THE ASSOCIATION BETWEEN THIOPURINE S-METHYLTRANSFERASE *3C POLYMORPHISM AND AZATHIOPRINE INDUCED MYELOSUPPRESSION IN THAI PATIENTS WITH RHEUMATOLOGIC DISEASES

Miss Janejira Kongpunvijit

A Thesis Submitted in Partial Fulfillment of the Requirements

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ความสัมพันธ์ระหว่างการแสดงออกของยีน Thiopurine S-methyltransferase ที่มีรูปแบบ *3C และการกดการทำงานของไขกระดูกจากการใช้ยา azathioprine ในผู้ป่วยไทยโรคข้อและรูมาติซั่ม

นางสาวเจนจิรา คงพันธุ์วิจิตร

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
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Thesis Title	THE ASSOCIATION BETWEEN THIOPURINE S-METHYLTRANSFERASE *3C POLYMORPHISM AND AZATHIOPRINE INDUCED MYELOSUPPRESSION IN THAI PATIENTS WITH RHEUMATOLOGIC DISEASES
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เจนจิรา คงพันธุ์วิจิตร : ความสัมพันธ์ระหว่างการแสดงออกของยีน Thiopurine S-methyltransferase ที่มีรูปแบบ *3C และการกดการทำงานของไขกระดูกจากการใช้ยา azathioprine ในผู้ป่วยไทยโรคข้อและรูมาติซั่ม (THE ASSOCIATION BETWEEN THIOPURINE S-METHYLTRANSFERASE *3C POLYMORPHISM AND AZATHIOPRINE INDUCED MYELOSUPPRESSION IN THAI PATIENTS WITH RHEUMATOLOGIC DISEASES) อ.ที่ปรึกษา วิทยานิพนธ์หลัก : รศ.นพ.ยิ่งยศ อวิหิงสานนท์, อ.ที่ปรึกษาวิทยานิพนธ์ร่วม: รศ.พญ.ดร. ณัฏฐิยา หิรัญกาญจน์, 74 หน้า.

โรคข้อและรูมาติซั่ม (Rheumatologic disease) มีสาเหตุของการเกิดโรคทั้งจากพันธุกรรมและการทำงานของเซลล์ภายในร่างกาย ซึ่งยาที่ใช้ในการรักษาได้แก่ยา azathioprine ที่มีคุณสมบัติกดภูมิคุ้มกันของร่างกายและมีเอ็นไซม์ที่สำคัญในการเมทาบอไลท์ยาคือ Thiopurine S-methyltransferase เป็นเอ็นไซม์ในไซโทพลาสซึม ซึ่งมีความหลากหลายทางพันธุกรรม คนไข้ที่ขาดการทำงานของเอ็นไซม์ TPMT จะมีความเสี่ยงต่อการกดการทำงานของไขกระดูก ในครั้งนี้ได้ทำการศึกษาลักษณะทางพันธุกรรมของเอ็นไซม์ TPMT ที่มีรูปแบบ *3C ด้วยวิธี PCR-RFLP และวัดระดับการทำงานของเอ็นไซม์ในเม็ดเลือดแดงด้วยวิธี HPLC ในผู้ป่วยโรคข้อและรูมาติซั่มที่ได้รับยา azathioprine จำนวน 112 ราย โดยเปรียบเทียบกับข้อมูลการรักษาทางคลินิกของคนไข้ย้อนหลัง จากการศึกษา พบคนไข้ 8 รายมีลักษณะทางพันธุกรรมแบบ heterozygous (TPMT*1/*3C) คิดเป็น 7.14% และ 104 รายเป็นแบบปกติ (wild type) homozygous TPMT*1 (A/A) คิดเป็น 92.86% ซึ่งมีความถี่ของ “G” allele คิดเป็น 3.57% และ “A” allele คิดเป็น 96.43%. และพบว่าลักษณะพันธุกรรมที่มีรูปแบบ TPMT*1/*3C มีความเสี่ยงสูงในการเกิด leucopenia จากการใช้ยา AZA ($P < 0.001$; odd ratio 26.0, 95%CI 4.783 – 141.333) เมื่อได้รับขนาดยาปกติคือ 0.65 – 2.50 mg/kg/day มีค่าเฉลี่ยเท่ากับ 1.54 ± 0.51 (SD) ($P = 0.186$) แต่ยังไม่พบความสัมพันธ์ที่ชัดเจนในการเกิด neutropenia ($P = 0.06$, odd ratio 8.889, 95%CI 1.237 – 63.878) และ lymphopenia ($P = 0.085$, odd ratio 6.583, 95%CI 0.995 – 43.553) โดยมีค่าเฉลี่ยการทำงานของเอ็นไซม์ TPMT เป็น 36.26 ± 13.40 nmol/gHb/hr โดย TPMT*1/*1 มีค่าเฉลี่ยเท่ากับ 37.90 ± 12.64 และ TPMT*1/*3C เท่ากับ 17.53 ± 4.96) เมื่อวิเคราะห์ค่าทางสถิติพบว่าค่าเฉลี่ยของทั้งสองกลุ่มแตกต่างกันอย่างมีนัยสำคัญทางสถิติ ($P<0.001$) เมื่อพิจารณาจากการทำงานของเอ็นไซม์ในเม็ดเลือดแดง โดยมีเกณฑ์ของการทำงานของเอ็นไซม์ระดับกลางคือ $\leq$ 23.01 nmol 6-MTG/gHb/hr และระดับสูงคือ > 23.01 nmol 6-MTG/gHb/hr พบว่ากลุ่มคนไข้มีความเสี่ยงสูงในการเกิด leucopenia ($P = 0.003$, odd ratio 21.5, 95%CI 2.961 – 156.128) จากการใช้ยา AZA แต่ยังไม่พบความสัมพันธ์ที่ชัดเจนในการเกิด neutropenia ($P = 0.059$, odd ratio 11.25, 95%CI 1.233 – 102.623) และ lymphopenia ($P = 0.391$, odd ratio 2.933, 95%CI 0.254 – 33.823)

สาขาวิชา วิทยาศาสตร์การแพทย์..... ลายมือชื่อนิสิต.....

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ลายมือชื่อ อ. ที่ปรึกษาวิทยานิพนธ์ร่วม.....

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KEYWORDS : THIOPURINE S-METHYLTRANSFERASE/ POLYMORPHISM/ PCR-RFLP/ RHEUMATOLOGIC DISEASE/ AZATHIOPRINE/ MYELOSUPPRESSION

JANEJIRA KONGPUNVIJIT: THE ASSOCIATION BETWEEN THIOPURINE S-METHYLTRANSFERASE *3C POLYMORPHISM AND AZATHIOPRINE INDUCED MYELOSUPPRESSION IN THAI PATIENTS WITH RHEUMATOLOGIC DISEASES. THESIS ADVISOR: ASSOC.PROF. YINGYOS AVIHINGSANON, M.D. THESIS CO-ADVISOR: ASSOC.PROF. NATTIYA HIRANKARN, M.D, Ph.D., 74 pp.

Rheumatic diseases are of unknown pathogenic origin; during of the molecular and cellular processes that lead to disease pathology. Drugs used of treatment include azathioprine as immunosuppressants. Thiopurine S-methyltransferase (TPMT) is an enzyme important in drug metabolism which located in cytoplasm and genetic variation. Patients inheriting TPMT deficiency is at high risk of myelosuppression. The aim of this study was to characterize the TPMT*3C polymorphism. TPMT genotypes were determined using PCR-RFLP assays and TPMT activity RBC using HPLC method in 112 Rheumatogic patients taking azathioprine treatment. Biochemical and clinical data were retrospective. The results of genotyping analysis that found eight patients (7.14%) were heterozygous (TPMT*1/*3C) and one-hundred and four patients (92.86%) were homozygous TPMT*1 (A/A). The allele frequencies were 3.57% for “G” allele and 96.43% for “A” allele. The result show TPMT*1/*3C polymorphism are statistic significantly different ($P < 0.001$) and odd ratio 26.0, 95%CI 4.783 – 141.333 higher risk for AZA-induced leucopenia. Both patients groups taking azathioprine dose between 0.65 – 2.50 mg/kg/day that mean 1.54 ± 0.51 (SD) between azathioprine dose that result show not significantly different ($P = 0.186$). But not significantly different induced neutropenia ($P = 0.06$, odd ratio 8.889, 95%CI 1.237 – 63.878) and lymphopenia ($P = 0.085$, odd ratio 6.583, 95%CI 0.995 – 43.553). The mean of TPMT activity between TPMT*1/*1 (37.90 ± 12.64) and TPMT*1/*3C genotypes (17.53 ± 4.96) that found both groups were significantly difference ($P<0.001$). Base on the enzyme activity in RBCs which the optimal cut-off activities for TPMT activity calculated intermediate activity was ≤ 23.01 nmol 6-MTG/gHb/hr and high activity was > 23.01 nmol 6-MTG/gHb/hr with AUC. The statistical represent association between leucopenia ($P = 0.003$, odd ratio 21.5, 95%CI 2.961 – 156.128). But not significantly different induced T neutropenia ($P = 0.059$, odd ratio 11.25, 95%CI 1.233 – 102.623) and lymphopenia ($P = 0.391$, odd ratio 2.933, 95%CI 0.254 – 33.823) same as compared with genotype.

Field of Study:…Medical Science……. Student’s Signature ……...……..…….

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Co-Advisor’s Signature…….

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LIST OF ABBREVIATIONS

A	adenine base
ALL	acute lymphoblastic leukemia
AZA	Azathioprine
bp	base pair
^{o}C	degree Celsius
DNA	deoxyribonucleic acid
et al	et alii
GMPS	guanidine-5-monophosphate synthase
GST	glutathione-S-transferase
GTP	guanosine-5'-triphosphate
HGPRT	hypoxanthine guanine phosphorybosyltransferase
HLA	human leukocyte antigen
HPLC	high performance liquid chromatography
IMP	inosine 5'-monophosphate
IMPDH	inosine 5'-monophosphate dehydrogenase
Kbp	kilo Base pair
kDa	kilo Dalton
MeTIMP	methylthioinosine monophosphate
MeTITP	methylthioinosine triphosphate pyrophosphate
ml	milliliter
mM	millimolar
MP	mercaptopurine
NF-kB	nuclear factor-kB
PBS	phosphate buffer saline

PCR	polymerase chain reaction
ROC	receiver operating characteristic
rpm	revolutions per minute
SAM	S-adenosyl methionine
SLE	systemic lupus erythematosus
SNP	single nucleotide polymorphism
TG	thioguanine
TGDP	thioguanine diphosphate
TGMP	thioguanine monophosphate
TGN	thioguanine nucleotide
TIMP	thioiosine monophosphate
TPMT	Thiopurine S-methyltransferase
μl	microlitter
μg	microgram
VNTR	variable number of tandem repeats
XO	xanthine oxidase

CHAPTER I

INTRODUCTION

Background and Rationale

The explosive increase in human genetic information has influenced the field of pharmacology; bring about to study of pharmacogenetics and pharmacogenomics. Pharmacogenetics will be used in reference to the study of genetic variation underlying differential response to drugs. Pharmacogenomics refer to the systemic application of genomics to discovery of drug-response markers. (1)

Genetic markers useful in predicting treatment response or toxicity may lie in genes whose proteins are the target of the drug, are directly involved in the pathogenesis of the disease itself, or are enzymes that influence the metabolic or pharmacokinetic pathways of the drug. (1)

The studies of role of inheritance in individual variation in response to drug that avoid adverse drug reaction, drug efficacy and select patients responsive to a particular therapeutic agent. Example of genetic variations in enzymatic pathways affecting drugs toxicity in the case of alleles in the thiopurine S-methyltransferase (TPMT) gene. This enzyme is important of the clinical relevant of genetic variation in drug metabolite that metabolized the immunosuppressive drug azathiopurine (as well as mercaptopurine and thiopurine), and genetic variants in its gene predict hematologic toxicity with used of the drug (2, 3).

Azathioprine is an immunosuppressant and purine synthesis inhibitor, inhibiting the proliferation of cells, especially leukocytes. It is an effective drug used alone in certain autoimmune diseases, or in combination with other immunosuppressants in organ transplantation. Azathioprine is used in organ transplantation, autoimmune disease such as pemphigus or inflammatory bowel disease and rheumatologic disorders (4) such as rheumatoid arthritis, ankylosing spondylitis, scleroderma, gout and systemic lupus erythematosus etc. Side effects are

uncommon, but include nausea, fatigue, hair loss, and rash. Because azathioprine suppresses the bone marrow, patients will be more susceptible to infection. (5)

Thiopurine drug are pro-drugs and have to be metobolised in order to exert their cytotoxic action (6) such as 6-mercaptopurine (6-MP), 6-thioguannine (6-TG) and azathioprine. Thiopurine drugs is associated with myelosuppression in individuals who poorly metabolited.

Azathioprine is non-enzymatically and converted to 6-MP and imidazole group (7). The active metabolite of azathioprine is also a substrate for TPMT enzyme. Both 6-MP and 6-TG undergo extensive metabolism before exerting cytotoxicity following incorporation into DNA as thioguanine nucleotide (TGNs) (8) causing DNA-protein cross-links and sister chromatid exchange (9). This event has been show to be recognized by the mismatch repair (MMR) system and associated with resistance to 6-TG (6).

The main enzymes competing for the initial metabolism of 6-MP and 6-TG are hypoxanthine guanine phosphoribosyltransferase (HGPRT), thiopurine methyltranferase (TPMT), aldehyde oxidase (AO) and xanthine oxidase (XO). Both XO and AO have a little product or no cytotoxic action (8). The Thiopurine Methyltransferase (TPMT) is a cytosolic enzyme that catalyzes the S-methylation of aromatic and heterocyclic sulfhydryl compounds including thiopurine drugs (10). S-methylation is an important catabolic path way for these drugs. Methylation of thiopurine drugs by TPMT competes with active form is 6-thioguanine nucleotide (6-TGNs) metabolite which is toxic effects of these drugs and there are individual variations in human RBC TPMT activity (11).

TPMT activity are controlled by genetic polymorphism can lead to excessive drug toxicity which results of population studies and of segregation analysis of data from families indicated that two alleles at a single locus, $TPMT^{L}$ for low and $TPMT^{H}$ for high activity, were responsible for most of the variation in human RBC TPMT activity. TPMT activity has been observed in Caucasians with 1/300 individuals having complete deficiency, 10% having intermediate activity and approximately 90% having high activity (10). To date 29 SNPs in the TPMT gene associated with altered enzyme activity (12). The wild-type allele for high TPMT activity has been designated TPMT*1/*1 (13) and first SNP to be described was TPMT*2 resulting in a G238C transversion leading to Ala80Pro amino acid substitution (14, 15). The most common of these in Caucasians, TPMT*3A, represents 55 – 70% of all variant alleles for very

low TPMT activity that contains two point mutation, G460A and A719G, resulting in Ala154Thr and Tyr240Cyr amino acid substitution (15, 16). And the most common variant alleles in African-American and Asian population is TPMT*3C resulting in A719G transversion leading to Tyr240Cyr amino acid substitution that the TPMT*3C allele is expressed in humans and associated with lower immunodetectable TPMT protein and catalytic activity (17).

The current study was conducted to compare both phenotype and genotype of TPMT in healthy Han and Yao Chinese children that result present erythrocyte TPMT activity of TPMT*3C heterozygote was 12.36 U/ml RBC. The frequency of the known mutant TPMT alleles was 0.2% (1/426) in Chinese children (18). After that determine the incidence of TPMT genetic polymorphism in the Thai population show the frequency of TPMT*3C mutant allale in Thai population was 0.050 (19) Japanese patients with SLE allele frequency of TPMT mutation was 2.9% that higher than in Japanese healthy (1.1%), although it did not reach statistically significant difference ($p = 0.23$) (20). In Korean patient with SLE taking azathioprine, TPMT polymorphism was detected in 12 heterozygous for TPMT*3C allele and 5 heterozygous for the TPMT*6 allele (21). The thiopurine S-methyltransferase allelic frequencies in Taiwan aborigines and Taiwanese of TPMT*1 were 99.88% and 98.72% for Taiwan aborigines and Taiwanese respectively. The allelic frequencies of TPMT*3C were 0.12% and 1.28% for Taiwan aborigines and Taiwanese respectively. No TPMT*2, 3A, 3B, 3D and 4–8 were found in these populations (22). TPMT*3C is the most prevalent mutant allele in Asian populations; the frequency of this defective allele is significantly higher in Thai than has been reported in other Asian populations. Recently, SLE Thai patient indicated AZA-induced severe myelosuppression associated with TPMT*3C heterozygous mutant allele. The patient presented with pancytopenia, sepsis, typhlitis and disseminated intravascular coagulopathy after a short period of AZA therapy (23). Therefore, we are interested TPMT*3C polymorphism in Thais Patients with Rheumatologic disorders taking azathioprine that have approximately prevalent 9.5% of Thai population (19).

We select TPMT*3C polymorphism in Thai population because it is the most prevalent mutant allele in Thai population. The genotyping method for TPMT*3C polymorphism was identified by PCR-restriction fragment length polymorphism (PCR-RFLP) and TPMT activity was determined by HPLC method and compare wild type TPMT activity with *3C polymorphism activity in Thai patients with Rheumatologic disorder.

Research Questions

Is the expression of TPMT*3C polymorphism in patients with Rheumatologic disorder and determination of TPMT activity can predict side-effects, myelosuppression in patients treated with azathioprine?

Objectives

To investigate the association between TPMT enzyme function in red blood cells and TPMT*3C polymorphism in Thai patients with Rheumatologic disorder taking azathioprine treatment.

And to investigate the association between TPMT*3C polymorphism and AZA-induced myelosuppression in Thai patients with Rheumatologic disorder.

Hypothesis

We hypothesized that TPMT*3C polymorphism was associated with decreased TPMT enzyme function and induced myelosuppression in Rheumatologic disorder patients with azathioprine treatment.

CHAPTER II

LITERATURE REVIEW

Rheumatologic disorders

Rhumatic diseases are of unknown pathogenic origin; during of the molecular and cellular processes that lead to disease pathology. Several biochemical steps have been identified in most of systemic diseases and the involved cells have been characterized. Their treatment has been the use of general measures without specificity. Such drugs as prednisone were used in the treatment of most of the diseases to suppress the inflammatory process and active immune system.

The major concepts underlying modern genetic approaches to the common rheumatic disorders. HLA-linked genes clearly play a major role in susceptibility to autoimmune disease, yet the exact identity of the predisposing alleles remains in dispute, as does the precise mechanism responsible for these associations. In addition, the HLA associations are not of themselves sufficiently predictive of disease to warrant routine incorporation of this information into clinical practice. It is likely that further advances in this area will depend in part on identifying other genes involved in susceptibility to rheumatic diseases, as well as basic research into disease mechanisms. Therefore, genetic information will be important at many levels, from basic research to population screening and clinical evaluation of specific patients. Both the basic and clinical researcher, as well as the practicing rheumatologist will need to have an understanding of these genetic concepts in the coming decade. (1)

The experimental evidence presented above reveals immunological crossreactivities between autoantigens and viruses. The concept that autoimmunity is triggered in genetically susceptible hosts by trivial environmental factors, possibly different from patient to patient is consistent with the general epidemiology (i.e., a relatively sporadic occurrence) of the disease (1). Moreover, proteins of commonly occurring viruses have profound effects on immune responses. Thus, molecular mimicry and immunomodulation by viral proteins may potentially

account for both crossreactivity with autoantigens and abnormal T- and B-cell functions in autoimmune disorders. Therefore, continued research on viral pathogenesis is likely to provide new clues for understanding the causation of rheumatic diseases.

The humoral immune response is an integral part of the immune system. It is an incredibly complex system yielding a vast repertoire of antibodies capable of binding and eliminating the millions of foreign challenges presented to it. This huge repertoire and the antigenic challenges presented to it provide a fertile ground for the development of autoreactivity. A number of checks and balances are in effect that prevent clinical autoimmunity in the vast majority of individuals. Autoantibodies are produced and appear to play a key role in normal immunity. It is not definitively clear, but the scientific evidence at present suggests that clinical autoimmunity does not arise from expansion or changes in the "normal autoimmune repertoire". Although we have gained great insight into the makeup and control of the humoral immune response, much remains to be learned, not only about normal immunity but also the defects that allow the humoral immune response to go away when "pathogenic autoantibodies" are produced. (24)

Thiopurine drug

Thiopurine are pro-drugs and have to be metobolised in order to exert their cytotoxic action (6) such as 6-mercaptopurine (6-MP, b-thiopurine), 6-thioguannine (6-TG) and Azathioprine (6-(1-methyl-4-nitro-5-imidazolylthio)purine) act as purine antagonists [fig.1] have been used since 1950s as immunosuppressants for neoplasia and autoimmune disease as well as Leukaemia and recipient of transplanted organs (10). Thiopurine drugs is associated with myelosuppression in individuals who poorly metabolited the drug. They interfere with biochemical process involving endogenous purines, which are essential components of DNA, RNA, and co-enzymes. They have cytotoxic and immunosuppressive properties that are variously due to the inhibition of the synthesis of protein, DNA and RNA with additional effects attributable to the reaction thiol group (8).

Azathioprine was introduced later in 1963 when it was discovered that used prolong renal allograft (10). Azathioprine is non-enzymatically and converted to 6-MP and imidazole group, so the active metabolite of azathioprine is also a substrate for TPMT (Thiopurine methyltransferase) enzyme. Both 6-MP and 6-TG undergo extensive metabolism before exerting cytotoxicity following incorporation into DNA as thioguanine nucleotide (TGNs) causing single-stand breaks (SSB), double stand breaks (DSB), DNA-protein cross-link (DPC), interstand cross-links (ICL), enhanced sister chromatid exchange and overt chromosome damage . This event has been show to be recognized by the mismatch repair (MMR) system and asso ciated with resistance to 6-TG (9, 25, 26).

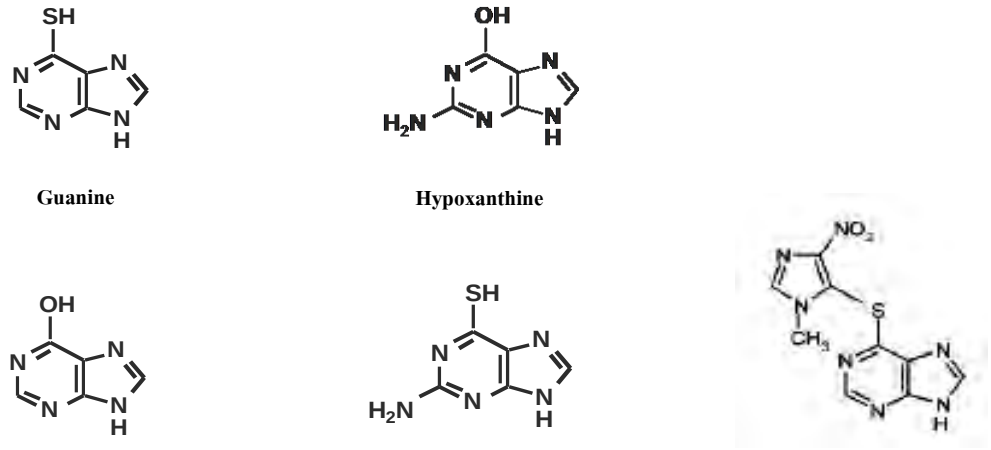

Figure 1. Structure of the thiopurine drugs. (8)

Azathioprine and Metabolism

Azathioprine is an immunosuppressant used in organ transplantation, autoimmune disease such as rheumatoid arthritis and pemphigus or inflammatory bowel disease such as Crohn's disease and ulcerative colitis. It is a pro-drug, converted in the body to the active metabolites 6-mercaptopurine and 6-thioinosinic acid. It is a purine synthesis inhibitor, inhibiting the proliferation of cells, especially leukocytes. It is an effective drug used alone in certain autoimmune diseases, or in combination with other immunosuppressants in organ transplantation. Side effects are uncommon, but include nausea, fatigue, hair loss, and rash. Because azathioprine

suppresses the bone marrow, patients will be more susceptible to infection. Caution should be exercised when it is used in conjunction with purine analogues such as allopurinol. The enzyme thiopurine S-methyltransferase (TPMT) deactivates 6-mercaptopurine. Genetic polymorphisms of TPMT can lead to excessive drug toxicity, thus assay of serum TPMT may be useful to prevent this complication (27).

The main enzymes competing for the initial metabolism of 6-MP and 6-TG are hypoxanthine guanine phosphoribosyltransferase (HGPRT), thiopurine methyltranferase (TPMT), aldehyde oxidase (AO) and xanthine oxidase (XO). Both XO and AO have a little product or no cytotoxic action (8).

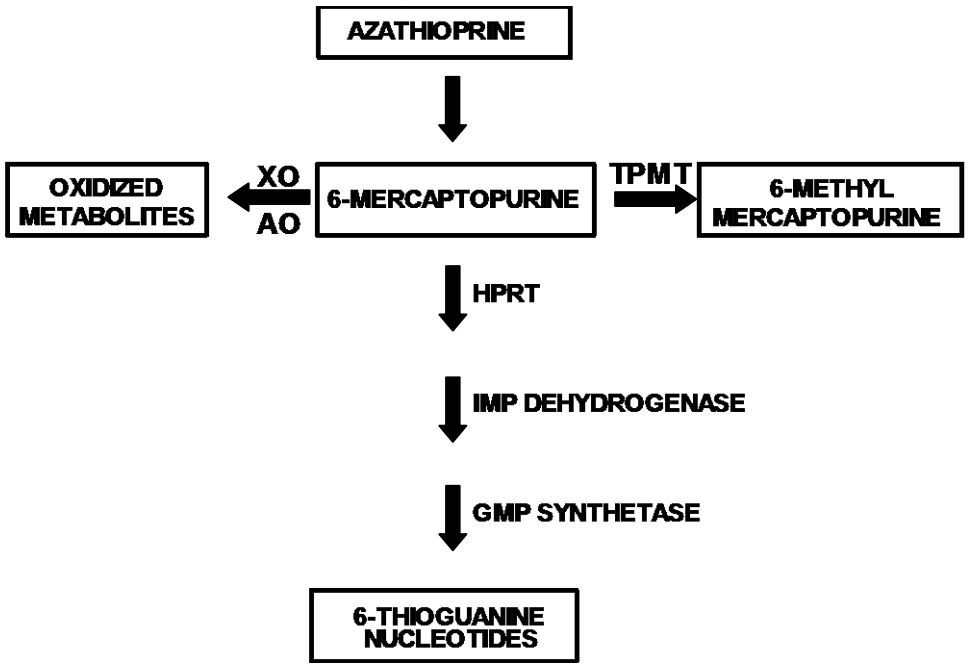

Figure 2 Azathioprine Metabolism pathways

The major metabolism in erythrocyte are 6-thioguanine nucleotide; mono-, di-, and tri-phosphates of the nucleoside 6-thioguanosine. Without prior dephosphorylation these metabolites cannot cross cell membranes.

The intestinal mucosa and liver are the primary sites for xanthine oxidase which is catabolism of mercaptopurine to the inactive metabolite 6-thiouric acid. The second metabolic pathway is thiol methylation, catalyzed by the enzyme thiopurine methyltransferase (6).

After an oral dose azathioprine is rapidly cleaved by sulphydryl- and amino-containing compounds to mercaptopurine and methyl-4-nitro-5-imidazole derivatives. The breakdown of azatioprine to 6-mercaptopurine is nonenzymeatic. After metabolite by HGPRT and PRPP, 6-TG froms thioguanine monophosphate (TGMP), this is metabolite to from di- and tri-phosphate TGNs, which become into DNA and RNA. The metabolite of 6-MP to TGMP is involving two additional enzyme: inosine monophosphate dehydrogenase (IMPDH) and guanosine monophosphate synthesis (GMPS). The 6-thioguanosine 5'-monophosphate (TIMP) is first intermediate in this pathway and act a substrate for thiopurine methyltransferase (TPMT) leading to the production of S-methyl-thioinosine 5'-monophosphate (MeTIMP) which is a strong inhibitor of de novo purine synthesis (DPNS). Increase in TPMT activity was associated with increased levels of MeTIPM and decrease DNPS inhibition. (6)

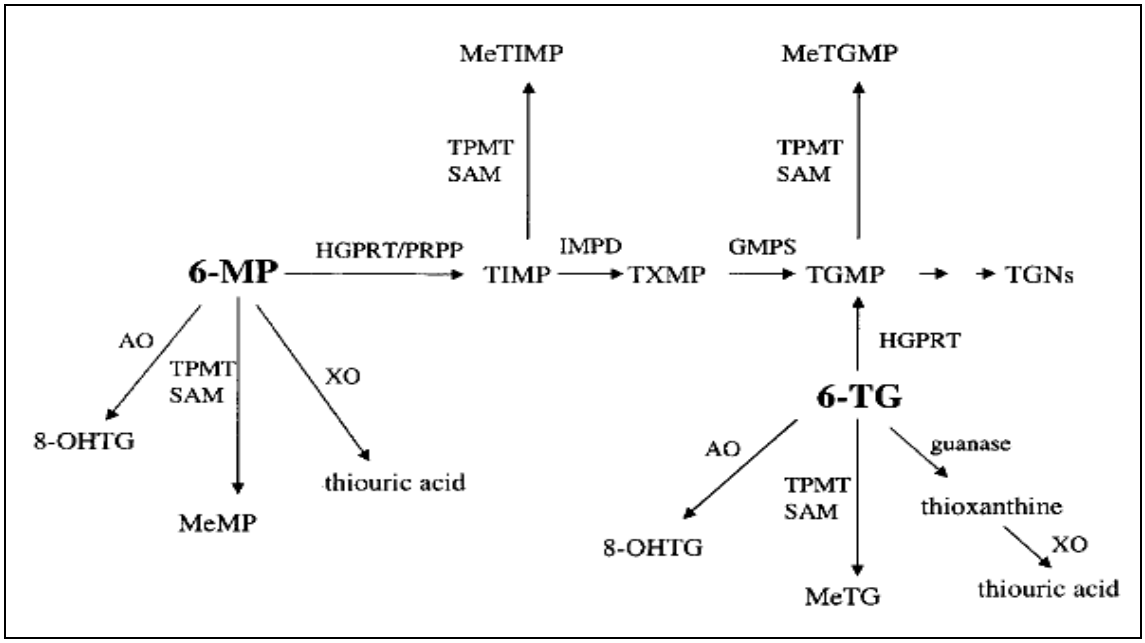

Figure 3 Metabolism of 6-MP and 6-TG in human ALL cells. PRPP, 5'-phosphoribosyl-1-pyrophosphate; GMPS, guanosine monophosphate synthase; HGPRT, hypoxanthine guanine phosphoribosyltransferase; IMPD, inosine monophosphate dehydrogenase; SAM, S-adenosine-L-methionine; TPMT, thiopurine methyltranferase; AO, aldehyde oxidase; 8-OHTG, 8-hydroxythioguanine; XO, xanthine oxidase; TGN, thioguanine nucleptide; TIMP, thioinosine 5'-monophosphate; TXMP, thioxanthine monophosphate; TGMP, thioguanosine monophosphate; MeTG, methylthioguanine; MeMP, methylmercaptopurine. (8)

Thiopurine S-methyltransferase (TPMT)

One major factor influencing nucleotide production after an oral dose for mercaptopurine or azathaioprine is the inherited activity of TPMT.

The Thiopurine Methyltransferase (TPMT) is a cytosolic enzyme that catalyzes the S-methylation of aromatic and heterocyclic sulfhydryl compounds including thiopurine drugs. S-methylation is an important catabolic path way for these drugs. Methylation of thiopurine drugs by TPMT competes with active form is 6-thioguanine nucleotide (6-TGNs) metabolite which is toxic effects of these drugs. TPMT activity is present in lysates of human red blood cells (RBC) and there are individual variations in human RBC TPMT activity (11). TPMT activity are controlled by genetic polymorphism which results of population studies and segregation analysis

of data from families indicated that two alleles at a single locus, $TPMT^{L}$ for low and $TPMT^{H}$ for high activity, were responsible for most of the variation in human RBC TPMT activity (11).

TPMT gene and toxicity

Leucopenia and hepatotoxicity are included among the serious effects of 6-MP. It has not been possible to predict individual variations either in the therapeutic response of patients to thiopurines or in the occurrence of side effects. The factor involved in differences in response to thiopurines might be individual variations in drug metabolism. There are many examples of significant variations in drug toxicity that are due to individual differences in drug metabolism. TPMT activity in rodent liver and kidney has been studies in 1963 but has not been characterized in human tissue. Until 1978, Studies to measure TPMT activity from human tissue and human RBC by radiochemical assay. In 1980, there were roles of inheritance in the regulation of human RBC TPMT activity, because it is easy. The level of human RBC TPMT activity is inherited and is mainly to a pair of alleles at a single locus. Approximately 88.6% (89%) of a randomly selected population is homozygous for an allele for high activity, about 11.1% (11%) is heterozygous and has intermediate activity and about 0.3% is homozygous for low activity. Subsequently, using classical segregation analysis of 213 individuals in 49 families they predicted that 66% of the total variance in TPMT controlled by a pair of alleles at a single locus, $TPMT^{H}$ for high activity and $TPMT^{L}$ for low activity. The gene frequencies of $TPMT^{L}$ and $TPMT^{H}$ are approximately 0.06 and 0.94, respectively in fig 5 (10). That results refer to inherited variation in S-methylation might represent one factor involved in individual differences in the clinical response or in the occurrence of serious side effects to thiopurines.

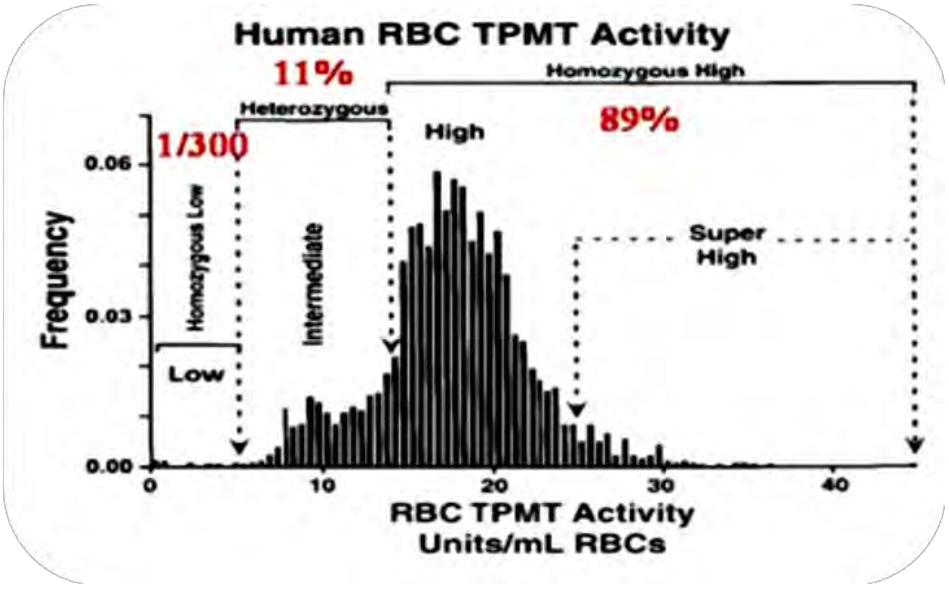

Figure 4 Human RBC TPMT activities by radiochemical assay. (10)

In 1982, Van Loon and Weinshilboum were studied genetically regulated level of TPMT activity in the RBC reflects the level of the enzyme activity in nucleated human cells as lymphocytes are nucleated cell type that can be obtained from large numbers of subjects. The result presented level of lymphocyte and platelet TPMT activity were regulated in parallel with the genetically determined level of activity in the RBC. The high degree of correlation among lymphocyte, platelet and RBC TPMT activity is compatible (11). After that Woodson and working group were determined genetic polymorphism regulating human RBC TPMT activity reflects variations in the enzyme activity in kidney because the kidney represents a potential target organ when thiopurine drugs such as azathioprine and 6-mercaptopurine are used clinically in the treatment of renal transplantation patients and of patients with diseases such as glomerulonephritis. Kidney TPMT enzymatic activity was determined by radiochemical assay. The results were compatible with the conclusion that the genetic polymorphism that regulates TPMT activity in the human erythrocyte plays a major role in the regulation of the enzyme activity in other human cells and tissue (28).

In 1986, Otterness and Weinshilboum (29) introduced an animal model for the studies of the biochemical basis of the genetic regulation of TPMT activity and toxicological experiment. Breeding experiments were studied by C57BL/6J (B6), AKR/J (AK) and DBA/2J

(D2) mice that mice stains B6 and AK for low TPMT activity and D2 for high TPMT activity that reported previously in 1985. TPMT activities were determined in liver and kidney tissue. The result presented TPMT activity in livers and kidneys of F1 and F2 mice from D2 x AK and D2 x B6 mating. TPMT activities in livers and kidney of F1 animals were also intermediate activity and F2 animals were included subgroups with low activity and subgroup with high activity that results were compatible with the autusomal monogenic inheritance of level of TPMT activity in livers and kidneys of these mice.

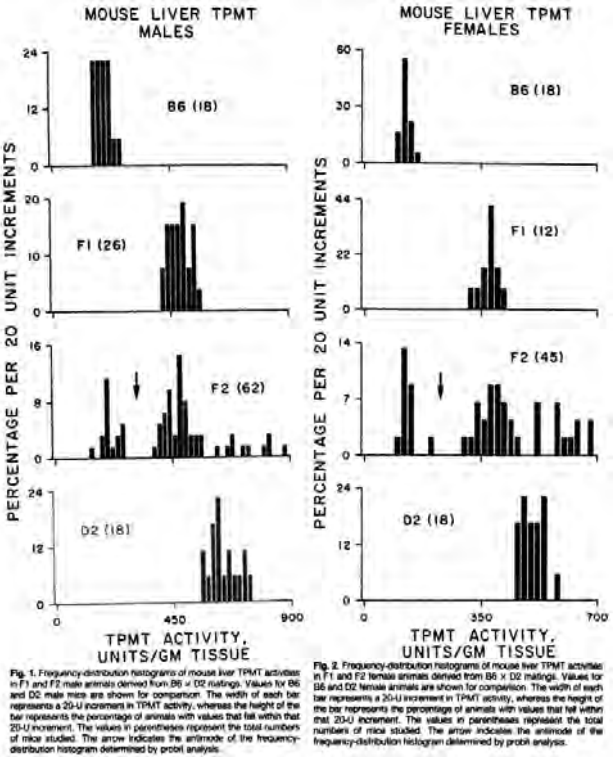

Fig. 1. Frequency-distribution histograms of mouse liver TPMT activities in F1 and F2 male animals derived from B6 × D2 matings. Values for B6 and D2 male mice are shown for comparison. The width of each bar represents a 20-U increment in TPMT activity, whereas the height of the bar represents the percentage of animals with values that fell within that 20-U increment. The values in parentheses represent the total numbers of mice studied. The arrow indicates the antimode of the frequency-distribution histogram determined by probit analysis.

Fig. 2. Frequency-distribution histograms of mouse liver TPMT activities in F1 and F2 female animals derived from B6 × D2 matings. Values for B6 and D2 female animals are shown for comparison. The width of each bar represents a 20-U increment in TPMT activity, whereas the height of the bar represents the percentage of animals with values that fell within that 20-U increment. The values in parentheses represent the total numbers of mice studied. The arrow indicates the antimode of the frequency-distribution histogram determined by probit analysis.

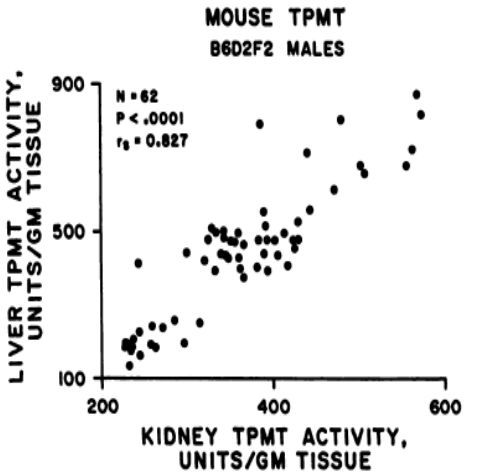

Fig. 4. Correlation of liver with kidney TPMT activities in F2 male animals derived from B6 × D2 matings. Each value is the average of three determinations.

Figure 5 Role of inheritance in the regulation of TPMT activities in liver and kidney tissue from B6, AK and D2 mice. (29)

TPMT gene

The TPMT gene has been localized to chromosome 6p22.3 and encodes a 245 amino acid protein with a predicted molecular mass of 35 kD. A processed pseudogene of TPMT has also been described with 96% homologous to the TPMT gene and is located on chromosome 18q21.1. Originally the TPMT gene was reported to be approximately 34 Kb in length made up of

10 exons 9 introns. Later this was modified in a reported the length to be 25 Kb with minor sequence difference including a 5 Kb difference in intron eight and an extra 17 nucleotides in the promoter region. However, reported the gene to be 27 Kb long and did find evidence of exon two. Within the 5' promoter region there is 71% GC content (from -873 to +736) and no consensus sequence for TATA box or CCAAT element but several binding Sp1, NF-κ , AP-2 and KROX-24. Variable number tandem repeats (VNTRs) have been identified in the 5'UTR and several studies have been carried out to elucidate the importance of their VNTRs. To date no strong link between the number of VNTRs and TPMT activity has been reported (8).

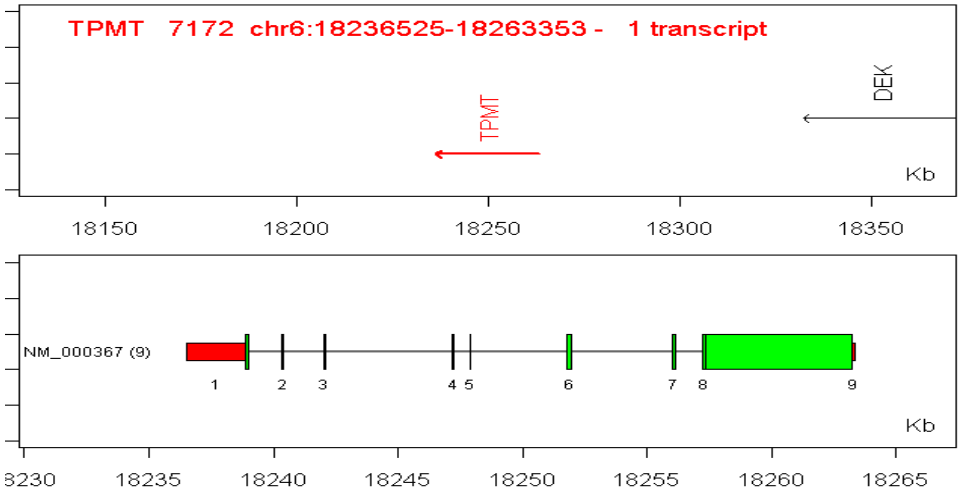

Figure 6 TPMT gene on chromosome 6p22.3 consists of 10 exons 9 introns.

TPMT Polymorphisms

Thiopurine methyltranferase (TPMT) activity in human RBC is regulated by inheritance. Approximately 90% are homologous for high enzyme activity, about 10% are heterozygous for intermediate activity and one in 300 subjects is homologous for low activity (10). Later, the study found single nucleotide polymorphisms (SNPs). Some of polymorphisms result in a loss of activity. The wild-type allele for high TPMT activity has been designated TPMT*1/*1 and first SNP to be described was TPMT*2 resulting in a G238C transversion leading to Ala80Pro amino acid substitution (14, 15). The following year TPMT*3A was result of two SNPs in exon 7; G460A leading to Ala154Thr and exon 10; A719G leading to Try240Cyr

(16). In 1996 Szumlanski et al. used site directed mutation to create expression containing the G460A (TPMT*3B) and the A719G (TPMT*3C) (16). TPMT*3A is the most prevalent deficiency-associated allele in Caucasians population and TPMT*3C is reported in Asian and African population (17). Later, the study found a new variant allele, TPMT*4 that contains a G$\rightarrow$A transition that disrupts the intron/exon acceptor splice junction at the final 3′ nucleotide of intron 9, the terminal intron of the TPMT gene (21). In addition TPMT*6 (A539T trasversion: Tyr180Phe) was reported in Korean and Japanese patients who have low TPMT activity (21).

In 1999, TPMT genotype was determined by using peripheral blood cell DNA obtained from 36 Japanese patients with rheumatic diseases. Study used polymerase chain reaction (PCR) technique that result showed TPMT*1/TPMT*3C in 3 (8.3%) individuals. All 3 patients (100%) with the mutant TPMTallele (TPMT*3C) discontinued AZAtreatment due to leucopenia while only 4 patients (12%) without mutant TPMTalleles showed leucopenia (p=0.0049, Fisher's exact test) (17). After that in 2005, to study the genotype status of TPMT in a group of Japanese SLE patients who may undergo a therapy of AZA, a substrate drug, toward TPMT The patient had low TPMT activity and TPMT*3C genotype and presented with pancytopenia, sepsis, typhlitis and disseminated intravascular coagulopathy after a short period of AZA therapy (20).

In 2004, to determine the incidence of TPMT genetic polymorphism in 200 Thai population by PCR-RFLP that result show TPMT*3C is the most prevalent mutant allele in Thai populations than has been reported in other Asian populations (19).

Table 1 Comparative allele frequencies of TPMT in various populations

Populations	N	Allele frequency *1	*2	*3A	*3C	*6	References
Thai	400	0.950 (0.924–0.969)	0	0	0.050 (0.031–0.076)	0	Present study
Chinese	384	0.977*	0	0	0.023*	ND	[14]
Japanese	1044	0.984**	0	0	0.016**	ND	[15]
Japanese	142	0.979**	0	0	0.014	0.007	[12]
Taiwanese	498	0.994**	0	0	0.006**	0	[17]
South-west Asians	198	0.990**	0	0.010*	0*	ND	[14]
South-east Asians	600	0.990*	0	0	0.010*	0	[17]
Kenyans	202	0.946	0	0	0.054	ND	[25]
Ghanaians	434	0.924	0	0	0.076	ND	[26]
American Caucasians	564	0.964	0.002	0.032*	0.002**	ND	[22]
British Caucasians	398	0.947	0.005	0.045*	0.003**	ND	[26]

N represents number of alleles detected. Data in parentheses represent 95% CI. ND, not detected. Different from Thai population (P < 0.05 and ** P < 0.005).*

In 2007, Gisbert, et.al. (30) studied the association between several clinical variables and TPMT activity was assessed by multiple linear regression and included 14,545 spain patients: autoimmune hepatitis (n=359 patients), inflammatory bowel disease (n=7,046), multiple sclerosis (n = 814), myasthenia gravis (n=344), pemphigus (n=133), and other diseases (n=5,849) that 0.5% had low TPMT activity, indicating a higher risk of myelotoxicity

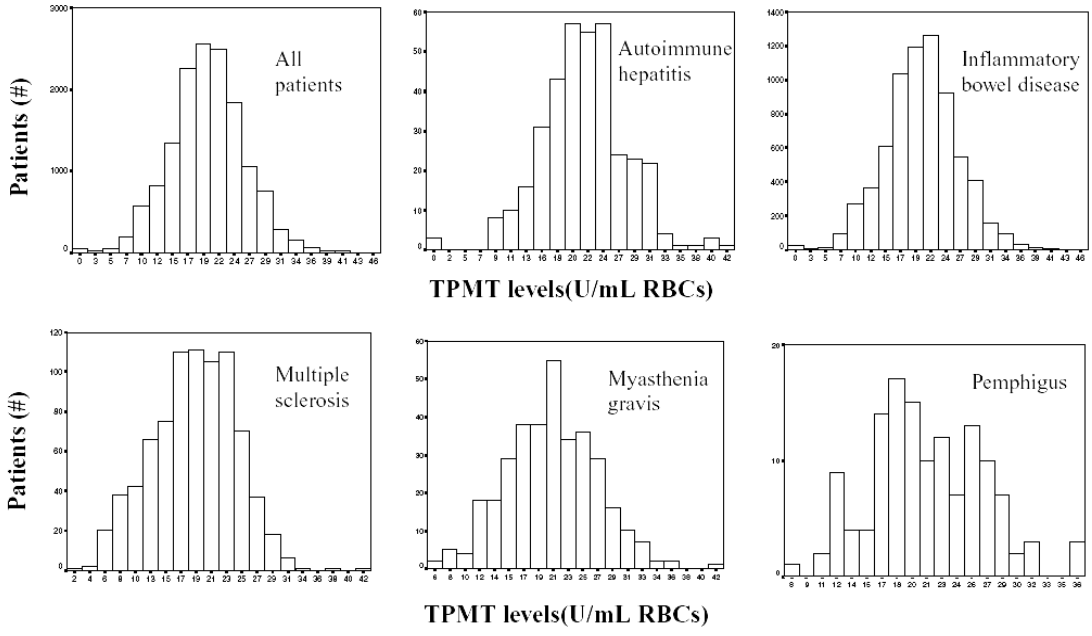

Figure 7 Distribution of thiopurine methyltransferase (TPMT) activity in the spain population study, RBCs red blood cells (30)

In 2008, the heterozygous TPMT*1/*3C genotype was found in 9 of the 139 kidney transplant recipients (6.47%) (95% CI, 3.00–11.94). The TPMT activity of those patients was significantly lower than that of patients with the homozygous wild-type genotype and risk for azathioprine-induced myelosuppression in the patients with the heterozygous TPMT*1/*3C genotype was significantly higher than that in patients with the wild-type genotype (adjusted OR, 14.18 [95% CI, 3.07–65.40]; $P < 0.005$).

After characterization of the TPMT cDNA and identification of several TPMT single nucleotide polymorphism (SNP) genotyping has been used to help individualize thiopurine drug therapy. To date as many as 24 SNPs in the TPMT gene associated with decreased enzyme activity have been identified (TPMT*2 to TPMT*25; Fig 8). Although systemic analysis of TPMT phenotype-genotype correlation in healthy individual, as well as in patients with thiopurine related side effects has lead to the identification of novel variants.

Other reported SNPs are rare and only reported in one individual. Seki et al. 2000 (31) have also described SNPs in the introns of the TPMT gene. In the study population of 48 Japanese and identified 30 SNPs in the non-coding regions that were detected one SNP in 870 bp promoter region, 26 SNPs in the intronic regions and 3 SNPs in the $3'$ untranslated region. It also found other characteristic such as deletion of exon six and nine and anomalously spliced RNA which resulted in intermediate TPMT activity and polymorphisms of the VNTR regions.

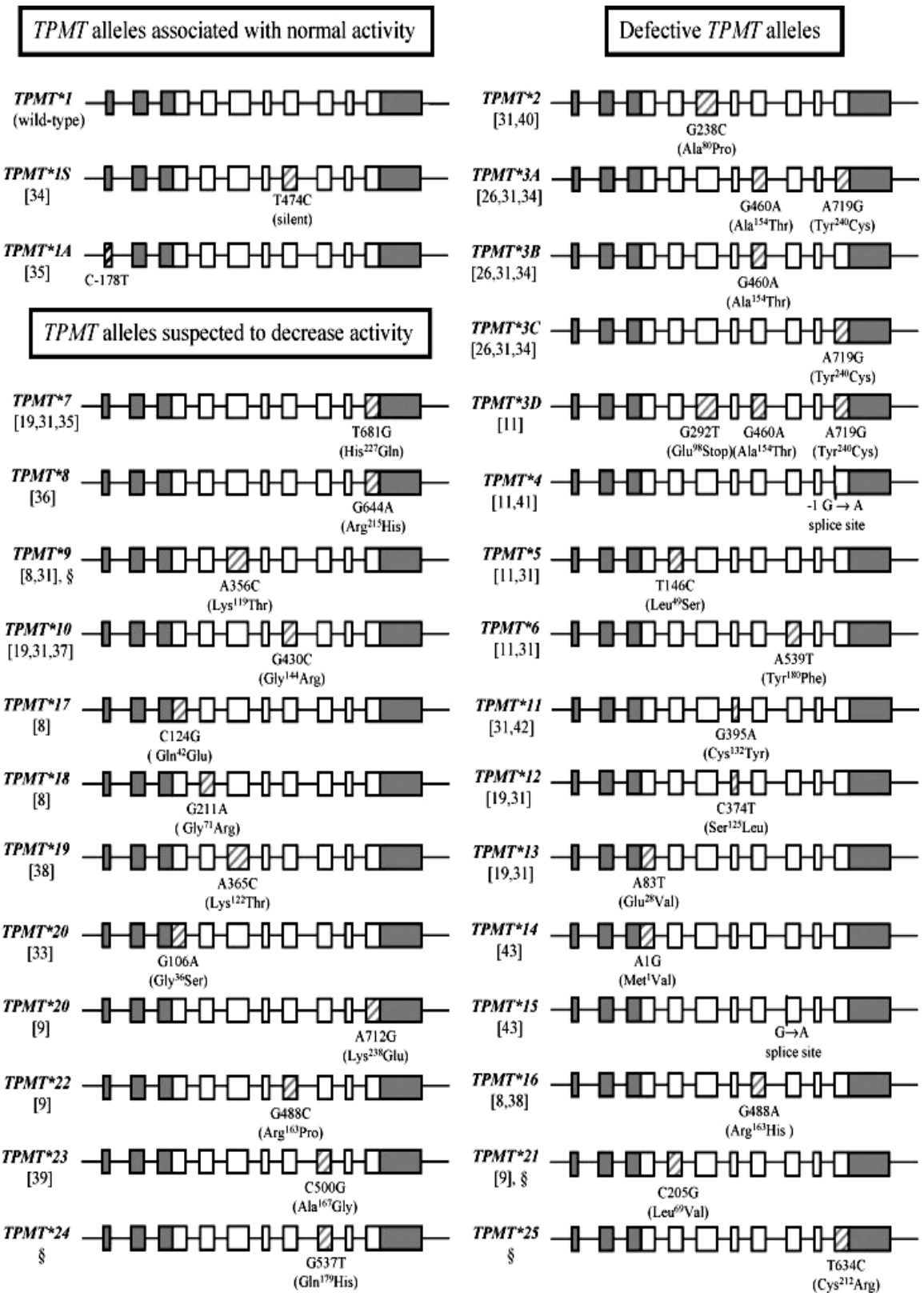

Figure 8 Summary of presently know allelic variations of the TPMT gene.

Adverse effects

Azathioprine is used as an immunosuppressant in preventing the rejection of organ and tissue transplants and in a variety of conditions which are considered to be autoimmune in character, e.g. lupus erythematosus, rheumatoid arthritis, chronic active hepatitis, ulcerative colitis, Crohn's disease, and pemphigus vulgaris.

Clinical trials in which azathioprine were significantly more common among treated patients compared with control patients. These included leucopenia, diarrhoea and vomiting, abnormal liver function, and skin rashes. The major adverse effect of treatment is bone-marrow depression. However, in immunosuppressive regimens morbidity due to haemopoietic suppression remains a problem (24).

Drug interaction

The therapeutic efficacy and cytotoxicity of mercaptopurine may be altered by many factors which can act at any point, from taking the drug and absorbing it to active metabolism formation and incorporation into DNA (6).

Allopurinol alters the pharmacokinetics of oral mercaptopurine by inhibition of its xanthine oxidase-mediated first-pass metabolism. Allopurinol pretreatment caused a 500% increase in both the peak plasma concentration and AUC of mercaptopurine, but did not effect the plasma kinetics of low dose i.v. mercaptopurine.

Mercaptopurine is an antimetabolite and endogenous purines can potentially modulate thiopurine toxicity.

Phosphoribosylpyrophosphate (PRPP) is a co factor in the HPRT reaction. Intercellular PRPP concentrations could potentially regulate cytotoxic metabolite formation. Increased PRPP formations stimulated nucleotide formation from azathioprine, mercaptopurine and thioguanine in human erythrocytes.

Methotrexate increases intercellutar PRPP concentration which increases nucleotide metabolite formation from mercaptopurine. Methotrexate can inhibit xanthine oxidase [R] and increase mercaptopurine concentration and promote the formation of active metabolites from mercaptopurine by inhibiting purine synthesis.

CHAPTER III

MATERIAL AND METHOD

Subjects

One hundred and twelve Thai patients with Rheumatologic diseases at King Chulalongkorn Memorial hospital and Nopparatrajchatanee hospital were included in this study. The ethics committee of the faculty of Medicine, Chulalongkorn University, Bangkok, Thailand approved the study and the subjects gave their informed consent.

Inclusion criteria

- Patients much be rheumatologic disease
- Thais citizen
- Much be treated with azathioprine drug at least 15 days

Exclusion criteria

- Patients with a bone marrow disease
- Missing clinical data

Clinical data information for myelosuppression

- WBC < 3,000 cells/mm^3 (Leucopenia) or
- Platelets < 100,000 /mm^3 (Thombocytopenia) or
- Neutrophil < 1,500 cells/mm^3 (Neutropenia) or
- Lymphocyte < 600 cells/mm^3 (Lymphopenia)

Determination of TPMT Activity

Chemical and samples

The substrates S-adenosyl-L-methionine (SAM, Sigma Chemical Co; St. Louis, MO, USA) and 2-amino-6-methyl mercaptopurine (6-MTG, Sigma Chemical Co; St. Louis, MO, USA) and 6-thioguanine (6-TG, GlaxoSmithKline UK; Stockley Park, Middlesex, UK)

A stock standard solution of 6-MTG was made by dissolving 10 mg of 6-MTG in 1 ml of 1 M NaOH and then diluting with distilled water to a final concentration 1 µg/ml. The solution was aliquot 300 µl per tube and store at -20 °C.

A stock solution of 6-TG was made by dissolving 14 mg of 6-TG in 1 ml of 1 M NaOH and then diluting with distilled water to a final concentration 3.5 mg/ml. The solution was centrifuged at 2500 rpm or 1400 × g for 5min. Supernatant was aliquot 1 ml per tube and store at -80 °C.

S-adenosyl-L-methionine (SAM) 16.8 mg was dissolved in 0.1 M phosphate buffer to a final concentration of 0.084 mg/ml. This solution was aliquot 6 ml per tube and store at -20 ^{O}C.

The substrate mixture comprised final concentration of 400 µl 6-TG and 5ml SAM in 10 ml of 0.1 M potassium phosphate buffer (pH 7.4). 0.5 ml of substrate mixture was pipette into incubation tube, which were capped and stored at -80 ^{O}C for up to 1 month piror to use.

The level of TPMT activity expected in a population, an addition 3 ml EDTA tube was collected from rheumatism patients for routine blood tests. Ethics committee approval was given and informed consent was obtained from patients who entered the study. To help with long-term quality assurance studies, blood samples were taken from volunteers. The lysates produced were aliquot, store at -80 ^{O}C and run on each assay as a quality assurance sample.

Samples collection, storage and RBC lysate preparation

Whole blood samples from volunteer were collected in 3 ml EDTA tubes and stored at 4 °C before analysis. The blood samples were centrifuged at 1400 × g for 5 min. The buffy coat was collected at -20 °C. 1.5 ml of Physiological saline added to wash the RBC. Samples were mixed vigorously for 5 min and centrifuged at 1400 × g for 5 min and the saline wash removed. The washed erythrocytes 0.5 ml was suspended in 2 ml of 0.02 mM phosphate

buffer, pH 7.4 and mixed for 30 seconds to lyses the cells. The lysate produced were aliquot 500 µl per tube and were kept frozen at -80 °C until the day of analysis. After thawing, the lysates was used to determine TPMT activity and the haemoglobin content (Hb).

Incubation conditions and HPLC analysis

Added 200 µl of RBC lysate and 500 µl of substrate mixture into 1.5 microtube were mixed and incubated in water bath at 37 °C for exactly 1 hr then the reaction was stopped by incubated for 10 min at 85 °C. Samples were cold on ice and centrifuged at 1400 × g for 5 min. The supernatant 50 µl were transferred to vial tube for analyzed by HPLC.

Standardization and blank

The standard curve was shown to be linear the range 0 – 3.3 nmol/ml. A stock standard was prepared with 1 mg/µl in 0.1 M NaOH, which was the dilute to give the working standard.

A standard blank was prepared by adding 200 µl NaCl to incubate. A sample blank was prepared by adding 200 µl of RBC lysate and then stopping the reaction at time zero.

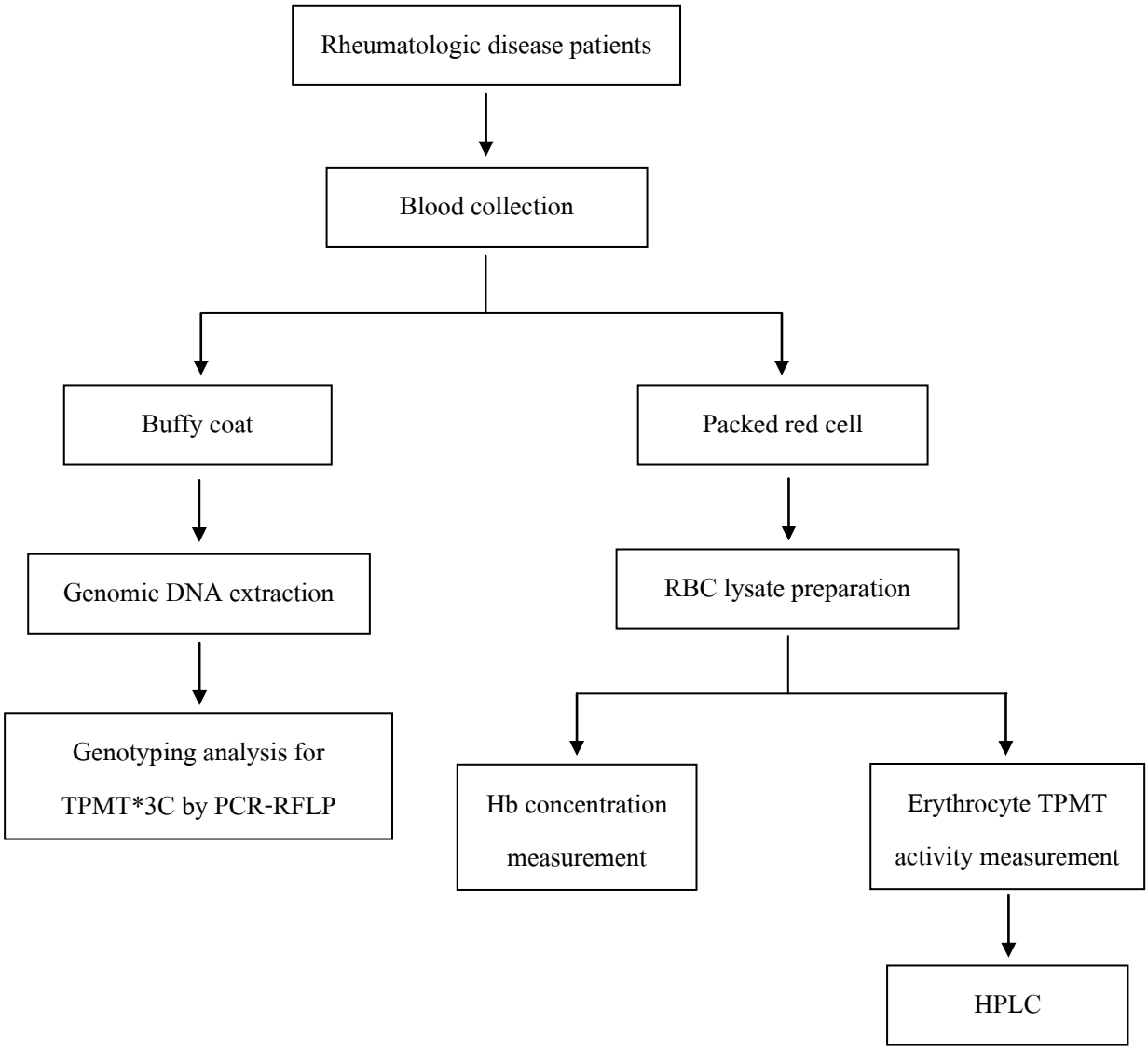

Figure 9 TPMT analysis protocol summery

Analysis of TPMT gene *3C polymorphism

DNA extraction

DNA was isolated from buffy coat collected with ethylenediaminetetraacetic acid (EDTA) and genomic DNA was extracted using a salting out method [R]. 1 ml of red cell lysis buffer (RCLB) was added to 0.5 ml of buffy coat, vortex for 30 seconds. This solution was centrifuged at 10,000 – 12,000 rpm for 30 second and the supernatant was discarded to obtain the pellet. Next step, add 200 µl of nuclei lysis buffer (NLB) and 50 µl of 10% SDS into pellet.

Pellet was broken up with pipette tip and vortex. The solution, 150 µl of NLB and 10 µl of proteinase K (10 mg/ml in H_2O stored frozen) were added, followed by incubation at 65^{O}C for 2 hours. Precipitation of proteins was obtained by adding 175 µl of 5.3 M NaCl and centrifuged at 10,000 – 12,000 rpm for 15 minutes in micro centrifuge. After centrifuge, the DNA in the supernatant was precipitated in 1 ml of cold absolute ethanol and invert tube 6 – 10 times to precipitate DNA, it will appear DNA pellet in ethanol solution. This solution was centrifuged at 10,000 – 12,000 rpm for 10 minutes and the supernatant was discarded to obtain the pellet. This pellet was washed in 1 ml of cold 70% ethanol, followed by centrifugation 1 – 2 minutes at 10,000 – 12,000 rpm. The supernatant was discarded to obtain the pellet and dried at 37 ^{O}C. The DNA pellet was dissolved in 200 µl of sterile distilled water and store at 4 ^{O}C.

Polymerase Chain Reaction-Restriction Fragment Length Polymorphism analysis of TPMT gene *3C polymorphism

We performed the Polymerase Chain Reaction-Restriction Fragment Length Polymorphism (PCR-RFLP) analysis were used to detect the TPMT*3C polymorphism (A719G) at exon 10. The genomic DNA of 113 Rheumatologic diseases were amplified with TPMT*3C gene specific primers to detect the A719G mutation.

Polymerase Chain Reaction of TPMT gene *3C polymorphism

A PCR assay using primers

P719R (5′-TGTTGGGATTACAGGTGTGAGCCAC-3′) and

P719F (5′-CAGGCTTTAGCATAATTTTCAATTCCTC-3′)

The final reaction volume for the amplification was 20 µl, containing 40 ng of blood genomic DNA as a template, PCR was done with primers P719R and P719F. The buffer contained 0.1 µl of FastTaq polymerase, 1X PCR buffer, 1X DMSO, 3mM $MgCl_2$, 0.2mM dNTP and 0.08 µM of each oligonucleotide primer. PCR was carried out using Eppendrop under specific PCR condition, that amplification was done for 35 cycles consisting of initiation denaturation at 95 °C for 5 minutes, followed by denaturation at 95 °C for 45 seconds, annealing at 60 °C for 45 seconds and extension at 72 °C for 1 minute and final extension at 72 °C for

10 minutes. The PCR products were digested with Acc I (New England Biolabs) for 2 hours at 37 °C. The resulting products were analyzed by electrophoresis in 2% tris-acetate agarose gel containing 50 µg/ml ethidiumbromide, in tris-acetate buffer at 100 volts for 40 minutes and visualized under UV light by camera Gel Doc™ MZL (BIO-LAD, USA). Nagative controls without DNA template were incubated in each experiment. A molecular ladder of 100-bp (Promaga Madison WI, USA) was used to estimate the size of the PCR fragment.

Detection of A719G by AccI restriction enzyme

PCR product size is 293 bp fragment. 10 µl of amplified DNA were digested with 5 U of specific restriction enzyme AccI (BioLabs), in 1X NEB buffer 4 in a total volume of 15 µl at 37 °C for 1-3 hours and heat inactivated at 80 °C for 15 min, followed by 3% agarose gel electrophoresis at 100 volts for 40 min. The amplified fragment was cut the 293 bp PCR product into two fragments; 207 and 86 base pairs. Wild-type (TPMT*1/*1) DNA yields an uncleaved fragment of 293 base pairs. The PCR products were detected under UV light by camera Gel Doc™ MZL (BIO-LAD, USA). Nagative controls without PCR product were incubated in each experiment. A molecular ladder of 100-bp (Promaga Madison WI, USA) was used to estimate the size of the PCR fragment.

AccI restriction site

5'…G T M K A C…3'

3'…C A K M T G…5'

M; adenine (A) or cytosine (C)

K; guanine (G) or thymine (T)

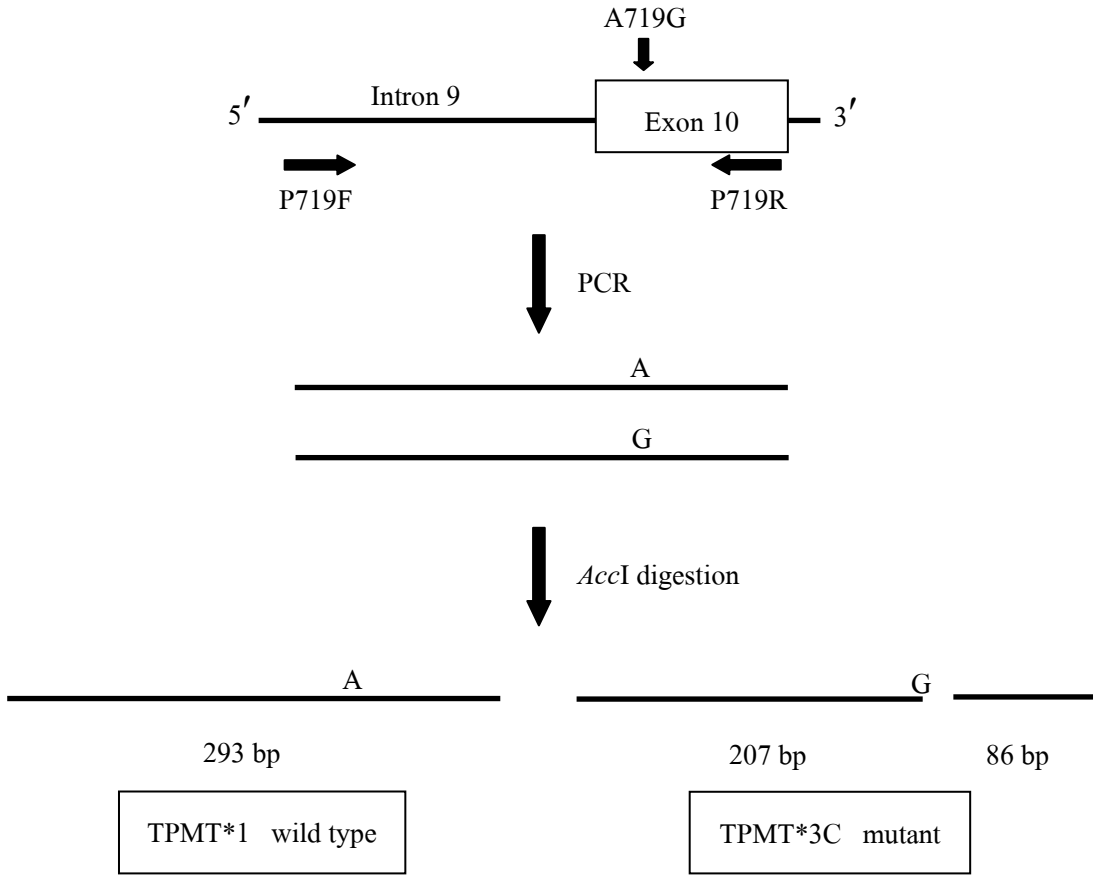

Figure 10 Polymorphism chain reaction-base methods to detect A719G mutation at human thiopurine S-methyltransferase (TPMT) gene locus.

Statistical Analysis

The statistical significance of the difference was tested by chi-square (χ^2) method. Fisher's exact test was applied if the expected frequency was less than 5. A p value of < 0.05 was considered to be significant. Odd ratio (OR) with 95% confidence interval (CI) were calculated using the statistical program (SPSS).

CHAPTER IV

RESULTS

1. Characteristic of patients with Rheumatologic disease

One hundred and twelve Thai patients with Rheumatologic disease taking azathioprine treatment attending at King Chulalongkorn Memorial hospital and Nopparatrajchatanee hospital were included in this study. The mean age was 38.45 ± 13.92 (SD) years, and 99.10% were women. The mean weight was 38.45 ± 13.92 (SD) kilograms and received azathioprine that mean was 1.51 ± 0.52 (SD) mg/kg/day. Azathioprine treatment attending at King Chulalongkorn Memorial hospital and Nopparatrajchatanee hospital were not difference (P = 0.357). Demographic data of subjects was summarized in table 2.

Table 2 Characteristic of Thai patients with Rheumatologic disease.

Demographic of subjects	**King Chulalongkorn Memorial hospital**	**Nopparatrajchatanee hospital**	**Total**
Gender (males/females)	-/40	1/71	1/112
Age (Mean ± SD, range; yr)	34.41 ± 10.96 (20 – 65)	40.66 ± 14.94 (13 – 76)	38.45 ± 13.92 (13 – 76)
Weight (Mean ± SD, range; kg)	55.59 ± 9.09 (43.6 – 84.2)	54.73 ± 10.01 (40 – 77)	55.05 ± 9.62 (40 – 84)
Dose(mg) /kg/day (Mean ± SD, range; mg)	1.47 ± 0.46 (0.77 – 2.29)	1.53 ± 0.55[ns] (0.65 – 2.5)	1.51 ± 0.52 (0.65 – 2.5)
Dose(mg) /kg/day (Median, mg)	1.53	1.56	1.78

Range; minimum - maximum

ns; not statical significantly, Fisher's Exact test (P < 0.05)

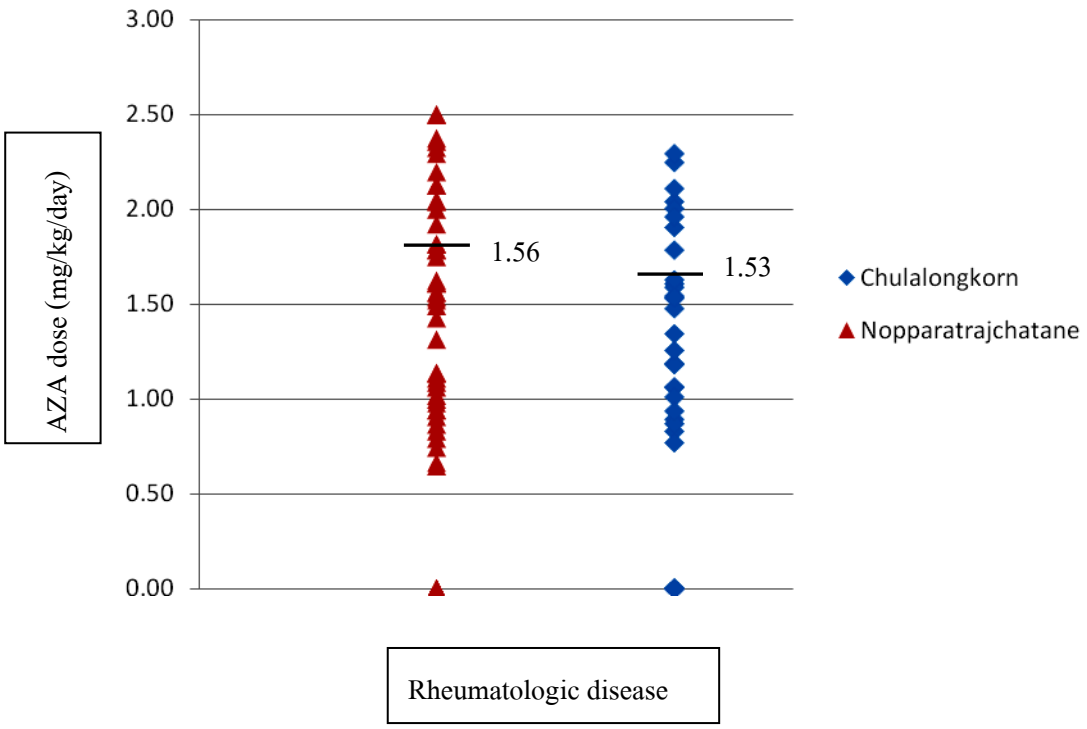

Figure 11 Azathioprine dose (mg/kg/day) for Thai patients with Rheumatologic disease taking azathioprine treatment attending at King Chulalongkorn Memorial hospital and Nopparatrajchatanee hospital

2. Polymerase Chain Reaction-Restriction Fragment Length Polymorphism analysis of TPMT *3C polymorphism

Polymorphism at 719A/G in the exon 10 region of TPMT gene were identified by the PCR-RFLP method. If a “G” present at this position, the AccI restriction enzyme would cut the 293 bp PCR product into two fragment; 207 and 86 bp. No digestion would occur if a “A” was present. (Figure 12)

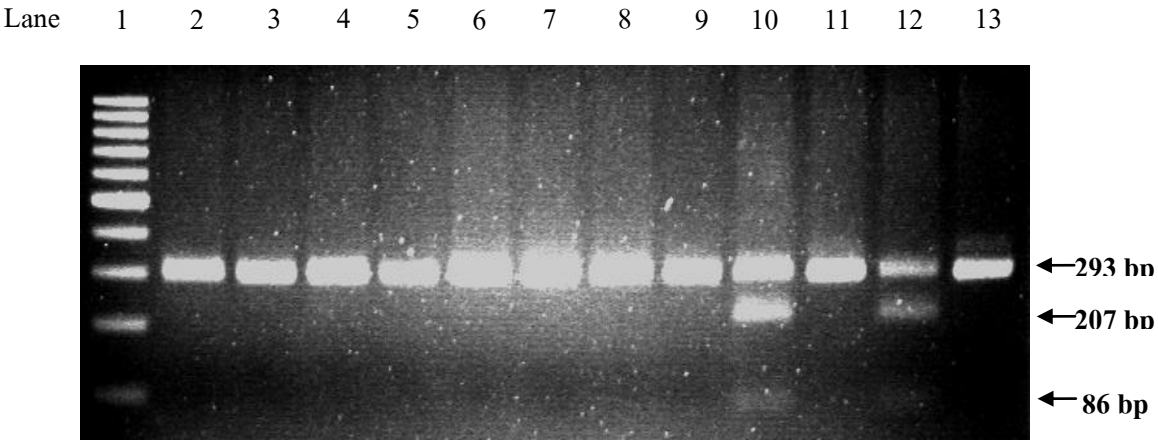

Figure 12 The representative of PCR-RFLP results from samples with homozygous of 719A and heterozygous 719G. Lane 1 is 100 bp molecular markers. Lane 10 and 12 are heterozygous of 719A/G. Lane 2,3,4,5,6,7,8,9,11 and 13 are homozygous of 719A.

The results of genotyping analysis were performed on 112 Thai patients with Rheumotologic diseases. This study, xero of 112 pateints were homozygous for the G/G genotype (TPMT*3C/*3C), eight patients (7.14%) were heterozygous of the A/G genotype (TPMT*1/*3C) and one-hundred and four patients (92.86%) were homozygous for the A/A genotype (TPMT*1/*1). The allele frequencies were 3.57% for “G” allele and 96.43% for “A” allele.

Table 3 Genotype and allele frequency for TPMT*3C polymorphism at position 719 A/G, exon 10 in Rheumatologic disease patients.

	King Chulalongkorn Memorial hospital	Nopparatrajchatanee hospital	**Total**
Genotype frequency			
G/G (TPMT*3C/*3C)	0 (0.0%)	0 (0.0%)	0 (0.0%)
A/G (TPMT*1/*3C)	4 (3.57%)	4 (3.57%)	8 (7.14%)
A/A (TPMT*1/*1)	36 (32.14%)	68 (60.72%)	104 (92.86%)
Allele frequency			
A	76 (33.93%)	148 (60.07%)	216 (96.43%)
G	4 (1.785%)	4 (1.785%)	8 (3.57%)

3. The association result of TPMT gene polymorphism with Thai patients Rheumatologic diseases taking azathioprine and myelosuppressive.

We analyze the association between clinical manifestation in patient with Rheumatologic disease (table 4) and polymorphism of TPMT*3C gene by using Fisher's Exact test and odd ratio, as show in table 5.

The result show association between myelosuppression (leucopenia) in patient with Rheumatologic disease and polymorphism of TPMT*1/*3C gene are statistic significantly different ($P < 0.001$) and odd ratio 26.0, 95%CI 4.783 – 141.333. Then test polymorphism of TPMT*1/*3C gene compared with neutropenia that result not significantly different ($P = 0.06$) and odd ratio 8.889, 95%CI 1.237 – 63.878 and compared with lymphopenia that result not significantly different either ($P = 0.085$) and odd ratio 6.583, 95% CI 0.995 – 43.553, as show in table 6.

Table 4 Summary of TPMT activity with genotype groups

genotype	TPMT activity (Cases)					
	Valid		Missing		Total	
	N	Percent	N	Percent	N	Percent
TPMT*1/*1	57	54.8%	47	45.2%	104	100.0%
TPMT*1/*3C	5	62.5%	3	37.5%	8	100.0%

Table 5 The association between myelosuppression in patient with Rheumatologic disease and polymorphism of TPMT*3C gene

	TPMT*1/*1	TPMT*1/*3C	Total
Myelosuppression	5 (6%)	5 (62.5%)**	10 (11%)
Non-toxic	78 (94%)	3 (37.5%)	81 (89%)
total	83	8	91

** Fisher's Exact test P < 0.001, odd ratio 26.0 (95%CI 4.783 – 141.333)

Table 6 Clinical manifestation of patients with Rheumatologic disease in this study

Clinical manifestation	TPMT*1/*1	TPMT*1/*3C	Total	p-value
Leucopenia (WBC < 3000 cells/mm^3)	5/83 (6%)	5/8 (62.5%)	10/91 (11%)	< 0.001**
Lymphopenia (Lymphocyte < 600 cells/mm^3)	4/83 (4.8%)	2/8 (25%)	6/91 (6.6%)	0.085ns
Neutropenia (Neutrophil< 1,500 cells/mm^3)	3/83 (3.6%)	2/8 (25%)	5/10 (5.5%)	0.060ns

** Fisher's Exact test; highly significantly different

ns Fisher's Exact test; not significantly different

4. Determination results of TPMT activity with Thai patients Rheumatologic diseases taking azathioprine

The enzyme activity was measured in 62 patients. Distribution of TPMT activity in our study population, which is graphically represented in Fig. 13, follow a normal distribution (P = 0.20; Kolmogorov-Smirnov). There is range of TPMT activity during 9.16 – 65.89 nmol 6-MTG/gHb/hr, that mean is 36.26 ± 13.403 (SD) nmol 6-MTG/gHb/hr (95%CI 32.86 – 39.67) and median is 35.20 nmol 6-MTG/gHb/hr.

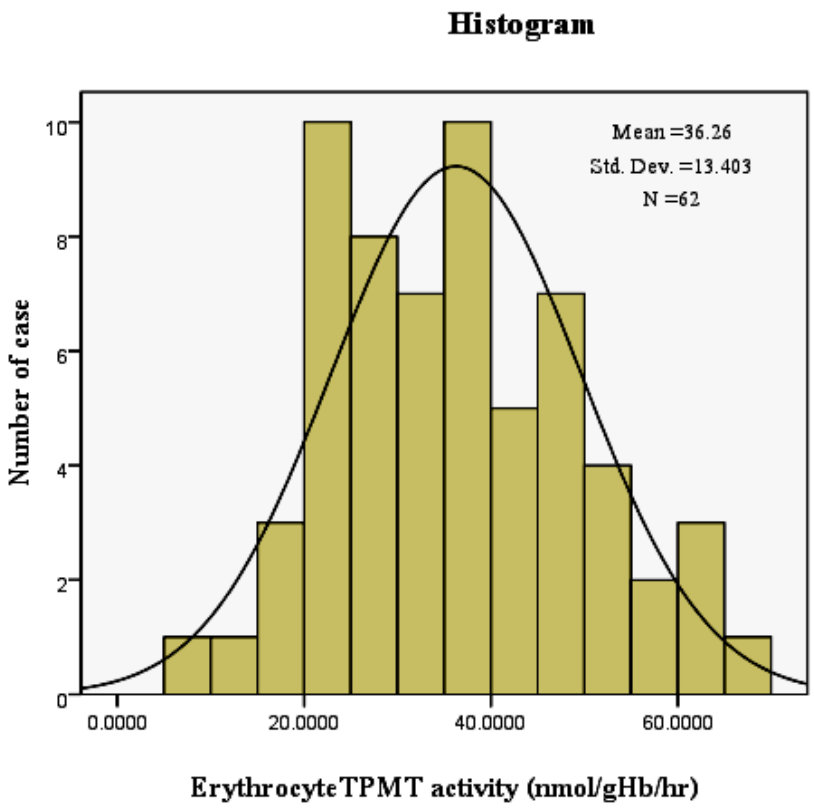

Figure 13 Distribution of thiopurine S-methyltransferase (TPMT) activity in the population study RBCs red blood cells

The TPMT*1/*3C genotype correlated with the intermediate TPMT activity and TPMT*1/*1 was some overlap of genotype result between the intermediate and normal activity distributions. Then test mean of TPMT activity both present statistic significantly P < 0.001 (t-test) that result show the both groups are difference (see table 7)

Table 7 Descriptive statistic of TPMT activity in patients red blood cell classify by genotype group.

TPMT genotype	Erythrocyte TPMT activity (nmol 6-MTG/ gHb/ hr)				
	Minimum	Maximum	Mean	Std. Deviation	Median
*1/*1 (n = 57)	13.01	65.89	37.90	12.64	35.65
*1/*3C (n = 5)	9.16	21.89	17.53**	4.96	18.45
Total (n = 62)	9.16	65.89	36.26	13.40	35.20

** Statistical significant P < 0.001 (t-test)

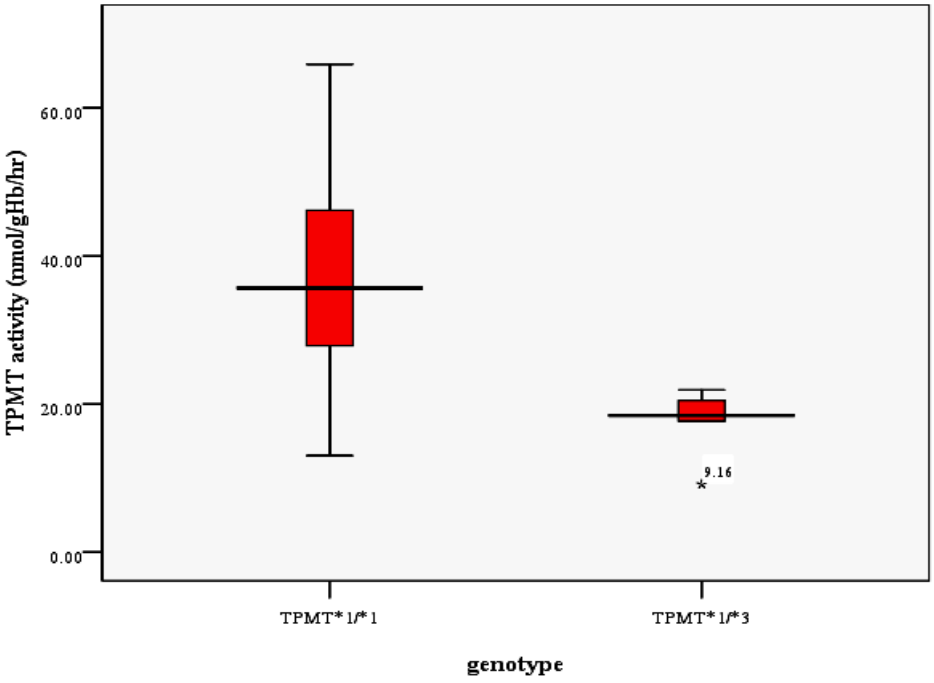

Figure 14 The boxplots of TPMT activity with genotype groups

In this study, patients taking azathioprine dose between 0.65 – 2.50 mg/kg/day that mean 1.54 ± 0.51 (SD) and then test statistic by Mann-Whitney U test between azathioprine dose with leucopenia that result show not significantly different P = 0.186 (exact sig. 2-tailed).

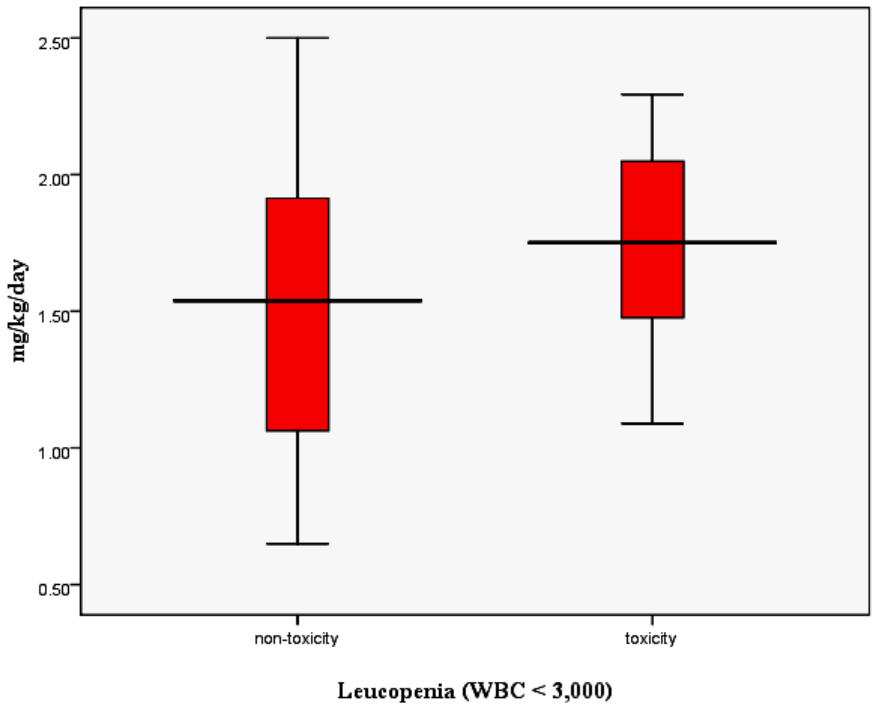

Figure 15 The boxplots of azathioprine dose (mg/kg/day) with toxicity groups (leucopenia)

This study, Rheumatologic disease patients taking azathioprine some overlap of TPMT activity result between the intermediate and normal activity distributions so receiver operating characteristic (ROC analysis) were applied for the calculation of the cut-off concentration for TPMT activity assay compared with TPMT genotype.

The optimal cut-off activities for TPMT activity assay predict a heterozygous TPMT*3C that calculated cut-off activity was 23.01 nmol 6-MTG/gHb/hr with a test sensitivity of 93.0%, a specificity of 100% and area under the curve (AUC) of 0.975 (95%CI; 0.939 – 1.012) see fig 16 and table 8.

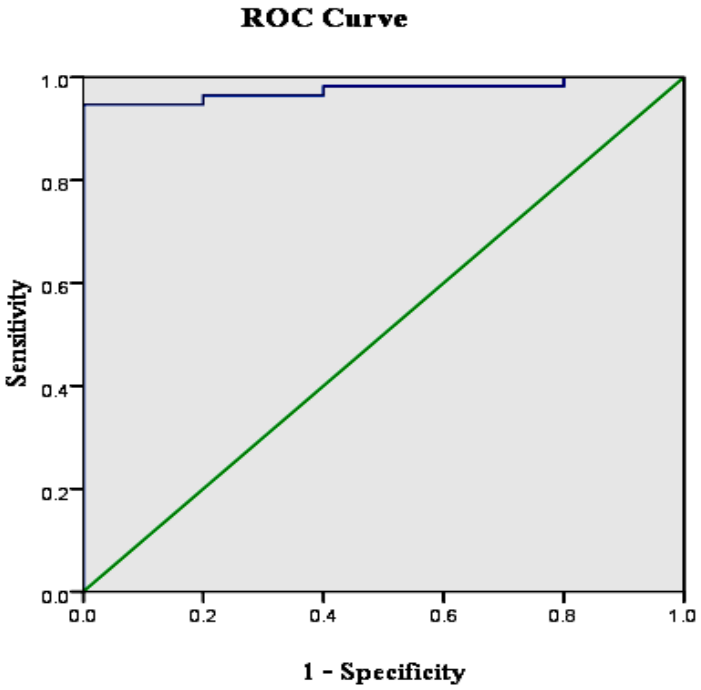

Figure 16 The ROC curve test TPMT genotype: TPMT activity (6-MTG nmol/gHb/hr)

Table 8 Area under The ROC curve test TPMT genotype: TPMT activity (6-MTG nmol/gHb/hr)

Area Under the Curve

Area	Std. Error[a]	Asymptotic Sig.[b]	Asymptotic 95% Confidence Interval	
			Lower Bound	Upper Bound
.975	.019	.000	.937	1.012

a. Under the nonparametric assumption

b. Null hypothesis: true area = 0.5

The optimal cut-off activities for TPMT activity calculated was 23.01 nmol 6-MTG/gHb/hr so that intermediate activity was ≤ 23.01 nmol 6-MTG/gHb/hr and high activity was > 23.01 nmol 6-MTG/gHb/hr. Then test mean of TPMT activity both present statistic significantly $P < 0.001$ (t-test) that result show the both groups are difference (see table 9).

The statistical represent association between myelosuppression (leucopenia) in patient with Rheumatologic disease and erythrocyteTPMT activity are statistic significantly different (P = 0.003) and odd ratio 21.5, 95%CI 2.961 – 156.128. Then test polymorphism of TPMT*1/*3C gene compared with neutropenia that result not significantly different (P = 0.059) and odd ratio 11.25, 95%CI 1.233 – 102.623 and compared with lymphopenia that result not significantly different either (P = 0.391) and odd ratio 2.933, 95%CI 0.254 – 33.823 (table 10).

Table 9 Descriptive statistic of TPMT activity in patients red blood cell classify by TPMT activity group

TPMT activity group	Erythrocyte TPMT activity (nmol 6-MTG/ gHb/ hr)				
	Minimum	Maximum	Mean	Std. Deviation	Median
High (n = 53) (> 23.01 nmol 6-MTG/gHb/hr.)	23.04	65.89	39.32	11.91	38.17
Intermediate (n = 9) (≤ 23.01 nmol 6-MTG/gHb/hr)	9.16	22.98	18.21**	4.46	19.50
Total (n = 62)	9.16	65.89	36.26	13.40	35.20

** Statistical significant P < 0.001 (t-test)

Table 10 The association between myelosuppression in patient with Rheumatologic disease and TPMT activity group

Erythrocyte TPMT activity group	TPMT activity (Cases)					
	Myelosuppression		Non-myelosuppression		Total	
	N	Percent	N	Percent	N	Percent
High (> 23.01 nmol 6-MTG/gHb/hr.)	4	8.50%	43	91.5%	47	100.0%
Intermediate ($\leq$ 23.01 nmol 6-MTG/gHb/hr)	4	66.7%	2	33.3%	6	100.0%
Total	8	15.1%	45	84.9%	53	100.0%

Fisher's Exact test P = 0.003, odd ratio 21.5 (95%CI 2.961 – 156.128)

Table 11 Clinical manifestation of patients with Rheumatologic disease in this study

Clinical manifestation	TPMT*1/*1	TPMT*1/*3C	Total	p-value
Leucopenia (WBC < 3000 cells/mm^3)	5/83 (6%)	5/8 (62.5%)	10/91 (11%)	< 0.001**
Lymphopenia (Lymphocyte < 600 cells/mm^3)	4/83 (4.8%)	2/8 (25%)	6/91 (6.6%)	0.085ns
Neutropenia (Neutrophil< 1,500 cells/mm^3)	3/83 (3.6%)	2/8 (25%)	5/10 (5.5%)	0.060ns

** Fisher's Exact test; highly significantly different

ns Fisher's Exact test; not significantly different

The other than test receiver operating characteristic (ROC analysis) were applied for the calculation of the cut-off concentration for TPMT activity assay compared with myelosuppressive. (fig17)

The optimal cut-off activities for TPMT activity assay predict a heterozygous TPMT*3C that calculated cut-off activity was 23.51 nmol 6-MTG/gHb/hr with a test sensitivity of 93.30%, a specificity of 50.0% and area under the curve (AUC) of 0.622 (95%CI; 0.377 – 0.867) table 12.

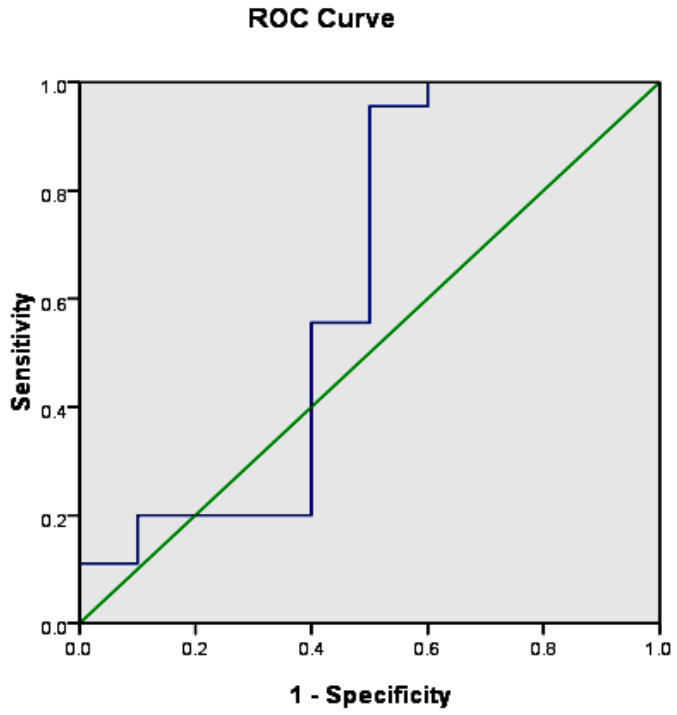

Figure 17 ROC curve test myelosuppressive phenomena (WBC < 3,000 cell/mm^3) : TPMT activity (6-MTG nmol/gHb/hr)

Table 12 Area Under the **ROC** curve test myelosuppressive phenomena (WBC < 3,000 cell/mm^3) : TPMT activity (6-MTG nmol/gHb/hr)

Area Under the Curve

Area	Std. Error[a]	Asymptotic Sig.[b]	Asymptotic 95% Confidence Interval	
			Lower Bound	Upper Bound
.622	.125	.230	.377	.867

a. Under the nonparametric assumption

b. Null hypothesis: true area = 0.5

Table 13 Case summary TPMT genotype with myelosuppressive

Case	TPMT genotype	TPMT activity (nmol 6-MTG/gHb/hr)	AZA (mg/kg/day)	myelosuppressive
Tn11	*1/*3C	Missing data	0.79	Non-toxicity
Tn64	*1/*3C	9.1648	2.06	Leucopenia, Lymphopenia
Tn67	*1/*3C	17.7014	2.05	Leucopenia
Tn70	*1/*3C	20.4607	1.59	Non-toxicity
Tc20	*1/*3C	18.4463	2.11	Leucopenia, Neutropenia
Tc21	*1/*3C	21.8852	2.00	Leucopenia, Neutropenia
SLE81	*1/*3C	Missing data	2.29	Leucopenia, Lymphopenia
SLE240	*1/*3C	Missing data	Missing data	Non-toxicity
Tn40	*1/*1	22.9816	Missing data	Missing data
Tn52	*1/*1	19.5041	Missing data	Missing data
Tn57	*1/*1	13.0083	Missing data	Missing data
Tc08	*1/*1	20.7787	1.54	Non-toxicity
Tn16	*1/*1	54.8476	1.61	Leucopenia
Tn48	*1/*1	47.4537	1.75	Leucopenia, Neutropenia
Tn49	*1/*1	35.0773	1.09	Leucopenia, Neutropenia
Tc23	*1/*1	46.7928	1.26	Leucopenia
SLE112	*1/*1	Missing data	1.48	Leucopenia, Neutropenia

CHAPTER V

DISCUSSION AND CONCLUSION

More than 30 polymorphisms were discovered in the TPMT gene. That TPMT*3C is the most common TPMT variant in South-east Asian population, including Japanese (2.9%), Chinese (0.2 – 3.0%), Filipinos (1.0%) and Indonesians (1.0%). In Thailand finding was consistent with the recent data of Hongeng et al. (32), who reported that the frequency of TPMT*3C observed in 75 Thai children with acute leukemia was 0.053. Afterwards to study in 200 Thais healthy found frequency of TPMT*3C was 0.05 (19) and 0.0324 in Thais kidney transplant recipients that higher risk for AZA-induced neutropenia after receiving the standard dose of AZA.

In this study, the results of the genotype analysis are summarized. Of 112 the results of genotyping analysis were performed on 112 Thai patients with Rheumotologic diseases. This study, 104 patients were homozygous for TPMT*1 (92.9%) and 8 patient were heterozygous of TPMT*1/*3C (7.1%) by PCR-RFLP. Similar to those previous reports in other Asian populations that TPMT*3C was the most prevalence. The allele frequencies of TPMT*1 and TPMT*3C in Thai patients Rheumatologic diseases population in this study were found to be 0.9643 and 0.0357, respectively (based on the Hardy– Weinberg equilibrium) that was similar to Thais kidney transplant recipients.

Results of TPMT genotyping in patient show a good correlation of TPMT*1/*1 and TPMT*1/*3C genotypes with high and intermediate enzyme activities ($P<0.001$), although there is some overlap between the groups. There was significant difference ($P<0.001$) in the mean TPMT activity between TPMT*1/*1 (37.90 ± 12.64) and TPMT*1/*3C genotypes (17.53 ± 4.96). The study suggested that the incidence of leucopenia from taking AZA in Rheumatologic disease patients with TPMT*1/*3C mutant were significantly higher than in Rheumatologic disease patients with TPMT*1/*1 (wild type) with odds ratio (OR) of 26 ($p<0.001$). Both patients groups taking azathioprine dose between 0.65 – 2.50 mg/kg/day that mean 1.54 ± 0.51 (SD) then

test statistic by Mann-Whitney U test between azathioprine dose that result show not significantly different (P = 0.186). But not significantly different induced neutropenia (P = 0.06, odd ratio 8.889, 95%CI 1.237 – 63.878) and lymphopenia (P = 0.085, odd ratio 6.583, 95%CI 0.995 – 43.553). This findings were found to be inconsistent with the previous study of SLE patients in the U.S. in 1999 (33) and in Japan in 2005 (20) which concluded that TPMT genotyping prior to AZA therapy could not predict myelosuppressive in SLE patients.

The optimal cut-off activities for TPMT activity calculated intermediate activity was ≤ 23.01 nmol 6-MTG/gHb/hr and high activity was > 23.01 nmol 6-MTG/gHb/hr with AUC. The statistical represent association between leucopenia (P = 0.003, odd ratio 21.5, 95%CI 2.961 – 156.128). But not significantly different induced T neutropenia (P = 0.059, odd ratio 11.25, 95%CI 1.233 – 102.623) and lymphopenia (P = 0.391, odd ratio 2.933, 95%CI 0.254 – 33.823) same as compared with genotype.

Due to the research was conducted with retrospective methodology; it was not possible to measure enzymatic activities of TMPT in all patients. Thus, only 62 activities of TMPT measurements were collected from the same number of patients. It is also evidenced that the enzymatic activity in TPMT*1/*3C mutant allele patient was statistically significantly lower than those in wild type patients ($p<0.001$). Limitations of this study include the absence of clinical information provided with each blood samples, some samples may have been from patients who are not responding to therapy.

However, 4 patients with TPMT*1/*1 (wild type) was found to have intermediate enzymatic activity of TPMT which 3 patients missing clinical data. The myelosuppression was also not found in patient. This might be resulted from AZA usage termination by doctor due to patient unstable conditions and may resulted from other enzymatic activities related to AZA transformation process such as xanthine oxidase which is an enzyme in the liver. Even though, the activity of TPMT was low without defective allele this incidence might be caused by the fact that this study was limited to the genotyping for TPMT*3C only where currently there were as much as 27 genotypes discovered. Recent case in Thailand, the

TPMT*27 mutant allele was identified in a renal transplantation recipient with reduced enzymatic TPMT activity which was also lower than those in wild type patient (34).TPMT*6 mutant allele were also found but with low incident rate in Korean and Japan (35, 36) which were not included in this study. Furthermore, the recent research found that the variable number of tandem repeats (VNTR) within the TPMT promoter affected the transcription and level of enzymatic activity of TPMT (37). Another 4 patients with TPMT*1/*1 mutant allele had normal level of enzymatic activity but myelosuppression was found which may resulted from abnormality of other enzymatic activities related to AZA transformation process. This included many other studies such as the study in IBD and rheumatoid arthritis patients which suggested that ITPA gene with ITPA93C>A mutant allele affected in low ITPA enzymatic activity and resulted in increasing 6-TITP level and eventually caused higher toxicity of AZA usage (38). More studies were conducted in patients with IBD with a wild type GST-M1 genotype presented high probability of higher 6-MP in cell, then higher risk of low WBC condition (39). Another study found low activity of XO enzyme in 20% of Caucasians and 11% of Japanese population (40). Xanthine oxidase is not present in heamapoietic tissue (41), there cannot be measured by blood. Nevertheless, those studies had never been conducted with Thai population. And phenotype should be performed in patients who had a recent blood transfusion as measurement of enzyme activity will be in correct in these patients. This did not correct information.

In addition to the relevant generic variation, there were also other related factors e.g. blood monitoring result was not accurate after blood transfusion, progression of disease, viral infection, patient's behavior, and drug interaction. Previous studied reported that drug interaction resulted in TPMT enzyme inhibition e.g. Sulphasalazine, Olsalazine, mesalamine, balsalazide (42-45), furosemide, bendroflumethiazide, trichlomethiazide (46) etc. However, in this study these issues were not included. Also may be due to relapse, viral infection and daily life of the patients.

In only 3 cases which patients with TPMT*1/*3C experienced no side-effects from drug taking and 2 cases no data of enzymatic activity was collected and reported and the patient could not be contacted for further investigation. Have only found that the intermediated

enzymatic level. It was suspected that patient did not take drug as prescribed or take low dose of drug.

The level of enzymatic activity varied significantly in each individual according to genetic polymorphism, only blood monitoring and blood cell counts seemed to be insufficient to prevent the toxicity from AZA use. The reason is the limitation that blood monitoring could not be done continuously real time. Genotyping prior to drug therapy is necessary and is an essential tool for doctor to decide the dosage and type of drug for each individual patient to maximize the treatment efficiency and patient safety simultaneously.

In conclusion, The study of genetic polymorphism of TPMT*3C and the enzymatic activity of TPMT revealed that Rheumatologic disease patients with TPMT*1/*3C mutant allele demonstrated significantly lower enzymatic activity of TPMT than normal wild type patient and high risk in myelosuppression from AZA usage. So the genetic polymorphism of TPMT gene may be predicting the toxicity from AZA usage. Prospective studies are needed to confirm this hypothesis. Determination of TPMT genotype before starting any thiopurine dosage regimen may also have clinical benefit to Thai patients.

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APPENDICES

APPENDIX A

Reagent for agarose gel electrophoresis

1. 50X Tris-acetate buffer (TAE)

Tris base	424.0	g
Glacial acetic acid	57.1	g
0.5M EDTA pH8.0	100	ml

Adjust volume to 1 liter with distilled water. The solution was mixed and sterilized by autoclaving at 121°C

2. 10 mg/ml Ethidium bromide

Ethidium bromide	1.0	g
Distilled water	100	ml

Mix the solution and store in the dark at 4°C

3. 2% Agarose gel

Agarose	0.6	g
1X TBE	40	ml

Dissolve by heating in microwave oven and occasion mix until no granules of agarose are visible.

4. 5X Loading buffer 100 ml

Tris HCL	0.6	g
EDTA	1.68	g
SDS	0.5	g
Bromphenol Blue	0.1	g
Sucrose	40	g

Adjust volume to 100 ml with distilled water. Mix the solution, aliquot into 1.5 microtube and store at 4°C

APPENDIX B

Reagent for DNA extraction

1. Red Cell Lysis Buffer (RCLB)

NH_4CL	1.875	g
Tris-HCL	0.25	g

Dissolve NH_4CL and Tris-HCL in 500 of distilled water. Adjust pH to 7.2. The solution was mixed and sterilized by autoclaving at 121°C for 15 minutes. Keep refrigerate at 4°C. Shelf lift is approximately 6 months.

2. Nuclei Lysis Buffer (NLB)

1M Tris (pH 8.0)	10	ml
5M NaCl	0.5	ml
0.5M EDTA (pH 8.0)	0.4	ml

Adjust volume to 100 ml with distilled water. Adjust pH to 7.2. Keep refrigerate at 4°C. Shelf lift is approximately 6 months.

3. 1M Tris

Tris base	12.11	g
Distilled water	100	ml

Adjust volume to 100 ml with distilled water. Adjust pH to 7.2. The solution was mixed and sterilized by autoclaving at 121°C for 15 minutes.

4. 5M NaCl

NaCl	29.22	g
Distilled water	100	ml

Adjust volume to 100 ml with distilled water. The solution was mixed and sterilized by autoclaving at 121°C for 15 minutes.

5. EDTA

EDTA	37.22	g
Distilled water	200	ml

Adjust volume to 200 ml with distilled water. The solution was mixed and sterilized by autoclaving at 121°C for 15 minutes. Keep refrigerated.

6. 5.3M NaCl

NaCl	15.5	g
Distilled water	50	ml

Adjust volume to 50 ml with distilled water. The solution was mixed and sterilized by autoclaving at 121°C for 15 minutes. Keep refrigerated.

7. Proteinase K 10 mg/ml

Proteinase K	100	mg
Distilled water	10	ml

Mix the solution and store at -20°C

8. 10% SDS

SDS	**10**	**g**
Distilled water	100	ml

Adjust volume to 100 ml with distilled water. The solution was mixed and sterilized by autoclaving at 121°C for 15 minutes.

APPENDIX C

Reagent for TPMT activity

1. Stock 6-TG (conc. 3.5 mg/ml)

6-TG (6-thioguanine)	79.8	mg
1M NaOH	1	ml

The solution was mixed by vortex and adjusts volume to 4ml with distilled water. The solution was centrifuged at 2500 rpm or 1400×g after that aliquot supernatant 1 ml into microtube and store at -80°C.

2. Stock SAM (0.084 mg/ml)

SAM (S-adenosyl-L-methionine)	16.8	mg
0.1M phosphate buffer	200	ml

The solution was mixed by vortex and adjusts volume to 200 ml with 0.1M phosphate buffer. This solution was aliquot 6 ml per tube and store at -20 °C.

3. 6-MTG (conc. 1 mg/ml)

6-MTG (2-amino-6-methyl mercaptopurine)	10	mg
1M NaOH	1	ml

The solution was mixed by vortex and adjusts volume to 10 ml with distilled water. The solution was centrifuged at 2500 rpm or 1400×g after that aliquot supernatant 300 µl into microtube and store at -20°C.

4. 1M NaOH

NaOH	4	g
Distilled water	100	ml

Adjust volume to 100 ml with distilled water. The solution was mixed and kept at room temperature.

5. 0.1M NaOH

1M NaOH	10	ml
Distilled water	90	ml

Adjust volume to 100 ml with distilled water. The solution was mixed and kept at room temperature.

6. Solution A: 0.1M K_2HPO_4

K_2HPO_4	17.418	g
Distilled water	1000	ml

Adjust volume to 1000 ml with distilled water. The solution was mixed and kept at room temperature.

7. Solution B: 0.1M KH_2PO_4

KH_2PO_4	4.083	g
Distilled water	1000	ml

Adjust volume to 1000 ml with distilled water. The solution was mixed and kept at room temperature.

8. 0.1M Phosphate buffer pH 7.4

Solution A	800	ml
Solution B	200	ml

Adjust pH to 7.4 with solution B. The solution was mixed and kept refrigerate.

APPENDIX D

Reagent for RBC lysate

1. 0.02 mM Phosphate buffer pH 7.4

0.1M Phosphate buffer	100	µl
Distilled water	499.9	ml

Adjust volume to 500 ml with distilled water. The solution was mixed and kept refrigerate.

2. NSS (Normal Saline Solution)

BIOGRAPHY

Miss. Janejira Kongpunvijit was born on July 29, 1980 in Nonthaburi, Thailand. She graduated with the Bachelor degree of Science Biology Major Biology from Kasetsart University in 2002 and then attended to particular in Medical Science Program, Graduate School, Chulalongkorn University for her master degree.