ORIGINAL ARTICLE

The Prevalence of SNP rs12517451 in Gene Transcribing Dihydrofolate Reductase and Drug Adverse Effects among MTX-treated Rheumatoid Arthritis Patients in Kelantan, Malaysia

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ABSTRACT

Introduction: Disease-modifying anti rheumatic drugs (DMARDs) provide the mainstay for the treatment of rheumatoid arthritis (RA). Adverse effects (AEs) in DMARDs among RA patients are usually related with methotrexate (MTX) use, the common conventional DMARDs. Genetic variant such as single nucleotide polymorphism (SNP) in gene transcribing dihydrofolate reductase (DHFR) (i.e, 829C>T, rs12517451) has been correlated with drug AEs in MTX-treated RA. The prevalence of the DHFR rs12517451 SNP has been reported in other populations, but not in Malaysian. The aim of this study was to determine the prevalence of the DHFR rs12517451 SNP and its association with drug AEs among MTX-treated RA patients from Kelantan, Malaysia. **Methods:** A total of 78 RA patients receiving MTX (alone or in combination) were included in this study. Based on evidence of clinically perceived drug AEs in MTX-treated RA patients, 33 and 45 samples were assigned as cases and controls, respectively. The genotype of the patients was determined using the polymerase chain reaction-restriction fragment length polymorphism method and validated by sequencing analysis. **Results:** Minor allele frequency (MAF) for DHFR rs12517451 in cases and controls were 28.8% and 32.2% but there was no significant difference (p=0.727) for the possession of the minor allele T between the two groups. The most reported AEs among cases were haematological effects, gastrointestinal toxicity, and skin problems resulting in 21% withdrawal of MTX. **Conclusion:** We did not find significant association of the DHFR rs12517451 with drug AEs in MTX-treated RA patients. Our findings warrant replication in a larger patient cohort.

Keywords: DMARD, Methotrexate, Rheumatoid arthritis, Dihydrofolate reductase, Polymorphisms, Adverse effects

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INTRODUCTION

Methotrexate (MTX) is widely used in the treatment of psoriasis, haematological malignancies such as acute lymphoblastic leukemia (ALL), chronic lymphocytic leukemia, lymphomas and multiple myeloma, and most notably, rheumatoid arthritis (RA). RA is an autoimmune disease and is characterised by chronic synovial joint inflammation leading to decreased mobility and disability (1). The risk of RA is related to genetic and environmental factors (2). Few drugs, including glucocorticoids, nonsteroidal anti-inflammatory drugs (NSAIDs) and disease-modifying anti rheumatic drugs (DMARDs), are used in RA treatment and are indicated to alleviate pain, decrease harm and retain the joint function caused by the disease (3). MTX, grouped under DMARDs, has been prescribed to RA patients and often considered to be first line treatment because of its proven efficacy and affordability (4).

MTX, an antifolate medication, competitively inhibits the activity of dihydrofolate reductase (DHFR), an important enzyme that plays a role in transforming dihydrofolate into tetrahydrofolate, an important active cofactor in the de novo synthesis pathway for the production of purines and pyrimidines (5). DHFR activity is inhibited by the introduction of MTX in RA treatment and consequently lead to altered folate metabolic pathway that is related to purine and pyrimidine production, the essential components of deoxyribonucleic acid make-up, thus suppressing cell proliferation (6). The clinical indication of MTX has been shown to have an anti-proliferative (DHFR-mediated) and anti-inflammatory (non-DHFR-mediated) effects (7). Despite its established portfolio of efficacy and effectiveness among RA patients, the outcome of MTX treatment is sometimes considered unpredictable. MTX is commonly used in combination with other DMARDs to increase the response percentage in the treatment of RA (8) and as a result, drug-drug

interaction may probably be underlying the occurrence of drug-related adverse effects (AEs) among MTX users. Symptoms such as gastrointestinal symptoms, hepatotoxicity and unexplained pulmonary toxicity are a significant cause of MTX-related AEs, in fact, with low dose of MTX (9).

Most studies reported multiple predisposing factors related to MTX-related AEs, such as demographic profiles of the patients, disease-specific factors and concomitant drugs (9,10). However, the genetic variation encoded within the metabolism pathway of MTX is mostly overlooked or less understood due to discrepancies in literature when it comes to drug-related AEs in MTX-treated RA patients. Due to a limited number of genetic study replication, no promising genetic predictive markers for drug-related AEs have been identified in MTX-treated RA patients to date.

Therefore, in this study, we aim to investigate the impact of selected single nucleotide polymorphism (SNP) in DHFR gene that is involved in folate metabolic pathway as it is believed to alter the drug response in MTX-treated RA patient (4). Several polymorphisms have recently been described in DHFR, including promoter polymorphisms, the 19-bp deletion allele and variations in 3'UTR (11). SNP in the DHFR enzyme i.e., rs12517451, is one of the potential markers associated with the accumulation of MTX resulting in its toxicity (4,12). DHFR rs12517451 SNP (829C>T) is a single nucleotide polymorphism that is located in chromosome 5 within the 3` -UTR of non-coding region and the SNP resulted in altered miR-24 function, and increased DHFR mRNA and protein levels which finally caused MTX resistance (13). Therefore, we hypothesise that the DHFR rs12517451 polymorphism results in MTX resistance, leading to the accumulation of the drug in the cellls for which this eventually manifested as drug AEs in MTX-treated RA patients. To the best of our knowledge, no research has yet been published in Malaysia that explicitly investigated the effect of the indicated SNP in association with AEs in MTX-treated RA. We believe that the information from this study has the potential to give prior evidence on the occurrence of drug AEs in MTX-treated RA patients and may provide more insight into risk stratification that will help to enhance MTX drug monitoring in RA patients in Malaysia and Asia as a whole.

MATERIALS AND METHODS

Study samples

This is a retrospective case-control study involving the examination of anonymous archived DNA samples of MTX-treated RA patients from Hospital Universiti Sains Malaysia, Kelantan, Malaysia. All information on the demographic and clinical characteristics of patients was derived from patient's medical records. The selected archived DNA samples of adult RA patients (>18 years old) were then assigned to the case and control group according to the following criteria; Cases: RA patients (were clinically evaluated as having RA according to the American College of Rheumatology 1987 criteria (14)) those who were presented with either seropositive and seronegative for rheumatoid factor, and had previously received MTX therapy (alone or in combination with other drug) for at least 3 months, at doses typically used for the treatment of RA i.e., 7.5mg-25 mg/week but had discontinued or re-challenged with lower dosage or with other DMARDs because of reported MTX-related AEs during the treatment (within 0-3 months from the start of drug therapy). The list of definitive classification for each MTX-related side effect stated in the supplementary file. Controls: RA patients whom had previously received MTX at doses typically used for RA treatment (as stated in the cases above) and have not reported any MTX-related AEs (responders) during the treatment (within 0-3 months from the start of drug therapy).

The archived DNA samples of the RA patients were all obtained from written informed consent subjects prior to inclusion in the previous study. Ethical clearance (USM/JEPeM/17090394) for this study was approved by local Human Research Ethics Committee of Universiti Sains Malaysia, and in accordance with the Declaration of Helsinki as well as local regulations and standard for ethical review.

Sample size calculation

Calculation of the sample size was based on the percentage of exposure (possessing at least one variant allele for rs12517451) determined by assuming a power of 80% and $P< 0.05$ (15) from independent subjects with a control and case ratio of 1:1. Previous data suggested that the probability of exposure among controls without AE was 35% (4). If the true probability of exposure among cases is predicted at 65%, at least 35 case patients and 35 control patients would be needed to be able to reject the null hypothesis that the exposure rates for case and controls are equal with probability (power) 0.8. The Type I error probability associated with this null hypothesis is 0.05. We used an uncorrected chi-squared statistic to evaluate this null hypothesis. The sample size was calculated using PS software using two proportion uncorrected chi square test (16). With respect to the sample size used, the use of 33 patients in the cases and 45 patients in the control group is not substantially affected the power of the study relative to the proposed subject number. Using a control with less than 5 controls per case did not result in different statistical power for a hypothetical case-control analysis according to Hannessy et al (17). In this study, the ratio resulting from 33:45 (case:control) of subjects is only 1.36, which is obviously lower than that of 5 as suggested by Hannessy and colleagues (17).

SNP genotyping

The SNP was selected on the basis of the following criteria; a genotype frequency of 10% or more,

showing functional evidence and clinical significance indication from previous publications (18). The SNP genotyping was performed by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP). The primers for DHFR rs12517451 (forward 5′ - CGGGTAACAGGAACAGCACT - 3′, reverse 5′ - CAAAGTGCTGGGGTTACAGG - 3′) were designed by BatchPrimer3 v1.0 software. The PCR master mix cocktail consisted of 2.5 µl of 10X PCR buffer, 2 µl 25mM of MgCl2, 0.5 µl 10mM dNTPs, 0.5 µl 10mM of both forward and reverse primers, 0.125 µl 1.6 U/µl of Taq DNA polymerase enzyme (ThermoFisher Scientific, USA) and 16.88 µl of sterile dH2O added to the final volume of 25µl of the reaction mixture. Approximately 50 ng of genomic DNA was used for genotyping of each sample. The PCR reaction consisted of an initial denaturation step of 5 min at 95°C followed by 35 cycles of denaturation steps of 30 sec at 95°C, annealing step of 30 sec at 61°C and extension at 72°C for 20 sec and post extension at 72°C for 5 min. The digestion of PCR products was checked at NEBcutter V2.0 (http://nc2.neb.com/NEBcutter2/) by inserting at least 10bp of gene sequences around the targeted site for variant sequences of interest. The commercial restriction endonuclease Tsp45i (NEB, England) was used for DHFR rs12517451 where the recognition nucleotide sequence 5'…↓GT(G/C)AC …3' was recognised and the enzyme digested at 65°C for 1 hour and 45 minutes. The Tsp45i enzyme cuts if the wild allele C is present on the nucleotide sequences and results in the following DNA fragments; cut DNA (homozygous wild type, CC), both uncut and cut DNA (heterozygous, CT) and uncut DNA (mutant allele, TT).

Both amplified PCR products and digestion products were subject to electrophoresis with 2% and 3% of agarose gels, respectively. SYBER Green (ThermoFisher Scientific, USA) was used for staining and the gel was photographed under UV illumination. The expected product size of undigested PCR is 316bp. The mutant TT genotype resulted in uncut DNA sequence, therefore the band will be appeared the same as undigested PCR. The non-template sample was used as negative control. 10 % of the total samples were sent for sequencing analysis, and the results were matched to that determined by the PCR-RFLP assay.

DNA sequencing

The unpurified PCR product was outsourced to the company (First BASE Research Laboratories Sdn. Bhd, Malaysia) for direct DNA sequencing. The PCR purification step was carried out by the company prior to sequencing. Sequencing results of the ABI file have been displayed using Chromas Lite 2.1.1 and aligned with Bioedit version 7.2.5.

Statistical analysis

Statistical analyses for all tested parameters (expressed as mean, range for 95 % confidence interval) were performed using SPSS software version 24. For univariate analysis, chi-square tests were used for categorical variables and t-tests for interval variables. Genotype frequencies between controls and cases were then compared using Fisher's exact (two-tailed) test in order to obtain p-value, odds ratio, 95% confidence interval and chi-square for trend (http://vassarstats.net/odds2x2.html). Genotype frequencies have been determined to be in Hardy-Weinberg equilibrium (HWE) and compliance with the HWE ($p>0.05$) was also determined for control groups to ensure that they met standard quality criteria using the available web-based calculator (http://www.oege.org/software/hardy-weinberg.shtml). For all analysis, a value of $p<0.05$ was considered statistically significant.

RESULTS

Clinical characteristics of the study population

A total of 78 RA patients met the inclusion criteria and were selected for this study. All selected subjects were then grouped into cases (n= 33) and controls (n=45). The clinical characteristics of the studied subjects is summarised in Table I. Based on Table I, there were more female subjects (n=36 and n=30, respectively) than male subjects (n=9 and n=3, respectively) in both control and case groups. The mean age of the cases and controls was 57.6 and 57.2 years old, respectively. The mean body weight of selected RA patients was 57.2 kg (31-79 kg) and 59.1 kg (31.9-83.6 kg) in cases and control, respectively. The number of patients with seropositive for rheumatoid factor in both cases and controls was more than 50%. The majority of RA patients in both groups had been suffering the disease and were on MTX therapy for more than five years. There was no difference between cases and controls in term of dosage and duration of MTX therapy. It was estimated that patients with RA had been treated with MTX for more than five years before the indicated AEs were detected, but this did not vary substantially from that of RA patient without side effects.

There were no significant differences between cases and controls for the effect of MTX alone and in combination either with corticosteroid or NSAIDs. The results from this study showed that the majority of RA patients were concomitantly administered with other medications, as most of them had multiple comorbidities, regardless of their clinical consequences with MTX therapy. Hypertension, hypercholesterolemia, and diabetes mellitus were the most commonly reported comorbidities in both groups.

The incidence of drug AEs in MTX-treated RA patients

A wide range of MTX-related AEs (Table II) have been described in this study. According to the patient's medical record, 33 (42.3%) of the total 78 RA patients were found to have had one or more MTX-related AEs. However, MTX discontinuation was only seen in

Table I: Demographic and clinical characteristics of MTX-treated RA patients with AEs (cases) and without AEs (controls) in this study.

Characteristic	Cases N= 33	Controls N= 45	P-value
Gender Male/Female	3/30	9/36	0.221
Age, years (range)	57.6 (23-79)	57.2 (23-87)	0.753
Body Weight, kg (range)	57.2 (31-79)	59.1 (31.9-83.6)	0.392
Race			
Malay, n (%)	30 (90.9)	39 (86.7)	0.471
Chinese, n (%)	3 (9.1)	4 (8.9)	
Indian, n (%)	0 (0)	2 (4.4)	
Seropositive for rheumatoid factor, n (%)	20 (60.6)	28 (62.2)	1.000
Duration of RA disease, mean months (range)	109.67 (8-274)	91.56 (5-227)	0.371
< 24 months n (%)	4 (12.1)	5 (11.1)	0.870
24-60 months, n (%)	8 (24.2)	13 (28.9)	
> 60 months, n (%)	21 (63.6)	27 (60)	
Dosage of MTX, mg/week (range)	12.5 (2.5-22.5)	13.56 (5-22.5)	0.563
Duration of MTX therapy, mean months (range)	80.21 (8-205)	67.76 (5-186)	0.448
< 24 months, n (%)	9 (27.3)	11 (24.4)	0.351
24-60 months, n (%)	12 (36.4)	11 (24.4)	
> 60 months, n (%)	12 (36.4)	23 (51.2)	
Concomitant Drugs with MTX			
Yes, n (%)	30 (90.9)	43 (95.6)	0.645[a]
Corticosteroids and NSAIDs	4 (13.3)	13 (30.2)	
Other DMARDs	8 (26.7)	17 (37.2)	
Corticosteroid, NSAIDs and other DMARDs	18 (60)	14 (32.6)	
No, n (%)	3 (9.1)	2 (4.4)	
Comorbidities, n (%)	28 (84.8)	33 (73.3)	0.2748

[a]The analyses compared the frequencies with those of having no concomitant drugs between cases and controls.
NSAID; Nonsteroidal Anti-inflammatory Drugs, DMARDs; drug modifying anti rheumatic diseases; N, total number of patients in the group.

Table II: Frequency of specific AE reported in RA patients (MTX-related AEs) and the resultant withdrawal due to the AE.

Types of AEs[a]	n	MTX withdrawal
Gastrointestinal toxicity	10	3
Elevation of ALT/AST	3	2
Skin problems (rashes, itching and alopecia)	6	1
General fatigue	3	0
Pulmonary toxicity	4	1
Haematological manifestation (neutropenia and leukopenia)	12[b]	0

[a]Some adverse effects were being reported redundant to each other.
[b]One patient suffered from both redness and haematological manifestation.
ALT, alanine transaminase; AST, aspartate transaminase.

seven patients (21.2%). In this study, haematological manifestation (n=12) was the most common AE observed in RA patients who were on MTX with indication of mild to moderate leukopenia and neutropenia. This occurred in patients with average MTX doses ranging from 7.5 to 17 mg per week. In this case, all of them were found to have a low number of total white cell count (TWC) (<3.6 X 10^9/ L) and they were considered to suffer from mild leukopenia or neutropenia except for one patient with moderate leukopenia with TWC of <1.75 X 10^9/L. This condition was managed by temporary drug withdrawal and re-challenged at a lower MTX dose at normal TWC level. Reduction of MTX dose was widely practiced as a way to address symptoms in patients experienced with haematological toxicity. Drug withdrawal was not recommended at this point. In addition, no case of severe bone marrow has been seen.

Ten patients had gastrointestinal (GI) toxicity, with a variety of nausea and vomiting symptoms. Three patients was discontinued the drug due to severe GI side effects and was replaced by other DMARDs. Other patients had to discontinue the drug on a temporary basis and restarted at lower dose after 4-6 weeks. Pulmonary toxicity, indicated by cough and lung fibrosis, occurred in four patients (12.1%) at 15 mg/week MTX dose. Six patients (18.1%) had skin rashes, itching and alopecia at MTX doses ranging from 5 to 15 mg/week.

Genotype and allele frequencies of DHFR rs12517451 polymorphism

The genotype of DHFR rs12517451 was verified by direct sequencing of PCR products (Fig. 1). Fig. 2 shows the representative hybridizing bands of the genotypes for DHFR rs12517451 determined by PCR-RFLP. Individuals with homozygous wild type (CC) displayed two fragments (87bp, 229bp), whereas individuals with homozygous mutant type (TT) displayed only one fragments (316bp). Results displayed the presence of all three fragments (87bp, 229bp, 316bp) were grouped as heterozygous individuals (CT). Table III shows the genotype and allele frequency of DHFR rs12517451 in both cases and controls. There was no significant difference (p=0.727) between the two groups for the carriage of minor allele T in DHFR rs12517451 SNP.

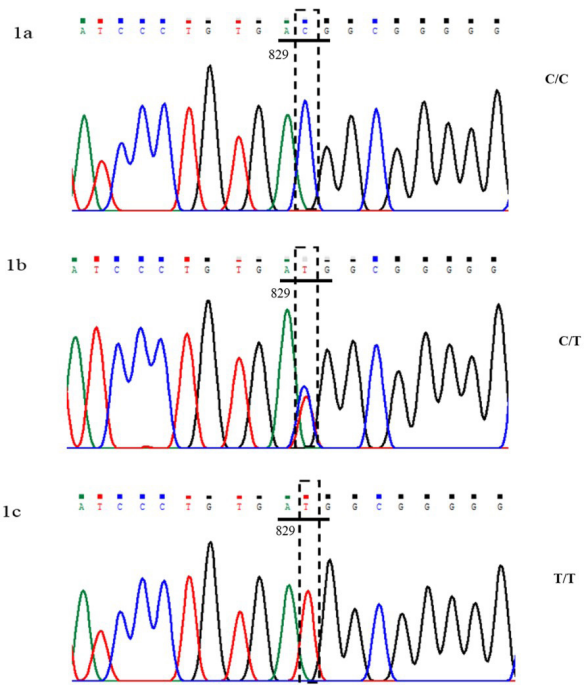

Figure 1: Chromatogram indicate the DNA sequence of (1a) homozygous wild-type (CC genotype), (1b) heterozygous (CT genotype) and (1c) homozygous mutant (TT genotype) of the DHFR rs12517451

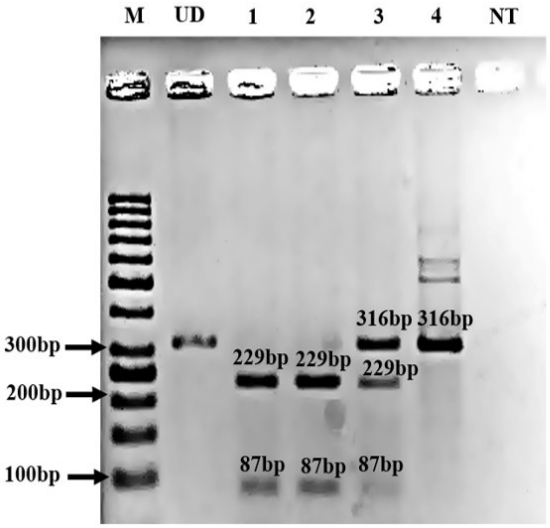

Figure 2: Genotyping of rs12517451 in DHFR gene by PCR-RFLP assay. Lane M, DNA ladder (50 bp); UD, undigested PCR product; lane 1 and lane 2, CC genotype (87- and 229 bp); lane 3, CT genotype (87bp, 229 bp and 316 bp); lane 4, TT genotype (uncut mutant, 316 bp), NT, non-template sample

Table III: Genotype and allele frequencies of DHFR rs12517451 in group of MTX-treated RA patient with AE and without AEs.

	Frequency of MTX-treated RA patient with AE, n (%) (N=33)	Frequency of MTX-treated RA patient without AE, n (%) (N=45)	P-value[a]	Reference East Asian population[b]	P-value[c] for HWE
Genotype					
CC	16 (48.5)	22 (48.9)	1.000	218 (43.3)	0.531
CT	15 (45.5)	17 (37.8)		231(45.8)	
TT	2 (6.0)	6 (13.3)		55 (10.9)	
Allele					
C allele	47(71.2)	61 (67.8)	0.727	667 (66.2)	0.751
T allele	19 (28.8)	29 (32.2)		341 (33.8)	

HWE, Hardy-Weinberg Equilibrium; n, frequency number; N= total number in the group.
[a]P-value indicates the association of carriage of minor allele T between MTX-treated RA patient with and without AEs.
[b]The reference population genotypes of the SNP in an East Asian population (derived from https://asia.ensembl.org).
[c] P-value for the HWE calculated between control group (MTX-treated RA patients without AEs) compared with the East Asian population.

DISCUSSION

While MTX-based therapy is the gold standard for the treatment of RA, researchers are still unable to predict precisely what type of genetic factor is likely to be at risk of developing drug AEs in MTX-treated RA patients. Pharmacogenetic approach has been shown to predict AEs of many clinical drugs including MTX (19). It is true that there is a large variation in the results for the pharmacogenetic data around the globe due to the difference in ethnicity, which clearly shows different minor allele frequency (MAF) for SNPs (https://www.ncbi.nlm.nih.gov/snp/).

With respect to drug-related AEs in MTX-treated RA patients, SNP in DHFR gene, one of the targets of interest in MTX metabolism pathways, is an important genetic predictor due to its association with DHFR expression and MTX responses (4). DHFR is a key enzyme in intracellular folate metabolism that essential for DNA synthesis and cell growth (20). Up to the present time, there is no study has investigated the regulatory functional role of SNP in DHFR regions associated with susceptibility towards MTX toxicity in East Asian subjects. DHFR rs12517451 829C>T, which is found in chromosome 5 within the 3'-UTR of non-coding region (13), has been regarded among the key SNP that has been significantly associated with MTX-related AEs in other populations such as found in white Caucasian ethnic origin like British and Southeast Europe (4,11). However, to the best of our knowledge, no such genetic study has been conducted in Malaysia to explore the impact of DHFR rs12517451 on the occurrence of AEs in low dosage MTX users to date. The allele frequency for this SNP is consistent with the Hardy-Weinberg law and is consistent with the reference East Asian population indicated by the genome browser (Table III).

The results from this study suggested that the allele frequency of DHFR rs12517451 was not significantly related to the occurrence of MTX-related AEs in our population. With regards to the association between the DHFR 829C>T SNP and MTX-related AEs, similar outcome was reported by another study in Thailand that investigated the risk of MTX toxicity in acute lymphocytic leukemia (ALL) patients who received a high dose of MTX (21). In the study, both dosage (i.e, high dose MTX) and genetic (i.e., DHFR rs12517451) factors were not associated with the MTX toxicity events and the reported frequencies of the SNP were 14% (CC), 86% (CT) and 0% (TT) respectively (21). Compared to the present findings, the allele frequencies of DHFR 829C>T among the Thai population (MAF = 43 %) was obviously different from that of our Malay population (MAF = 28.8 %) which thus suggests that each ethnicity had distinct allele frequencies of the SNP even though we originate from the same Asia continent.

There was considerable inter-individual variation in response to MTX, with large number of patients did not respond to the drug, while others (10-30%) developed drug side effects requiring drug discontinuation (22). As the current study found insignificant association between DHFR rs12517451 and MTX toxicity, other studies such as found in European and Indian (which also studied other SNP in the DHFR gene) reported differently (4,22-25). A study involving 309 British RA patients cohort reported rs12517451 was significantly associated with a higher risk of MTX-related side effects with odds ratio of 1.68 (95% CI 1.03–2.75) (4). Albeit at

a lower odds ratio for the association with MTX-related AEs, the influence of other SNPs (i.e., rs1643657 and rs10072026) in DHFR gene have also been described (4). Thererefore, we consider that the rs12517451 is the best representative SNP in the DHFR gene that contributed to the drug AEs in MTX-treated RA patients. Whilst other SNPs in the DHFR gene, although lack of evidence for their association with MTX-related AEs, are also worth to study since their known functional consequences in DHFR expression. The DHFR rs12517451 resulted in high DHFR expression, although in different related disorders, similar to that found in other polymorphisms in both DHFR minor promoter (i.e., (rs1650694, rs408626) and major promoter (i.e., rs1105525, rs1650697, rs3045983/ -), respectively (12). There were, however, conflicting results between the abovementioned SNPs and MTX response in different disease susceptibility with different population. Wessels and colleagues (24) found no association between between DHFR rs1650697 and DHFR rs1232027 (35289A>G located at downstream to 3' UTR with MTX efficacy and toxicity among early RA patients in European subjects. DHFR rs408626 was found to be associated with increased risk of ALL relapse and severe leucopenia caused by MTX in South Indian (25). In another study among Indians (North Indian, n=213 MTX tolerants vs n=68 MTX intolerants), DHFR rs7387 instead, was found to be associated with MTX response (26).

In terms of genotype frequencies of DHFR SNP rs12517451in MTX-treated non-RA patients, there have been conflicting results in several previously published studies. In Japan, Goto and colleagues (27) reported that the MAF of SNP rs12517451 in 32 cases of acute leukemia in children (25 cases in ALL and 7 cases in acute myelogenous leukemia, AML) was 10.8 % and 15.7% in healthy control group, respectively. Whereas in Thailand, as studied by Komdeee et al (21), the MAF in both ALL cases and healthy children were 47 % and 43 %, respectively. If we compare the MAF in the ALL case group for both populations, the MAF value was higher in Thai (21) compared to that observed by Goto et al (27) in Japanese children (i.e., MAF value of 47 % vs 10.8%). Whereas, in terms of genotype frequencies of DHFR SNP rs12517451in MTX-treated RA patients, a more consistent MAF value is seen between this study and a previously studied case-control study that also looking at the association of the SNP with MTX-related AEs (MAF value of 28.8 % vs 29.7 %, respectively). Goto and colleagues (27) have shown that there was no association between the rs12517451 and their studied clinical outcome. We stipulate that the use of relatively small sample size (n= 32) to detect the actual association has contributed to this observation. This is probably true because the MAF value obtained in the control group, and also in the cases, by Goto's study was significantly lower (P<0.05) when compared with other reliable healthy Japanese cohort data such as found in (https://asia.ensembl.org/index.html) indicating the MAF value of 28.4 % (CC genotypes = 50%, CT genotypes = 43.3 % and TT genotype = 6.7 %). Likewise, the observation also suggests that the genotype frequencies of DHFR 829C>T polymorphisms in Goto's study (27) were not in the HWE. The validity of genetic association studies is highly dependent on the use of appropriate controls (i.e., control group should follow the HWE) and the key inferences from the genetic-association analysis may be compromised if the HWE is deviated (28). In the present study, since the use of 33 cases resulted in no statistical power difference with 35 cases as suggested earlier (17), and the controls used follow the HWE and resulted in no difference in MAF compared to the cases, we can infer that using a higher number of cases, providing a similar ratio of its control counterparts, would lead to the same outcome of the gene association with the MTX-related AEs in RA. Nonetheless, the extent of successful replication of the study and validation of the postulated associations from the genetic-association study is not yet known (29).

In this study, the demographic profiles of patients and all clinical variables studied were not associated with the development of drug AEs among MTX-related RA patients. Previous pharmacogenetics studies found no association between clinical variables, other than that determined by patient's genetic, and MTX response (30-32). Several previous studies showed that certain clinical variables, such as MTX dose and length of treatment duration, were associated with the risk of MTX toxicity. For example, the study by Ghodke-Puranik et al. (33) reported period of MTX treatment (2.4 ± 1.7 years) with variation in MTX dose was significantly correlated with hepatotoxicity and bone marrow AE, while a higher number of toxicities was observed in RA patients receiving ≥15mg/week of MTX dose. Yanagimachi et al. (34) have reported a higher risk of hepatotoxicity in patients with longer duration of MTX therapy. In addition, a retrospective analysis of Japanese RA patients found that MTX-related AEs were also significantly associated with age (p=0.013), C-reactive protein (p=0.028) and MTX dosage (p=0.019) (35). Moreover, a Singaporean study (36) involving higher cases:controls reported significant (p<0.05) discrepancies between cases (MTX non-tolerant) and controls (MTX tolerant) in terms of cumulative dose. In their study,the ratio of cases to controls was controlled with age- and gender-matched and fixed at 1:2 instead, thus contributing to the included subjects of 46:92 for the groups, respectively (36).

The overall incidence of MTX-related AEs in our study was not similar to that found in most of the related studies. Inconsistency findings can be affected mainly by variation in sample size, study design and marked differences in ethnicity. The smaller sample size in this study is not comparable to other pharmacogenetic studies of MTX (22,37). Majority of the previous studies had recruited a relatively larger number of patients, which might have more significant findings.

For example, DHFR rs12517451 was found to be significantly associated with higher risk of MTX-related side effects in a cohort study involving 309 British RA patients (4). Considering different ethnicity, the impact of the DHFR rs12517451 on the toxicity of MTX was evident in Caucasian population (4), and no conclusion can be made for Asian population because the SNP has been less studied among the Asian population (38).

We found that haematological AE was the most common MTX-related AEs with 36 % of reported cases. Various studies have demonstrated a lower prevalence (i.e, 3.8% - 19.4%) of haematological AEs manifested as mild leukopenia and neutropenia with TWC values of less than 3.5 x 10^9/L (25,39). This is accompanied by gastrointestinal toxicity (30%) with three patients discontinued from the MTX therapy. The majority of patients with gastrointestinal symptoms were elderly (≥60 years old), as stated by Bologna et al. (40). Although all patients in the this study had received folic acid supplementation, most of them were still prone to a higher risk of gastrointestinal toxicity as this supplement had no effect on the development of MTX-related AEs (41). Considering the effect of DHFR rs12517451 on MTX-related AEs, the SNP was directly associated with the incidence of liver problems in two separate studies (4,24). Likewise, the consequence of two MTX withdrawal cases due to elevation of ALT/AST levels, a specific signal for liver problems, was seen in this study, although we were unable to correlate the cases with the SNP.

Pulmonary toxicity, a life-threatening complication, has also been observed in this study. This AE has been shown to result in a large number of cases (i.e, 4.1%-10%) (35,42) thus preventive measures may need to be considered during patient management possibly by monitoring drug-drug interactions. The incidence of MTX-induced pulmonary toxicity was unusually high (12%) in this study. Some patients with lung fibrosis were believed to be either due to other DMARDs or due to comorbidity, since the majority of cases of pulmonary toxicity were over more 60 years of age. In addition, the aetiology of pulmonary fibrosis is probably due to drug hypersensitivity (43). In order to rule out any pulmonary comorbidities prior to diagnosis of MTX-related pulmonary toxicity, it is recommended that patients should have chest X-rays and the determination of interstitial pneumonia markers such as KL-6 serum (44).

Last but not least, the elevation of liver enzyme level was also observed in this study (9.1%). Surprisingly, liver toxicity is the second most common MTX-related AEs in previous studies, with a prevalence ranging from 7.46 to 38.4% (30,39). Low incidence of MTX-induced hepatotoxicity as indicated in this study may be due to folic acid supplementation as suggested previously (41). Numerous evidences has shown the beneficial effects of folic acid in the protection of individual against MTX-related AEs. Without a protective effect of folic acid, it has been proposed that the mechanism of liver toxicity is likely to be triggered by intracellular aggregation of MTX-polyglutamates in the liver, which is subsequently subjected to higher risk of hepatotoxicity (45).

The generalisability of these findings is subject to certain limitations. For instance, we consider a general, non-specific effect of MTX (since MTX is often used concomitantly with other DMARDs) in association with the drug-related AEs observed in the MTX-treated RA patients in this study. As regards the association between DHFR rs12517451 and drug-related AEs in the MTX-treated RA patients, the contribution of the SNP to a specific type of drug AE was not considered. Undeniably, it is however a good approach to demonstrate the association between the SNP and the specific type of drug AEs. Nevertheless testing a SNP marker to detect genetic association requires a higher case number i.e., 248 cases, based on the assumption of an odds ratio of 2 and 5 % disease prevalence (46). In this study, some AEs were being reported redundant from one type of drug AE to another, so it was not possible to link the specific type of drug AE with the SNP. Therefore, we decided to only report the prevalence of the SNP studied and relate it with the general drug AEs in MTX-treated RA patients.

CONCLUSION

The prevalence of minor allele T of the SNP rs12517451 in drug-related AEs among MTX-treated RA patients was 28.8%. While not subtantially different from controls without drug AEs, the presence of T allele in the SNP is higher in cases and may be a predictive genetic factor in drug AEs in MTX-treated RA thus warrants further analysis in a larger subject number and involves multicentre settings to validate the association of the SNP.

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