Polymorphisms in Folate, Pyrimidine, and Purine Metabolism Are Associated with Efficacy and Toxicity of Methotrexate in Psoriasis

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Methotrexate is the gold standard therapy for moderate to severe psoriasis, but there is marked interpersonal variation in its efficacy and toxicity. We hypothesized that in psoriasis patients, specific common polymorphisms in folate, pyrimidine, and purine metabolic enzymes are associated with methotrexate efficacy and/or toxicity. DNA from 203 retrospectively recruited psoriasis patients treated with methotrexate was collected and genotyped by restriction endonuclease digestion or length polymorphism assays. The reduced folate carrier (RFC) 80A allele and the thymidylate synthase (TS) 3′-untranslated region (3′-UTR) 6 bp deletion were associated with methotrexate-induced toxicity ($P=0.025$ and $P=0.025$, respectively). RFC 80A and 5-aminoimidazole-4-carboxamide ribonucleotide transformylase (ATIC) 347G were associated with methotrexate discontinuation ($P=0.048$ and $P=0.038$). The TS 5′-UTR 28 bp 3R polymorphism correlated with poor clinical outcome ($P=0.029$), however, this was not the case when patients with palmoplantar pustular psoriasis were not included in the analysis. Stronger associations between specific polymorphisms and methotrexate-induced toxicity and discontinuation were found in a subanalysis of patients on methotrexate not receiving folic acid supplementation. We have demonstrated preliminary evidence that specific polymorphisms of enzymes involved in folate, pyrimidine, and purine metabolism could be useful in predicting clinical response to methotrexate in patients with psoriasis.

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INTRODUCTION

Methotrexate is considered the gold standard therapy for moderate to severe psoriasis. However, there is a considerable interpersonal variation in the therapeutic response and toxicity profile of this drug. Methotrexate therapy can be complicated by acute myelosuppression, gastrointestinal symptoms, and hepatotoxicity, resulting in up to 30% of patients having to discontinue therapy (Roenigk *et al.*, 1998; Haustein and Rytter, 2000). A pharmacogenetic study of the use of methotrexate in psoriasis would allow the development of individually tailored drug therapy, therefore maximizing efficacy and minimizing toxicity.

Although the mechanism of action of methotrexate is not fully understood, it appears clear that once transported into the cell by folate-specific carrier mechanisms, methotrexate interferes with folate metabolism (Figure S1), and that polymorphisms in the carriers and enzymes involved in this biological pathway could potentially be responsible for methotrexate resistance or an increased risk of adverse events in patients with psoriasis. There is evidence from research in hematological malignancies (Krajinovic *et al.*, 2002, 2004; Laverdiere *et al.*, 2002; Aplenc *et al.*, 2005), osteosarcoma (Ifergan *et al.*, 2003; Flintoff *et al.*, 2004; Serra *et al.*, 2004), and recently, rheumatoid arthritis (RA) (Kumagai *et al.*, 2003; Berkun *et al.*, 2004; Dervieux *et al.*, 2004), that polymorphic variants in some enzymatic targets of methotrexate play an important role in the individual's sensitivity to the drug, but very little is known about their effect in psoriasis patients taking methotrexate.

The reduced folate carrier (RFC) 80G>A (Arg27His) single-nucleotide polymorphism (SNP), was significantly associated with a worse outcome in children with acute lymphoblastic leukemia who received chemotherapy with methotrexate (Whetstine *et al.*, 2001; Laverdiere *et al.*, 2002). More recently, patients diagnosed with RA carrying the

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Abbreviations: ADA, adenosine deaminase; ALT, alanine transaminase; ATIC, 5-aminoimidazole-4-carboxamide ribonucleotide transformylase; CI, confidence interval; MTHFR, methylenetetrahydrofolate reductase; OR, odds ratio; P3P, procollagen III; RA, rheumatoid arthritis; RFC, reduced folate carrier; SNP, single-nucleotide polymorphism; TS, thymidylate synthase; UTR, untranslated region

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homozygous variant genotype (RFC 80AA) were found to have a better clinical response to methotrexate (Dervieux *et al.*, 2004).

There is evidence that a polymorphism in the promoter region of the thymidylate synthase gene (TS), a double or triple tandem 28 bp repeat (2R or 3R), might influence therapeutic response to methotrexate. The homozygous 3R/3R genotype was associated with increased expression of the TS gene (Horie *et al.*, 1995; Kawakami *et al.*, 1999). However, a guanine to cytosine SNP recently identified within the triple repeat allele (3R G>C) results in a TS transcriptional activity similar to that of the double repeat (2R) allele. The 3R/3R genotype was associated with a poor response to methotrexate therapy in acute lymphoblastic leukemia (Krajinovic *et al.*, 2002) and RA (Kumagai *et al.*, 2003; Dervieux *et al.*, 2004). A homozygous 6 bp deletion (6bp del) at nucleotide 1,494 in the 3′-untranslated region (3′-UTR) (TS 3′-UTR 6bp ins/del) of the TS gene correlated with a better therapeutic response to methotrexate in RA (Kumagai *et al.*, 2003).

The 677C>T (Ala222Val) and 1298A>C (Glu429Ala) variants in the methylenetetrahydrofolate reductase (MTHFR) gene have been associated with adverse drug events related to methotrexate (Chango *et al.*, 2000; Rosenberg *et al.*, 2002). The MTHFR 677T allele leads to a thermolabile enzyme variant, with decreased activity and subsequent increased plasma homocysteine levels, and has been associated with increased methotrexate toxicity in patients treated for acute lymphoblastic leukemia (Ulrich *et al.*, 2001; Chiusolo *et al.*, 2002) and RA (van Ede *et al.*, 2001). The MTHFR 1298A>C polymorphism leads to a protein variant with 60% of the activity, which is neither thermolabile nor associated with increased plasma homocysteine levels. However, when combined with the 677T SNP in a compound heterozygous genotype, MTHFR activity was significantly decreased with an increased frequency of methotrexate-induced adverse events (Weisberg *et al.*, 1998).

Inhibition of 5-aminoimidazole-4-carboxamide ribonucleotide transformylase (ATIC) by methotrexate results in the accumulation of adenosine, a potent anti-inflammatory agent (Baggott *et al.*, 1986; Cronstein *et al.*, 1994; Montesinos *et al.*, 2003). Although the functional consequences of the ATIC 347C>G (Thr116Ser) SNP are unknown, the homozygous variant genotype 347 GG was associated with better therapeutic response to methotrexate in patients with RA (Dervieux *et al.*, 2004).

A functional significance in relation to methotrexate has also been suggested for adenosine deaminase (ADA), not directly related to folate metabolism but also involved in purine metabolism. Activity of this enzyme falls during methotrexate treatment with a subsequent increase in adenosine levels (van Ede *et al.*, 2002). A 22G>A SNP leads to the synthesis of ADA2, a polymorphic variant with a significantly reduced enzyme activity (Hirschhorn *et al.*, 1994). ADA2 is predicted to result in increased adenosine concentrations, and, therefore, might be associated with a better therapeutic response to methotrexate.

We set out to investigate the potential role of these common polymorphisms in folate, pyrimidine, and purine metabolic enzymes in predicting clinical outcome and/or toxicity owing to therapy with methotrexate in patients with psoriasis.

RESULTS

203 patients with psoriasis (192 with chronic plaque, 8 with palmoplantar pustular (PPP), 2 with erythrodermic, and 1 with guttate psoriasis), who had previously or currently been treated with oral methotrexate were recruited in this study. Among patients with PPP, four were classified as non-responders, two as responders, and two as indeterminate. In total, 193 patients completed 3 months therapy with methotrexate, and of these, 114 were defined as methotrexate responders, 44 as non responders, and 35 as indeterminate. Patients were classified as indeterminate if clinical information was not available, incomplete, or did not fulfil the criteria for the definition of response or non response. Seven patients out of 203 did not finish a 3-month course with methotrexate because of intolerable side effects. Clear information regarding the presence or absence of methotrexate-induced toxicity was available for 188 patients. One hundred and four patients experienced at least one adverse event, with 67 having to discontinue the drug (Table S1). Only two out of 203 patients experienced bone marrow toxicity and were therefore not analyzed as a separate subgroup. Ninety-six patients received folic acid 5 mg either daily or weekly. In each group the majority of patients were white Caucasian (80–84%), with too few non-Caucasian patients for allele frequencies to be compared by ethnicity. Patients who responded to methotrexate or experienced an adverse event and/or discontinued therapy did not receive higher doses of the drug (Table S1). Folic acid was not associated with poor response to methotrexate, although there was a negative correlation between folate supplementation and methotrexate withdrawal, with patients not receiving folic acid significantly more likely to discontinue therapy ($P=0.011$, odds ratio (OR) = 2.26[1.19–4.30] 95% confidence interval (CI)). The therapy discontinuation rate was 33%, the most frequent reason being nausea (35%), closely followed by abnormal transaminase levels (30%) (Table S2).

Impact of RFC, MTHFR, TS, ATIC, and ADA polymorphisms on methotrexate efficacy

The TS 5′-UTR 3R allele was significantly ($P=0.029$) more frequent in patients who did not respond to methotrexate (64%) than in responders (50%). Although the frequency of the 3R/3R variant homozygous genotype did not significantly vary between responders and non-responders, the presence of one or two 3R alleles (homozygous or heterozygous variant genotype) was significantly associated with non-response to therapy ($P=0.048$, OR = 2.96 [0.96–9.06] 95% CI). However, when patients with PPP were excluded, the significance for the TS 5′-UTR 3R allele was lost. The frequency of the TS 5′-UTR 3RC allele or the TS 3′-UTR 6bp del allele did not differ significantly between the two patient

groups. A subanalysis of patients not receiving folic acid showed that TS 5′-UTR 3R and TS 3′-UTR 6bp del alleles were more frequent in non-responders (58 and 37%, respectively) compared with responders (43 and 25%, respectively), but not significantly so. The MTHFR 677C>T and MTHFR 1298A>C, ATIC 347C>G, RFC 80G>A and ADA 22G>A allele or genotype frequencies did not significantly differ between the two groups, regardless of whether a dominant or recessive model was applied.

Impact of RFC, MTHFR, TS, ATIC, and ADA polymorphisms on methotrexate-induced toxicity

The TS 3′-UTR 6bp del polymorphism was significantly more frequent in patients who experienced an adverse event ($P=0.025$), and this was more likely to be a symptomatic side effect ($P=0.044$) than an alanine transaminase (ALT) elevation ($P=0.126$) (Table 1), irrespective of folic acid supplementation. This was a dominant effect as the frequency of the variant homozygous and heterozygous genotypes was significantly higher than the frequency of the wild-type genotype ($P=0.046$, OR = 1.86 (1.00–3.44) 95% CI).

Patients with one or more adverse events had a significantly higher percentage of RFC 80A alleles than those tolerant to methotrexate ($P=0.025$) and this effect was dominant ($P=0.049$, OR = 1.89[0.99–3.61] 95% CI). When the adverse event group was broken down into specific events, the RFC 80A allele was more frequent both in patients with hepatotoxicity ($P=0.053$) and those with a symptomatic side effect ($P=0.043$) than in tolerant patients, but analysis of genotype frequencies failed to show any associations (Table 1). In patients with symptomatic side effects, a joint analysis of RFC and TS 3′-UTR polymorphisms showed evidence for epistasis between the loci ($P=0.013$), with an OR of 2.86 for the RFC 80A/TS 3′-UTR 6bp del genotype, compared to the RFC 80G/TS 3′-UTR 6bp ins genotype. The frequency of the TS 5′-UTR 2R or 3R alleles, as well as the 3RG>C SNP did not differ significantly between patients who experienced adverse events and those who did not.

In a subanalysis of patients not on concomitant folic acid supplementation, allele and genotype distributions were altered even more (Table S3). The TS 5′-UTR 3R allele was significantly more frequent in patients who experienced an adverse event ($P=0.0025$), hepatotoxicity ($P=0.015$), or a symptomatic side effect ($P=0.0034$). Genotypes with at least one TS 5′-UTR 3R allele were significantly more frequent in these three groups except among patients with hepatotoxicity. ORs calculated for the different genotypes were greater for the variant homozygous genotype than for variant homozygous and heterozygous genotypes combined, suggesting a recessive effect: the TS 5′-UTR 3R/3R genotype was significantly more frequent in patients with any adverse event ($P=0.0052$, OR = 13.12 [1.59–107.9] 95% CI), hepatotoxicity ($P=0.0083$, OR = 15.75 [1.63–152] 95% CI), and symptomatic side effects ($P=0.011$, OR = 11.8 [1.35–103]95%CI). The TS 5′-UTR 3RC allele was also more common in patients with an adverse event ($P=0.027$) and with a symptomatic side effect ($P=0.013$) but not in those with hepatotoxicity. However, there was no difference in genotype distribution. Patients with hepatotoxicity had an increased frequency of the TS 3′-UTR 6bp del mutation ($P=0.012$) and any variant genotype carried a significantly risk of this adverse event ($P=0.015$, OR = 8.4 [1.5–46.1] 95% CI).

The RFC 80A allele was significantly more common in patients with an adverse event ($P=0.024$). The frequency of any RFC variant genotype was also higher in this group of patients but not significantly so. The frequency of the MTHFR 1298C allelic variant was significantly reduced if hepatotoxicity had occurred ($P=0.042$), whereas the distribution of the 677T allele was not changed. The frequency of MTHFR variant genotypes did not differ between groups.

The occurrence of abnormal serum procollagen III (P3P) results was investigated separately (data not shown). The ATIC 347G allele was found to be more frequent in patients with abnormal serum P3P values ($P=0.043$), who also had an increased number of the RFC 80A allele if they were not taking folic acid ($P=0.027$). No other mutation was found to be more common in this group.

Patients who discontinued methotrexate owing to intolerable side effects (Table S4) had a higher frequency of the RFC 80A allele ($P=0.048$), and any RFC variant genotype was associated with a significant increase in risk of discontinuing the drug ($P=0.01$, OR = 2.40[1.21–4.75] 95% CI). The ATIC 347G allele frequency was also significantly increased in these patients ($P=0.038$), but no specific genotype was significantly more frequent. A joint analysis of RFC and ATIC confirmed the risk effect of both loci ($P=0.0076$) with an OR of 2.9 for the RFC 80A/ATIC 347G genotype; there was no evidence of epistasis between the loci. In patients who discontinued methotrexate and had not received folic acid supplementation, the increase in RFC 80A frequency was even more significant ($P=0.00021$), with a higher risk of discontinuation conferred by both variant homozygous and heterozygous genotypes ($P=0.002$, OR = 4.87 [1.69–14.05] 95% CI). In this group of patients, TS 5′-UTR 3R was also significantly more common than in the group who continued therapy with methotrexate ($P=0.033$), but genotype distribution did not significantly differ between the two groups.

Haplotype analysis

The frequencies of MTHFR haplotypes were as follows: 677C/1298A 0.35, 677C/1298C 0.28, and 677T/1298A 0.36. The 677T/1298C haplotype was very rare, with a frequency of approximately 0.004. MTHFR SNPs are therefore in strong linkage disequilibrium ($D'=1$). The double heterozygous genotype (677CT/1298AC) was significantly less frequent in patients who experienced a raised ALT and were not taking folate supplementation compared with patients with no abnormal liver function tests ($P=0.033$). Linkage disequilibrium analysis of the TS loci showed four haplotypes: 5′-UTR 2R/3′-UTR 6bp ins 0.37, 5′-UTR 3R/3′-UTR 6bp ins 0.28, 5′-UTR 3R/3′-UTR 6bp del 0.25, and 5′-UTR 2R/3′-UTR 6bp del 0.096. A D' score of 0.4 meant that these two loci are in some but not strong disequilibrium. The double homozygous genotype was less frequent in patients who experienced an adverse event, but not significantly so.

Table 1. Methotrexate-induced toxicity and allele frequencies[1]

Genotypes and alleles	Controls (%)	Overall toxicity (%)	*P*-value	OR (95% CI)	Hepatotoxicity (%)	*P*-value	Symptomatic side effects (%)	*P*-value	OR (95%CI)
RFC 80G>A									
80A allele	58 (40)	108 (52)	**0.025**	**1.63 (1.06–2.51)**	37 (54)	0.053	80 (52)	**0.043**	**1.60 (1.01–2.53)**
80G allele	86 (60)	98 (48)			31 (46)		74 (48)		
80 AA	15 (20)	32 (31)	0.132		13 (38)	0.057	23 (30)	0.205	
80 GA + 80 GG	57 (80)	71 (69)			21 (62)		54 (70)		
80 GA + 80 AA	43 (59)	76 (74)	**0.049**	**1.89**	24 (70)	0.279	57 (74)	0.063	
80 GG	29 (41)	27 (26)			10 (30)		20 (26)		
MTHFR 677C>T									
677 T allele	54 (36)	75 (36)	0.92		30 (44)	0.319	55 (35)	0.751	
677C allele	92 (64)	131 (64)			38 (56)		101 (65)		
MTHFR A1298A>C									
1298C allele	43 (29)	48 (23)	0.193		13 (19)	0.109	40 (26)	0.502	
1298A allele	103 (71)	158 (77)			55 (81)		114 (74)		
TS 5′-UTR 28 bp repeats									
3R allele	71 (51)	116 (56)	0.305		41 (60)	0.193	84 (55)	0.511	
2R allele	69 (49)	90 (44)			27 (40)		70 (45)		
TS 5′-UTR 3RG>C									
3RC allele	30 (22)	59 (30)	0.092		19 (31)	0.193	44 (31)	0.106	
All other alleles (inc. 2R)	106 (78)	135 (70)			43 (69)		100 (69)		
TS 3′-UTR 6bp ins/del									
6bp del allele	42 (30)	84 (41)	**0.025**	**1.67 (1.06–2.63)**	27 (40)	0.126	62 (40)	**0.044**	**1.63 (1.01–2.65)**
6bp ins allele	102 (70)	122 (59)			41 (60)		92 (60)		
del/del	6 (8)	17 (16)	0.115		7 (21)	0.109	12 (16)	0.174	
ins/del+ins/ins	66 (92)	86 (84)			27 (79)		65 (84)		
ins/del+del/del	36 (51)	67 (65)	**0.046**	**1.86 (1.00–3.44)**	20 (59)	0.396	50 (64)	0.065	
ins/ins	36 (49)	36 (35)			14 (41)		27 (36)		
ATIC 347C>G									
347G allele	87 (60)	136 (66)	0.283		45 (66)	0.42	104 (68)	0.2	
347C allele	57 (40)	70 (33)			23 (34)		50 (32)		
ADA 22G>A									
22A allele	10 (7)	16 (8)	0.698		3 (4)	0.556	12 (8)	0.806	
22G allele	134 (93)	182 (92)			65 (96)		144 (92)		

ADA, adenosine deaminase; ATIC, 5-aminoimidazole-4-carboxamide ribonucleotide transformylase; CI, confidence interval; MTHFR, methylenetetrahydrofolate reductase; OR, odds ratio; RFC, reduced folate carrier; UTR, untranslated region.
[1]Genotype frequencies are provided only for alleles with a significantly different distribution.

DISCUSSION

This is the first study of methotrexate pharmacogenetics in psoriasis. Eight polymorphisms in five enzymes involved in folate, purine, and pyrimidine metabolism were correlated with methotrexate efficacy and toxicity in patients with severe psoriasis. We found consistently significant associations between polymorphisms in the RFC and TS genes and clinical response to methotrexate.

Our results show that the TS 5′-UTR 3R allele was associated with poor therapeutic response to methotrexate. When all patients were analyzed, those with variant 5′-UTR 3R genotypes were almost three times less likely to respond to

treatment than wild types. However, the exclusion of PPP patients resulted in the loss of a correlation between the TS 5′-UTR 3R allele and poor response to methotrexate, making it difficult to interpret this finding. It is possible that loss of significance is due to lower statistical power or perhaps, may reflect different pathophysiological mechanisms present in PPP.

An unexpected finding was the significant association between the TS 5′-UTR 3R allele and the increased incidence of methotrexate-induced toxicity in patients not receiving folic acid. TS 5′-UTR 3R/3R homozygotes in this group were 13 times more likely to experience any adverse event, 15 times more likely to develop hepatotoxicity, and almost 12 times more likely to suffer a symptomatic side effect than other genotypes. When a dominant model combining homozygotes and heterozygotes was applied, the overall risk of developing toxicity decreased to 3-fold and there was no significant association with hepatotoxicity. Thus, homozygosity for the 3R allele seems necessary for this mutation to significantly increase the risk of hepatotoxicity in patients not supplemented with folic acid. We suggest that the correlation of this variant with a higher incidence of side effects could be due to the increased transcriptional and/or translational activity associated with the 3R allele, and possibly the consequent depletion of the TS substrate 5,10-methylene-THF, necessary for the methylation of homocysteine. In addition, the 3R/3R genotype was associated with elevated plasma homocysteine levels among individuals with low dietary folate intake (Trinh *et al.*, 2002; Kumagai *et al.*, 2003).

However, recent evidence suggests that the addition of a tandem repeat alone is not sufficient for enhanced transcriptional activity of the TS gene, but that a specific consensus element binding an upstream-stimulating factor is required within the extra repeat to enhance transcription and hence expression (Mandola *et al.*, 2003). A G>C SNP at the 12th nucleotide of the second repeat of the TS 5′-UTR 3R allele abolishes upstream stimulating factor binding by disrupting the upstream stimulating factor consensus sequence, resulting in decreased transcriptional activity compared with wild types (3RG), but similar to the 2R allele (Mandola *et al.*, 2003). In our study, the distribution of the C and G SNPs in the second repeat of the 3R allele did not differ according to the methotrexate efficacy or toxicity. However, when we compared the frequency of the 3RC allele with that of all the other alleles combined (3RG and 2R), we found that when folic acid was not administered, patients carrying the 3RC allele had an almost 3-fold increased risk of developing an adverse event and a more than a 3-fold increased risk of experiencing symptomatic side effects, but were not more likely to have hepatotoxicity. These findings would suggest that the mechanism by which TS contributes to the development of toxicity varies with different adverse events, and that increased TS expression may be related to the development of hepatotoxicity, but not to that of symptomatic side effects.

We are the first to report that the TS 3′-UTR 6bp del allele is more frequent in patients with an adverse event or a symptomatic side effect, and in the absence of folate supplementation is associated with an 8-fold increased risk of developing a raised ALT level. This could be explained by decreased TS , as the TS 3′-UTR 6bp del mutation leads to lower TS mRNA expression in tumor cells (Ulrich *et al.*, 2000; Kumagai *et al.*, 2003).

In our study, the RFC 80G>A SNP was not associated with response to methotrexate. This is in conflict with the finding reported by Dervieux *et al.* (2004) but is consistent with the observation that the amino-acid sequence change (Arg27His) in RFC resulting from the 80G>A SNP did not alter uptake rates of methotrexate in pharmacokinetic studies (Chango *et al.*, 2000; Whetstine *et al.*, 2001). The 80G>A SNP was significantly and consistently associated with occurrence of methotrexate-induced side effects. Altered methotrexate uptake through the cell membrane now seems an unlikely explanation for the observed increased incidence of adverse events, and it may be that other mechanisms are involved.

No association was found between the MTHFR 677T SNP and methotrexate toxicity, a finding supported by some studies of RA patients, one prospective, (Kumagai *et al.*, 2003; Berkun *et al.*, 2004), but contrasting with others (van Ede *et al.*, 2001; Urano *et al.*, 2002). However, in those studies which showed a significant association between the 677T allele and methotrexate-induced toxicity, the relative risk was very small (1.25–2.38), suggesting a small effect for this allele, and possibly reflecting insufficient power in our study. In contrast, patients with the MTHFR 1298C allele and those with the double heterozygous genotype (677CT/1298AC) not receiving supplementation with folic acid were less likely to develop hepatotoxicity, consistent with the results of a previous study (Berkun *et al.*, 2004). We speculate that decreased enzyme activity may result in accumulation of 5,10-methylene-THF, necessary for thymidylate synthesis, therefore counteracting the methotrexate-induced depletion of THF and conferring protection against side effects.

The ATIC 347G allele was associated with abnormal serum P3P results, an effect which followed a recessive model, and increased 1.6-fold the likelihood of discontinuing methotrexate because of side effects. However, this polymorphism was not associated with any other adverse events, making the significance of these results uncertain.

We did not find any relationship between the ADA 22G>A SNP and methotrexate efficacy or toxicity. However, in our study, the frequency of the 22A allele was much lower (0.07) compared to that of the other polymorphisms studied and power may be an issue.

Our findings confirm the importance of administering concomitant folic acid to patients receiving methotrexate, as (1) patients not receiving folic acid supplementation were more than twice as likely to discontinue the drug and (2) in most cases the impact of polymorphisms associated with methotrexate-induced adverse events was attenuated or disappeared if folic acid had been prescribed.

There are important limitations to this study. First of all this is a retrospective study, which prevents objective and systematic assessment of clinical progress and routine documentation of adverse events. For the same reason it was not possible to obtain pharmacokinetic data and correlate polymorphisms with methotrexate or homocysteine

blood levels. Furthermore, some of the study subgroups are small and when results are corrected for multiple testing, significance for some associations is lost. However, allowing a correction factor for each polymorphism when the correction for multiple testing is applied, the TS 5′-UTR 3R and RFC 80 A alleles are still significantly associated with methotrexate-induced toxicity and discontinuation in the no-folate group. It is a concern that RFC genotypes were not in Hardy–Weinberg equilibrium within the study population. However, when patients with adverse events and patients without adverse events when analyzed separately, both were found to be in Hardy–Weinberg equilibrium. We cannot exclude the possibility that this finding results from genotyping errors. Alternatively, RFC alleles may be in disequilibrium with a locus conferring increased risk for psoriasis. In our view, the lack of Hardy–Weinberg equilibrium is likely to be the result of putting together two patient phenotypic groups with very different genotype distribution and may indicate a strong association between RFC 80 A and adverse events.

Despite important limitations, our findings are consistent within the study and/or are supported by previous studies. We have thus identified several polymorphisms in enzymes involved in folate, purine, and pyrimidine metabolism, especially TS and RFC, which have a significant impact on clinical response to methotrexate, and therefore are potentially useful in determining valid predictive markers of methotrexate efficacy and toxicity in psoriasis. Future work should be directed toward exploring the differential impact of these polymorphisms in a large prospective cohort of psoriasis patients commencing systemic therapy with methotrexate.

MATERIALS AND METHODS

This study adheres to the Declaration of Helsinki Principles and was approved by the Research Ethics Committee of Guy's and St Thomas' Hospitals in London. Informed written consent was obtained from all patients included in this study.

Study population

Adult patients (age 18 years and over) with psoriasis who had currently or previously been treated with methotrexate monotherapy were recruited via dermatology clinics, pharmacy lists, and a procollagen III test database at St Thomas' Hospital, London, UK. Patients were retrospectively classified according to clinical response to therapy with methotrexate, development of methotrexate-induced adverse events, and/or methotrexate discontinuation owing to any intolerable side effect. Patients who had completed at least 3 months of therapy with methotrexate were classified as responders (if there was an explicit statement in the case notes documenting significant improvement or clearance of psoriasis and/or >75% improvement in the Psoriasis Area Severity Index), non-responders (if there was an explicit statement in their case notes that therapy was stopped because of lack of efficacy and/or <50% improvement in Psoriasis Area Severity Index), or indeterminate (patients failing to fulfil the above criteria). Methotrexate-induced adverse events were categorized as (1) hepatotoxicity, that is, raised ALT (a raise in ALT more than three times the upper limit of normal within 3 years of starting methotrexate), resulting in dose reduction or termination of therapy; (2) bone marrow toxicity, that is, a decrease in hemoglobin, white cell count, or platelets to <75% of baseline, or white cell count <3.5, and/or platelets <120 within 3 years of starting methotrexate, resulting in dose reduction or termination of therapy; (3) symptomatic side effects, that is, nausea, diarrhea, hair loss, mouth ulcers, depression, headache, dizziness, or other side effects sufficiently significant to be documented in the clinical case notes; (4) abnormal serum P3P, that is, P3P>8 μg/l on two consecutive occasions, or >4.2 μg/l at least three times in a year, within 3 years of starting methotrexate (Chalmers *et al.*, 2005). General methotrexate-induced toxicity was defined as the presence of at least one adverse event excluding abnormal serum P3P. The abnormal serum P3P category was analyzed separately in view of the relatively recent introduction of this monitoring test for methotrexate-induced hepatic fibrosis, and its unavailability in many dermatology centres. Methotrexate withdrawal was defined as discontinuation of methotrexate owing to any adverse event. Concomitant therapy with folic acid was documented for each patient.

Methods

Venous blood for DNA extraction was collected in EDTA tubes and stored at –70°C. Genomic DNA was isolated from whole blood using standard techniques (Young *et al.*, 2003). DNA was amplified with recombinant *Taq* DNA polymerase recombinant (Invitrogen Ltd, Paisley, UK) in a total volume of 20 μl with 0.5 μM of each primer. Reactions were supplemented with 5% DMSO.

Methods for genotyping the TS 5′-UTR (Marsh *et al.*, 1999) TS 5′-UTR 3R G>C (Mandola *et al.*, 2003) RFC-1 80G>A (Dervieux *et al.*, 2004), MTHFR 677C>T and MTHFR 1298A>C (Breen *et al.*, 2005) polymorphisms have been published previously.

ATIC 347C>G. The ATIC 347 C>G mutation was amplified using primers forward 5′-TCCCTTTGTAAAGACAGTGGCTTCTC CAG*CA*GTAA-3′ and reverse 5′-AATTATAGTAATCCCAAAACACA ATCCAGAAGTAG-3′. The mismatch forward primer creates an *Alw*NI site when the 347 C>G mutation is present. The thermocycler profile consisted of 35 cycles of 94°C 30 seconds, 54°C 30 seconds, and 72°C 30 seconds. The PCR product was microdialysed for 3 minutes on Millipore VSP filters and digested with *Alw*NI at 37°C overnight. Samples were separated on a 2.5% Agarose 1000 (Invitrogen Ltd, Paisley, UK).

ADA 22G>A. The ADA 22 G>A mutation destroys a *Taq*I site and was amplified with primers forward, 5′-CACGAGGGCACC ATGGCCCAGA-3′ and reverse 5′-CCCATTGTCCCTGATTAGCCC GCAAG-3′. Thermocycler profile consisted of 35 cycles of 94°C 30 seconds, 63°C 30 seconds, and 72°C 30 seconds. The PCR product was microdialysed for 3 minutes on Millipore VSP filters and digested with *Taq*I at 65°C overnight. Products were separated on a 2.5% Agarose 1000.

The TS 3′-UTR 6bp ins/del. Sample were amplified twice. Both amplifications shared the same forward primer 5′-ATTACAA CAGGTCGTACAATTATGGC-3′, and differed in the reverse primer. The positive 5′- CTTTATTATAGCAACATATAAAACAACTATAACT-3′ primer was designed to anneal when the 6-bp sequence was present, and the negative 5′-TTTATTATAGCAACATATAAAACAACTATA AAGT-3′ reverse primer annealed specifically to the DNA sequence when the 6-bp fragment was absent. The fragments amplified with each set of primers was approximately 673-bp long. Each sample was run on consecutive lanes on a 2% standard agarose gel and scored for the presence or absence of the 673-bp band.

A PCR blank and genotype controls were included in each series of PCR reactions.

The genotyping success rate for all polymorphisms studied was 98–99.5%, with the exception of the TS 5′-UTR 3R G>C SNP (93.5%).

Statistical analysis

Patients were assigned to different groups according to their outcome to therapy (clinical response, development of side effects, and/or drug withdrawal following therapy with methotrexate). A subanalysis comparing these different groups was also carried out in patients not receiving folic acid. When analysing methotrexate efficacy, a further analysis was carried out with the exclusion of patients with palmoplantar pustular psoriasis, as there is increasing evidence that, although associated with psoriasis, this is a separate disease with different pathophysiological mechanisms. The number of alleles for each polymorphism was counted in each patient group. Allele frequencies were compared between groups in 2×2 contingency tables using the χ^2 test or the two-tailed Fisher's exact test (if <5 patients in the study group). A *P*-value of <0.05 was considered significant. Genotype frequencies were calculated and compared when a significant difference in allele distribution was detected. All genotypes were in Hardy–Weinberg equilibrium except for the RFC 80 genotypes. However, when RFC 80G>A genotypes of patients with adverse events and those without adverse events were analyzed as separate groups, genotypes were in Hardy–Weinberg equilibrium for both groups.

For the phenotypes where at least two SNPs or mutations were significant, both polymorphisms were analyzed together to test for an interaction or a joint effect (where each SNP increases the risk of the phenotype occurring, independently). This was carried out using Unphased (Dudbridge, 2003) software, which does not provide confidence intervals for ORs. The same program was used to carry out linkage disequilibrium analysis of the MTHFR and TS gene.

CONFLICT OF INTEREST

The authors state no conflict of interest.

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SUPPLEMENTARY MATERIAL

Figure S1. Overview of folate, purine and pyrimidine metabolic enzymes and methotrexate intracellular interactions.

Table S1. Demographic and clinical details of methotrexate patients.

Table S2. Adverse events leading to discontinuation of methotrexate.

Table S3. Methotrexate-induced toxicity and genotype and allele frequencies in patients not on folic acid.

Table S4. Genotype and allele frequencies and methotrexate discontinuation.

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