ANALYSIS OF THE ASSOCIATION BETWEEN POLYMORPHISM IN DHFR AND SLC19A1 GENES, CLINICAL FACTORS AND METHOTREXATE-RELATED SIDE EFFECTS IN RHEUMATOID ARTHRITIS PATIENTS FROM HUSM, KELANTAN.

by

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LIST OF ABBREVIATION

Abbreviation	Name
ACPA	Anti-citrullinated protein antibodies
AE	Adverse effects
ALT	Alanine aminotransferase
AST	Aspartate aminotrasferase
ABC	ATP-binding cassette
ATIC	5-aminoimidazole-4-carboxamide ribonucleotide (AICAR) transformylase
ACR	American College of Rheumatology
bDMARDs	Biologic DMARDs
bsDMARDs	Biosimilar drug modifying antirheumatic disease
BMI	Body mass index
CD 4^+	Cluster of differentiation 4
CCR6	C-C chemokine receptor type 6
CD40	Cluster of differentiation 40
csDMARDs	Conventional synthetic DMARDs
CXR	Chest X-ray
DC	Dendritic cells
ddH_2O	Deionised distilled water
dNTP	Deoxynucleotide
DMARDs	Drugs modifying antirheumatic disease
DHFR	Dihydrofolate reductase
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
dUMP	Deoxyuridine monophosphate
dTMP	Deoxythymidine monophosphate
EBV	Epstein-Barr virus

EDTA	Ethylenediaminetetraacetic acid
ERSD	End-stage renal disease
FPGS	Folypolyglutamate synthase
GIT	Gastrointestinal tract
GGH	γ-glutamyl hydrolase
gDNA	Genomic DNA
HSP	Hypersensitivity pneumonia
HLA-DR	Human Leukocyte Antigen – antigen D Related
HLA-DRP1	Human Leukocyte Antigen – antigen D Related P1
Kg	Kilogram
IL-6	Interleukin 6
IL-1β	Interleukin 1β
IL-8	Interleukin 8
LEF	leflunomide
mg	miligram
MTHFR	5,10-methylene-tetrahydrofolate reductase
MTX-PG	MTX-polyglutamate
$MgCl^2$	Magnesium chloride
MHC	Major histocompatibility complex
Non-MHC	Non Major histocompatibility complex
NSAIDs	Nonstreroidal anti-inflammatory drugs
NAFLD	Non-alcoholic fatty liver disease
PCR	Polymerase chain reaction
PTPN22	Protein tyrosine phosphatase, non-receptor type 22
RFLP	Restriction fragment length polymorphism
RF	Rheumatoid factors
RFC-1	Reduce folate carrier-1
STAT4	Signal transducer and activator of transcription 4
SHMT	Serine hydroxymethyltansferase

SLC19A1	Solute carrier family 19 (folate transporter), member 1
SLE	Systemic lupus erythematosus
TLR	Toll-like receptor
TNF-α	Tumor necrosis factor α
Th17	Helper T cell 17
TCDD	Tetrachlorodibenzo-P-dioxin
TsDMARDS	targeted synthetic DMARDs
TYMS	Thymidylate Synthase
THF	Tetra hydrofolate
TBE	Tris-boratte-EDTA
UV	Ultraviolet
UTR	Untranslated region
%	Percentage
α	Alpha
β	Beta
γ	Gamma

ANALYSIS OF THE ASSOCIATION BETWEEN POLYMORPHISM IN DHFR AND SLC19A1 GENES, CLINICAL FACTORS AND METHOTREXATE-RELATED SIDE EFFECTS IN RHEUMATOID ARTHRITIS PATIETS FROM HUSM, KELANTAN.

ABSTRACT

Methotrexate (MTX) is folic acid antagonist that widely used for treatment of rheumatoid arthritis (RA) nowadays. Despite its proven efficacy in RA patients, the occurrence of adverse effects is a major reason for drug discontinuation. Generally, more than 30% of patients on MTX are susceptible to risk of MTX-related adverse effects (AEs) in which 19% of them had discontinued the drug. In clinical setting, it is currently challenging to predict which patients on MTX are possibly developing drug toxicity. Most studies speculate demographic characteristics and clinical factors are associated with risk of MTX-related AEs. Besides that, the genetic polymorphisms in the folate pathway are also implicated in MTX outcome. Two single nucleotide polymorphisms (SNPs) in dihydrofolate reductase (DHFR) (i.e; rs12517451) and solute carrier family 19 folate transporter member 1, SLC19A1 (i.e: rs1050266) genes were reported as promising predictive genetic markers for MTX toxicity. Until now, no genetic association study among Malaysian population has explored the influence of both SNPs on MTX toxicity. Therefore, we aim to study the association of SLC19A1 rs1051266 and DHFR rs12517451 variants, together with other clinical factors on MTX-related AEs among RA patients from HUSM, Kelantan. This is a case-control study, and involves 72 RA patients, 27 patients were assigned as cases and 45 patients were classified as control. All information regarding demographics and clinical characteristics of patients were obtained through examination of patient's medical records. The genotyping of selected archived DNA

samples of RA samples were performed by using PCR-RFLP (Polymerase Chain Reaction-Restriction Fragment Length Polymorphism) technique and followed by sequencing. The results indicated that, the most frequent MTX toxicity was Gastrointestinal (GI) toxicity (37%), followed by haematological toxicity (29.6%), skin toxicity (18.5%), pulmonary toxicity (14.8%), fatigue (11.1%) and only one patient with elevated liver enzyme level was identified. The results also indicated that there were no difference in demographic variables and clinical factors analysed between cases and controls. Besides that, no significant different in the genotype and allele frequencies for both DHFR rs12517451 and SLC19A1 rs1051266 variant between cases and controls was found suggesting that both SNPs were not associated to the risk of MTX-related AEs. Due to small sample size, our finding warrant further investigation and replication in larger patient cohort and in multicentre trials.

Analisis perkaitan di antara polimorfik dalam gen DHFR dan SLC19A1, faktor klinikal dan kesan buruk Methotrexate dalam kalangan pesakit rheumatoid arthritis dari HUSM, Kelantan.

ABSTRAK

Methotrexate (MTX) adalah antagonis asid folat yang digunakan secara meluas bagi rawatan rheumatoid arthritis (RA) pada masa kini. Walaupun telah terbukti keberkesanannya, kesan buruk MTX adalah penyebab utama pesakit diberhentikan daripada rawatan. Secara amnya, lebih daripada 30% pesakit mengambil MTX terdedah kepada risiko kesan buruk terkait dengan MTX, di mana 19% daripada mereka berhenti mengambil MTX. Dalam aturan klinikal, pada masa ini, amatlah sukar untuk kita mengenal pasti pesakit yang terdedah kepada kesan buruk berkaitan MTX. Kebanyakan kajian mencadangkan karekter demografi pesakit dan faktor klinikal berhubung kait dengan risiko mendapatkan kesan buruk MTX. Selain itu, polimorfisme nukleotid tunggal (SNPs) dalam *dihydrofolate reductase* (DHFR) (rs12517451) dan *solute carrier family 19 folate transporter member* 1, SLC19A1 (rs1050266) telah dilaporkan sebagai penanda genetik bagi mengenal pasti kesan buruk MTX. Sehingga kini, tiada kajian genetik kaitan hubungan di antara kedua-dua SNPs tersebut dan kesan toksik MTX dalam kalangan penduduk Malaysia. Oleh itu, tujuan kajian ini adalah mengkaji perkaitan di antara varian SLC19A1 rs1051266 dan DHFR rs12517451, bersama faktor klinikal lain keatas kesan buruk MTX, dalam kalangan pesakit RA dari HUSM, Kelantan. Ini adalah kajian kes-kawalan yang melibatkan 72 pesakit RA, dimana 27 pesakit dikategorikan sebagai kes manakala 45 pesakit dikategorikan sebagai kawalan. Kesemua maklumat mengenai demografi dan ciri klinikal pesakit diperolehi melalui pemeriksaan rekod perubatan pesakit. Kaedah genotip sampel DNA arkib telah dilakukan menggunakan teknik PCR-RFLP

(Polymerase Reaction-Restriction Fragment Length Polymorphism) dan diikuti dengan penjujukan DNA. Keputusan menunjukkan, kesan toksik MTX paling kerap ialah gastrousus(GI) (37%), diikuti dengan kesan toksik hematologi (26.9%), kesan toksik kulit (18.5%), ketoksikan paru-paru (14.8%), keletihan (11.1%), dan hanya seorang pesakit mengalami kenaikan tahap enzim hati dikesan. Keputusan juga menunjukkan bahawa tiada perbezaan bagi demografi dan faktor klinikal yang dianalisis di antara kes dan kawalan. Selain itu, tiada perkaitan yang dikenal pasti bagi taburan genotip dan alel kedua-dua varian DHFR rs12517451 dan SLC19A1 1051266 yang dianalisis di antara kes dan kawalan. Ini mencadangkan bahawa kedua-dua SNPs tidak mempunyai perkaitan dengan risiko kesan buruk MTX. Oleh kerana saiz sampel yang kecil, hasil kajian ini boleh disyorkan untuk dikaji selidik dalam kajian susulan yang mempunyai saiz sampel pesakit yang lebih besar dan melibatkan banyak lokasi penyelidikan (*multicentre)*.

CHAPTER 1

INTRODUCTION

1.1 Introduction

Rheumatoid arthritis (RA) is a multi-systemic autoimmune disorder characterized by chronic synovial joint inflammation which leads to disability and irreversible damage as well as limited mobility (Atic, Muralidharan and Jain, 2016). Nowadays, it is estimated to affect 1-5% of worldwide population (Atic, Muralidharan and Jain, 2016). According to Global Burden of Disease 2010 study regarding burden of RA, the prevalence of RA is the highest in the Australasian region followed by Western Europe and North America (high-income population), whereby the lowest is in Asia (East and Southeast region) and North Africa/Middle East. RA is listed at 42nd of the highest contributor among disabilities disorders to global disability (Cross *et al.*, 2014). It is also known that the risk of RA is associated with both genetic and environmental factors (Ramamoorthy, Abraham and Isaac, 2014). Frequently, RA is diagnosed among middle-aged adults and incidence is two times higher in females compared to males (Cross *et al.*, 2014). The detection of RA at an early stage of disease to receive appropriate intervention is crucial to a good prognosis.

Several types of Disease Modifying Antirheumatic Drugs (DMARDs) have been used for treatment of RA, but methotrexate (MTX) is the first-line treatment because of its established efficacy and affordability (Owen *et al.*, 2012). MTX is an antimetabolite agent that specifically inhibits dihydrofolate reductase (DHFR) activity and also some other enzymes and leads to suppression of cell growth. It is also generally used for treatment of other chronic inflammatory disorders such as

psoriasis, systemic lupus erythematosus (SLE) and various malignancies. Initially, MTX was introduced as an anticancer agent and then started being used as primary treatment for RA after it was discovered effectively improved disease activity in RA patient. Besides, a large scale study also found more than 75% RA patients went into remission after receiving 7.5-mg/week of MTX (Buhroo and Ortho, 2006).

Currently, the literature regarding the mechanism of MTX was still scarce. It is well known that, MTX is an active drug that is a highly selective competitive inhibitor of dihydrofolate reductase that converts dihydrofolate to tetrahydrofolate (THF), which is an important precursor for biologically active folate cofactors. MTX mainly enter cells by an active transport mechanism via the reduced folate carrier (RFC). Within the cell, MTX is activated into polyglutamate form of MTX (MTX-PG) by the enzyme folypolyglutamate synthase (FPGS). MTX-PG can be hydrolysed by the enzyme γ-glutamyl hydrolase (GGH) and transported out cells by ABC protein transport. MTX-PG also inhibits dihydrofolate reductase (DHFR) together with thymidylate synthase (TYMS) and 5-aminoimidazole-4-carboxamide ribonucleotide (AICAR) transformylase (ATIC). This results in increasing intracellular adenosine levels which appears to be the mechanism for the anti-inflammatory activity of methotrexate (Stamp and Roberts, 2011).

Despite its proven efficacy in RA patients, the outcome of MTX treatment is unpredictable. Interpatient variability in response to treatment may be associated with several factors such as demographic factors (age and sex), disease specific factors (disease duration and severity) as well as other factors such as concomitant drugs, folic acid supplementation and genetic variation. Number of studies have

demonstrated some of these factors affect both efficacy and toxicity of MTX in RA patients (Owen *et al.*, 2012; Wessels *et al.*, 2006). Furthermore, despite the complexity of MTX response among RA patients, there is still no available test to predict the treatment outcome. Current routine tests such as blood and liver function testing are relatively costly and regular checking of patients using these tests is inconvenient and burdensome (Owen *et al.*, 2012).

Even though MTX is relatively effective compared to other DMARDs, the presence of side effects is a major factor in discontinuation of treatment rather than lack of efficacy. The most common side effects experienced by RA patients including gastrointestinal intolerance, hepatotoxicity, pancytopenia, alopecia and sometimes unexpected pulmonary and bone marrow toxicity. Approximately 30% of RA patients treated with low dosage of MTX having side effects after at least 6 months treatment duration (Li *et al.*, 2016). Commonly, those who experiences MTX-related side effects either withdraw from MTX therapy or are change to other available DMARDs with good safety profiles. Alternatively, MTX-related side effects will be resolved after reducing the MTX dosage to fit to patient conditions. Besides that, folic acid supplementation can alleviate the side effects due to folate deficiency without interfering with the therapeutic activity of MTX in RA patients (Buhroo and Ortho, 2006).

1.2 The Significant of Study and Contribution in Clinical Setting

This study is relevant to be conducted in order to provide a data regarding the prevalence and incidence of MTX-related adverse effects in RA patients specifically among our population. To date, limited data and evidence describing the occurrence of MTX toxicity in our population. Since MTX has been prescribed as the first line treatment for most RA patients, the major concerns about the risk of side effects become the main issues in clinical setting.

Various studies have reported multiple potential predisposing factors linked to MTX outcome. Most of authors have speculated there are several factors predisposing to MTX-related side effects which including demographics factors (age and sex), disease specific factors (disease duration and severity) and concomitant drugs (Romão *et al.*, 2014). An evidence has indicated advanced age of RA patient concurrent with renal failure increase a risk of MTX toxicity (Jerzy and Jacek, 2008). Besides that, RA patients concomitant on other medication like steroids, NSAIDs and other DMARDs were at higher risk of developing MTX-related side effects. Since administration of folic acid is crucial to alleviate the side effects, therefore lack of folic acid supplementation will increases the risk of hepatotoxicity and GIT intolerance (Report, 2003). The limited literature described the influence of MTX dosage on the development of MTX-related side effects. Some studies found high MTX dose and cumulative dose linked to development of non-alcoholic liver disease (NAFLD) and elevated transaminase with unknown underlying mechanism (Hyrich, 2012; Sakthiswary *et al*., 2014).

Genetic variation encoded within MTX metabolism is the most prominent genetic factors associated with MTX toxicity and efficacy. However, due to discrepancy of findings among published studies, no accurate genetic predictive markers for MTX toxicity have been identified so far. Hence, the current study intended to investigate the impact of selected SNPs in genes encoded in folate metabolic pathway and drug transport protein in development of MTX adverse effects. Genetic polymorphism in Dihydrofolate reductase (DHFR) enzyme and Solute carrier family 19 folate transporter member 1/Reduced folate carrier-1 (SLC19A1/RFC-1) are the potential genetic markers that associated with MTX toxicity. Many of studies showed both SNPs of DHFR (rs12517451) and SLC19A1 (rs1051266) were significantly associated with MTX-related side effects (Grabar and Lestan, 2008, Owen *et al.*, 2013). Moreover, to best of our knowledge, there are no genetic study has reported from Malaysia, which has explored the influence of variants in DHFR and SLC19A1 on MTX toxicity.

Besides that, the findings from this study also will assist the development of personalized therapy which is one of the main goals of modern medicine. In addition, this study also possibly discovered the potential pathophysiological explanation about studied gene in order to elucidate the underlying mechanism of MTX toxicity.

1.3 Objectives of the research

1.3.1 General Objective

To evaluate the association between genetic polymorphism, clinical factors and methotrexate-related side effects among RA patients from HUSM, Kelantan.

1.3.2 Specific Objectives

1. To determine the prevalence of the most commonly observed side effects of MTX among RA cases by retrospective examination of patient clinic files and medical report.

2. To assess the association between DHFR rs12517451 and SLC19A1 rs1051266 variants with MTX-related side effects by using Restriction Fragment Length Polymorphism (RFLP) method.

3. To determine the extent by which factors (demographic factors, clinical variables and genetic variants) contribute to the risk of MTX-related side effects in the RA patients by logistic regression of data generated 1 and 2 above.

CHAPTER 2

LITERATURE REVIEW

2.1 Rheumatoid Arthritis (RA)

2.1.1 Epidemiology of RA

The considerable variation in prevalence and incidence of RA has been reported from various studies indicated the distribution of disease was widely vary across different populations (Alamanos and Drosos, 2005). The majority of studies on prevalence and incidence of RA that have been conducted among North Europe and North American population has shown the prevalence of 0.5- 1.1% .This also demonstrate the incidence rate of RA at this region which ranged from 20 to 50 cases per 100,000 populations (Alamanos, Voulgari and Drosos, 2006). The prevalence and incidence of RA in South East Asian region has been reported the lowest so far. The highest prevalence of RA in 2006 has was reported from India with 0.5%, followed by China (Beijing) 0.34%, Indonesia (urban) 0.3%, Japan 0.3%, Vietnam 0.28%, Philippines (urban) 0.17%, Malaysia 0.15% and Thailand 0.12% (Davatchi, 2006). The lowest frequency of RA was found in some areas of rural Africa (Silman and Pearson, 2002). In fact, RA occurs at twice the rate in women compared with men, with a prevalence of 1.06% in women compared with 0.61% in men (Rudan *et al.*, 2015).

The differences of prevalence and incidence of RA across different population can be explained by certain potentials predisposing risk factors of RA such as genetic and environmental factors that influence susceptibility towards development of disease. Besides that, the method identification for diagnosis of RA also has contributed this

variation. The higher mortality rate of RA patients has been reported due to decreasing life span in RA patients (Gabriel *et al.*, 2003). By treating RA patients with anti-rheumatic drugs, the treatment only manage the symptoms of RA which relate to lowering a person lifespan. However, this expected survival of RA patients also might depend on severity of the disease and the disease duration (Alamanos, Voulgari and Drosos, 2006).

2.1.2 Pathogenesis of RA

The precise mechanism for initiation of RA is still unknown. It was speculated that the initiation of this disease was due to production of autoantibodies against specific citrullinated proteins (ACPA) and activation of innate immunity. It has been known the detection of rheumatoid factor (RF) is predictive markers to identify patients with rheumatoid arthritis. Nowadays, a new alternative predictive marker has been discovered. Anti-citrullinated protein antibodies (ACPAs) is a new potential marker to detect the disease activity. It has been found in almost 50% of RA patients which may facilitate for identification of patients with early arthritis (Arend and Firestein, 2012).

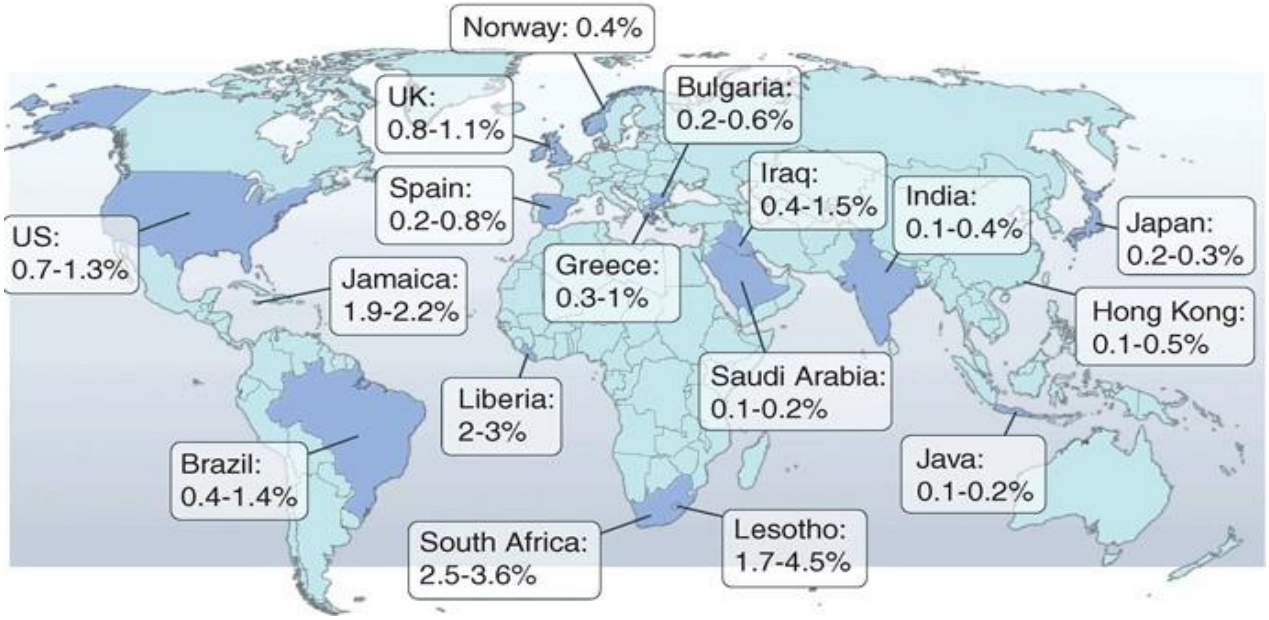

Figure 2.1: The prevalence of rheumatoid arthritis worldwide (Cross *et al.*, 2014).

The mechanism of initiation of RA is known to be related to stimulation of immune cells and inflammatory events. Initially, innate immune system is stimulated by several initiating agents including pathogenic microorganism, toll like receptor (TLR) or environmental factors, in which subsequently activate various immune cells such as mast cells, neutrophils, dendritic cells and possibly chondrocytes. The production of ACPAs in many mucosal-lined organs was occurred in response to inflammatory cytokines. ACPAs, the specialized antibody towards is circulated within synovial tissues, it will bind to specific epitope in cartilage and synovial that leads to destruction of cartilage components and other synovial tissues through formation of complement cascade pathway (Komatsu and Takayanagi, 2012). The damage in synovial tissue is also related to higher expression of pro-inflammatory cytokines especially tumor necrosis factor (TNF) and IL-6 that result in proliferation of synovial cells in joints and formation of pannus which cause the destruction of cartilage and bone erosions (Wasserman, 2011). Moreover, activation of helper $CD4^+$ T-cells, specifically Helper T (Th17) cells, also plays a major role in initiation of immune response in RA.

2.1.3 Risk Factors of RA

RA is known as multifactorial disease that result from interaction between genetic and environmental factors (Tobon, Youinou and Saraux, 2010). Several risk factors of RA that has been proven by various studies linked to disease such as genetic variants encode for the functional of immune systems, smoking, anti-citrullinated protein antibodies (ACPAs) and rheumatoid factors (RF).

2.1.3.1 Genetic risk factor

Until now, researchers are still uncertain whether the impact of genetic factors can be linked with risk of RA or the severity of the disease or both of them. In development of RA, the genetic variant in MHC and non-MHC genes is believed associated with immune response and inflammation. Class II region of MHC locus was the most prominent genetic risk that linked to RA, specifically genes encoding HLA-DR molecules. The association between different HLA DRB1 alleles and risk to RA have been demonstrated in several population groups. Besides that, the "shared epitope" (SE) is the conserved 5 amino acid sequence found in HLA DRB1 alleles and also other variety of alleles play an important role in pathogenesis of RA. Vast majority of RA patients carried shared epitope were associated other variety autoimmune disorder (Konda Mohan, Ganesan and Gopalakrishnan, 2014).

Various genes of non-MHC molecule also linked to RA. The most prominent non-MHC gene is a single nucleotide polymorphism in the gene PTPN22 which relatively can be found in many populations. The presence of PTPN22 allele has been showed increase risk of development of RA. If fact, least frequent of Asian populations have detected with PTPN22 polymorphism, thus this allele may only partly explained the occurrence of RA among Asian. Whereas, those individuals from other populations present with PTPN22 polymorphism have greater risk of RA development. The association between PTPN22 polymorphism and expression ACPAs has been described (Begovich *et al.*, 2004).

Furthermore, there are also other several of non-MHC genes have been studied which related to risk of RA and other autoimmune disease, including STAT4 (T-cell response), TNFAIP3 (TNF protein, cytokine), CCR6 (chemokine receptor) and CD40 (B cells, monocytes and DCs) (Silman and Pearson, 2002; Wasserman, 2011). Even though lack of evidence regarding the role of genetic factors in the occurrence of RA, it may possibly explain the significant variation in prevalence and incidence of RA among different populations and ethnic groups.

2.1.3.2 Gender and Hormonal Factors

Commonly, women are two times likely diagnosed with RA compared to men. This can be explained why the incidence of RA in women was higher than in men (Rudan *et al.*, 2015). Some of studies have speculated hormonal imbalance and pregnancy are major factors contribute to increase risk of RA in females (Alamanos and Drosos, 2005; Silman and Pearson, 2002).

Many of studies have found the influence of exogenous hormone may correlate with risk of RA. The follow-up study has discovered the women who taking oral contraceptive pill have low incidence of RA. However, no clear explanation was found. They have speculated oral contraceptive pill might able to reduced risk of RA and provide a protective effects against the development of disease, or maybe it just delay the disease progression (Silman and Pearson, 2002). Moreover, women who are undergo hormone replacement therapy (HRT) may have lower risk of RA. HRT has been shown possess protective effect against the development of RA by preventing the production of ACPAs with unknown underlying mechanism (Salliot *et al.*, 2010).

2.1.3.3 Ethnicity

The differences in prevalence and incidence of RA among different populations or ethic groups have been described. Some ethic and racial groups are at higher risk for RA than others. Ethnicity was considered as independent risk factor which involved between the interaction of genetic and environmental factors. Comparison of population with similar genetic backgrounds but different lifestyles clearly explained about the influence of non-genetic factors. Genetic variation associated with pathogenesis of RA possibly demonstrated the significant different of risk and severity of RA among ethic groups (Silman and Pearson, 2002).

2.1.3.4 Environmental Factors

There are several environmental factors linked to risk of RA including smoking, infections, dietary factors and pollutants. Long term exposure to these predisposing factors may promote the development of disease until it was clinically apparent.

Smoking

The effect of smoking to risk of RA has been reported in various studies. Smoking has showed an increase risk of RA especially in those who are carried the "shared epitope". An evidence has showed smoking can promote the expression of RF and ACPAs production, which explained the higher risk of RA in individual having "shared epitope" (Stolt, 2003).

Tetrachlorodibenzo-P-dioxin (TCDD) is one of the harmful chemical in cigarettes, which is believed to play a role in pathogenesis of disease. TCDD binds to arylhydrocarbone receptor and regulates the expression of several cytokine such as

IL-1β, IL-6, IL-8 through extracellular signal-regulated kinase signalling cascades (Kobayashi *et al.*, 2008). In addition, synergistic interaction between polymorphism of PTPN22 genes and heavy smoking towards the risk of RA also has been reported (Begovich *et al.*, 2004).

Infection

Several potential infectious agent may implicated to RA development such as Human parvovirus B19, rubella virus, human retrovirus 5 and Epstein-Barr virus (EBV) have been identified (Silman and Pearson, 2002; Tobón, Youinou and Saraux, 2010). The presence of viral genome, specific antigens and antibodies production are indicator to determine infected individual with RA. The detection of high level of EBV antigens and production antibodies against latent and replicative virus has been found in some RA patients. Besides that, the presence of EBV RNA in B cell of synovial fluids was strongly indicated integration of EBV viral genome may trigger for RA development. Other viral DNA of pathogenic microorganism such as parvovirus B19 also have been found in synovial tissue and cells of RA patients (Tobón, Youinou and Saraux, 2010).

Dietary Factors

Dietary intake is important factors determine the susceptibility one individual towards various illnesses. The consumption of food rich with omega 3 fatty acids and vitamin D such as fish, olive oil and cooked vegetables able to protect individual from occurrence of RA. Eicosapentaenoic acid is one of omega 3 fatty acids is responsible for anti-inflammatory effect by competing with arachidonic acid to reduce inflammatory response in RA (Ariza-Ariza, Mestanza-Peralta and Cardiel,

1998). Besides that, vitamin K is also another important nutrient mainly found in various vegetables able to alleviate inflammatory response in RA by inhibiting the proliferation of fibroblast-like synoviocytes, specialized cells involve in pathogenesis of chronic inflammatory disease (Silman and Pearson, 2002).

2.2 Drug Modifying Anti Rheumatic Diseases (DMARDs)

2.2.1 Overview

Nowadays, we are still unable to find a cure for RA. Usually, RA patients were treated with variety of drugs including glucocorticoids and drug-modifying anti-rheumatic diseases (DMARDs). The current treatment was seen to alleviate the symptom and improves the disease progression.

DMARDs are the group of medication that commonly used for treatment in patients with rheumatoid arthritis and management of other inflammatory arthritis. There are few types of DMARDs which include (1) conventional synthetic DMARD (csDMARDs) (methotrexate (MTX), cyclosporine, azathioprine, sulfasalazine and hydroxychloroquine), (2) biologic DMARDs (bDMARDs) (Infliximab, Etanercept, Adalimumab, and Rituximab), (3) biosimilars DMARDs (bsDMARDs) and (4) targeted synthetic DMARDs (tsDMARDs) (Singh *et al.*, 2012). The main function of synthetic DMARDs is to subdue the autoimmune response while the biological DMARDs was acting on individual molecules to inhibit the production and expression of inflammatory mediators such as TNF, IL-1, and IL-6 (Umićević Mirkov and Coenen, 2013). Even though biological DMARDs is more efficient compared to synthetic DMARDs, the application of these drugs as first-line treatment in clinical practice was limited due to much higher cost required. The use

of biological DMARDs was commonly implied to those patients fail to respond with DMARDs monotherapy or sometimes combination with other conventional DMARDs (Choy et al., 2005).

The list of common prescribed cDMARDs for treatment of RA are including methotrexate (MTX), leflunomide, sulfasalazine, hydroxychloroquine, and azathioprine (Singh *et al.*, 2012). However, MTX is the first choice for RA treatment because of its established efficacy and affordability compared to others (Owen *et al.*, 2012). Currently, this drugs also widely used in other related-arthritis disorder such as ankylosing spondylitis, psoriasis, and systemic lupus erythematosus. In rheumatoid arthritis disease, mechanism of action of MTX is mainly to alleviate pain and inflammation as well as to prevent joint damage by sustaining the structure and function of joint.

2.2.2 Methotrexate (MTX) as First-line of Treatment in RA

Methotrexate (4-amino-4deoxy-N^{10}-methyl-pteroyl-L-glutamic acid) is known as antimetabolite agents that structurally similar to folic acid (Bannwarth *et al.*, 1996). It has been synthesized in 1948 and initially introduced as an anticancer agent (Braun, 2011). A few years later, MTX was started use as primary treatment for RA after rheumatologist has discovered significant improvement in RA patients who are taking MTX. Until now, MTX is used as first-line therapy for most of patients with RA. The primary role of MTX is to alleviate symptoms of disease and prevent the progression of joint damage (Griffiths H et al., 2014).

As many other DMARDs have been used in treating RA, MTX is most well tolerated with considerable toxicity profile. Gastrointestinal toxicity is the most common MTX-related side effect present in RA patients. Rare side effects are bone marrow, lung or liver toxicity (Weinblatt, 2013). Comparison of MTX with other DMARDs suggested that MTX therapy exhibit more reliable improvement in disease activity of RA patients. The 52 weeks randomized double-blind trial that evaluating the efficacy of MTX and leflunomide (LEF) has found group of patients on MTX therapy has greater improvement in disease activity than patients on leflunomide alone. Compared to MTX, many of RA patients on azathioprine had withdrawn from treatment due to lack of efficacy and adverse effects (Braun, 2011). Besides that, even though advanced of complex biological DMARDs has been develop recently, MTX still remained as anchor drugs in majority of patients with RA. This may explain why the combination of anti-TNF therapy with MTX is far more effective as compared to anti-TNF alone (Griffiths H et al., 2014).

Despite the long establishment of MTX as gold therapy of RA, the optimum dosage for every individual patient is still an issue. Commonly, rheumatologist will prescribe MTX to patients at a starting dose of 7.5-10 mg weekly either orally or by subcutaneous injection. The increase of dose at 5 mg per month is required for patients who are did not show good improvement in disease activity. However, escalation of dosage was only tolerated until 25-30 mg weekly as higher dose can lead to drug toxicity (Griffiths H, 2014).

2.2.3 Mechanism of Action of MTX

The precise mechanism of action of MTX is still poorly understood. MTX is an active drug that is a highly selective competitive inhibitor of dihydrofolate reductase (DHFR). DHFR is enzyme crucial in catalyzes the conversion of dihydrofolate to tetrahydrofolate which is active cofactor involved in the *de novo* synthetic pathway for purine and pyrimidine of DNA and RNA required for cell proliferation. As MTX primarily supress the function of DHFR, it also acts on another various key enzymes in folate metabolic pathway (Swierkot and Szechinski, 2006).

Figure 2.2 shows the mechanism of MTX in mammalian cells. MTX mainly enter cells by active transport mechanism via drug transport protein, namely reduced folate carrier (RFC) or also known as SLC19A1. Within the cell, MTX is converted into polyglutamate form (MTX-PG) by the enzyme folypolyglutamate synthase (FPGS). Polyglutamation is process addition of glutamic acid moieties by FPGS enzyme to form an activated MTX. MTX-PG can be hydrolysed by enzyme γ-glutamyl hydrolase (GGH) and transported out cells by ATP-binding cassette (ABC) protein transport (Griffiths H, 2014). Within the cells, MTX-PG will bind to and inhibit several important enzymes crucial in biochemical of cell growth including DHFR, thymidylate synthase (TYMS) and 5-aminoimidazole-4-carboxamide ribonucleotide (AICAR) transformylase (ATIC). Pyrimidine biosynthesis was suppressed due to inhibition of TYMS by MTX-PG, an essential enzyme for conversion deoxyruridylate to deoxythymidylate.

The inhibition of 5-aminoimidazole-4-carboxamide ribonucleotide (AICAR) transformylase (ATIC) was result in increasing intracellular adenosine levels which appears to be the mechanism for anti-inflammatory activity of methotrexate (Stamp and Roberts, 2011). The activation of adenosine receptors through the binding of adenosine subsequently inhibits the production of pro-inflammatory cytokines involved in RA development such as TNF-α, interferon-γ and IL-1β (Cronstein, 2005; Stamp and Roberts, 2011). MTX-PG also indirectly inhibit 5,10-methylene-tetrahydrofolate reductase (MTHFR) which key enzyme in folic acid pathway and responsible for the conversion of 5,10-,ethylene THF to 5 methyl-THF in process of rementhylation of homocysteine to methionine (Ranganathan, 2008). Other putative mechanisms are including increase apoptosis of T cells, alteration of expression of cellular adhesion molecules, reduction in expression of cellular adhesion molecules and anti-angiogenesis. However, it was speculated that the anti-inflammatory activity mediated by release of adenosine is probably more important than the anti-proliferative effects (Hyrich, 2012)

2.5 The Development of MTX-related side effects (AEs)

2.5.1 Prevalence and incidence of MTX-related side effects in RA patients

RA patients treated with MTX are more likely to withdraw from therapies due to adverse effects rather than lack of efficacy. For instance, a double-blind randomized clinical trial study has demonstrated that 15% of RA patients were withdrawn from MTX therapy due to side effects while only 3% of them stop taking MTX as first-line treatment due to inefficacy of drug (Ishaq *et al.*, 2011). Usually, adverse effects of low-dose MTX are mild, self-limited and preventable. However, some of patients may expose to other severe or life-threatening type of adverse effects which required

drug discontinuation (Tian, Henghe, Cronstein, 2007). During the last two decades, numerous epidemiological studies have been established on the efficacy and side effects of MTX therapy. The reported prevalence of MTX-related side effects worldwide ranges from 30% to 80% of patients. According to published studies, overall data regarding prevalence of MTX-related side effects among Asians and Caucasians patients showed approximately more than half of RA patients on MTX were likely to suffer from one or more adverse effects.

Among Asian population, the wide variation in prevalence of MTX adverse effects was reported by numerous studies. On the basis of retrospective and prospective cohort studies, the prevalence of MTX-related side effects in Asian population ranged between 20% and 80% of patients. Specifically, the prevalence of MTX-related side effects observed within patients of East Asia was 50% in China (Xiao *et al.*, 2010), 38% in Japan (Fujii *et al.*, 1997), 40% in Korean (Kim *et al.*, 2006) and 25% in Taiwan, whereas among patients of South Asia were 20% in India (Atic, Muralidharan and Jain, 2016) and 80% in Pakistan (Ishaq *et al.*, 2011). A Singaporean study also reported the incidence of MTX associated-liver toxicity and Non-alcoholic Fatty Liver Disease (NAFLD) was 11.4% and 4.6% in patients respectively (Sakthiswary *et al.*, 2014). The variation in data indicated ethnicity may affect the development of MTX-related side effects.

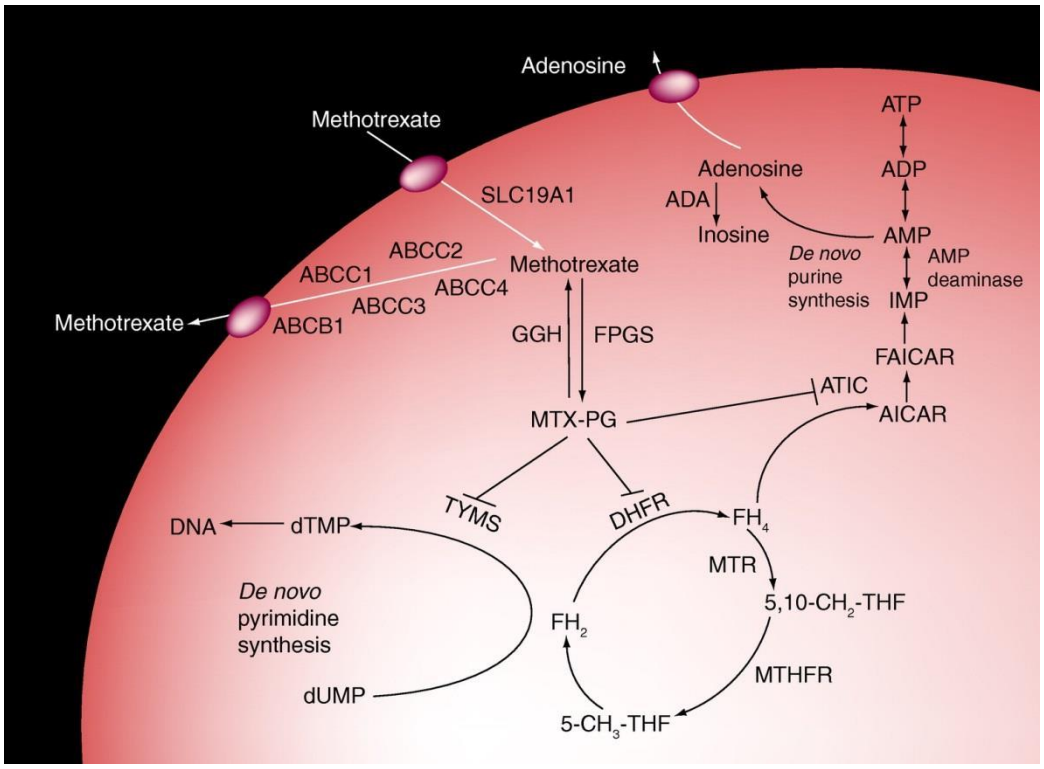

Figure 2.2: The diagram above show a complete cellular pathway of methotrexate within the mammalian cells. 5,10-CH_2-THF: 5,10-methylene tetrahydrofolate; 5-CH_3-THF: 5-methyl tetrahydrofolate; AICAR: 5-aminoimidazole 4-carboximade ribonucleotide; ATIC: AICAR formyltransferase; FAICAR: 10-formyl AICAR, FH_2 : dihydrofolate; MTX-PG : Methotrexate polyglutamate (Ranganathan *et al*, 2008).

2.5.2 Mechanism of action of MTX toxicity

The exact mechanism of MTX toxicity is still obscure. Mostly overall side effects have been directly related to MTX involvement in the previously mentioned metabolic pathways (van Ede *et al.*, 1998). The inhibition of various folate metabolizing enzyme and other folate-related pathway by MTX may partly explain the occurrence of adverse effects in RA patients. The anti-proliferative activity of MTX associated with depletion of purines, thymine and methionine that crucial in cell growth mainly affected by rapidly dividing tissue such as bone marrow and gastrointestinal tract. Different type of adverse effects can be related to various MTX metabolic pathways. The toxicities from folate antagonism, including anemia, neutropenia, stomatitis and oral ulcers can be prevented or alleviated by folic acid supplementation. On the hand, prescribing physician should aware of toxicities unrelated to suppression of folate metabolisms such as MTX-induced nodulosis, hepatic fibrosis, pulmonary fibrosis, lethargy, fatigue, and renal failure (Tian, Henghe, Cronstein, 2007).

The expression of intracellular adenosine by MTX acting on 5-aminoimidazole-4-carboxamide ribonucleotide (AICAR) transformylase (ATIC), the folate metabolizing enzyme was the main mechanism related to its anti-inflammatory action in RA patients (Cronstein, 2005). Many of studies have intended to investigate the putative role of adenosine pathway as potential mechanism involved in MTX toxicities (Tian, Henghe, and Cronstein, 2007). An *in vitro* study had shown purified monocyte treated with methotrexate has formed a multinucleated giant cell. This was indicated the possible mechanism of MTX-induced nodulosis present in RA patient.

Besides that, the adenosine pathway also associated with the development of MTX-induced hepatic fibrosis. Hepatoma cells that treated with methotrexate also lead to increase of adenosine production. Another *in vivo* study also has found the higher of adenosine level in mice treated with hepatotoxin, an adenosine agonist that binding to adenosine A_{2A} receptor. This result demonstrated the role adenosine A_2 receptor may involve in pathogenesis of hepatic fibrosis (Chan and Cronstein, 2010; Tian, Henghe, and Cronstein, 2007). Moreover, the effect ethanol stimulates the release of adenosine by hepatocytes was also well documented. Thus, this evidence has shown alcohol consumption in MTX-treated patients will likely increase the likelihood of developing liver cirrhosis (Swierkot and Szechinski, 2006).

The occurrence of MTX toxicity is also associated with homocysteine-methionine-polyamine pathway. In the homocysteine and methionine pathway, MTHFR enzyme plays an important role in conversion of homocysteine to methionine. Inhibition of MTHFR enzyme through indirect effect of MTX will result in elevated level of homocysteine in the plasma. The accumulation of homocysteine level will lead to increased risk of various disorders such as vascular and neurodegenerative diseases, autoimmune disease, osteoporosis and cancer (van Ede *et al.*, 1998). However, no plausible explanation regarding the deleterious effect hyperhomocysteinemia associated with MTX toxicity has been discovered yet. A study has found those patients present with GIT toxicity had a significantly higher level of homocysteine after 52 weeks of MTX treatment. In addition, the interference of MTX in homocysteine-methionine pathway also will result in decreased level of SAM and SAH, which is a marker of methylation reactions. Diminished level of SAM and SAH might explain the development of adverse effect in some RA patients treated

with MTX. Finally, the inhibition of polyamine synthesis by MTX also may cause toxicity, because polyamines are important for cell function (van Ede *et al.*, 1998).

2.5.3 Specific MTX related side effects

Recently, a considerable literature has grown up around the theme of safety among MTX users. Methotrexate related adverse effects (AEs) can be defined as one or combination of the following side effects including nausea/vomiting, abnormal liver function (greater than twice the upper limit of normal values), aggravated skin nodules, oral ulcers and cytopenia or any other known MTX related AE documented in drug literature (Ghodke *et al.*, 2008). Usually, RA patients would start to experience low dose of MTX-related side effects within first 6 months of therapy (Mittal *et al.*, 2012).

2.5.3.1 GIT toxicity

Gastrointestinal (GIT) toxicity/intolerance is the most frequently observed in RA patients on MTX and this accounted for more than half of overall side effects (Xiao *et al.*, 2010; Muralidharan *et al.*, 2015). The reported side effects included a range of GIT toxicity symptoms like abdominal pain, vomiting, diarrhea, nausea, and dyspepsia (Patil *et al.*, 2014). Previous studies have shown GIT toxicity was commonly present in obese patients and those previously diagnosed with GIT adverse effects (Hoekstra *et al.*, 2003; Report, 2003). The highest rate of GIT toxicity among RA patients can be explained presumably due to the primary effects of MTX on rapidly dividing mucosal cells lining the GI tract (Kremer, 2004; J., 2008). However, the causes of GIT toxicity also might be associated with other predisposing factors such as concomitant drugs especially NSAIDs. Several studies have