to treat rheumatoid arthritis.
Objective: To determine the allele frequencies of five common coding single-nucleotide polymorphisms (SNPs) in the methylenetetrahydrofolate reductase (*MTHFR*) gene in African-Americans and Caucasians with rheumatoid arthritis and controls to assess whether there are differences in allele frequencies among these ethnic or racial groups and whether these SNPs differentially affect the efficacy or toxicity of MTX.
Methods: Allele frequencies in the 677, 1298 and 3 additional SNPs in the *MTHFR* coding region in 223 (193 Caucasians and 30 African-Americans) patients with rheumatoid arthritis who previously participated in one of two prospective clinical trials were characterised, and genotypes were correlated with the efficacy and toxicity of MTX. Another 308 subjects with rheumatoid arthritis who participated in observational studies, one group predominantly Caucasian and the other African-American, as well as 103 normal controls (53 African-Americans and 50 Caucasians) were used to characterise allele frequencies of these SNPs and their associated haplotypes.
Results: Significantly different allele frequencies were seen in three of the five SNPs and haplotype frequencies between Caucasians and African-Americans. Allele frequencies were similar between patients with rheumatoid arthritis and controls of the same racial or ethnic group. Frequencies of the rs4846051C, 677T and 1298C alleles were 0.33, 0.11 and 0.13, respectively, among African-Americans with rheumatoid arthritis. Among Caucasians with rheumatoid arthritis, these allele frequencies were 0.08 ($p<0.001$ compared with African-Americans with rheumatoid arthritis), 0.30 ($p=0.002$) and 0.34 ($p<0.001$), respectively. There was no association between SNP alleles or haplotypes and response to MTX as measured by the mean change in the 28-joint Disease Activity Score from baseline values. In Caucasians, the 1298 A (major) allele was associated with a significant increase in MTX-related adverse events characteristic of a recessive genetic effect (odds ratio 15.86, 95% confidence interval 1.51 to 167.01; $p=0.021$), confirming previous reports. There was an association between scores of MTX toxicity and the rs4846051 C allele, and haplotypes containing this allele, in African-Americans, but not in Caucasians.
Conclusions : These results, although preliminary, highlight racial or ethnic differences in frequencies of common *MTHFR* SNPs. The *MTHFR* 1298 A and the rs4846051 C alleles were associated with MTX-related adverse events in Caucasians and African-Americans, respectively, but these findings should be replicated in larger studies. The rs4846051 SNP, which is far more common in African-Americans than in Caucasians, can also be proved to be a useful ancestry informative marker in future studies on genetic admixture.

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The anti-folate drug methotrexate (MTX) is commonly used to treat rheumatoid arthritis and other inflammatory autoimmune diseases.[1] Most patients with rheumatoid arthritis have improvement in disease activity with MTX treatment, but side effects and incomplete responses are common.[2] Multiple mechanisms of action of MTX may exist in rheumatoid arthritis, including an inhibitory effect on folic-acid metabolism and increases in extracellular adenosine,[3] which has local anti-inflammatory properties.[4] Methylenetetrahydrofolate reductase (*MTHFR*, EC 1.5.1.20) catalyses the irreversible reduction of 5,10-methylenetetrahydrofolate to 5-methyltetrahydrofolate, the predominant circulating form of folate.[5] 5-Methyltetrahydrofolate participates in the vitamin B_{12}-dependent remethylation of homocysteine to methionine. Methionine is converted to S-adenosylmethionine, which serves as a methyl group donor in methylation reactions of nucleotides in DNA, RNA and proteins.[6]

Because of its critical importance in folate metabolism, *MTHFR* has been studied in multiple diseases, including cardiovascular disease,[7] neural-tube defects,[8] pre-eclampsia,[9] and malignancies such as colon cancer[10] and leukaemia.[9] The human *MTHFR* gene has been mapped to chromosomal

Abbreviations: ACR, American College of Radiology; cDNA, complementary DNA; DAS28, 28-joint Disease Activity Score; ERA, early rheumatoid arthritis; MTHFR, methylenetetrahydrofolate reductase; MTX, methotrexate; MTXPG, methotrexate polyglutamate; PCR, polymerase chain reaction; SNP, single-nucleotide polymorphism; UAB, University of Alabama at Birmingham

region 1p36.3 and contains 11 exons.[5] The two most commonly analysed *MTHFR* polymorphisms are the 677 C/T (rs1801133) and 1298 A/C (rs1801131) single-nucleotide polymorphisms (SNPs). The 677 T (minor) allele leads to a thermolabile MTHFR variant with decreased enzyme activity and is associated with mildly elevated plasma homocysteine levels.[11 12] Compared with single heterozygosity for 677 T or for 1298 C, combined minor allele heterozygosity (677 T/1298 C) is associated with reduced MTHFR specific activity, higher homocysteine levels and decreased plasma folate levels.[13]

A key clinical aspect with regard to *MTHFR* SNPs is the pharmacogenetics of MTX. In a study of Caucasian patients with rheumatoid arthritis, the presence of at least one copy of the 677 T allele was associated with increased risk of discontinuation of MTX from adverse events, mainly because of raised alanine aminotransferase values.[14] Japanese patients with rheumatoid arthritis with the 1298 C allele received considerably lower doses of MTX compared with patients without this allele, whereas a higher rate of overall MTX toxicity was observed in patients with 677 T than in those without.[15] In the same study by Urano *et al*,[15] patients with the 677C/1298C haplotype received lower doses of MTX than those without the haplotype, and subjects with the 677T/1298A haplotype had a higher frequency of side effects from MTX than those without the haplotype. In a study of Jewish subjects with rheumatoid arthritis, the 1298 C allele was associated with a reduction in MTX-related adverse events. There was no association of the 677C/T polymorphism with MTX toxicity, nor was there any correlation of either of these polymorphisms with disease activity or plasma homocysteine levels.[16]

The goal of this study was to characterise allele frequencies of common coding SNPs in Caucasians and African-Americans, to deduce haplotypes in the *MTHFR* gene defined by these SNPs, and to identify associations of SNP alleles or haplotypes with efficacy and toxicity of MTX in rheumatoid arthritis.

PATIENTS AND METHODS

Subjects and controls

Our analysis included 531 well-characterised people with rheumatoid arthritis defined by the American College of Radiology (ACR) criteria.[17] This included 108 African-American patients from the Grady African-American Rheumatoid Arthritis cohort,[18] 200 Caucasians from the Iowa rheumatoid arthritis cohort,[19] 77 subjects with rheumatoid arthritis (59 Caucasians, 18 African-Americans) from a prospective clinical trial of folate supplementation during MTX treatment,[20] and 146 MTX-treated patients (134 Caucasians, 12 African-Americans) who participated in the Immunex early rheumatoid arthritis (ERA) trial comparing MTX with two doses of etanercept.[21] In addition, 103 healthy controls comprising 53 African-Americans from Birmingham, Alabama and Atlanta, Georgia, and 50 Caucasians from Birmingham, Alabama, were included.

Data on MTX toxicity

Each of the 223 subjects (193 Caucasians and 30 African-Americans) with rheumatoid arthritis who participated in one of the two clinical trials (University of Alabama at Birmingham (UAB) folate trial and Immunex ERA trial) was prospectively evaluated for toxicity of MTX. In the UAB folate trial, laboratory data and detailed information regarding toxicity of MTX were evaluated at approximately 12-week intervals for 52 weeks.[20] Participants received a mean MTX dose of 9.1 mg a week and 5 mg a week of folic acid, 27.5 mg a week of folic acid, or placebo.[20] In all, 68% of the participants experienced some form of toxicity of MTX, most commonly nausea with indigestion (39%), diarrhoea (14%), oral ulcers or stomatitis (11%), and rash (11%).[20] Events were more in the placebo group than in the folic acid groups ($p \leqslant 0.001$). Each of the 77 patients with rheumatoid arthritis in the UAB folate trial was assigned a cumulative MTX toxicity score determined by detailed information on the organ system, duration and intensity of side effects.[20]

In the Immunex ERA trial, one third of the participants received MTX and placebo for etanercept. DNA and clinical data were available for 146 participants. MTX doses were escalated according to a standard protocol; the mean MTX dose was 19 mg a week. All participants received 1 mg of folic acid a day.[21] Adverse events and laboratory data were obtained at scheduled visits occurring at intervals between 2 and 8 weeks for 52 weeks. Data on toxicity were obtained from all participants and defined categorically according to the common toxicity criteria of the National Cancer Institute.[21] In all, 96% of the MTX-treated participants experienced toxicity, most commonly nausea or indigestion (35.1%), skin rash or pruritis (21.2%), and fatigue or malaise or headache (19.1%). Table 1 shows the specific toxicities according to organ group.

Data on MTX efficacy

Therapeutic response was evaluated in the 146 participants in the ERA trial through analysis of the ACR 20% (ACR20), 50% (ACR50) or 70% (ACR70) response criteria, and the serial 28-joint Disease Activity Score (DAS28) values.[22 23] The ACR response and DAS28 scores were calculated at the initiation of MTX treatment, 2 and 4 weeks after treatment was started, and 6, 8, 10 and 12 months after treatment was started. A mean change in DAS28 score was determined by subtracting the baseline DAS28 from the mean DAS28 during weeks 16–52 of the trial. The mean (standard deviation (SD)) baseline DAS28 for the participants treated with MTX was 5.52 (1.01), with 73% categorised as having high disease activity (DAS28 >5.1), 25% as having moderate disease activity (DAS28 >3.2 but $\leqslant 5.1$) and 2% as having low disease activity

Table 1 Number and organ-specific types of toxicity from 223 participants in the University of Alabama at Birmingham folate and Immunex early rheumatoid arthritis trials

MTX toxicity by organ system	Number of events (n = 362)	Total events (%)
General	69	19.1
Gastrointestinal	127	35.1
Ear, nose, throat	52	14.3
Skin	77	21.2
Pulmonary	23	6.4
Neuropsychiatric	2	0.6
Blood chemistry	8	2.2
Haematological	4	1.1
No of subjects with any toxicity	196	87.9

MTX, methotrexate.
Toxicities were categorised according to the common toxicity criteria of the National Cancer Institute. Each toxicity was counted only once for each subject. General: fatigue/malaise, fever (>37.7˚C), headache, rigours or chills, sweating, weight gain and weight loss. Gastrointestinal: anorexia, diarrhoea, dyspepsia, gastrointestinal bleed, hepatitis, nausea or vomiting, pancreatitis. Ear, nose, throat: stomatitis, tinnitus, xerostomia. Skin: alopecia, bullous eruption, petechiae, pruritis, rash. Pulmonary: cough, dyspnoea, pleurisy, pneumonitis, decreased pulmonary function (<90% diffusing capacity of the lung for carbon monoxide or forced vital capacity of pretreatment value). Neuropsychiatric: anxiety or depression, somnolence, inability to concentrate, insomnia, decreased libido, peripheral neuropathy, vertigo. Blood chemistry: serum creatinine>1.3×upper limit of normal (ULN); serum aspartate transaminase>1.5×ULN; serum alanine transaminase>1.5×ULN; serum alkaline phosphatase>2.0×ULN. Haematological: anaemia (haemoglobin decrease>1.4 g/dl from baseline); leucopenia (total white cell count<3900/mm^3); thrombocytopenia (platelet count<75 000/mm^3).

(DAS28 ⩽ 3.2). The mean (SD) DAS28 during weeks 16–52 was 3.73 (0.82), representing a mean (SD) change in DAS28 score of −1.80 (1.24). In contrast, by the end of the study, 30% of the participants had a good DAS28 response (change in DAS28 of >1.2, with a mean DAS28 of ⩽3.2), 52% had a moderate response (change in DAS of >1.2, with a mean DAS28 of ⩾3.2, or change in DAS28 of ⩽1.2, with a mean DAS28 of 0.6) and 18% had no response. At the end of the study, approximately 65% of subjects achieved an ACR20, 40% an ACR50 and 20% an ACR70 response.[21]

SNP identification

By using public domain and Celera SNP databases, we identified 27 coding SNPs in *MTHFR*. To have a crude estimate of the frequency of these alleles and to identify novel SNPs, we performed sequence analysis using methods optimised for heterozygote detection on 10 normal Caucasians and 10 normal African-Americans. For complementary DNA (cDNA) sequencing, we used the primers 5′-GGA ACC CAG CCA TGG TGA ACG-3′ (sense) and 5′-ACT CTC CTT CGT GTC TCT CCC ACC-3′ (antisense) to amplify the 2085-base-pair (bp) *MTHFR* cDNA. The polymerase chain reaction (PCR) was carried out in a total volume of 50 μl, with 5 U expanded long-template PCR polymerase mix (Roche, Nutley, New Jersey, USA) in buffer 3 provided by the manufacturer, 2.75 mM magnesium chloride ($MgCl_2$), 0.2 mM deoxynucleoside triphosphate, primers at a final concentration of 0.4 μmol/l and 1 μl cDNA product from a total volume of 20 μl. This PCR product was used as a template for two subsequent nested PCR amplifications to generate two amplicons for sequencing. Sequencing was performed using the BigDye™ Terminator Cycle Sequencing Ready Reaction kit (PE Applied Biosystems, Foster City, California, USA) on an ABI 377 automated sequencer.

Only 4 of the 27 SNPs had a minor allele frequency of ⩾0.05 (at least one minor allele in the 20 participants). Haplotypes were defined by the following four SNPs: 677 C/T (rs1801133) (exon 4); rs2066462 C/T (exon 6); 1298 A/C (rs1801131) (exon 7); and rs2274976 A/G (exon 11). In addition, we identified a T/C SNP 18 bp upstream of the 1298 A/C SNP in exon 7, with a minor allele frequency of 0 in Caucasians (0/20 alleles) and 0.55 in African-Americans (11/20 alleles). Although this SNP was not listed in public databases or the Celera databases during our initial experiments, it was subsequently identified in these databases (rs4846051). We genotyped all subjects from the Grady African-American Rheumatoid Arthritis and Iowa rheumatoid arthritis cohorts, the UAB folate and Immunex ERA trials, and normal controls.

SNP genotyping

The 677 C/T SNP was analysed by *Hin*fI restriction fragment length polymorphism as reported previously.[24] Sense and antisense PCR primers were 5′-TGA AGG AGA AGG TGT CTG CGG GA-3′ and 5′-AGG ACG GTG CGG TGA GAG TG-3′, respectively. PCR conditions were 95℃ for 2 min, followed by 35 cycles of 95℃ for 30 s, 55℃ for 30 s and 72℃ for 30 s, followed by a final extension of 7 min at 72℃. To analyse the rs2066462 C/T and rs2274976 G/A SNPs, we used a pyrosequencing approach (PSQ 96 instrument, Pyrosequencing AB, Uppsala, Sweden). To allow simultaneous determination of genotypes at the rs1801131 A/C (1298) and rs2274976 T/C SNPs, we amplified by PCR a 152-bp fragment from exon 7 of *MTHFR*. In all, 20 ng of genomic DNA was used in each PCR, which included 200 μM deoxynucleoside triphosphate, 1.5 mM $MgCl_2$, 0.4 μM of each primer and 5 U of Taq polymerase in standard PCR buffer. The PCR primers used were 5′-CTT TGG GGA GCT GAA GGA CTA CTA C-3′ (sense) and 5′-CAC TTT GTG ACC ATT CCG GTT TG-3′ (antisense). PCR conditions were 95℃ for 2 min, followed by 35 cycles of 95℃ for 30 s, 55℃ for 30 s and 72℃ for 30 s, followed by a final extension of 7 min at 72℃. PCR products were analysed by electrophoresis on 2% agarose gel, followed by excision of bands from the gel and purification using the Qiaquick gel extraction kit (Qiagen, Valencia, California, USA). Direct cycle sequencing of the amplified product was performed on an ABI 377 automated sequencer, using the Applied Biosystem BigDye™ Terminator Cycle Sequencing Ready Reaction kit (PE Applied Biosystems, Foster City, California, USA).

Construction of haplotypes

Haplotypes defined by the five *MTHFR* SNPs were constructed using the PHASE V.2.0 program (http://www.stat.washington.edu/stephens/software.html).[25] This program is based on the expectation–maximisation algorithm, which allows maximum-likelihood estimates of molecular haplotype frequencies under the assumption of Hardy–Weinberg proportions, as described by Excoffier and Slatkin.[26] PHASE V.2.0 incorporates SNP position while deducing haplotypes to account for linkage disequilibrium and is capable of making accurate haplotype calls with up to 5% missing data (alleles). In our analysis, <1% of alleles were missing. The degree of linkage disequilibrium between these 677 and 1298 SNPs was determined using Ldmax using the Graduates of the Last Decade program, which uses the expectation–maximisation algorithm[27] to estimate the maximum likelihood of pairwise disequilibrium (http://www.sph.umich.edu/csg/abecasis/GOLD/docs/stats.html). The distribution of linkage disequilibrium is estimated by the D′ values, where D′ ranges from 0 to 1 (greater values indicate stronger linkage disequilibrium).

Statistical analyses

Hardy–Weinberg equilibrium was assessed using χ^2 analysis. Differences in allele and haplotype frequencies between sample populations were evaluated using the χ^2 test. For the subset of 223 participants from the folate and Immunex ERA trials, we performed a multivariable logistic regression analysis based on the presence or absence of the minor allele for each SNP and MTX toxicity events. MTX dose and folic-acid dose were adjusted for in the analyses. Because there were significant differences in SNP allele and haplotype frequencies between Caucasian and African-American patients, separate analyses were carried out according to ethnic group. A similar analysis was carried out on the basis of the presence or absence of the six most common haplotypes (which defined 97.3% of African-American and 94.7% of Caucasian haplotypes) and MTX toxicity, also adjusting for MTX and folic acid dose in the analyses. For the 146 patients in the ERA trial for whom we had ACR20, ACR50, ACR70 and DAS28 response data, logistic and linear regressions were used to assess clinical response, respectively. As this study was exploratory in nature and not all comparisons were independent (haplotypes are defined by individual SNP alleles), we did not perform explicit correction for multiple testing.

RESULTS

SNP allele or haplotype frequencies in Caucasians and African-Americans

Table 2 shows the genotype frequencies; all SNPs were in Hardy–Weinberg equilibrium. There were no significant differences in genotype frequencies of individual *MTHFR* SNPs between patients and controls, regardless of race or ethnicity. Striking differences were, however, seen in allele frequencies between African-Americans and Caucasians (independent of disease status) in three of the five SNPs. The rs1801133 (677) T allele frequencies were 0.30 among Caucasians with rheumatoid arthritis and 0.11 among

African-Americans with rheumatoid arthritis (p = 0.002). The 1298 C allele frequency was 0.34 among Caucasians with rheumatoid arthritis and 0.13 among African-Americans with rheumatoid arthritis (p<0.001). The most striking difference in rheumatoid arthritis between Caucasians and African-Americans was in the frequency of the rs4846051 C allele (0.33 among African-Americans with rheumatoid arthritis *v* 0.08 among Caucasians with rheumatoid arthritis, p<0.001).

As expected from the differences in allele frequencies, there were significant differences in haplotype distributions between Caucasians and African-Americans. Table 3 gives a complete list of haplotypes. The most common haplotypes (haplotypes 1–6) accounted for ~98% of all haplotypes. The overall haplotype distributions were significantly different between African-Americans and Caucasians, regardless of disease status (p<0.001).

SNP alleles or haplotypes and MTX efficacy and toxicity

We found no association between SNP alleles or haplotypes and efficacy as defined by the mean change in DAS28 or achievement of ACR20, ACR50 or ACR70 response among the 146 participants in the Immunex ERA trial.[21 22] Our analysis of adverse events in 223 participants in the UAB folate and Immunex ERA trials, however, showed several important findings. Among Caucasians (n = 193), there was a significant association between the presence of the 1298 A allele and the presence of at least one MTX-related adverse event (odds ratio 15.9, 95% confidence interval 1.5 to 167.0; p = 0.021). We found no other significant associations between SNP alleles or haplotypes and the presence of overall MTX toxicity, and no SNP or haplotype associations with specific types of MTX toxicity as defined by organ systems. No correlations were found between SNP alleles or haplotypes and the presence or absence of MTX toxicity or specific types of MTX toxicity in 30 African-Americans from these two trials. We, however, found an association with the quantitative MTX toxicity score (see below).

In addition to the approach described above, we analysed *MTHFR* genotypes and cumulative toxicity scores in the UAB folate trial.[20] The toxicity scores of all subjects in the three groups combined (27.5 mg folate, 5 mg folate and 0 mg placebo) ranged from 0 to 9.29, with a mean score of 1.519 and a median score of 0.058. Among the African-Americans (n = 18), the mean and median toxicity scores were 0.73 and 0.056, respectively. The mean and median scores among the Caucasians (n = 59) were 4.643 and 0.063, respectively. Linear regression was used to assess genetic effects on toxicity. Analysis of the residuals indicated a slight departure from normality. A log transformation was used and the results were extremely similar; therefore, we report the results based on the untransformed variables. The presence of the C allele at the exon 7 rs4846051 SNP was associated with a higher mean toxicity score among African-Americans than Caucasians (0.371 *v* 0.078, p = 0.050). The presence of at least one copy of haplotype 4 (which contains the rs4846051 C allele) was also associated with a higher toxicity score among the African-Americans (p = 0.03). No other significant associations were seen between SNP alleles or haplotypes and MTX toxicity scores in the Caucasians or African-Americans in this trial.

DISCUSSION

We have defined racial or ethnic differences in frequencies of SNP alleles and novel SNP haplotypes in the coding region of *MTHFR*. Analyses of *MTHFR* SNP haplotypes by other investigators have focused on the 677 and 1298 SNPs, and more recently the rs2274976 SNP.[28] One study of Israelis, Ghanaians and Japanese showed substantial differences in 677 T allele frequencies in Africans (0.08) compared with Caucasians (0.34) and Japanese (0.42).[12] Similar frequencies of the 677 T allele were reported in a study evaluating 95 European and 95 African blood donors, with a 677 T allele frequency of 0.316 in Europeans compared with 0.038 in Africans.[28] The 677 T allele frequency in our African-American population (n = 179) was slightly higher at 0.12 than Africans, probably reflecting genetic admixture with the Caucasian population.

Our study corroborates the finding of linkage between the 677 T allele and the 1298 A allele in Caucasians.[12] Of the 15 haplotypes shown in table 2, 5 contain the 677 T allele (haplotypes 2, 10, 13–15), which account for 311 of the 1268 haplotypes (24.5%). Of the 311 haplotypes containing the 677 T allele, 307 (98.7%); (2, 13, 14 and 15) also contained the

Table 2 Number and frequency of methylenetetrahydrofolate reductase gene single-nucleotide polymorphism genotypes in Causasians and African-Americans with rheumatoid arthritis and controls

SNP genotype	African-Americans with RA (n = 138)	Caucasians with RA (n = 393)	African-American controls (n = 53)	Caucasian controls (n = 50)
677 CC	109 (0.79)	196 (0.50)	39 (0.75)*	25 (0.50)
CT	27 (0.20)	157 (0.40)	12 (0.23)	22 (0.44)
TT	2 (0.01)	40 (0.10)	1 (0.02)	3 (0.06)
rs2066462 CC	117 (0.85)	320 (0.81)	42 (0.79)	37 (0.74)
CT	21 (0.15)	68 (0.18)	10 (0.19)	13 (0.26)
TT	0	5 (0.01)	1 (0.02)	0
1298 AA	102 (0.74)	178 (0.45)	36 (0.68)	25 (0.50)
AC	35 (0.25)	165 (0.42)	16 (0.30)	19 (0.38)
CC	1 (0.01)	50 (0.13)	1 (0.02)	6 (0.12)
rs4846051 TT	63 (0.46)	387 (0.98)	25 (0.47)	49 (0.98)
TC	60 (0.43)	6 (0.02)	23 (0.43)	1 (0.02)
CC	15 (0.11)	0	5 (0.09)	0
rs2274976 GG	123 (0.93) *	355 (0.90)	43 (0.84) *	46 (0.92)
GA	9 (0.07)	37 (0.09)	8 (0.16)	4 (0.08)
AA	0	1 (0.002)	0	0

RA, rheumatoid arthritis; SNP, single-nucleotide polymorphism.
Caucasians with rheumatoid arthritis were from the Iowa rheumatoid arthritis cohort (n = 200),[19] the University of Alabama at Birmingham folate trial in rheumatoid arthritis (n = 59)[20] and the Immunex rheumatoid arthritis trial (n = 134).[21] African-Americans with rheumatoid arthritis were from the Grady African-American Rheumatoid Arthritis (GAARA) cohort (n = 108),[18] the aforementioned folate trial (n = 18) and Immunex trial (n = 12). *In all, 102 subjects from GAARA were genotyped at the rs2274976 GA SNP; 52 African-American controls were genotyped at the 677 CT SNP and 51 subjects at the rs2274976 GA SNP.

Table 3 Number and frequency of methylenetetrahydrofolate reductase gene single-nucleotide polymorphism haplotypes in Causasians and African-Americans with rheumatoid arthritis and controls

SNP haplotype	African-Americans with RA (n = 138)	Caucasians with RA (n = 393)	African-American controls (n = 53)	Caucasian controls (n = 50)
1. C/C/A/T/G	115 (0.417)	272 (0.346)	39 (0.368)	38 (0.380)
2. T/C/A/T/G	28 (0.102)	233 (0.296)	14 (0.132)	28 (0.280)
3. C/C/C/T/G	20 (0.073)	186 (0.237)	6 (0.057)	20 (0.200)
4. C/C/A/C/G	88 (0.321)	5 (0.010)	33 (0.311)	1 (0.010)
5. C/T/C/T/G	12 (0.044)	42 (0.053)	6 (0.057)	8 (0.080)
6. C/T/C/T/A	6 (0.024)	28 (0.036)	5 (0.047)	3 (0.030)
7. C/C/C/T/A	0	6 (0.012)	1 (0.009)	0
8. C/T/A/T/A	2 (0.008)	2 (0.004)	1 (0.009)	1 (0.010)
9. C/T/A/T/G	2 (0.008)	4 (0.005)	0	1 (0.010)
10. T/C/C/T/G	0	4 (0.008)	0	0
11. C/C/A/T/A	1(0.004)	2 (0.004)	1 (0.009)	0
12. C/T/A/C/G	0	1 (0.002)	0	0
13. T/T/A/T/G	1 (0.004)	0	0	0
14. T/C/A/T/A	0	1 (0.002)	0	0
15. T/C/A/C/G	2 (0.007)	0	0	0

RA, rheumatoid arthritis; SNP, single-nucleotide polymorphism.
Haplotypes were defined by the five SNPs shown in table 1: 677 (rs1801133), rs2066462, 1298 (rs1801131), rs4846051 and rs2274976. The difference in haplotype distribution between Caucasians and African-Americans with rheumatoid arthritis was significant (p = 0.001).

1298 A allele Haplotypes. In Caucasians, the D′ value for the 677 and 1298 SNPs was 0.955, indicating strong linkage disequilibrium. In African-Americans, however, the D′ value was much lower (0.408), indicating less linkage disequilibrium between the two SNPs in this racial or ethnic group. We found no associations between SNP alleles or haplotypes and efficacy of MTX as defined by the mean change in DAS28, despite 82% of the subjects having a good to moderate response by the end of the study.

We found an association between the 1298 A allele and the presence of at least one MTX-related toxicity event in 193 Caucasians with rheumatoid arthritis (including both the Immunex ERA trial and the UAB folate trial). We found no such association among the 18 African-American patients who had MTX toxicity (of a total of 30 African-Americans from the Immunex ERA and the UAB folate trials). The lack of association in African-Americans is probably because of a lack of statistical power. We did not find an association between the 677 SNP and toxicity of MTX in either Caucasians or African-Americans. We do not believe that this is due to a concealing effect by linkage disequilibrium, as there was no evidence of two-way interaction found between the 677 and 1298 SNPs. The association of the 1298 A allele with MTX toxicity corroborates the findings in a Jewish population with rheumatoid arthritis[15 16] and those in a Japanese population with rheumatoid arthritis. We did not find the 1298 A allele to be over-represented in either our Caucasian or African-American patients with rheumatoid arthritis compared with the controls, as reported by Berkun *et al*.[16]

This characterisation of *MTHFR* SNP allele and haplotype frequencies (independent of MTX toxicity) included 138 African-Americans and 393 Caucasians with rheumatoid arthritis. Significant differences in SNP allele frequencies in Caucasian and African-American patients and controls were found in the 677C/T 1298A/C and rs4846051 T/C SNPs. The minor allele frequency of the rs4846051 SNP differs by ~30% between the African-American and Caucasian populations with rheumatoid arthritis and therefore may prove to be a useful ancestry informative marker in future analyses on admixtures.[29]

One potential limitation of this study is that our toxicity data are derived from two clinical trials in which the mean dose of MTX and folic-acid supplementation differed. Although we accounted for MTX and folic-acid dose in our statistical analyses, these may have affected the data on both toxicity and efficacy, as suggested by data from Khanna *et al*.[30] In addition, we do not have detailed information on other factors such as weight that may influence MTX pharmacokinetics. Another limitation is our lack of statistical power among African-Americans with rheumatoid arthritis, which highlights the need for cohorts of African-Americans with rheumatoid arthritis.[31] Finally, there may have been some confounders of the reportedly MTX-related adverse events—for example, fatigue and malaise may be due to rheumatoid arthritis disease activity and dyspepsia may be due to concurrent drugs such as non-steroidal anti-inflammatory drugs. Thus, these findings should be replicated in larger studies, particularly those that include African-Americans.

Because of the many actions of MTX, multiple genes may influence its efficacy and toxicity in patients with rheumatoid arthritis. In addition, the ability of cells to accumulate methotrexate polyglutamates (MTXPG), important metabolites of MTX, is an important determinant of the effects of the drug. Candidate genes that have been explored for an effect on the efficacy or toxicity of MTX include methionine synthase,[32] methionine synthase reductase,[33] adenosine 2A receptor,[34] dihydrofolate reductase,[35] 5-aminoimidazole-4-carboxamide ribonucleotide formyltransferase transformylase,[36 37] aldehyde oxidase,[38 39] ATP-binding cassette,[28] reduced folate carrier,[37] folylpolyglutamyl synthase (FPGS),[28] thymidylate synthetase,[28 37] adenosine monophosphate deaminase-1, adenosine deaminase-1 and others. In a cross-sectional study of 102 patients with rheumatoid arthritis (race/ethnicity unspecified) on low-dose MTX for at least 3 months, Dervieux *et al*[37] showed that a combination of common genetic polymorphisms in reduced folate carrier-1 (*SLC19A1*), aminoimidazole-4-carboxamide ribonucleotide formyltransferase transformylase (*ATIC*), and thymidylate synthetase (*TYMS*) and MTX polyglutamate levels in RBCs influences the effect of MTX in rheumatoid arthritis.

Evans *et al*[40] found that acute lymphoblastic leukaemia subtype-specific patterns of folate pathway gene expression were significantly related to MTXPG accumulation. They found that lower MTXPG accumulation in these acute lymphoblastic leukaemia subtypes correlated with lower expression of the reduced folate carriers (*SLC19A1*, an MTX uptake transporter), higher expression of breast cancer resistance protein (ABCG2, an MTX efflux transporter) and lower expression of folylpoly glutomyl synthese (which catalyses the formation of MTXPG). The role of SNPs in these genes in the efficacy and toxicity of

MTX in rheumatoid arthritis is unknown. Further studies are needed to clearly define the functional role of SNPs and haplotypes in candidate genes, and differences in allele frequencies according to race or ethnicity, to move forward to the goal of providing useful pharmacogenetic markers for all patients with rheumatoid arthritis.

In conclusion, our results highlight racial or ethnic differences in frequencies of common *MTHFR* SNPs. The *MTHFR* 1298 A allele was associated with MTX-related adverse events in Caucasians, whereas the rs4846051 C allele appears to be related to MTX toxicity in African-Americans. These findings should be tested in larger, prospective studies of MTX in rheumatoid arthritis. The rs4846051 SNP, which is more common in African-Americans than Caucasians, may prove to be a useful ancestry informative marker in future studies on genetic admixture.

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