Review

The pharmacogenetics of methotrexate

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Methotrexate (MTX) is a cornerstone of therapy for rheumatoid arthritis. However, it is not universally effective and up to one-third of patients fail to respond to treatment, either because of inefficacy or adverse events, although at present it is not possible to predict therapy response accurately. Pharmacogenetics is the study of variability in drug response due to heredity. MTX has a complex intracellular metabolism and acts via a number of key enzymes. This review critically appraises the studies of MTX pharmacogenetics and highlights the need for further work in this area.

Key words: Rheumatoid arthritis, MTX, Pharmacogenetics.

Introduction

Methotrexate (MTX) is a cornerstone of therapy for rheumatoid arthritis (RA) since it can be prescribed as monotherapy or in combination with other agents, including biologics. However, like other DMARDs, it is not universally effective and up to one-third of the patients fail to respond to treatment, either because of inefficacy or adverse events. At present it is not possible to predict with any accuracy which patients will respond to therapy.

Given the variable effectiveness and significant toxicity of MTX (requiring expensive regular monitoring), together with the cost of biologics, it is an attractive choice for pharmacogenetic testing. If it were possible to identify patients at low risk of adverse events, such patients could be treated with a higher (and rapidly escalating) dose of MTX (either orally or parenterally) to ensure a swift clinical response. Patients failing to respond to rapid dose escalation could then be switched to another DMARD/biologic. This model of genotyping-based dose adjustment is already proposed for azathioprine, where thiopurine methyltransferase (TPMT) genotyping is being tested as a way to optimize drug dose, which ultimately may improve safety and reduce drug-induced morbidity [1].

Pharmacogenetics

Pharmacogenetics is the study of variability in drug response due to heredity. The commonest form of genetic variation is the single nucleotide polymorphism (SNP) where a single nucleotide base is altered or deleted or an additional nucleotide base inserted. It is estimated that such a change (or polymorphism) occurs every 500–1000 bases in the human gene [2]. SNPs may occur within the coding or non-coding region of the gene. SNPs within the coding region may lead to changes in the amino acid produced (known as a non-synonymous polymorphism). SNPs within the non-coding region may lead to changes in the amino acid produced (known as a non-synonymous polymorphism). SNPs within the non-coding region may lead to changes in the amino acid produced (known as a non-synonymous polymorphism). SNPs within the non-coding region may lead to changes in the amino acid produced (known as a non-synonymous polymorphism). SNPs within the non-coding region may lead to changes in the amino acid produced (known as a non-synonymous polymorphism).

Intracellular metabolism of MTX

MTX enters the cell via active transport across the reduced folate carrier (RFC) [3]. It is effluxed from the cell by several of the ATP-binding cassette (ABC) transporters, especially ABCC1-5 and ABCG2 [4–7]. This is summarized in Fig. 1. Once inside the cell, MTX undergoes polyglutamation by the addition of two to seven glutamic acid groups. This process is catalyzed by the enzyme folylpolyglutamate synthetase (FPGS). The polyglutamated form is not as readily transported across the cell membrane, and thus the intracellular half-life of MTX is increased [3]. This polyglutamation process is reversed via the enzyme gamma-glutamyl hydrolase (GGH), which removes the glutamic acid groups.

The MTX polyglutamates (MTXPGs) act on several key enzymes including dihydrofolate reductase (DHFR) and thymidylate synthase (TYMS-which is involved in pyrimidine synthesis). Studies suggest that MTX is a 'pro-drug', and that it is the polyglutamated form, rather than the parent drug, which is responsible for its efficacy in RA [8]. It is, therefore, suggested that defective polyglutamation represents one potential mechanism of non-response to MTX [3, 9]. It has been shown in both adult leukaemias and RA that MTX-polyglutamate levels correlate well with therapeutic efficacy [10–12]. In addition, MTX also influences the activity of the enzyme methylenetetrahydrofolate reductase (MTHFR), via its effects on intracellular folate metabolism. However, the enzyme reaction most potently inhibited by methotrexate polyglutamates is the conversion of 5-aminoimidazole-4-carboxamide ribonucleotide (AICAR) to formyl-AICAR, which is mediated by the enzyme AICAR transformylase (ATIC) [13, 14]. The accumulation of AICAR and its metabolites has a direct inhibitory effect on several other enzymes within the adenosine pathway, including adenosine deaminase and adenosine monophosphate deaminase (AMPD1). The net result of this is the release of adenosine, a potent anti-inflammatory mediator [13, 14]. There are therefore, a number of key enzymes that mediate MTX metabolism and may be potential pharmacogenetic targets (Fig. 1). This review will consider first single gene polymorphisms at both the transport and cellular metabolism level before reviewing what is known to date on polygenic interactions.

Transporter pharmacogenetics

MTX is transported intracellularly by the reduced folate carrier (RFC). A polymorphism leading to substitution of arginine for histidine at codon 27 of the RFC protein has been identified (RFC G80A), although the functional effects of this SNP are unknown. Studies suggest this polymorphism may influence MTX...
efficacy; in one study of 204 children with leukaemia receiving MTX [15] patients who were homozygous for the RFC 80A variant had higher plasma MTX levels than patients with other genotypes. In addition, Dervieux et al. [16, 17] showed that RA patients with the RFC 80AA genotype had higher MTX polyglutamate levels than other genotypes. In a further cross-sectional study of 108 RA patients, they also found that patients with the RFC 80AA genotype had lower MTX polyglutamate levels compared with the other genotypes [11]. However, Wessels et al. [18], in a prospective clinical trial, found no association between RFC genotypes and either MTX efficacy or toxicity.

Although MTX is effluxed from the cell by several efflux transporters, especially ABCC1-5 and ABCG2 and studies suggest that polymorphisms within these transporters occur frequently [19], to date these have not been systematically studied with respect to outcome of MTX therapy.

**Cellular pharmacogenetics**

**MTX polyglutamation**

Dervieux et al. [16] illustrated that a polymorphism within the promoter of the GGH gene (GGH C401T) influences MTX polyglutamate levels. Patients carrying the GGH 401TT genotype had lower MTX polyglutamate levels compared with the other genotypes [Odds ratio (OR) 4.8; 95% Confidence interval (CI) 1.8–13.0, \( P=0.002 \)], although this was not confirmed in a prospective analysis of 48 patients [12]. A second polymorphism in GGH has been described (C452T) which is suggested to reduce GGH activity, although van der Straaten et al. [20] found no association between this polymorphism and efficacy or toxicity of MTX.

**Thymidylate synthase (TYMS)**

Thymidylate synthase (TYMS) is a key enzyme involved in the synthesis of thymidylate, which is required for cellular proliferation. Inhibition of TYMS leads to depletion of deoxothymidylate monophosphate (dTMP), meaning that the uracil base is instead incorporated into DNA, leading to chromosome damage and cell death [21]. A tandem repeat sequence has been identified within the 5’-untranslated region (UTR) of the TYMS gene, containing a variable number of 28 base pair repeats. These repeats appear to function as enhancers, since with increased numbers of repeat sequences both mRNA expression and enzyme activity are increased [22]. Dervieux et al. [17] found that patients who were homozygous for two copies of the repeat had a better response to MTX, with response defined on the basis of a physician’s VAS score; however, this finding was not confirmed in a smaller prospective study [12]. In addition, a Japanese study [23] found that patients homozygous for three copies of the repeat required higher doses of MTX (>6 mg/week) than patients homozygous for two copies (\( P=0.033 \)). They also identified a second polymorphism, a 6 bp deletion in the 3’UTR, and found that patients homozygous for the deletion allele had greater CRP reduction than patients with other genotypes (\( P=0.024 \)). Functional studies suggest that this deletion polymorphism is associated with reduced mRNA stability, and hence reduced enzyme expression [24]. However, further studies are needed to confirm these associations in other ethnic groups, since Japanese patients respond very differently to MTX than Caucasians and typically require much lower doses.

**MTHFR**

MTHFR is the best studied gene to date with respect to MTX metabolism. However, the inconsistencies in the published studies also highlight the problems of studying MTX pharmacogenetics. MTHFR is a central regulatory enzyme in the folate pathway and catalyses the conversion of 5,10-methylenetetrahydrofolate to 5-methyltetrahydrofolate, which is a co-substrate for homocysteine remethylation [25]. Severe MTHFR enzyme deficiency leads to hyperhomocysteinaemia with subsequent damage to the central nervous system and vascular system [26]. However, it is the genotypes that lead to mild MTHFR deficiency that are of most interest with respect to MTX metabolism, since in this scenario patients are phenotypically normal although there may be biochemical defects within folate metabolism. At least 15 polymorphisms within the MTHFR gene have been described [27] although functional data does not exist for all of these. Two non-synonymous SNPs (C677T and A1298C) have been extensively studied and the results are summarized in Table 1.

The C677T polymorphism was identified by Frost et al. in 1995 [28]. This polymorphism leads to an alanine to valine amino acid change at codon 222. This has functional effects, leading to the formation of an enzyme with reduced activity. Approximately, 50% of a Caucasian population carry at least one copy of the variant allele [29]. Heterozygotes (CT) have ~60% in vitro enzyme activity and make up ~40% of the Caucasian population. The homozygous variant TT genotype is present in ~10% of Caucasians and has only 30% of the homozygous wild-type (CC) enzyme activity [21]. A second common polymorphism (A1298C) is a glutamic acid to alanine substitution at codon 429. This has functional effects, leading to the formation of an enzyme with reduced activity. Approximately, 50% of a Caucasian population carry at least one copy of the variant allele [29]. Heterozygotes (CT) have ~60% in vitro enzyme activity and make up ~40% of the Caucasian population. The homozygous variant TT genotype is present in ~10% of Caucasians and has only 30% of the homozygous wild-type (CC) enzyme activity [21]. A second common polymorphism (A1298C) is a glutamic acid to alanine substitution at codon 429 and was shown by Weisberg et al. [30] to lead to reduced enzyme activity. The C allele has a frequency of 32% in a Caucasian population. Interestingly, patients who are heterozygous for both SNPs (~15% of a Caucasian population) are clinically similar to individuals homozygous for the C677T polymorphism, suggesting an interaction between the two SNPs [29]. One might predict that patients with reduced MTHFR enzyme activity would be more susceptible to toxicities from MTX because of the effects on homocysteine metabolism, although it is more difficult
to determine how such polymorphisms would influence drug efficacy.

Several studies have examined the effect of MTHFR polymorphisms and outcome of treatment with methotrexate in RA. Van Ede et al. [31] studied 236 RA patients on MTX as part of a clinical trial. They found that 48% of the patients carried at least one T allele and that carriage of the variant allele conferred an increased risk of stopping MTX for adverse events (RR 2.01; 95% CI 1.01–4.02), mainly due to an increased risk of abnormal LFTs (RR 1.38; 95% CI 1.09–1.75), mainly due to an increased risk of abnormal LFTs (RR 1.38; 95% CI 1.09–1.75) when compared with patients with the CC genotype. No difference in MTX efficacy was seen between the groups [25]. The predictive power of the genotype was independent of folate supplementation status.

Urano et al. [32] studied the effect of both the C677T and A1298C polymorphisms in a cohort of 106 Japanese RA patients. They found that MTX toxicity was more frequent in patients with the 677T allele compared with those without the T allele (27% compared with 8.6%; RR 1.25), again no correlation was observed between this polymorphism and treatment efficacy. Considering the A1298C polymorphism, patients carrying the variant C allele required significantly lower doses of MTX for adverse events (RR 2.01; 95% CI 1.06–4.06, 

\[ \text{OR} = 15.86; \text{CI} 1.5–167, P = 0.02. \]

In contrast, Wessels et al. [18] demonstrated associations between MTHFR SNPs and MTX efficacy (measured by change in DAS score at 6 months) in 205 RA patients recruited for a clinical trial. They found an association between MTX response and the MTHFR 1298AA genotype relative to those carrying the 1298C allele (OR 2.3; 95% CI 1.18–4.41). In addition, carriers of the 1298C allele developed more adverse events (OR 2.5; 95% CI 1.32–4.72). Patients with the 677CC genotype also showed greater clinical improvement on MTX. However, this study was based on clinical response at 6 months and to date no longer term outcome data is available.

Thus, the results for MTHFR suggest that C677T may be a marker for MTX toxicity, particularly hepatotoxicity. The results for the A1298C SNP are inconsistent, with some studies suggesting an association between 1298AA genotype and toxicity [34, 35] and others with the 1298CC genotype and toxicity [18]. To date, however, all of the studies have reported single cohorts where different definitions of drug efficacy and toxicity are used. In particular, different definitions for drug toxicity are used, with some authors reporting mild adverse events not requiring drug cessation (meaning some adverse events may not be directly attributable to MTX) and others reporting severe adverse events. In addition, many of the studies reported are retrospective which may have led to errors in the estimation of MTX effectiveness, a problem that can be resolved by using clinical trial subjects. Therefore, further studies (using similar definitions for drug efficacy and toxicity) are required to clarify these apparent inconsistencies.

### DHFR

Whilst this has not been studied systematically in RA, in vitro studies in lymphocytic leukaemia suggest that low level gene amplification of DHFR or mutations in the enzyme may provide a mechanism of acquired resistance to MTX [10]. Studies within a Japanese population [36] suggests that an SNP within the 3’UTR of the DHFR (C829T) gene may be functional and lead to increased expression of DHFR. However, to date the effect of this polymorphism has not been studied in other populations or correlated with outcome of MTX treatment.

### Adenosine pathway

MTX has potent effects on adenosine metabolism and this provides a novel target for pharmacogenetic studies. Wessels et al. [37] studied five polymorphisms within five genes coding for enzymes in the adenosine pathway. In an analysis of 186 patients...
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Toxicity (MTHFR/TYMS/ATIC/SHMT)

Serine hydroxyl methyltransferase (SHMT) is an enzyme that is important in the synthesis of 5,10 methylene tetrafolate. Weisman et al. [39] reported a toxicity index on a cohort of 214 RA patients. Patients were genotyped for four SNPs (MTHFR C677T, ATIC C347G, the TYMS 28 bp tandem repeat and a SNP within SHMT, C1420T). A ‘toxicogenetic index’ of the sum of the homozygous variant genotypes was calculated. MTX side effects were recorded (cross-sectionally at a single-study visit), and occurred in 67 patients (32%). For each unit increase of the index there was a 1.9-fold increase in the likelihood of side effects (OR 1.9; 95% CI 1.1–3.1, \( P = 0.004 \)). However, since the adverse events were mild (not requiring treatment cessation), again replication in a prospective cohort is required to determine the positive predictive value of such a measure in clinical practice.

In their prospective analysis of 48 patients, however, Dervieux et al. [12] found that the MTHFR C677T, TYMS 28bp repeat and SHMT C1420T did not contribute significantly to toxicity, instead demonstrating associations between the ATIC C347G SNP and other adenosine pathway genes.

Summary and future directions

To date, a number of studies have reported potential genes that may be associated with toxicity or response to MTX. However, to date many of these associations have been reported in single cohorts and have either not been replicated or show inconsistent findings (as exemplified by MTHFR).

There are a number of explanations for these apparent inconsistencies. First, the majority of studies have been from single cohorts (with no replication sets) and with relatively small numbers of patients, which both limit the study power and increases the likelihood of false positive associations. Given the small effect size quoted for many of the SNPs (with reported ORs of around 2), large sample sizes are required to prevent false negative results. In addition, different populations have been studied using varying definitions of drug toxicity and efficacy, which may lead to selection bias. These different outcome criteria mean that it is difficult to compare results between studies. Other issues which need to be considered when interpreting genetic association studies include how the SNPs typed are selected (since many studies report single or selected SNPs rather than whole gene coverage), genotyping quality, relevant data analysis (with appropriate correction for the number of analyses performed) together with the likely validity of interpretation.

These issues are reviewed in detail by Hattersley et al. [41].

However, given the strength of association observed for many of the individual SNPs, the predictive value of single SNPs may ultimately be limited. Given the complex cellular metabolism of MTX, it is more likely that a combination of genotypes may be necessary. Given the complex cellular metabolism of MTX, it is more likely that a combination of genotypes may be necessary. Ultimately pharmacogenetics alone may not have sufficient predictive power, and therefore post-translational and biochemical changes around the MTX pathway such as polyglutamation and cytokine changes may need to be included to fully understand MTX response.

From a clinician’s standpoint we are still a long way from being able to accurately predict an individual’s likely outcome on MTX. Nevertheless, given its central importance in the treatment of RA and related diseases a major effort is justified to allow us to personalize the use of this therapy more effectively and safely.

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Polygenic analyses

Several recent studies have examined the effect of a number of genetic polymorphisms on MTX efficacy and toxicity.

MTX efficacy (TYMS/ATIC/RFC)

Dervieux et al. [17] studied the combined effects of three SNPs (C347G SNP in ATIC, TYMS 28 bp tandem repeat and G80A in RFC) by determining a ‘pharmacogenetic index’ of the sum of the homozygous variant genotypes. 108 MTX-treated RA patients were classified as responders or non-responders to MTX, according to a physician’s assessment of response using a VAS. Patients with at least 1 homozygous variant were 3.7 times more likely than patients without a homozygous variant to respond to MTX (OR 3.7; 95% CI 1.7–9.1; \( P = 0.01 \)). When they expanded their study population to 226 (including 107 patients on low-dose steroids) and refined their scoring of the index, a lower pharmacogenetic score (i.e. less variant SNPs) was associated with higher tender (\( P = 0.002 \)) and swollen joint count (\( P = 0.003 \)) [11]. Further prospective studies are required to determine the clinical utility of such a measure in predicting drug response. Dervieux et al. [12] also performed a prospective study on 48 RA patients to evaluate the effect of polyglutamate and pharmacogenetic markers on response to MTX and demonstrated an association between MTX-polyglutamate levels and response. Clearly, with such a small cohort there is limited power to detect modest genetic effects although within this group only the MTHFR 677TT genotype and SHMT1 1420CT/TT genotypes were associated with poor response to therapy (measured using the DAS28). None of the other genetic markers were associated with efficacy/inefficacy outcomes.
The authors have declared no conflicts of interest.

References