Concise Report

Polymorphisms within the adenosine receptor 2a gene are associated with adverse events in RA patients treated with MTX

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Objective. To examine the role of adenosine receptor 2a gene (ADORA2a) polymorphisms on outcome of MTX treatment in RA.

Methods. Subjects included 309 RA patients with a defined response to MTX. Patients were included if they were (i) good responders ($n = 147$) (ESR <20 for $>6/12$ on stable dose of MTX) (ii) inefficacy failures ($n = 101$) (physician statement and failure to reduce ESR/CRP by 20%) or (iii) adverse event (AE) failures ($n = 61$) (verified by medical record review). AEs were sub-divided into gastrointestinal (GI) ($n = 24$), abnormal LFTs ($n = 20$) or other ($n = 17$). 8 single nucleotide polymorphisms (SNPs) within ADORA2a were genotyped using the Sequenom MALDI-TOF platform.

Results. Five SNPs within ADORA2a were associated with stopping MTX for AEs (OR 2.1–3.07, $P < 0.05$ for all). Analysis by AE type showed that the association was specific for GI toxicity. No association was observed between ADORA2a and inefficacy outcomes.

Conclusion. Genetic variation within ADORA2a is significantly associated with AEs on MTX, specifically GI AEs. Knowledge of the ADORA2a genotype may help to improve identification of patients at high risk of GI toxicity with MTX.

KEY WORDS: Rheumatoid arthritis, Methotrexate, Adenosine, Polymorphism.

Introduction

Methotrexate (MTX) is a cornerstone of therapy for RA. However, its clinical efficacy is variable, with between 46% and 65% patients gaining a 20% improvement [1, 2]. MTX has anti-proliferative effects, via effects on purine synthesis and folate metabolism [3]. However, this is not its only mechanism of action in RA, since co-administration of folic acid does not significantly reduce efficacy although it does abrogate certain adverse effects [4].

Studies suggest that its anti-inflammatory effects are mediated via adenosine release, a potent anti-inflammatory nucleoside [5–7]. MTX polyglutamates inhibit the action of amino-imidazole-carboxamidoadenosine-ribonucleotide transformylase (AICAR transformylase) more potently than enzymes involved in purine synthesis, causing an accumulation of AICAR and its metabolites. These inhibit adenosine deaminase and adenosine monophosphate (AMP) deaminase, leading to increased levels of endogenous adenosine [3, 6]. Adenosine agonists have been shown to be effective in murine models of arthritis [8]. Adenosine binds to several receptor subtypes including A1, A2a, A2b and A3 [6]. Murine studies suggest a pivotal role for the A2a and A3 receptors [8] with the administration of adenosine receptor antagonists, caffeine and theophylline, being shown to effectively block the effects of MTX [9]. Similarly, adenosine receptor 2a (ADORA2a) agonists (e.g. CGS-21680) have anti-inflammatory actions similar to MTX [10].

There is increasing interest in the potential of pharmacogenetics to guide therapeutic decision making. Understanding MTX pharmacogenetics may determine as to which patients are most likely to respond to treatment and/or which patients may be more susceptible to toxicity. Recent reports suggest that genetic polymorphisms within the folate pathway may influence both MTX toxicity [11] and efficacy [12, 13]. Within newly diagnosed RA patients, Wessels *et al.* [14] demonstrated an association between genes coding for adenosine release and good response to MTX. In addition, they used a clinical pharmacogenetic model to predict MTX efficacy in 205 newly diagnosed DMARD-naïve RA patients, with 60% of patients being classified as responders or non-responders [15], although the utility of such a model in patients with established disease has yet to be determined. Our aim was to examine the association between MTX outcomes in a well-characterized group of established RA patients and single nucleotide polymorphisms (SNPs) within the adenosine receptor 2a (ADORA2a) gene.

Patients and methods

Study subjects and outcomes

Patients were retrospectively recruited from two hospital centres—the University Hospital of North Staffordshire (UHNS) and Central Manchester and Manchester Children's University Hospitals Trust (CMMC). UHNS has a computerized monitoring database which records all drug histories, blood results and clinical correspondence for each patient. Reasons for stopping DMARDs are also entered into this database that records all patients treated with MTX since 1986. At CMMC, case notes were analysed prior to all RA follow-up clinics to identify patients exposed to MTX monotherapy. Patients were considered eligible for inclusion if they were aged over 18 yrs, of White Caucasian ethnic origin, classified as having RA according to ARA criteria [16] and had one of three defined outcomes to MTX (see subsequently). Such patients were then approached to take part in the study, although it should be noted that only one patient considered eligible declined to take part.

(i) MTX responder: defined as patients who fulfilled three criteria—physician statement of good response and ESR ≤20 and/or normal CRP with a stable dose of MTX for at least 6 months.

(ii) MTX inefficacy: defined as physician statement of inefficacy plus failure to reduce ESR and/or CRP by at least 20% from baseline. Patients had to have received a minimum dose of 15 mg/week for at least 3 months.

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(iii) Adverse event (AE) failure: for an AE to be attributed to MTX it had to occur on treatment, resolve on treatment cessation and in the case of gastrointestinal side-effects recur on MTX rechallenge. AEs were further divided into subtypes:

(a) Gastrointestinal: nausea, vomiting or diarrhoea precluding MTX continuation.
(b) Abnormal liver function: liver transaminases $\geq$ twice the upper limit of normal on at least two occasions, or biopsy changes suggestive of toxicity.
(c) Other: haematological AEs, headaches, rashes or pneumonitis.

None of the patients were taking oral steroids or other DMARDs and all patients were taking at least 5 mg folic acid per week. Ethical approval for the study was obtained from North Staffordshire and Central Manchester Local Research Ethics Committees (Refs 03/20 and 03/CM/315, respectively) and all patients provided written informed consent prior to taking part in the study. Anonymized clinical data was abstracted from case notes using a standard proforma by a single observer (S.L.H.). Data collection and recruitment was completed using these pre-defined outcomes prior to any genetic analysis taking place.

Genetic polymorphisms

SNPs within ADORA2a (Chr22q 11.2, two exons) were identified using HapMap Phase I data and National Center for Biotechnology Information. Eight validated SNPs with a reported minor allele frequency (MAF) of >5% were selected (rs5760410, rs2298383, rs3761422, rs2267076, rs4822489, rs2236624, rs17650937, rs8192446). Genotyping was performed using MassARRAY high-throughput DNA analysis with matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometry (Sequenom, San Diego, CA, USA, www.sequenom.com).

Statistical analysis

Quality control. Hardy–Weinberg equilibrium (HWE) was determined using STATA v8 software (Stata Corp). SNPs were removed from analysis using pre-defined quality control (QC) standards (i) if deviation from HWE ($P \leq 0.05$) was observed in the responders (used as controls), (ii) if the genotyping success rate was <85% or (iii) if the MAF within our cohort was <5%.

Single-point analysis. Genotype frequencies were compared between the three groups using a chi-squared test, then analysed as a nested case–control study using the responders as the referent category. For significant results ($P < 0.05$) 2×2 contingency tables were used to generate odds ratios (ORs with 95%CIs), based on carriage of the rare allele. For SNPs showing a significant genotypic association ($P < 0.05$), permutation testing was performed to limit type II error. Empirical P-values were generated using PLINK, a Monte Carlo-based method for assessing significance of case–control association studies (http://pngu.mgh.harvard.edu/~purcell/plink/). Ten thousand simulations were performed. PLINK generates both uncorrected (EMP1) and corrected (EMP2) empirical P-values. The corrected empirical P-value (EMP2) calculates the proportion of permutations in which any of the test statistics exceeded that particular observed statistic. It does not assume that the tests are independent, but it still controls the probability of observing at least one false positive per experiment and hence is more stringent than the conventional EMP1 P-value. In both cases, P-values of <0.05 were taken as significant.

Results

We studied 309 RA patients (for clinical data, see supplementary table 1, available as supplementary data at *Rheumatology* Online), including 147 good responders, 101 inefficacy failures and 61 AE failures. The AEs included gastrointestinal ($n = 24$), abnormal liver function tests ($n = 20$) and other ($n = 17$, including haematological ($n = 7$) and skin rashes ($n = 6$).

Of the eight SNPs tested within ADORA2a, three failed QC (rs4822489 [genotyping success <85%, rs8192446 (non-polymorphic) and rs17650937 (MAF <5%)] leaving five SNPs for further analysis. These SNPs provided 100% pairwise coverage of the ADORA2a gene based on HapMap Phase I data (for linkage disequilibrium data, see supplementary table 2, available as supplementary data at *Rheumatology* Online). There was a significant difference across the three outcome groups for SNPs rs3761422 and rs2236624 (Table 1). All five SNPs were significantly associated with AEs (Table 1). Even applying the more stringent corrected permutation P-values (EMP2 in Table 1); two of the SNPs remained significantly associated with AEs. The AE group was then stratified by type and a consistent association was observed between gastrointestinal (GI) AEs and all five SNPs (Table 1). After corrected permutation P-values were applied, one SNP (rs3761422) remained significant and two other SNPs were borderline (rs5760410 and rs2298383, $P = 0.06$ for each). Table 2 illustrates the odds of having any AE (or GI AE) compared with responders with carriage of the variant allele.

TABLE 1. Genotype frequencies for ADORA2a SNPs

Genotype (% success)		R n (%)	IE n (%)	AE n (%)	Global chi	R vs IE P	Responders vs AEs			Responders vs GI AEs			
							P	EMP1	EMP2	Genotype GI AE n (%)	P	EMP1	EMP2
rs 5760410 (85.1)	AA	38 (31)	26 (30)	7 (13)	0.09	0.75	0.033	0.03	0.1	2 (9)	0.03	0.02	0.06
	AG	63 (52)	43 (49)	37 (69)						12 (56)			
	GG	20 (17)	18 (21)	10 (19)						8 (36)			
rs 2298383 (92.6)	CC	53 (38)	37 (40)	12 (22)	0.12	0.87	0.04	0.04	0.1	4 (19)	0.03	0.02	0.06
	CT	71 (52)	45 (48)	32 (58)						11 (52)			
	TT	14 (10)	11 (12)	11 (20)						6 (29)			
rs 3761422 (94.8)	CC	62 (44)	39 (42)	16 (27)	0.05	0.87	0.012	0.01	0.03	7 (29)	0.001	0.002	0.004
	CT	68 (48)	45 (48)	31 (53)						9 (38)			
	TT	11 (8)	9 (10)	12 (20)						8 (33)			
rs 2267076 (95.4)	CC	68 (49)	44 (45)	18 (31)	0.12	0.88	0.036	0.03	0.1	8 (35)	0.05	0.04	0.1
	CT	59 (42)	44 (45)	29 (50)						9 (39)			
	TT	13 (10)	9 (9)	11 (19)						6 (26)			
rs 2236624 (95.1)	CC	86 (62)	57 (59)	22 (37)	0.02	0.39	0.004	0.004	0.01	9 (38)	0.05	0.03	0.1
	CT	44 (32)	36 (38)	33 (56)						11 (46)			
	TT	9 (6)	3 (3)	4 (7)						4 (17)			

The table illustrates the genotype frequencies for the ADORA2a SNPs together with chi-squared results among the three groups, and responders (R) vs inefficacy (IE) and then AEs. All data is presented as n (%). The results of the permutation testing are displayed as with uncorrected P-values (EMP1) and with the permuted P-values corrected for the number of tests performed (EMP2). Data is then presented for GI AEs compared with responders, again with uncorrected P-value, and then EMP1 and EMP2.

TABLE 2. Odds of AEs and GI AEs with carriage of the ADORA2a variant allele

| | Carriage of variant allele (1 or 2 copies) | | | |
| | Any AE OR (95% CI) | P | GI AE OR (95% CI) | P |
SNP				
rs 5760410	3.07 (1.22, 8.76)	0.01	4.58 (1.01, 42.05)	0.03
rs 2298383	2.23 (1.03, 5.07)	0.03	2.65 (0.8, 11.35)	0.09
rs 3761422	2.11 (1.04, 4.39)	0.02	1.91 (0.69, 5.77)	0.17
rs 2267076	2.1 (1.05, 4.27)	0.023	1.77 (0.65, 5.13)	0.22
rs 2236624	2.73 (1.4, 5.39)	0.002	2.7 (1.02, 7.49)	0.04

Overall, these SNPs were significantly associated with any AEs at all five loci (OR 2.1–3.07, $P < 0.05$ for all). In subgroup analysis, carriage of the variant allele was significantly associated with GI AEs at two of the five loci. Although the numbers are small, homozygosity of the variant allele was associated with GI AEs at four loci (OR 2.9–5.9, $P < 0.05$).

Discussion

A key anti-inflammatory mechanism of MTX is via the release of adenosine acting at several receptors. Our analysis demonstrates an association between five SNPs in the ADORA2a gene and AEs (specifically GI) on MTX. No association was observed between ADORA2a polymorphisms and MTX inefficacy. While this is the first study of ADORA2a receptor polymorphisms in RA, others have shown associations between ATIC polymorphisms and AEs on MTX [14], specifically GI AEs [11] supporting the concept that the adenosine pathway is important in determining AEs on MTX.

There are several possible mechanisms for MTX-induced GI AEs such as nausea. First, it may be due to local anti-proliferative effects on gut tissue, although the risk of this is reduced by co-prescription of folic acid, which all of our patients received. Alternatively, MTX-induced nausea may be due to sensitization of central chemoreceptors. MTX-associated nausea may be treated using 5HT3 antagonists, such as ondansetron and granisetron, which act via central nervous system (CNS) receptors around the basal ganglion [17, 18]. It is of interest that the ADORA2a receptor is strongly expressed within the CNS, especially around the basal ganglia [19]. Our study raises the hypothesis that central sensitization to MTX limits its tolerability and that variation in the ADORA2a receptor may mediate this. Further studies are now needed, both to replicate our findings and to examine the mechanisms behind this observation. ADORA2a polymorphisms have previously been associated with panic disorder [20] and anxiety response to amphetamine [21] again suggesting important central effects of this receptor.

There are several strengths and weaknesses of the study which need to be considered when interpreting the results. With regard to study design, patients were identified retrospectively and our phenotypes were pre-defined using data that was available on all patients (thus ESR and CRP were used as surrogates for defining response rather than formal joint counts that were not available for all patients). Clearly drug response is a spectrum, and in a prospective design, a composite measure of response such as the disease activity score 28 (DAS28) would be preferable. However, our patient populations were selected on the basis of pre-defined, well-characterized phenotypes in an attempt to minimize variation in the clinical phenotype and improve our ability to detect important genetic effects.

Establishing causality of AEs is difficult, and therefore we chose to recruit only patients with AEs severe enough to require treatment cessation so that those recorded were likely to be correctly attributable to the MTX. We recognize that other factors (both clinical and psychosocial) may influence both the likelihood of and reporting of AEs. As the majority of patients were monitored in hospital we feel that significant AE unreporting is unlikely. Further studies are, however, needed to determine the

positive predictive value of these polymorphisms and determine whether there is any association with patients who experience but tolerate milder AEs.

A key limitation of our study is the small numbers in each group. However, this cohort is one of the largest studied to date in the context of RA MTX pharmacogenetics. Importantly, the observed associations remained significant after permutation testing, suggesting that the findings are robust and not simply due to Type II error. Nevertheless, given the size of the cohort we felt that further haplotype analysis was not appropriate. However, given that the SNPs are in relatively strong linkage disequilibrium it is difficult to separate the effects and determine which is the functionally relevant SNP. To date the functional effects of these SNPs are not known. Further work is therefore required both to replicate these findings and determine the functional effect of these polymorphisms.

In summary, we report a novel association between ADORA2a polymorphisms and AEs in MTX-treated RA patients. Specifically, associations were observed between several ADORA2a SNPs and GI AEs on MTX. These results support the work of others suggesting that the outcome of MTX therapy is in part, genetically determined. Further studies are required to confirm and refine these observations and examine the pragmatic value of pharmacogenetic testing in RA patients commencing therapy with MTX.

Rheumatology key messages

- Polymorphisms within ADORA2a are associated with GI AEs on MTX therapy.
- Knowledge of the ADORA2a genotype may help identify patients at high risk of GI toxicity with MTX.

Supplementary data

Supplementary data are available at *Rheumatology* Online.

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