ความสัมพันธ์ระหว่างรูปแบบความหลากหลายของยีน CTLA-4 (ตำแหน่ง+49A/G และ CT60A/G) กับความเสี่ยงทางพันธุกรรมต่อการเกิดโรคเอส แอล อี ในประชากรไทย

นางสาวสิริวลี แซ่โง้ว

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THE ASSOCIATION BETWEEN CTLA-4 GENE POLYMORPHISMS (+49A/G AND CT60A/G) AND THE GENETIC SUSCEPTIBILITY OF SYSTEMIC LUPUS ERYTHEMATOSUS IN THAI POPULATION

Miss Siriwalee Sae-Ngow

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สิริวลี แซ่โง้ว : ความสัมพันธ์ระหว่างรูปแบบความหลากหลายของยีน CTLA-4(ตำแหน่ง+49A/GและCT60A/G) กับความเสี่ยงทางพันธุกรรมต่อการเกิดโรคเอส แอล อี ใน ประชากรไทย (THE ASSOCIATION BETWEEN CTLA-4 GENE POLYMORPHISMS(+49A/G AND CT60A/G) AND THE GENETIC SUSCEPTIBILITY OF SYSTEMIC LUPUS ERYTHEMATOSUS IN THAI POPULATION)

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Systemic lupus erythematosus หรือโรค SLE เป็นโรค autoimmune ที่มีความผิดปกติของระบบภูมิคุ้มกันทำให้เกิดการสร้างภูมิต้านทานต่อเนื้อเยื่อของตนเองจนทำให้เกิดพยาธิสภาพได้ทั่วทุกอวัยวะ SLE สามารถพบได้ในทุกชนชาติทั่วโลก ถึงแม้ว่ายังไม่ทราบถึงสาเหตุที่แน่ชัดของโรคนี้แต่มีหลายปัจจัยในการเกิดโรคไม่ว่าจะเป็นปัจจัยทางด้านสิ่งแวดล้อม สารเคมี การติดเชื้อ รวมถึงปัจจัยทางพันธุกรรมซึ่งเชื่อว่าเป็นปัจจัยสำคัญของโรค ดังนั้นจึงมีการศึกษาปัจจัยทางพันธุกรรมมากมายไม่ว่าจะเป็นยีนที่มีความเสี่ยงต่อเกิดโรคและยีนที่มีความสำคัญต่อการเกิดโรค การศึกษาทางพันธุกรรมมากมาไม่ว่าจะเป็น linkage study และ candidate gene มีรายงานมากมายที่กล่าวถึง susceptibilities gene หนึ่งในนั้นคือ CTLA-4 ซึ่งเป็นยีนที่อยู่บนโครโมโซมที่ 2q33 เนื่องจากเป็นยีนที่มีบทบาทสำคัญในกระบวนการ immunoregulation จึงเป็นยีนที่มีความสำคัญต่อโรค autoimmune หลายๆโรค รวมทั้ง SLE ในการศึกษานี้ได้ศึกษารูปแบบความหลากหลายของยีนนี้ที่ตำแหน่ง+49A/G และ CT60 โดยทั้งสองตำแหน่งเป็นตำแหน่งที่มีหน้าที่สำคัญภายในยีน ในการศึกษานี้เป็นเปรียบเทียบรูปแบบความหลากหลายของยีนนี้ที่ตำแหน่ง +49A/G และ CT60 ต่อความเสี่ยงทางพันธุกรรมต่อการเกิดโรคในคนไทย ด้วยการศึกษาแบบ population-base case-control รวบรวมผู้ป่วย 150 คน และ คนปกติ 150 คนซึ่งมีเชื้อชาติเดียวกัน ใช้วิธี PCR-RFLP หารูปแบบความหลากหลายในยีน CTLA-4 ทั้ง 2 ตำแหน่ง ผลการศึกษาไม่พบความแตกต่างอย่างมีนัยสำคัญทางสถิติทั้งที่ตำแหน่ง +49A/G และ CT60 alleleและ genotype ในยีนCTLA-4 เมื่อเปรียบกันระหว่างผู้ป่วยและคนปกติ จากการวิเคราะห์ haplotype ของยีนนี้ทั้งของตำแหน่งเข้าด้วยกันพบว่ารูปแบบ +49*A;CT60*G haplotype มีความแตกต่างอย่างมีนัยสำคัญทางสถิติเมื่อเปรียบเทียบกับ haplotype รูปแบบอื่นๆระหว่างผู้ป่วยและคนปกติในแง่ความเสี่ยงต่อการเกิดโรคที่มีลักษณะการถ่ายทอดยีนแบบยีนด้อย (OR= 0.0,95%CI=0.50-0.9,P=0.39) โดยสรุปว่าจากการศึกษาในครั้งนี้ถึงแม้ว่าจะไม่พบความสัมพันธ์ของรูปแบบความหลากหลาย SNP ทั้งสองตำแหน่งของยีนกับความเสี่ยงต่อการเกิดโรคSLE ในประชากรไทย แต่จากการวิเคราะห์โดย haplotype พบว่า+49*A;CT60*G haplotype มีความสัมพันธ์ต่อกับความเสี่ยงต่อการเกิดโรค ทั้งนี้เนื่องจาก SLE เป็นโรคที่มีความซับซ้อนมากและมีปัจจัยจากยีนหลายตัวที่เกี่ยวข้องกับการเกิดโรคดังนั้นจึงต้องมีการศึกษาเพื่อหา genetic marker ตัวอื่นซึ่งอาจเป็น marker ที่เหมาะสมกับประชากรไทยต่อไปและควรมีการศึกษาต่อถึงหน้าที่ของ SNP ทั้งสองตำแหน่งของยีนเพื่อยืนยันบทบาทหน้าที่ที่แท้จริงของยีนทั้งสองตำแหน่งในประชากรไทยต่อไป

สาขาวิชา จุลชีววิทยาทางการแพทย์ ลายมือชื่อนิสิต.....

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SIRIWALEE SAE-NGOW: THE ASSOCIATION BETWEEN CTLA-4 GENE POLYMORPHISMS(+49A/G AND CT60A/G)AND THE GENETIC SUSCEPTIBILITY OF SYSTEMIC LUPUS ERYTHEMATOSUS IN THAI POPULATION. THESIS ADVISOR : ASSOCIATE PROF.NATTIYA HIRANKARN, MD, Ph.D. 73 pp. ISBN: 974-14-2131-1

Systemic lupus erythematosus(SLE) is a prototype for systemic autoimmune disease, affecting most major organ systems and characterized by the production of various autoantibodies. Although the etiopathology of SLE remains only partially elucidated, SLE is genetically complex, with contributions anticipated from environmental factors in the pathogenesis of SLE. From many genome wides and linkage studies showed that CTLA-4 gene is the potential susceptibility gene for many autoimmune disease including SLE. CTLA-4 is important in peripheral tolerance and its regulation has the potential to affect the pathogenesis of disease. The aim of this study was to identified the association of CLA-4 gene polymorphisms at position +49A/G in exon 1 and CT60 in 3' UTR with genetic susceptibility and /or disease between in SLE and normal control in Thai population. Population –base case-control study included 150 SLE patients and 150 healthy controls with similar ethnic background. CTLA-4 gene polymorphisms were identified by PCR-restriction fragment length polymorphism. The results of this study demonstrated that no significant association in CTLA-4 polymorphisms from both +49A/G and CT60 when compared by used single SNP with SLE patient and healthy control. Intestingly, from haplotype analysis, we found that +49*A-CT60*G haplotype, especially in recessive mode of inheritance is a risk genotype in our population(OR=0.00,95%CI=0.09,p=0.039). However, SLE is very heterogenous syndrome and these SNP might not effect to function of the CTLA-4 gene, to exploring this possibility, other SNPs and more genetic markers and haplotype analysis need to be performed to find the most suitable genetic marker for Thai populations and functional effect of these SNPs should be further studied.

Field of Study Medical Microbiology Student's signature.....

(Inter-Department) Advisor's signature.....

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ABBREVIATIONS

APCs	antigen-presenting cells
bp	base pair
CD	Cluster of Differentiation
CTLA-4	Cytotoxic T-lymphocyte antigen-4
95% CI	95% Confidence Interval
°C	degree Celsius
et al	et all
GD	Graves' disease
Ig	Immunoglobulin
kDa	Kilodalton
HLA	Human Leukocyte Antigen
μl	microliter
μg	microgram
ml	milliliter
mM	millimolar
MS	Multiple sclerosis
MW	molecular weight
ng	nanogram
OR	Odd Ratio
PCR	Polymerase Chain Reaction
RA	Rheumatoid arthritis
RFLP	restriction fragment-length polymorphism
SSP	sequence specific primer
SDS	Sodium Dodecyl sulfate
SNPs	Single Nucleotide Polymorphisms
TSH	Thyroid-stimulating hormone

T1DM	Type 1 Diabetes melitus
U	Unit
VNTR	variable numbers of tandem repeats

CHAPTER I

INTRODUCTION

Systemic lupus erythematosus (SLE) is a prototype of human autoimmune disease various characterized by the production of autoantibodies against self-antigens. The disease affects different tissues and organs, the patients vary in age at onset, and the outcome is unpredictable from severe to relatively benign (Hochberg 1997; Mok and Lau 2003).

The overall disease prevalence 1.5-250 in 100,000. It's less common in Europeans than Asian and Afro-Caribbeans populations and is approximately 10 fold more common in women of childbearing age than men (Hochberg 1997).

Both environmental and genetic factors are thought to contribute to continuous autoimmune process. Although the etiopathology of SLE remains only partially elucidated, but genetic factor seems to be important. Twins Studies show higher concordance in monozygotic than dizygotics over 10 times increased risk of SLE (Hochberg 1997).

In addition to many genome-wide linkage studies have been performed and discover loci important in the SLE. More than 20 loci showing suggestive linkage to SLE were identified that indicated that SLE is multiple gene disease. One of the important loci is 2q33-35 that contains CTLA-4 gene which is the susceptible gene in many autoimmune diseases include SLE (Moser, Neas et al. 1998; Tsao 2003; Nath, Kilpatrick et al. 2004).

CTLA-4 (cytotoxic T-lymphocte-associated antigen-4) is structural homologue of CD28 but plays a negative regulatory role in T-cell responses CTLA-4 controls the adaptation of T-cell to a state of proliferative unresponsiveness and tolerance. Therefore

CTLA-4 is a positional and functional candidate gene for susceptibility to SLE. CTLA-4 polymorphism have been associated with several autoimmune disorders, such as type I diabetes, autoimmune thyroids disease, celiac disease, Graves' disease (GD), rheumatoid arthritis (RA), and multiple sclerosis to the first degree relatives (Nistico, Buzzetti et al. 1996; Kristiansen, Larsen et al. 2000; Vaidya and Pearce 2004).

Several polymorphisms have been described in CTLA-4, and these include -1722T/C and -319C/T, both of them are in promoter region, +49A/G in exon 1, microsatellite (AT)n polymorphism in 3'-untranstrated region (3'UTR) (D'Alfonso, Rampi et al. 2000; Ahmed, Ihara et al. 2001; Aguilar, Torres et al. 2003; Barreto, Santos et al. 2004; Fernandez-Blanco, Perez-Pampin et al. 2004) and recently described CT60A/G dimorphisms, which are within 3'UTR (Torres, Aguilar et al. 2004). However the functional polymorphism is believed to be mainly in +49A/G on exon 1 region, which causes an amino acid exchange (threonine to alanine) in the peptide leader sequence of the CTLA-4 protein (Chistiakov and Turakulov 2003). Recently Ueda and workers demonstrated that the CT60A/G in 6.1-kb of the 3' region of the CTLA-4 is the strongest susceptibility marker to autoimmune disease. They suggest that this SNP determines the efficiency of the splicing and production of sCTLA-4, and may plays role in mRNA stability of sCTLA-4 (Ueda, Howson et al. 2003).

The aims of this study were to investigate the association of proposed functional CTLA-4 polymorphisms (+49A/G in exon1 and CT60A/G in 3'UTR) and/or susceptibility to SLE in Thai populations.

CHAPTER II

OBJECTIVE

The objective of this study was;

To identify the polymorphisms of CTLA-4 gene(+49A/G in exon 1 and CT60 A/G) in patient with SLE compared with control group and to determine the association with disease susceptibility and/or severity of SLE in Thai population.

CHAPTER III

LITERATURE REVIEW

Systemic lupus erythematosus (SLE)

Systemic lupus erythematosus (SLE) is an autoimmune disease characterized by autoantibody production and immune complex formation. The tissue deposition of antibodies and immune complexes (ICs) could cause inflammation and injury of multiple organs (Hochberg 1997; Mok and Lau 2003). Clinical manifestations of SLE could be diverse, including glomerulonephritis, dermatitis, thrombosis, vasculitis, seizures and arthritis (Hochberg 1997).

Epidemiology

SLE primarily affects women during their childbearing ages. The gender difference in SLE prevalence is striking, with a female to male ratio of 8:1. The prevalent of lupus in the general US population is 1 in 2000 but it varies among ethic groups (be more prevalent in non Caucasian than Caucasian populations) (Hochberg 1997).

Classification of SLE

The criteria for the classification of SLE promulgated by the American College of Rheumatology support the likelihood of many phenocopies for this phenotype (Tan, Cohen et al. 1982; Hochberg 1997). They allow the classification as SLE by satisfying any four of eleven criteria. The criteria are for the classification of the disease rather than for use as a diagnostic tool, although in practice there is a blurring of this distinction in systemic lupus erythematosus (Hochberg 1997).

Table 1. Classification criteria for SLE (Hochberg 1997)

1. Malar rash
2. Discoid rash
3. Photosensitivity
4. Oral ulcers
5. Arthritis
6. Serositis
    a) pleuritis, or
    b) pericarditis
7. Renal disorder
    a) proteinuria > 0.5g/24 h or 3+, persistenly,or
    b) cellular casts
8. Neurological disorder
    a) seizures or
    b) psychosis (having excluded other causes)
9. Hematological disorder
    a) haemolytic anemia or
    b) leucopaenia or < 4.0 x 10^9 /1 on two or more occasions
    c) lymphopaenia or < 1.5 x 10^9/1 on two or more occasions
    d) thombocytopaenia < 100 x 10^9/1
10. Immunological disorders
    a) raised anti-native DNA antibody binding or
    b) anti-Sm antibody or
    c) positive finding of anticardiolipin antibodies based on
        i. an abnormal serum level of IgG or IgM anticardiolipin antibodies
        ii. A positive test result for lupus anticoagulant using a standard method
        iii. A false-positive serological test for syphilis, present for at least 6 months
11. Anti-nuclear antibody in raised titer

Etiology

The etiology of SLE remains unknown. A genetic predisposition, sex hormones, and environmental trigger(s) likely result in the disordered immune response that typifies the disease.

A role for genetics is suggested by the increased percentage of two histocompatibility antigens in patients with SLE, HLA-DR2 and HLA-DR3. In addition, there is an increased frequency of the extended haplotype HLA-A1, B8, DR3. The role for heredity is further supported by concordance for this illness among monozygotic twins, the twins who shared the same environment. The disease concordance rate is 2-5% for dizygotics and 24-58% for monozygotic twins. This 10 fold difference in the disease concordance rate between identical twins and fraternal twins suggested that multiple genes shared between each pair of twins greatly influence the susceptibility to SLE. Because of the high concordance rate in monozygotics twins but less than 100%, non-genetics factors also contribute to the pathogenesis. Taken together, susceptibility to SLE is multifactorial, as result of the combination of hormonal factor, environmental factors, infection and genetic factor (Gaffney, Kearns et al. 1998; Lindqvist and Alarcon-Riquelme 1999; Nath, Kilpatrick et al. 2004).

Genetics of SLE

Several techniques including association studies with specific alleles and genome scans, have been used to analyze the genetic basis for SLE susceptibility in humans. The genetic approaches in SLE can be broadly divided into two strategies; the genome-wide linkage studies and hypothesis-driven candidate gene association analysis (Nath, Kilpatrick et al. 2004).

Genome-wide linkage studies

There are several different study design approaches with variable validation approaches that have been used for genome wide scanning to identify novel

susceptibility loci for SLE. Some of the study designs involve: sibling pairs, which might not have parental available; and small and large pedigrees with several generations available for study. Several genome scans have been carried out by the four scientific groups (located in California, Oklahoma, Minnesota and Sweden), revealing many susceptible loci spread across the genome (Gaffney, Kearns et al. 1998; Moser, Neas et al. 1998; Wakeland, Wandstrat et al. 1999).

To date, 13 major cytogenetic locations show significant evidence of linkage to SLE based on the recommended criteria for genome scan, and can be confirmed in independent sample. These key regions, together with several suggested regions identified by at least two independent groups of pedigrees, are summarized in the table 2. Not surprisingly linkages to many loci are ordinarily replicated across different population groups study sites. Among the identified linkages are eight SLE susceptibility regions that have also been replicated independently using lupus phenotype only. These are 1q23, 1q41, 2q33-37, 4p16, 6p21, 11p13, 12q24 and 16q13 (Moser, Neas et al. 1998; Nath, Kilpatrick et al. 2004). Each of these linkages is best-detected in families from single ethnicity or racial groups. In addition, some of these linked regions were linked to other autoimmune diseases, suggesting that the same genes can be involved in related disorders (Gaffney, Kearns et al. 1998; Moser, Neas et al. 1998; Lindqvist and Alarcon-Riquelme 1999; Tsao 2003).

Candidate gene studies in SLE

Link, however, is fundamentally a statistical process testing for the co-inheritance of genetic markers such as DNA polymorphisms, with the disease phenotype in families with multiple affected members. Consistent co-inheritance of the marker with the disease in many families indicated that it is close proximately to the actual disease gene, and might be in linkage disequilibrium which can provide evidence of gene identity than the assortment of individual alleles (Lindqvist and Alarcon-Riquelme 1999).

In the candidate gene analysis an allele or haplotype, or many DNA polymorphism, is directed assessed and, usually, a different in frequency of allele is demonstrated (hopefully repeatedly in independently ascertained samples) between

affected patients and appreciate controls. Therefore, genetic association with candidate gene suggests that the polymorphism(s) being measured is the actual disease-causing allele, or one located very closely to the responsible gene (Tsao 2003).

Because of the loss of immune tolerance to self components is the basis of SLE etiology, many genes encoding protein with regulatory or adaptive functions in the immune system have been considered as candidates. Several candidate genes have been studied and found to be associated with SLE (table 2). Some of the important candidate genes are discussed below (Nath, Kilpatrick et al. 2004).

Table 2. Association studies and candidate genes for SLE (Nath, Kilpatrick et al. 2004).

Gene	Cytogenetic location	Associated allele	Statistical significance
FcGR2A	1q22-23	R131	0.18(0.05-0.69)
FcGR3A	1q22-23	F176	x^2=9.87,p≤0.01
IL10	1q31-32	Multiple alleles	X^2=33.20,p<0.0001
CTLA-4	2q33	49G	p=0.003
PDCD-1	2q37	PD-1.3A	2.6(1.6-4.4)
HLA-DR-3,DR2	6p21	DR2/DR3	X^2=35.0/76.0, both p <0.0005
TNFα	6p21	TNF2	p=0.04,OR=1.6
TNFβ	6p21	TNFB'2	RR=3.4,p<0.0001
C4	6p21	AQ0	1.6(1.0-2.8)
MBL	10q11,2-q21	23QA	p<10-6
FASL	1q23	844c	p=0.0024
FAS	10q24	297C/416G	RR=5.0
Bcl-2	18q21	Multiple alleles	X^2=34.95,p=0.0001

One of the potencial susceptibility regions in humans SLE is located in 2q33. Recently, a linkage study located an association region in 2q34 in patient with autoimmune. In addition, lingkage to 2q34-35 (SLEN2) was found in patients with renal disease.

SLE is an autoimmune multisystem disorder characterized by the production of immunoglobulin G autoantibodies. Inappropriate T cell dependent expansion of autoreactive B cell is considered to play a role in the production of pathogenic autoantibodies against nuclear, cytoplasmic and cell-surface autoantigens (Hochberg 1997).

T cell activation requires two discrete signals: a signal delivered by the T- cell receptors and an accessory signal that occurs when costimulatory recepters interact with their ligands. CD28 a major costimulatory molecule, binds to CD80/CD86 on antigen presenting cells and delivers a potent costimulatory signal to T cells. CTLA-4, a related receptor of CD28, also binds to CD80/CD86 on antigen presenting cells but delivers negative signals to T cells, depending on both the T-cell activation state and strength of the T cell receptor signal. Thus, CD28 and CTLA-4 molecules regulate the immune responses to self and foreign antigens by controlling antigen –specific T cell activation (Chistiakov and Turakulov 2003; Vaidya and Pearce 2004).

The CTLA-4 molecule is homolog for CD28, and both molecules and their common ligands (B7-1 and B7-2) constitute the B7/CD28-CTLA-4 costimulatory pathway for T-cell activation. Whereas the CD28-ligand interaction plays a critical role in increasing and maintaining the T-cells response initiated through engagement of the T-cell antigen receptor, CTLA-4 ligand contribute to peripheral tolerance. The CTLA-4 gene is located with in the described risk region 2q33. Thus, CTLA-4 is a position and functional candidate gene for susceptibility to SLE (Harper, Balzano et al. 1991).

Structure and CTLA-4 gene

The Human CTlA-4 gene starts from 202 949-6 kb about 6.2kb on chromosome region 2q33. It exists as a single copy per haploid genome and consist of four exons and each exons encode for different protein according to their functions (Chistiakov and Turakulov 2003).

The 1ST exon encodes the the leader sequence of 37 amino acids

The 2nd exon encodes an immunoglobulin (Ig)V-like domain of 116amino acids

The 3rd exon encodes hydrophobic transmembrane region of 37 amino acids

The 4th exon encodes 34 amino acids cytoplasmic domain

Introns 1,2 and 3 span 2.5,0.5 and 1.1 kb respectively. The 5'region of the gene contains a Kozak consensus sequence with the TG iniatiaion codon, an in frame stop codon 26bp upstream of this ATG and TATA box 75bp upstream of stop codon(Harper 1991)(Chistiakov and Turakulov 2003).

CTLA-4 has two main isoforms;

1. Full-length CTLA-4 (ftCTLA-4)

2. Soluble isoform (sCTLA-4)

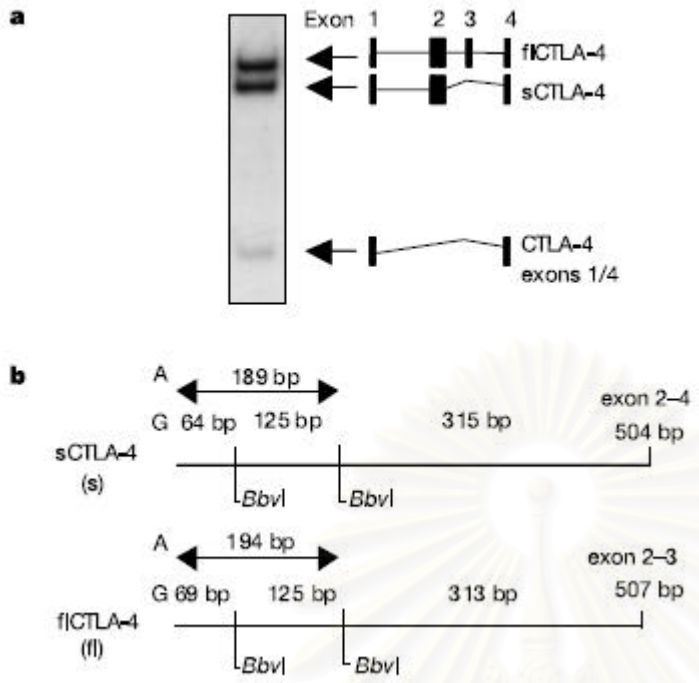


Figure 1. CTLA-4 isoforms (Ueda, Howson et al. 2003)

sCTLA-4 is generated by alternative splicing of CTLA-4 mRNA, which induces a frame shift by deletion of transmebrane region of CTLA-4 resulting in a native soluble protein(Magistrelli, Jeannin et al. 1999; Oaks, Hallett et al. 2000). sCTLA-4 is constituitively expressed on nonstimulated T –cell and its expression is downregulated after T-cell activation (Oaks, Hallett et al. 2000). The soluble form of surface protein is belived, in most cases to play an inhibitory role which due to competition for ligands with their surface counterparts. The finding that the sCTLA-4 expression level remains at sustained levels suggested that sCTLA-4 blocks the B7 mCTLA-4 interaction, therby enhancing T-cell and autoactivity by inhibiting the induction of anergy (Oaks, Hallett et al. 2000; Chistiakov and Turakulov 2003).

Role of CTLA-4 in autoimmunity

CTLA-4 helps regulating T-cells, CTLA-4 signaling mediates a negative regulator in both the cellular and humeral immune responses and mediates antigen-specific apoptosis. Loss of actively maintained tolerance to self-antigens and the generation of autoimmunity in the context of immune ignorance are possible mechanisms of autoimmunity(Kristiansen, Larsen et al. 2000).

CTLA-4 is important in peripheral tolerance and its dysregulation has the potential to affect the linkage at 2q33-35 in an interval that includes CTLA-4(Gaffney, Kearns et al. 1998; Quintero-Del-Rio, Kelly et al. 2002). The polymorphisms of the CTLA-4 gene have been associated with several autoimmune diseases suggesting a shared pathogenesis in autoimmune diseases such as type1, Graves'disease, rheumatoid arthritis(Kristiansen, Larsen et al. 2000).

Negative signaling via CTLA-4 plays an active role in regulating autoreactive T-cell. Disruption of the normal physiologic control provided by the CTLA-4 can contribute to the pathogenesis of autoimmune disease, as demonstrated by gene knockout studied in mice(Waterhouse, Penninger et al. 1995). Its predominant role in suppressing T-cell function is best reflected in the phenotype of CTLA-4-/- mice which rapidly develop lymphoproliferatioive disease with massive multiorgan lymphocyte infiltrations, tissuse destruction; splenomegaly, lymphadenopathy, and elevated serum immunoglbulin with early death (Tivol, Borriello et al. 1995; Waterhouse, Penninger et al. 1995; Chambers, Sullivan et al. 1997; Brunner, Chambers et al. 1999) .

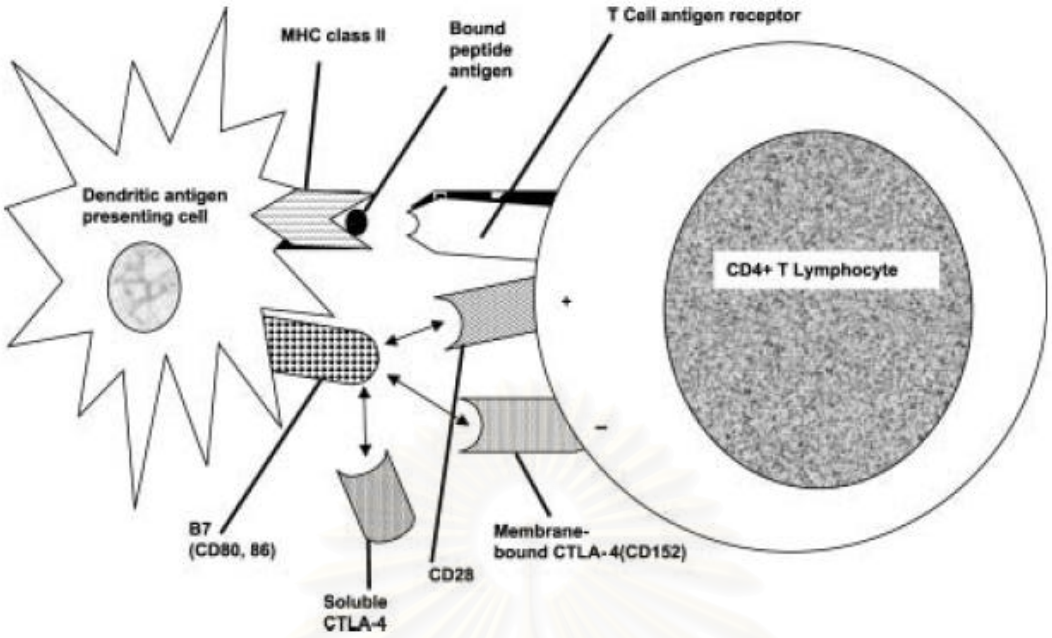


Figure 2. T-cell activation and co-stimulatory signals (Vaidya and Pearce 2004).

CTLA-4Ig (immunoglobulin) can suppress lupus-like illness in the New Zealand Black/New Zealand White (NZB/NZW)F1 mouse models and prolong life. CTLA-4 Ig is a chimeric fusion protein consisting of the CTLA-4 extracellular domain and the FC portion of human IgG. Variants in the expression, regulation, and ligand binding for CTLA-4 are, therefore strong candidates for a role in the pathogenesis of SLE(Mihara, Tan et al. 2000).

Four different polymorphisms on the CTLA.-4gene (-318C/T, -1722C/T, o+49 A/G and CT 60A/G) have been studied in various populations for an association with SLE. Of these, the most widely used SNP is the A/G polymorphism at position +49, which results in an amino acid change (Threonine to Alanine) in the leader sequence. Thus, this exon -1 +49A/G SNP may have a functional significance for susceptibility. Several laboratory experiment also suggested reduce up-regulation of CTLA-4 from individuals with GG at this locus upon T-cell activation(Kouki, Sawai et al. 2000; Anjos, Nguyen et al. 2002; Maurer, Loserth et al. 2002; Ueda, Howson et al. 2003). How the G allele at +49 is a risk for SLE is unclear. In the theory, reduced expression or function of CTLA-4 may lead to autoimmune T-cell proliferation and contribute to the pathogenesis of SLE. Because of the inhibitory role of the CTLA-4 in the immune response and observed hyperactivities of

both T and B cells in SLE, perhaps these polymorphism influences the expression of the CTLA-4.

Recently, a significant association of the CT60 (A/G) polymorphism with lupus was found in Spanish collection (Torres et al 2004). The CT60 polymorphism has also been reported to be associated with other autoimmune diseases, such as GD and type 1 diabetes families(Ueda, Howson et al. 2003). Moreover, the CT60 polymorphism also reported to have functional significance. Ueda and co-workers (2003) showed that the ratio of sCTLA-4 to full-length isoform (flCTLA-4) mRNA splice forms in unstimulated CD4 T cell is 50%lower in CT60 GG positive disease-susceptible individuals with AA protected individuals. This observation raises another possibility that the exon1 +49 association with disease might be a result of having strong linkage disequibrium (LD) with CT60(A/G) polymorphism.

Table 3. Characteristics of the individual studies with CTLA-4 polymorphism with SLE (ns= nonsignificance)

Study	Population	sample size		polymorphisms	Finding
		SLE	control		
(Mehrian, Quismorio et al. 1998)	Mexican	125	223	3'UTR	ns
(Matsushita, Tsuchiya et al. 1999)	Japanese	71	150	Exon 1+49A/G	ns
(Heward, Allahabadia et al. 1998)	English	124	355	promoter-318C/T	ns
(Heward, Gordon et al. 1999)	English	126	363	Exon 1+49A/G	ns
(Pullmann, Lukac et al. 1999)	Slovac	102	76	Exon 1+49A/G	OR for allele G>1, p=0.05
(D'Alfonso, Rampi et al. 2000)	Italian	99	99	Exon 1+49A/G	ns
(Liu, Wang et al. 2001)	Chinese	81	81	Exon 1+49A/G promoter-318C/T	genotype p=0.03
(Lee, Kim et al. 2001)	Korean	80	86	Exon 1+49A/G promoter-318C/T	+49A/G OR>1, p=<0.05
(Ahmed, Ihara et al. 2001)	Japanese	113	200	Exon 1+49A/G promoter-318C/T	OR for 49 allele G=1.72 (p=0.003)
(Hudson, Rocca et al. 2002)	Korean	130	200	Exon 1+49A/G promoter-318C/T	OR for -1722 allele T =2.06 (p=.00003)

Table 3 Characteristics of the individual studies with CTLA-4 polymorphism with SLE (ns= nonsignificance) (continue)

Study	Population	sample size		polymorphisms	Finding
		SLE	control		
(Aguilar, Torres et al. 2003)	Spanish	276	194	Exon 1+49A/G promoter-318C/T promoter-1722T/C	ns
(Takeuchi, Kawasugi et al. 2003)	Japanese	47	107	Exon 1+49A/G promoter-318C/T	ns
(Fernandez-Blanco, Perez-Pampin et al. 2004)	Spanish	214	235	promoter-1722T/C	OR for alleleC=2.45 (p=0.0004)
(Barreto, Santos et al. 2004)	Portugal	118	173	Exon 1+49A/G 3'UTR	Exon 1; ns OR for106bp allele=0.27(p <0.01)

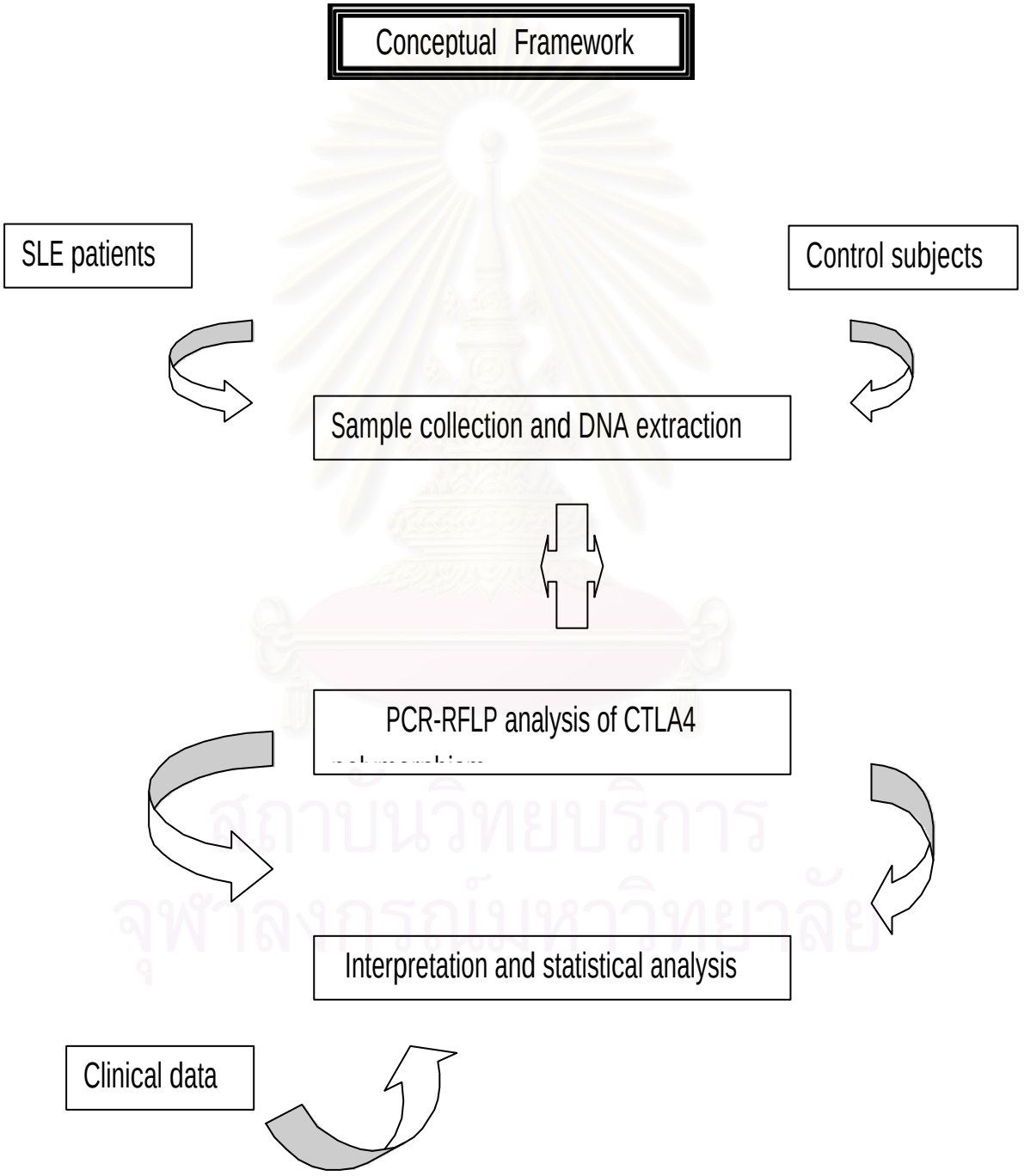
Table 4. The association studies of CT 60 polymorphism of CTLA-4 gene to autoimmune diseases.

Study	Population	sample size SLE	control	Finding
(Torres, Aguilar et al. 2004)	Spanish	SLE=395	N=293	OR for G allele=1.32 (1.06-1.65) P=0.01
(Ban, Tozaki et al. 2005)	Japanese	AITD=264	N=179	OR for G allele=1.5 P=0.009
(Suppiah, Alloza et al. 2005)	Flander	MS=120	N=120	OR for G allele=1.18 (0.83-1.69) P=0.361
(Orozco, Torres et al. 2004)	Spanish	RA=433	N=398	No significant
(Furugaki, Shirasawa et al. 2004)	Japanese	AITD=380	N=266	OR for G allele=1.4 (1.30-1.88) P=0.016
(Goswami, Gupta et al. 2005)	Indian	SIH=73	N=73	No significant
(Petrone, Giorgi et al. 2005)	Italian	GD=150	N=301	OR for G allele=1.42 (1.07-1.87) P=0.02

The aims of this study was to identify the suitable genetic marker within CTLA-4 gene that associate with the susceptibility to SLE in Thai population. Although there are many studies about association previously, variable results were obtained. Since genetic marker for SLE in each ethic group may be different and single SNP in different population can not be the best genetic marker to every population in this world. In this study we chose two SNPs in the CTLA-4 with the most functional significance(+49A/G in exon 1 and CT 60 A/G in 3' UTR) to identify genetic susceptibility to SLE in Thai population compared with normal controls.

CHAPTER IV

MATERIALS AND METHODS



Subjects

One hundred and fifty Thai patients from outpatient and inpatient service of King Chulalongkorn Memorial hospital, who fulfilled at least 4 of the American College of Rheumatology (ACR) revised criteria for SLE (Table 1), were included in this study. One hundred and fifty normal controls for the population based case-control association study were recruited from volunteer unrelated healthy donors. The ethics committee of the faculty of Medicine, Chulalongkorn University, Bangkok, Thailand approved the study and the subjects gave their informed consent.

DNA extraction

DNA was isolated from buffy coat collected with ethylenediaminetetraacetic acid (EDTA) as anticoagulant, using a salting out method (Miller, Dykes et al. 1988). For the genomic DNA extraction, 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 seconds and the supernatant was discarded to obtain the pellet. The pellet remaining should be white to pink. This step may be repeated if necessary. To this pellet, 200 μl nuclei lysis buffer (NLB) and 50 μl 10% SDS were added. Pellet was broken up with pipette tip and vortex to get powdery, tiny flakes. 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 °C for 2 hours. Precipitation of proteins was obtained by adding 175 μl of 5.3 M NaCl. This solution was centrifuged at 10,000-12,000 rpm for 15 minutes in micro centrifuge. After centrifugation, the DNA in the supernatant was precipitated in 1 ml of cold absolute ethanol. Invert 6-10 times to precipitate DNA, it will appear as a white to translucent stringy mass. 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 resuspend in 1 ml of cold 70% ethanol (break pellet by tapping), followed by centrifugation 1-2 minutes at 10,000-12,000 rpm and the supernatant was discarded to obtain the pellet. After removal of the ethanol, the pellet was

dried at 37 °C with the cap open to evaporate the ethanol. This pellet was dissolved in 200 µl of sterile distilled water, followed by incubation at 65 °C for 15 minutes. Use gentle vortexing to resuspend. If clumps of undissolved DNA are present, it will be in 65 °C until completely resuspended .

Genotyping methodology

Polymerase Chain Reaction-Restriction fragment Length Polymorphism Analysis of CTLA-4

We performed the Polymerase Chain Reaction-Restriction fragment Length Polymorphism (PCR-RFLP) analysis on the polymorphism of CTLA-4 at exon1 position +49A/G (Heward, Gordon et al. 1999) and CT 60A/G in 3'UTR as previously describe (Torres, Aguilar et al. 2004). The genomic DNA of 150 SLE patient and 150 healthy controls were amplified with the use of the CTLA-4 gene specific primers as shown in Table 5.

Table 5. Primer used for analysis of the CTLA-4 polymorphism at +49A/G position in exon 1 and CT60A/G

Gene polymorphism and primer	Sequence of primers (5' .....3')
+49A/G	
Sense	GCT-CTA-CTT-CCT-GAA-GAC-CT
Antisense	AGT-CTC-ACT-CAC-CTT-TGC-AG
CT60A/G	
Sense	CAC-CAC-TAT-TTG-GGA-TAT-ACC
Antisense	AGG-TCT-ATA-TTT-CAG-GAA-GGC

The reaction volume for the amplification reaction was 30 μl, containing 100 ng of genomic DNA, 0.15 μl of 5.0 Taq polymerase (Promega Madison WI, USA), 3 μl of 10x PCR buffer (20mM Tris-HCL pH 8.0, 100 mM KCL), 1.8 μl of 25 mM $MgCl_2$, 0.6 μl of 10 mM dNTP and 1.5 μl (20 pmol) of each oligonucleotide primer. PCR was carried out using Perkin Elmer/GeneAmp PCR system 2400 or Applied Biosystems/ GeneAmp PCR system 9600 (Applied Biosystems, USA) under specific PCR condition, that consisted of an initiation denaturation at 94 °C for 4 minutes followed by 30 cycles of denaturation (94 °C, 20 seconds), annealing (60 °C, 50 seconds) and extension (72 °C, 20 secounds) and final extension at 72 °C for 7 minutes. The resulting products were futher analyzed by electrophoresis in 1.5 % Tris-acetate agarose gel containing 50 μg/ml ethidium bromide, in Tris-acetate buffer at 100 volts for 40 minutes and visualized under UV light by Camera Gel Doc™ MZL (BIO-LAD, USA). Negative controls without DNA template were included in each experiment. A molecular ladder of 100-bp (Promega Madison WI, USA) was used to estimate the size of the PCR fragments. Successful amplification PCR products for 2 positions:

1.At position +A49(A/G)

PCR product size is 162 bp fragment and then, 10 μl of amplified DNA were digested with 5U of specific restriction enzyme *BbvI* (New England Biolabs, Hitchin, UK) in 1X NEBuffer 1 in a total volume of 15 μl at 37°C for 14-16 hours, followed by 3% agarose gel electrophoresis at 100 volts for 50 minutes . If an G was present at this position, the enzyme would cut the 162 bp PCR product into two fragment; 91 and 71 bp. No digestion would occur if a A was present. A molecular ladder of 100-bp (Promega Madison WI, USA) was used to estimate the size of the PCR fragments.

2. At position CT60(A/G)

PCR product size is 216 bp fragments and then, 10 μl of amplified DNA were digested with 10U of specific restriction enzyme *NcoI* (New England Biolabs, Hitchin, UK) in 1X NEBuffer 4 in a total volume of 15 μl at 37°C for 14-16 hours, followed by 3% agarose gel electrophoresis at 100 volts for 60 minutes. If a A was present at this position, the enzyme would cut the 216 bp PCR product into two fragment; 216 and 196 bp. No digestion would occur if a G was present. A molecular ladder of 100-bp (Promega Madison WI, USA) was used to estimate the size of the PCR fragments.

Additionaly, the selected PCR products were analyzed to confirm the results of 2 SNPs site for CTLA4 gene genotyping by DNA sequencing.

DNA sequencing

DNA sequencing were used to validate the results of CTLA-4 gene polymorphism by PCR-RFLP. For direct cycle sequencing, 40 μl of the PCR products were purified by the QIAquick PCR purification Kit (QIAGEN Inc., USA) to obtain clean double-standed DNA amplicated . Cycle sequencing was performed on an ABI prism 310 Genetic Analyzer using a cycle sequencing chemistry with base-specific fluorescence-labeled dideoxynucleotide termination reagents, BigDye Terminator Ready Reaction Mix (Applied Biosystems, USA). Thus, each sequencing reaction mixture of 10 μl final volume contained 2.5 μl of 5 pmol primer, 3 μl of template and 3 μl of BigDye Terminator Ready Reaction Mix. Each sample mixture was then subjected to a cycle sequencing reaction in a Perkin Elmer/GeneAmp PCR system 2400 or Applied Biosystems/ GeneAmp PCR system 9600(Applied Biosystems, USA). The condition of cycle sequencing reaction consisting of 25 cycles of denaturation at

96 °C for 30 seconds, annealing at55 °C for 10 seconds and extension at 60 °C for 4 minutes were carried out . Then each sequencing reaction product was pooled into 2 μl of 3 M sodium acetate (NaOAc, pH 4.6) and 50 μl of 95% ethanol (EtOH) mixture in 1.5 microcentrifuge tubes, incubated at room temperature for 15 minutes to precipitate the extension products and centrifuged at 13,000 rpm for 20 minutes. The products were washed with 70% ethanol (EtOH) and centrifuged for 5 minutes at 13,000 rpm. The DNA pellet was then dried by place the tubes with the lids open in a heat block or thermal cycler at 90°C for 1 minute. Finally, the samples were resuspended in 15 μl of TSR (template suppression reagent), heat the samples at 95°C for 2 minutes and then chill on ice. The samples were loaded into an ABI Prism 310 Genetic Analyzer. Data collection was performed using the software package provided with ABI 310 a sequencing system.

Statistical Analysis

Allele and genotype frequencies were compared between groups using the Chi-square (χ^2) test or Fisher's extract probability test, where appropriate. Gene frequencies were determined by gene counting. A *P* value of < 0.05 was considered significant. Odds ratio (OR) with 95% confidence interval (CI) were calculated using the statistical program Epi Info version 6(Centers for Disease Control and Prevention [CDC], 1994). The mode of inheritance analysis was also included. Furthermore, when one element in the 2x2 table (Chi-square (χ^2) test) was zero, OR was calculated with the Haldane' s modified formula [$RR_h=(2a+1)(2d+1)/(2b+1)(2c+1)$]. Haldane' s correxion for the OR was used when either all patients were positive or controls were negative for a particular specificity or allele (Haldane 1956)

Haplotype and linkage disequilibrium analysis

The program PHASE were used to reconstructing haplotypes from population genotype data (Stephens and Donnelly 2003). The software can deal with SNP, microsattellite, and other multi-allelic loci, in any combination and missing data are allowed. The remaining ambiguous sites are assigned by PHASE, and the uncertainly associated with each PHASE assignment is calculated (see Appendix V). In addition, linkage disequilibrium was calculated using the LDPlotter tool(http:/innateimmunity.net/llPGA2/Bioinformatics/index_html) (see Appendix D and E).

Table 6. Characteristics of healthy controls and patients with SLE

Characteristics	healthy controls	SLE
Number of patients	150	150
Female/males	85/58	146/4
Mean age±SD yr	26±13.8	36±11.9

CHAPTER V

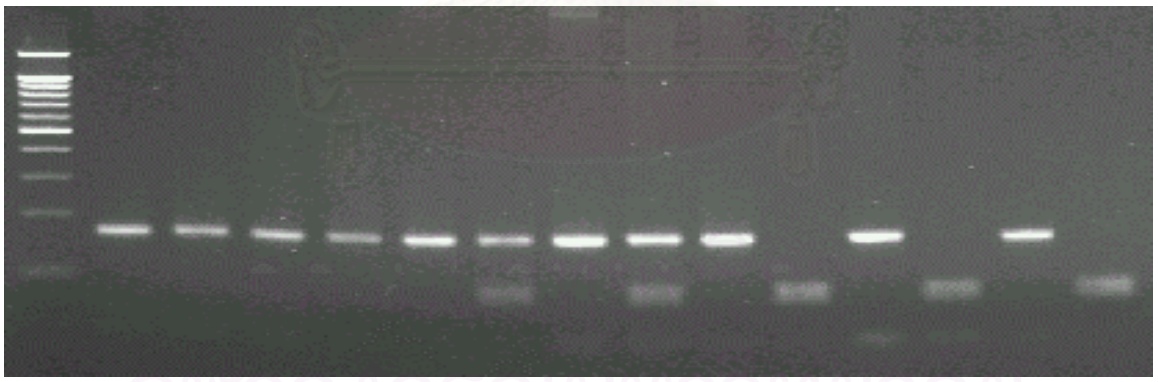
RESULTS

1. Polymerase Chain Reaction-Restriction Fragment Length polymorphism(PCR-RFLP) analysis of CTLA-4

1.1 PCR-RFLP analysis of CTLA-4 at position +49A/G

Polymorphism at +49A/G in exon 1 position were identified by the PCR-RFLP method. If a G was present at this position, the *BbvI* restriction enzyme would cut the 162 bp. PCR product into two fragments 88 bp and 74 bp. No digestion would occur if a A was present (Heward, Gordon et al. 1999)(Figure 3.)

Figure 3. The representative result from samples with homozygous of 49 A/A, homozygous of 49G/G and heterozygous of +49 A/G



Lane	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
		U	C	U	C	U	C	U	C	U	C	U	C	U	C

Lane 1 is 100bp molecular marker

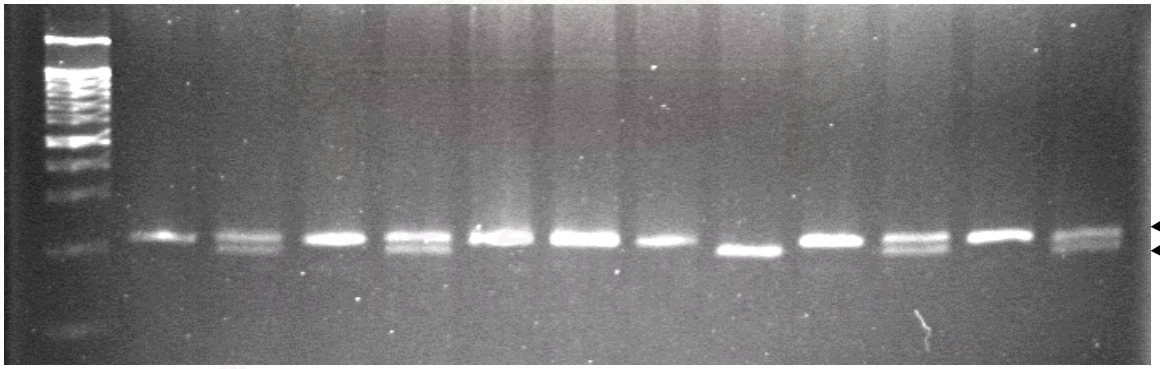
Lane 2, 4, 6, 8, 10, 12, 13 and 14 are samples without restriction enzyme

Lane 3, 5 are homozygous of+ 49 A/A
Lane 7, 9 are heterozygous of +49 A/G
Lane 11, 13 and 15 are homozygous of +49 G/G
U=not add restriction enzyme, C=add restriction enzyme

1.2 PCR-RFLP analysis of CTLA-4 at position CT60A/G in 3'UTR

Polymorphism of CT60A/G in 3'UTR position were identified by the PCR-RFLP method. If a A was present at this position, the *NcoI* restriction enzyme would cut the 216 bp PCR product into two fragments 216 bp and 196 bp. No digestion would occur if a G was present (Torres, Aguilar et al. 2004)(Figure 4).

Figure 4. The representative of PCR-RFLP results from samples with homozygous of CT60A, homozygous of CT60G and heterozygous of CT60A/G



Lane	1	2	3	4	5	6	7	8	9	10	11	12	13
		U	C	U	C	U	C	U	C	U	C	U	C

Lane 1 is 100bp molecular markers
Lane 2, 4, 6, 8, 10, 12 are samples without restriction enzyme

Lane 3, 5, 11 and 13 are heterozygous A/G
Lane 7 is homozygous G/G
Lane 9 is homozygous A/A

2. The association results of CTLA-4 gene polymorphism with SLE

We assessed the quality of the genotype data by testing for Hardy-Weinberg equilibrium in the control sample, using Chi-square (χ^2) test ($p>0.05$). There are no significant deviation from Hardy-Weinberg equilibrium in all SNPs in the study.

2.1 CTLA-4 gene exon1 polymorphism at position +49(A/G)

Genotype and allele frequencies for the +49(A/G) in the exon1 of CTLA-4gene in healthy controls and SLE patients were shown in table 7 and 8. Twenty-four of 150 healthy controls (16%) were homozygous for A/A genotype, 53(35.3%) were homozygous for G/G genotype and 73(48.7%) for heterozygous. The allele frequencies were 121(40.3%) for A allele and 179(59.7%) for G allele. In comparison, thirty-two of 150 SLE patients (21.33%) were homozygous for A/A genotype, 40(26.67%) were homozygous for G/G genotype and 78(52%) were heterozygous. The allele frequencies were 47.33% for A allele and 52.67% for G allele. There were no statistically significant difference in allele frequency of +49(A/G) polymorphism in the exon1 of CTLA-4 gene between SLE patients and healthy controls.

2.2 CTLA-4 gene polymorphism at position CT60 (A/G) in 3'UTR region

Genotype and allele frequencies for the CT60 in 3' UTR region of CTLA-4gene in healthy controls and SLE patients were shown in table 9 and 10. Eleven of 150 healthy

controls (7.3%) were homozygous for A/A genotype, 58(38.7%) were homozygous for G/G genotype and 81(54%) for heterozygous. The allele frequencies were 103(34.3%) for A allele and 197(65.6%) for G allele. In comparison, eight of 150 SLE patients (5.33%) were homozygous for A/A genotype, 69(46%) were homozygous for G/G genotype and 73 (48.67%) were heterozygous. The allele frequencies were 29.66% for A allele and 70.34% for G allele. There were no significant difference in allele frequency of CT60 (A/G) polymorphism in the 3'UTR region of CTLA-4 gene between SLE patients and healthy controls.

3. Haplotype analysis of CTLA-4 polymorphism at position (+49A/G and CT60(A/G)

The haplotype frequencies of CTLA-4 polymorphism were also calculated by PHASE program. The haplotype frequencies in patient s with SLE and normal controls were shown in table 12. We found 4 haplotypes; AA, AG, GG and GA in patients with SLE and normal controls. After comparing haplotype frequencies of the CTLA-4 polymorphism (position +A49A/G and CT60 A/G) between normal controls and SLE patients, the GG haplotype was fond to be significantly associated in patients with SLE compared with normal controls as protective haplotype(p=0.0209,OR=1.49,95%CI=1.06-2.10) but we found that +49A;CT60G haplotype according to model of inheritance was associated with SLE patients when with controls(p=0.039,OR=1.49,95%CI=1.06-2.10). (table 13)

3.1 The linkage disequilibrium coefficients (|D'| and r^2) among CTLA-4 SNPs

We performed the LD by using the plotter, the |D'| and r^2 among both SNPs is 0.3570 and 0.8230 as shown in table 14.

4. Pattern of CTLA-4 gene polymorphism in various population

This study will provide the basic knowledge of allele distribution for CTLA-4 gene polymorphism in healthy controls Thai individuals, as compared with other populations from previous reports.

4.1 Pattern of CTLA-4 gene polymorphism at position +49A/G in exon 1 in various populations.

The analysis of +49A/G in exon 1 showed no significant difference in the genotype distribution between study population in Thai, Chinese and Japanese. There were significant difference in allele frequencies between Thai population with Caucasian (Table 15).

4.2 Pattern of CTLA-4 gene polymorphism at position CT60 in 3'UTR in various populations.

The analysis of CT60 in 3'UTR showed no significant difference in the distribution between study population in Thai and Japanese. There were significant difference in allele frequencies between Thai population with Spanish, Italian, Flander, and Indian .(Table 16)

5. The association results of CTLA-4 (+49A/G and CT60) gene polymorphisms with clinical manifestation SLE

We analyze the association between clinical manifestation inpatients with SLE and polymorphism of CTLA-4 (+49A/G and CT60) by using chi-square test and odds ratio.

5.1 Clinical manifestation of SLE patients

The clinical expression of SLE is tremendously varied among individuals. In this study, we obtained clinical data of 107 patients, as shown in table 17 and 18.

5.2 +49A/G in exon 1 polymorphisms and clinical presentation of SLE

There is weakly significant association between A dominance mode of inheritance with Malar rash (OR=2.61, 95%CI=1-6.85, p=0.0496) in table 19 and 20.

5.3 CT60 in 3'UTR polymorphisms and clinical presentation of SLE

There is weakly significant association between A dominance mode of inheritance with Malar rash (OR=2.64,95%CI=1.1-6.38,p=0.02) in table 21 and 22.

Table 7. Genotype and allele frequencies for CTLA-4 polymorphisms at position +49 in exon 1 in healthy control and SLE patients (ns= non significant)

+49 A/G	SLE N=150	NORMAL N=150
Genotype frequencies		
A/A	32(21.33%)	24(16%)
G/G	40(26.67%)	53(35.3%)
A/G	78(52%)	73(48.7)
Allele frequencies		
A	142(47.3%)	121(40.3%)
G	158(52.7%)	179(59.7%)

ns

Table 8. Risk of SLE associated with CTLA-4(+49 A/G) genotype according to different models of inheritance

+49 A/G	SLE N=150	NORMAL N=150
G dominance, A wild type		
G/G or A/G	118(78.7%)[a]	126(84%)
A/A	32(21.3%)	24(16%)
G recessive, A wild type		
G/G	40(26.7%)	53(35.3%)
A/A or A/G	110(73.3%)	97(64.7%)

ns

Table 9. Genotype and allele frequencies for CTLA-4 polymorphisms at position CT60A/G in 3' UTR in healthy control and SLE patients

CT60A/G	SLE N=150	NORMAL N=150
Genotype frequencies		
A/A	8(5.33%)	11(7.3%)
G/G	69(46%)	58(38.7%)
A/G	73(48.67%)	81(54%)
Allele frequencies		
A	89(29.66%)	103(34.3%)
G	211(70.34%)[a]	197(65.6%)

P^a=0.255, OR=1.24, 95%CI=0.87-1.77

Table 10. Risk of SLE associated with CTLA-4(CT60A/G) genotype according to different models of inheritance

CT60A/G	SLE N=150	NORMAL N=150
G dominance, A wild type		
G/G or A/G	142(94.7%)[a]	139(92.6%)
A/A	8(5.3%)	11(7.3%)
G recessive, A wild type		
G/G	69(46%)[b]	58(38.7%)
A/A or A/G	81(54%)	92(61.3%)

P^a=0.63, OR=1.40, 95%CI=0.51-1.20
P^b=0.242, OR=1.35, 95%CI=0.83-2.20

Table 11. Haplotype frequencies of the CTLA-4 polymorphism at position +49A/G in exon 1 and CT60A/G in 3'UTR between normal controls and SLE patients

Haplotype frequencies	SLE patients n=300	normal controls n=300
AA	90(30%)	82(27.3%)
AG	53(17.6%)	39(13%)
GG	152(50.66%)	178(59.3%)
GA	5(1.6%)	1(0.4%)

Table 12. Association of CTLA-4(+49A/G,CT60A/G) between healthy controls and SLE patients

Haplotype frequencies	SLE patients. n=300	normal controls n=300	p-values
AA/other haplotypes	90	82	*p*=0.527 OR=0.1.14 (0.79<OR<1.65)
AG/other haplotypes	53	39	*p*=0.140 OR=1.44 (0.90<OR<2.30)
GG/other haplotypes	152	178	*p*=0.040 OR=0.70 (0.50<OR<0.95)
GA/other haplotypes	5	1	*p*=0.218 OR=5.07 (0.58<OR<115.32)

Table 13. Risk of SLE associated with CTLA-4(+49 A/G.CT60A/G) haplotype according to different models of inheritance

haplotypeA49,CT60	SLE N=150	Normal N=150	p-value
GG,GG	36	52	p=0.057**
AG,GG	23	27	p=0.642
GA,GG	4	-	p=0.131
AG,AG	6	-	p=0.039*
AA,GG	53	47	p=0.540
AA,GA	1	1	p=0.470
AA,AG	18	12	p=0.335
AA,AA	9	11	P=0.816

Table 14.Linkage disequilibrium coefficients (|D'| and r^2) among CTLA-4 SNPs

|D'| r^2	+ 49A/G	CT 60
+ 49A/G	-	0.8230
CT 60	0.3570	-

Table15. Comparison between genotype and frequencies of CTLA-4(+49A in exon 1) gene polymorphism in the different population

Cytokine	Position	Genotype	Control						
		Author	Heward	Pullman	Liu	Ahmed	Matsushita	Aguilar	This study
		Year	1999	1999	2001	2001	1999	2003	2005
		Ethnic	English[a]	Slovac[b]	Chinese[c]	Japanese[d]	Japanese[e]	Caucasian[f]	Thai
		N	363	76	81	233	150	194	150
CTLA-4	+49	A/A	164(45.1%)	41(53.9%)	10(12%)	33(16.5%)	22(15%)	110(56.7%)	24(16%)
	(exon1)	A/G	171(47.1%)	31(40.8%)	50(62%)	105(52.5%)	72(48%)	67(34.5%)	73(48.7%)
		G/G	28(7.8%)	4(5.3%)	21(26%)	62(31%)	56(37%)	17(8.8%)	53(35.3%)
		Allele							
		A	498(68%)	113(74.3%)	70(43%)	171(57.2%)	116(38.7%)	101(26%)	121(40.7%)
		G	228(32%)	39(25.7%)	92(57%)	229(42.8%)	184(61.3%)	287(74%)	179(59.7%)

[c,d,e] The genotype distribution is not significantly different when compared with Thai.

[a] The genotype distribution is significantly different when compared with Thai (χ^2=75.99,p<0.00000001)

[b] The genotype distribution is significantly different when compared with Thai (χ^2=44.02,p<0.00000001)

[f] The genotype distribution is significantly different when compared with Thai (χ^2=69.47,p=0.00000001)

Table 16. Comparison between genotype and allele frequencies of CTLA-4 CT60 gene polymorphism in the different population

Cytokine	Position	Genotypes	Control						
		Author	Orozco	Goswami	Suppiah	Petrone	Yoshiyuki	Torres	This study
		Year	2004	2005	2005	2005	2005	2004	2005
		Ethic	Spanish	Indian[b]	Flander[c]	Italian[d]	Japanese*	Spanish[e]	Thai
		N	398	228	120	301	179	293	150
CTLA-4	CT60	A/A	98(24.6%)	88(38.6%)	31(25.8%)	83(28%)	14(7.8%)	83(28.3%)	11(7.3%)
	(3' UTR)	A/G	199(50%)	92(40.4%)	57(47.5%)	149(49%)	70(39.1%)	147(50.2%)	81(54%)
		G/G	101(25.41%)	48(21%)	32(26.7%)	69(23%)	95(53.1%)	63(21.5%)	58(38.7%)
		Allele							
		A	395(49.6%)	134(58.8%)	119(49.6%)	315(52%)	98(27.4%)	313(53.4%)	103(34.3%)
		G	409(50.4%)	94(41.2%)	121(50.4%)	287(48%)	260(72.6%	273(46.6%)	197(65.65%)

* The genotype distribution is not significantly different when compared with Thai.

[a] The genotype distribution is significantly different when compared with Thai (χ^2=47.46,p<0.05)

[b] The genotype distribution is significantly different when compared with Thai (χ^2=47.46,p<0.05)

[c] The genotype distribution is significantly different when compared with Thai (χ^2=18.10,p=0.00011745)

[d] The genotype distribution is significantly different when compared with Thai (χ^2=28.89,p=0.00000053)

[e] The genotype distribution is significantly different when compared with Thai(χ^2=31.59,p=0.00000014)

Table 17. Clinical manifestation of patients with SLE in this study

Clinical manifestation	No. of patient withs SLE(%)
1. Malar rash	66(61.68%)
2. Discoid rah	33(39.2%)
3. Photosensitivity	47(43.9%)
4. Oral ulcers	1(0.93%)
5. Pleuritis	2(1.87%)
6. Pericarditis	66(61.68%)
7. Proteinuria	9(8.41%)
8. Seizures	4(3.739%)
9. Psychosis	1(0.93%)
10. Anemia	70(65.42%)
11. Leukemia	51(47.66%)
12. Lymphopenia	61(57%)
13. Thrombocytopenia	8(7.47%)
14. Anti-DNA antibodies	31(28.97%)
15. Anti-Sm antibodies	6(5.6%)
16. Anti-cardiolipin antibodies	4(3.73%)

.

Table 18. Comparison between genotype of CTLA-4 polymorphism and clinical manifestations.

Clinical manifestation (N)	+49A/G Genotype Total n=107			CT60 Genotype Total n=107		
	A/A	A/G	G/G	A/A	A/G	G/G
Malar rash(66)	16	37	13	4	39	23
Discoid rash(33)	4	18	11	2	17	14
Oral ulcer(47)	11	25	11	3	27	17
Photosensitivity(42)	8	25	9	-	28	14
Pleuritis(1)	-	1	-	-	1	-
Pericarditis(2)	1	1	-	1	1	-
Proteinuria(66)	17	33	16	4	35	27
Seizure(4)	2	1	1	1	2	1
Psychosis(6)	2		-	-	4	2
Anemia(70)	18	37	15	4	35	31
Leukopenia(51)	13	26	12	3	28	20
Lymphopenia(61)	12	30	19	1	33	27
Thrombocytopenia(8)	1	3	4	4	4	-
Anti-DNA antibodies(31)	4	15	12	1	13	17

*There is not significant after correction for multiple comparison.

Table 19. Genotype and allele frequencies for CTLA-4(+49A/G) polymorphism in exon 1 in SLE patients with and without Malar rash

	SLE patients with Malar rash n=66	SLE patients without Malar rash n=41
Genotype frequencies		
A/A	16(24.24%)	7(17%)
A/G	37(56%)	18(44%)
G/G	13(19.7%)	16(39%)
Allele frequencies		
A	69(52.3%)	32(39%)
G	63(47.7%)	50(61%)

ns

Table 20. Risk of Malar rash associated with CTLA-4(+49A/G) genotype according to different models of inheritance.

	SLE patients with Malar rash n=66	SLE patients without Malar rash n=41
G dominance, A wild type		
G/G or A/G	50(75.8%)	34(83%)
A/A	16(24.2%)	7(17%)
G recessive, A wild type		
G/G	13(20%)	16(39%)
A/A or A/G	53(80%)	25((61%)

ns

Table 21. Genotype and allele frequencies for CTLA-4(CT60) polymorphism in 3'UTR in SLE patients with and without Malar rash

	SLE patients with Malar rash n=66	SLE patients without Malar rash n=41
Genotype frequencies		
A/A	4(6%)	2(4.8%)
A/G	39(59%)	15(36.5%)
G/G	23(35%)	24(59.7%)
Allele frequencies		
A	47(35.6%)	19(23%)
G	85(64.4%)	63(77%)

ns

Table 22. Risk of Malar rash associated with CTLA-4(CT60) genotype according to different models of inheritance

	SLE patients with Malar rash n=66	SLE patients without Malar rash n=41
G dominance, A wild type		
G/G or A/G	62(94%)	39(95.2%)
A/A	4(6%)	2(4.8%)
G recessive, A wild type		
G/G	23(34.9%)	24(58.5%)
A/A or A/G	43(65.1%)	17(41.5%)

ns

CHAPTER VI

DISCUSSION

Systemic lupus erythematosus (SLE) is a human prototype of autoimmune disease, characterized by sustained abnormal immune activation and autoantibodies production. Genetic factors are known to play role in the disease. SLE is genetically complex, with contribution anticipated from environmental and stochastic factors in the pathogenesis of SLE (Hochberg 1997).

Loss of actively maintained tolerance to self-antigens and the generation of autoimmunity in the context of immune ignorance are possible mechanisms of autoimmunity. CTLA-4, which is important in peripheral tolerance and dysregulation, has the potential of affect the pathogenesis of SLE. CTLA-4 signaling mediated negative regulation in both the cellular and the humeral immune responses and also mediated antigen specific apoptosis. Chromosome 2q33 which contained CTLA-4 gene is linked to may autoimmune diseases included SLE. CTLA-4 polymorphisms have been studied with several autoimmunity such as GD, RA, Type 1DM, multiple sclerosis and SLE (Kristiansen, Larsen et al. 2000). The most widely reported SNP is the A/G polymorphism at position of exon 1 in CTLA-4 gene which results in an amino-acid change (threonine to alanine) in the leader sequence.

In the theory, reduced expression or function of CTLA-4 may lead to autoimmune T-cell proliferation and contribution to the pathogenesis of SLE. Because of the inhibitory role of CTLA-4 in the immune response and observed hyperaction of both B and T cell in SLE, perhaps this polymorphism influence the expression of CTLA-4.

Although the +49A/G polymorphism of the CTLA-4 is associated with GD, Type1 Diabetes, most association with SLE show negative result (table 3). We found no association between +49A/G and SLE in this study. This finding is in correlation with most studies that found no association of the +49A/G in exon 1 of CTLA-4 gene (Heward, Gordon et al. 1999; Matsushita, Tsuchiya et al. 1999; Pullmann, Lukac et al. 1999; D'Alfonso, Rampi et al. 2000; Aguilar, Torres et al. 2003; Takeuchi, Kawasugi et al. 2003; Barreto, Santos et al. 2004).

Lack of association between the +49A/G polymorphism with SLE may be explained by many reasons. First the polymorphism not is being in linkage disequilibrium with a predisposing allele of a disease causing mutation in SLE. This possibility is based on one assumption that the threonine to alanine subtibution is not affect the function of the leader peptide or simply because of this CTLA-4 gene polymorphism is not sufficient to influence disease expression in SLE. Another possibility might be due to the difference in the enrolled patient since SLE is a heterogenous disease and gene polymorphism might correlated with some specific clinical features. However, meta-analysis can demonstrate a positive association between +49A/G allele and SLE susceptibility, although most single studies reported negative result (Lee, Harley et al. 2005). This observation might suggest that the effect of +49 G allele is very small.

Recently, genotyping data in multiple SNPs obtained in a study of other autoimmune disease (Graves' disease, autoimmune hypothyroidism, and type 1 diabetes) suggest the presences of a common locus of susceptibility in the 6.1kb 3' region of CTLA-4 gene. The strongest association in the region corresponds to the marker CT60(SNP 3087243) which is located more than 800 nucleotide after the(AT)n microsatellite region, whereas association with the promoter and exon 1 region was ruled out . The CT60 allelic variation was reported to be correlated with lower mRNA levels of the soluble form of CTLA-4 (sCTLA-4), suggesting that differential expression of alternatively spliced forms of CTLA-4 might have an important role in determining susceptibility to autoimmune diseases. The

CTLA-4 protein has sCTLA-4 (soluble) and flCTLA-4 (full length) isoform and their mRNA ratio is 50% lower in unstimulated CD4+ T-cell in autoimmune disease-susceptible individuals with G alleles at CT60SNPs. The lower levels of sCTLA-4 would leave a larger number of these B7 ligand molecules free for binding to CD28. A significantly higher frequency of G allele (autoimmunity predisposed) at CTLA-4 +49A/G and CT60 A/G sited has been reported in patients with autoimmune thyroid disease when compared to healthy controls (Ueda, Howson et al. 2003; Vaidya and Pearce 2004). After the paper was published there were studies of the association of CT60 in 3'UTR polymorphism to various autoimmune disease such as RA, Type 1 Diabetes, MS and SLE (Aguilar, Torres et al. 2003; Ban, Tozaki et al. 2005; Suppiah, Alloza et al. 2005). Most studies report that CT60 G allele is associated with susceptibility to disease(table 4). Our results do not support an association between CT60A/G SNPs polymorphism and susceptibility to SLE in Thai population. However after performing haplotype analysis, we found that the +49A;CT60G, especially in recessive mode of inheritance is a risk genotype in our population similar to haplotype study in Spanish population (table). We also found that +49G;CT60G is a protective haplotype for SLE in our population. Therefore, our study did not support the role of CT60 G allele as causative genetic factor for SLE. Infact, later functional study cannot confirm the direct role of CT60 marker on activity and expression of CTLA-4 (Anjos, Nguyen et al. 2002). More studied focusing on CT60 and SLE and meta-analysis should be done to confirm this hypothesis.

To further study, other markers within CTLA-4 possibly closely linked with +49A;CT60G haplotype that contribute to disease susceptibility should be further characterized to find the most suitable marker in Thai population. Second, we should study the functional of both SNPs CTLA-4(+49A/G and CT60) in Thai population to demonstrate that both SNPs did not associate to function in Thai.

When we performed the analysis between CTLA-4 genetic marker and clinical manifestation, none of the comparison shows statistically significant result after the

correction of multiple comparison. This is not unexpected result because CTLA-4 has important role regulation of immunity, so it is an important susceptibility gene for autoimmunity rather than gene that determine specific clinical manifestation.

We also compare genotype and allele frequency of both SNPs in CTLA-4 with results from other populations. The genotype distribution is not significantly different between Chinese, Japanese and Thai but there were significantly different when compared with English, Slovac and Spanish in +49A/G polymorphism. When we compared genotype and allele frequencies of CT60, we fond there is no significantly different between Thai and Japanese but the different in genotype were found when compared with Spanish, Indian, Flander and Italian.

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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
0.5 M EDTA pH 8.0	100	ml

Adjust volume to 1 liter with distilled water. The solution was mixed and sterilized by autoclaving at 121°C for 15 min.

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. 1.5% Agarose gel

Agarose	0.3	g
1x TAE	20	ml

Dissolve by heating in microwave oven and occasional mix unit 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 ml of distilled water. Adjust pH to 7.2. The solution was mixed and sterilizes by autoclaving at 121°C for 15 min. Keep refrigerated. Shelf life is approximately 6 months.

2. Nuclei Lysis Buffer (NLB)

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

Adjust volume to 100 ml with distilled water. Adjust pH to 7.2. Keep refrigerated. Shelf life is approximately 6 months.

3. 1 M Tris

Tris base	12.11	g
Distilled water	100	ml

Adjust volume to 100 ml with distilled water. Adjust pH to 8.0. The solution was mixed and sterilizes by autoclaving at 121°C for 15 min.

4. 5 M NaCl

NaCl	29.22	g
Distilled water	100	ml

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

5. EDTA

EDTA	37.22	g
Distilled water	200	ml

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

6. 5.3 M NaCl

NaCl	15.5	g
Distilled water	50	ml

Adjust volume to 50 ml with distilled water. The solution was mixed and sterilizes by autoclaving at 121°C for 15 min.

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 sterilizes by autoclaving at 121°C for 15 min.

APPENDIX C

Haplotype analysis by PHASE

PHASE program is produced by the Mathematical Genetics Group, University of Oxford, Oxford, UK. The software is available online at http://www.stats.ox.ac.uk/mathgen/ (Stephens and Donnelly 2003)

Input file format

The input file is supplied by the user to specify how many individuals there are to be analyzed, how many loci/sites each individual has been typed at, What sort of loci/sites these are (SNP or microsatellite), and the genotypes for each individual. The default format input file, as illustrated in the accompanying file test.inp . The default structure for the input file can be represented as follows:

NumberOfIndividuals

NumberOfLoci

P Position (1) Position (2) Position (NumberOfLoci)

LocusType (1) LocusType (2) ...LocusType (NumberOfLoci)

ID (1)

Genotype (1)

ID (2)

Genotype (2)

:

:

:

:

:

ID (numberOfIndividuals)]

Genotype (NumberOfIndividuals)

Where the quantities above are as follows:

-Number of Individuals An integer specifying the number of individuals who have been genotyped.

-Number Of Loci An integer specifying the number of loci or sites at which each individual has been typed.

-P The character ‘P’ (upper case, without quotations marks) .

-Position (i) A number indicating the position of locus I, relative to some arbitray reference point (typically in units of base pairs ,but any unit can be used: if you use a unit other than base pairs, see the documentation on the –R option). The loci must be in their physical order along the chromosome (ie these positions must be increasing).

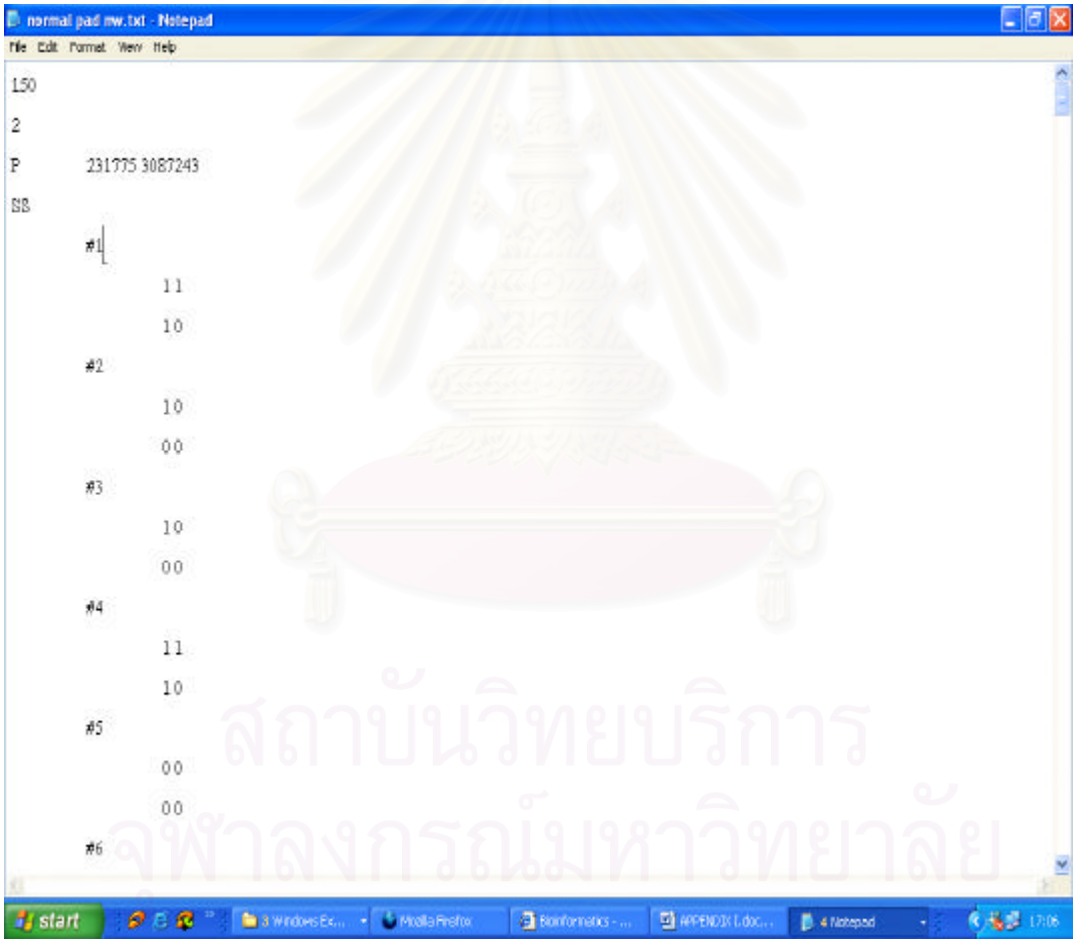
-LocusType (i) A letter indicating the type of locus i. The options are (a) S for a biallelic (SNP) locus, or biallelic site in sequence data. (b) m for microsatellite, or other multi-allelic locus (eg trallelic SNP, or HLA allele). The default assumption is that this denotes a microsatellite locus with stepwise mutation mechanism.

-ID (i) A string, giving a lable for individual I .

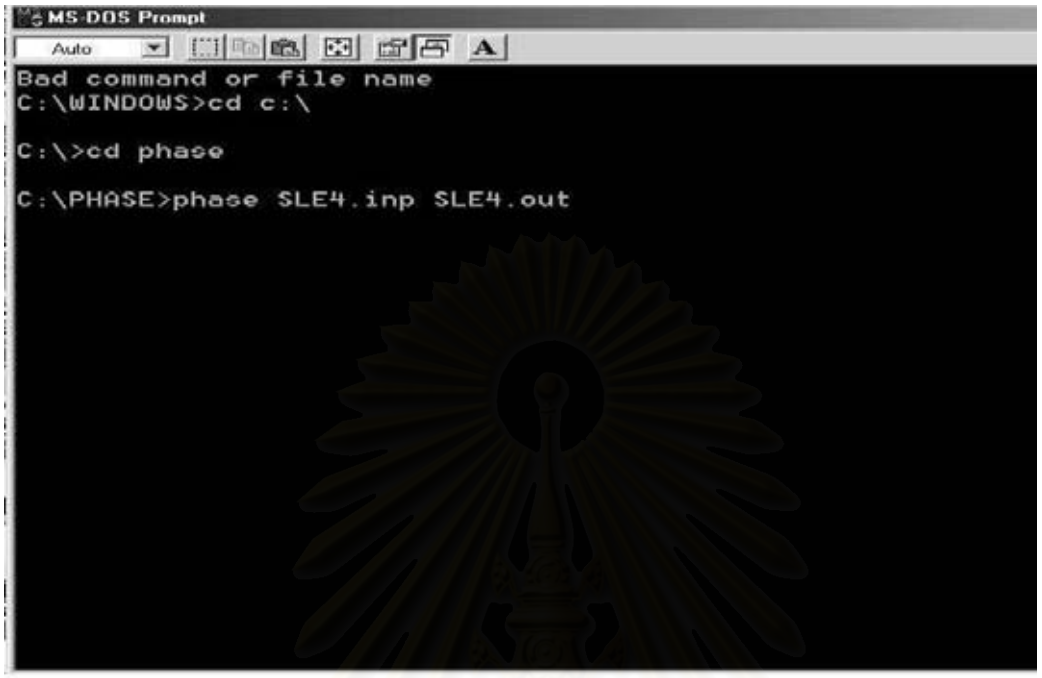
-Genotype (i) The genotypes for the ith individual. This is given on two consecutive rows. At each locus, one allele is entered on the first row, and one on the second row. It does not matter which allele is entered on each row. For biallelic loci, any two characters (e.g. A/C, G/T, 0/1) can be used to represent the two alleles, and they do not need to be separated by a space. Missing alleles at SNP loci should be entered as? . For multiallelic loci a positive integer must be used for each allele (representing the number of repeats at microsatellite loci), and data for each locus should be separated by a space. Missing alleles at multialleleic loci should be represented by –1 .

This study, considered the example input file, SLE.inp and Normal.inp, which is as follows:

The example of input file (SLE.inp) was shown below .



Running of PHASE program was shown below.



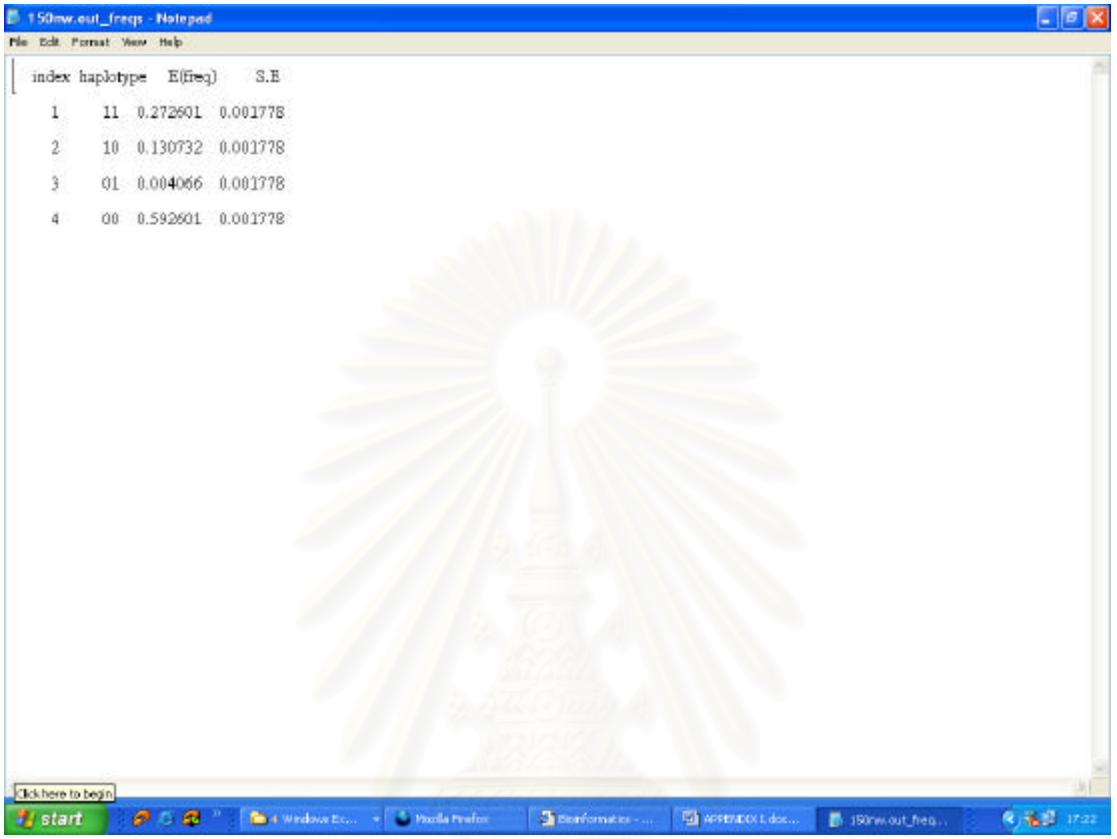
Output file

When run, the program initially outputs the data it has read from input file . The program produces a number of output files. The first, which has the user-specified name, and a similar format to previous versions of PHASE, contains a summary of the individual haplotype estimates for each individual.

Type of output file, which were used in this study, consists of Two types .

1. Output frequencies : defined as how many haplotype in this study group and used the number (frequencies) to estimate how many the people ,who have these haplotypes .

The example of output file (output frequencies) was shown below .



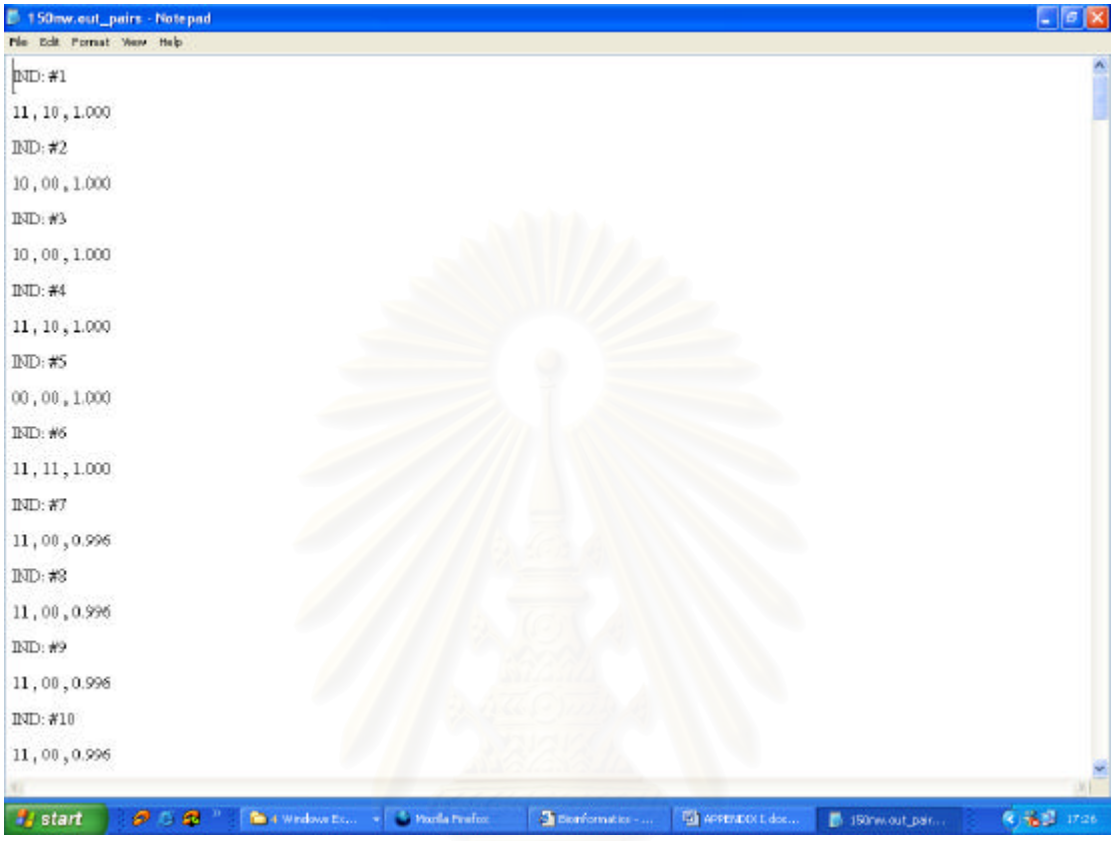
150nw.out_freqs - Notepad

File Edit Format View Help

index	haplotype	E(freq)	S.E
1	11	0.272601	0.001778
2	10	0.130732	0.001778
3	01	0.004066	0.001778
4	00	0.592601	0.001778

2. Output pairs: defined as the individual haplotype

The example of output file (outputpairs) was shown below.



APPENDIX D

Linkage disequilibrium analysis

The values of linkage disequilibrium (LD) were determined using LDplotter (). The LDPlotter tool allowed conversation of a Lickerson Lab Prettybase format file into aplot showing pairwise LD of various types (r^2, r, D' and absolute D).

Example

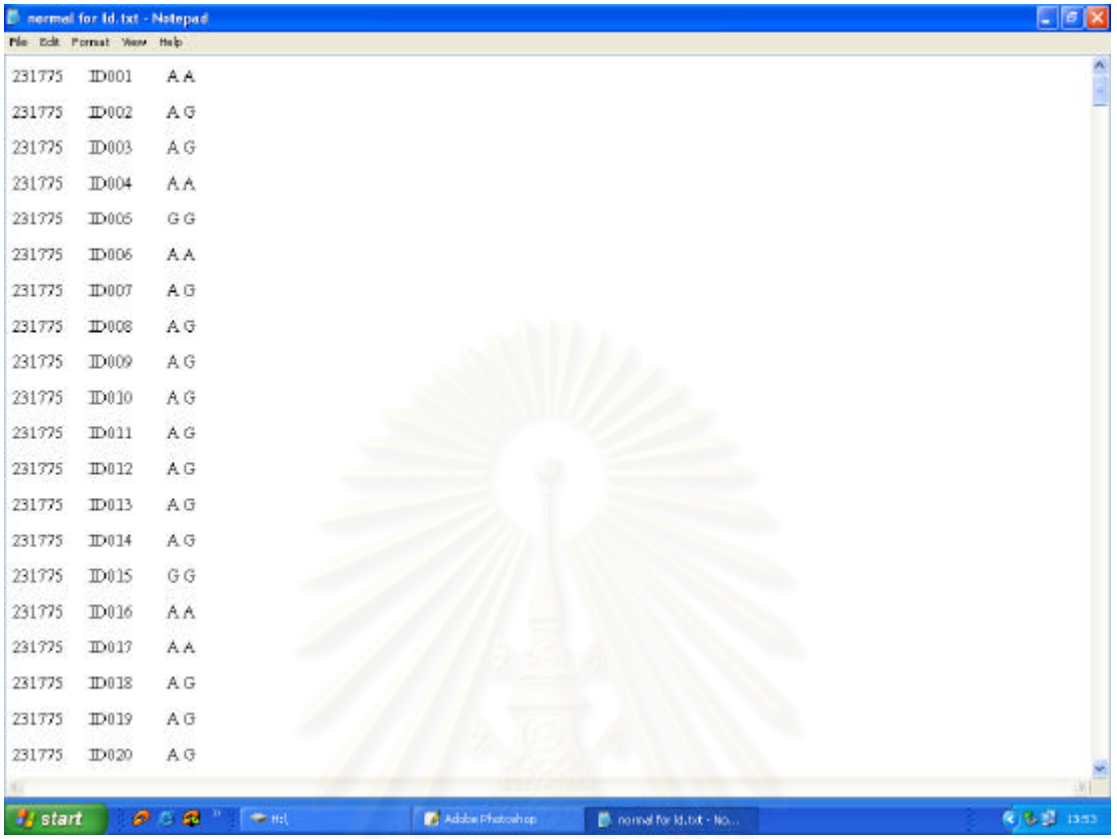
This example will walk through the generation of a plot like the one below:

I. Genename

The gene name can automatically be included in the plot title, and is also used to label the optional SNP Map which is drawn along the diagonal of the LD Plot. You can change the gene name in the gray box below to anything you like:

II. Prettybase

Input is a standard prettybase file. This study, consider the example input file, total 2 position (+49A/G and CT60A/G)



III. LD Type

Indicates which measure of LD you would like to plot:

- ☐ $r^2 = D^2/P_A * P_B * (1 - P_A) * (1 - P_B)$
- ☐ $r = sqrt(r^2)$
- ☐ $D' = D/D_{max}$
- ☐ $abs(D) = | P_{AB} - (P_A * P_B) |$

IV. Configure Populations

This text area allows you to configure how the LD Plotter will split the samples in the prettybase file into different populations. The field should have one line for each population represented in your dataset. Each line should be a population identifier, followed by a colon, followed by a description of the population. Leaving this textarea

empty will indicate that you do not wish to partition your sample set, but instead, would like to consider all of the samples to be part of a single population.

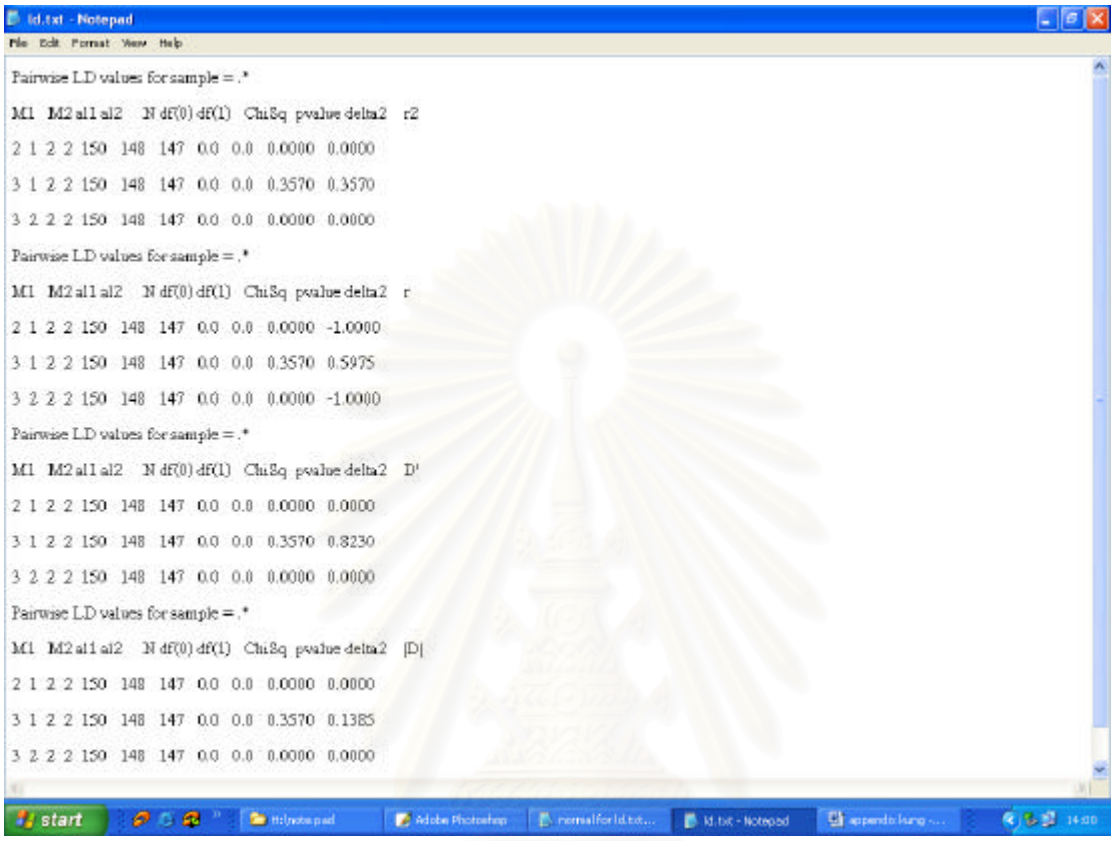
V. Plot Title

The plot title has two modes of operation, one for simple use, and the other for more advanced purposes for users who are familiar with Python format strings, and would to specify exactly what the plot title should look like.

VI. Miscellaneous

- Miniraf: You can set an arbitrary threshold for minimum allele frequence. The default value of 0.0 will not exclude SNPs based on rare frequency. If you specify a higher value, SNPs with frequency for the rare allele below this threshold in any for the rare population will NOT appear in the plot.
- Color Scheme: Several color schemes can be used to indicate the extend of LD between two loci.

The output file: The output file (total 2 positions.txt) was shown below.



Linkage disequilibrium coefficients (|D'| and r^2) among CTLA-4 SNPs

|D'| / r^2	+ 49A/G	CT 60
+ 49A/G	-	0.8230
CT 60	0.3570	-

APPENDIX E

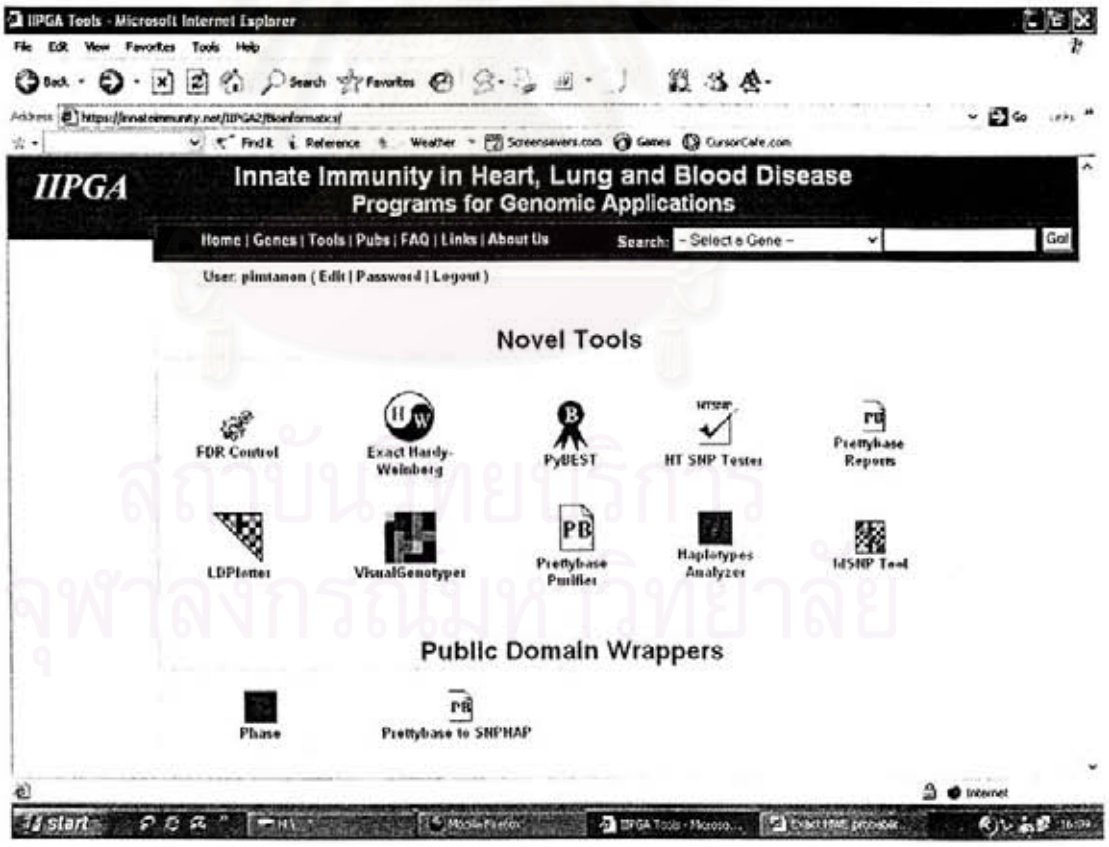
Exact Hardy-Weinberg Equilibrium Test

Exact Hardy-Weinberg Equilibrium Test for genotype counts, biallelic locus. This program will estimate the statistical significance of a test that the genotype counts are in Hardy-Weinberg equilibrium.

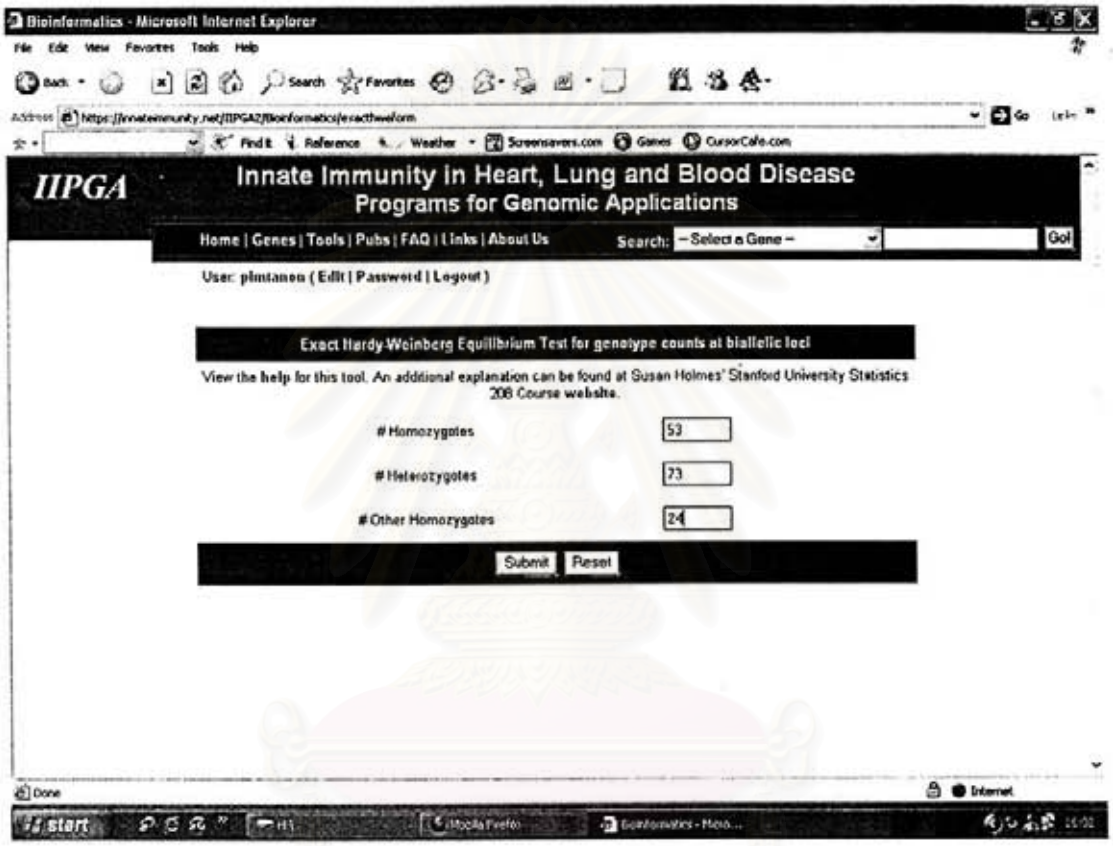
The software is available online at

I Counts

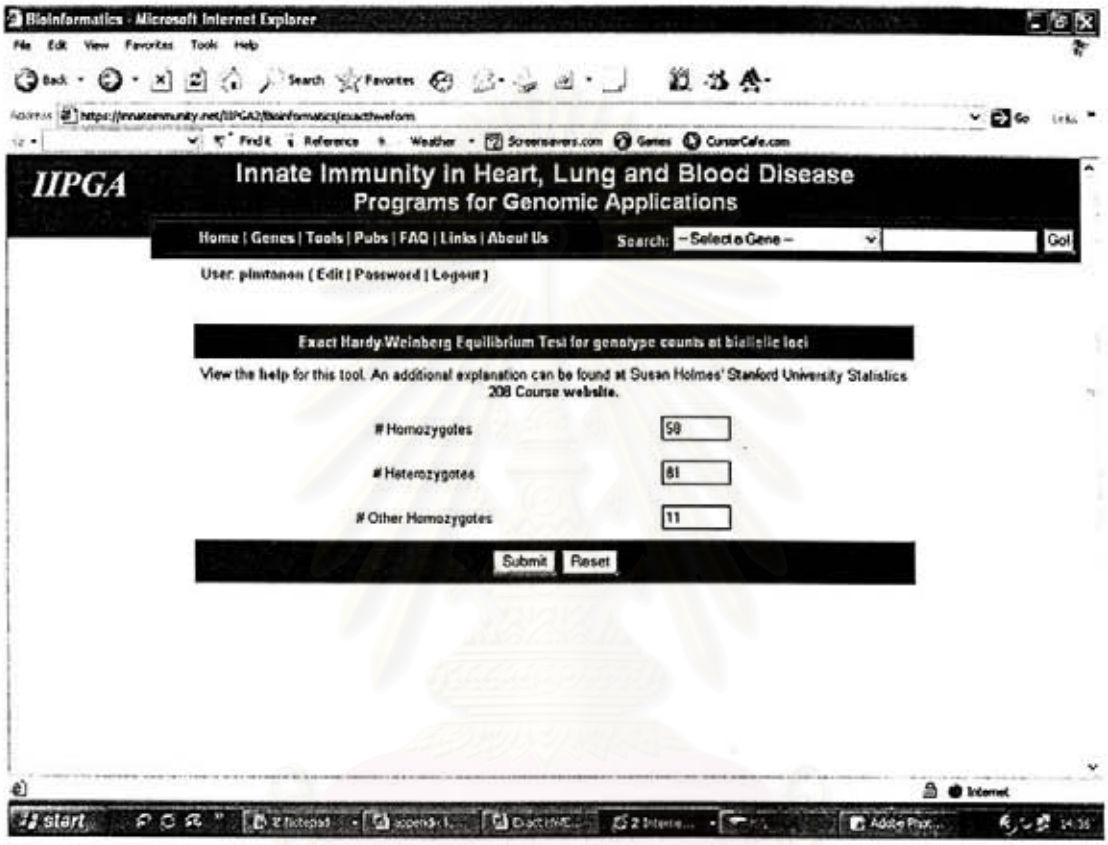
The input to this tool is simply three integer counts representing the number of heterozygotes, common homozygotes, and rare homozygotes at a given locus in your data set.



This study, the genotype counts of CTLA-4(+49A/G) was shown

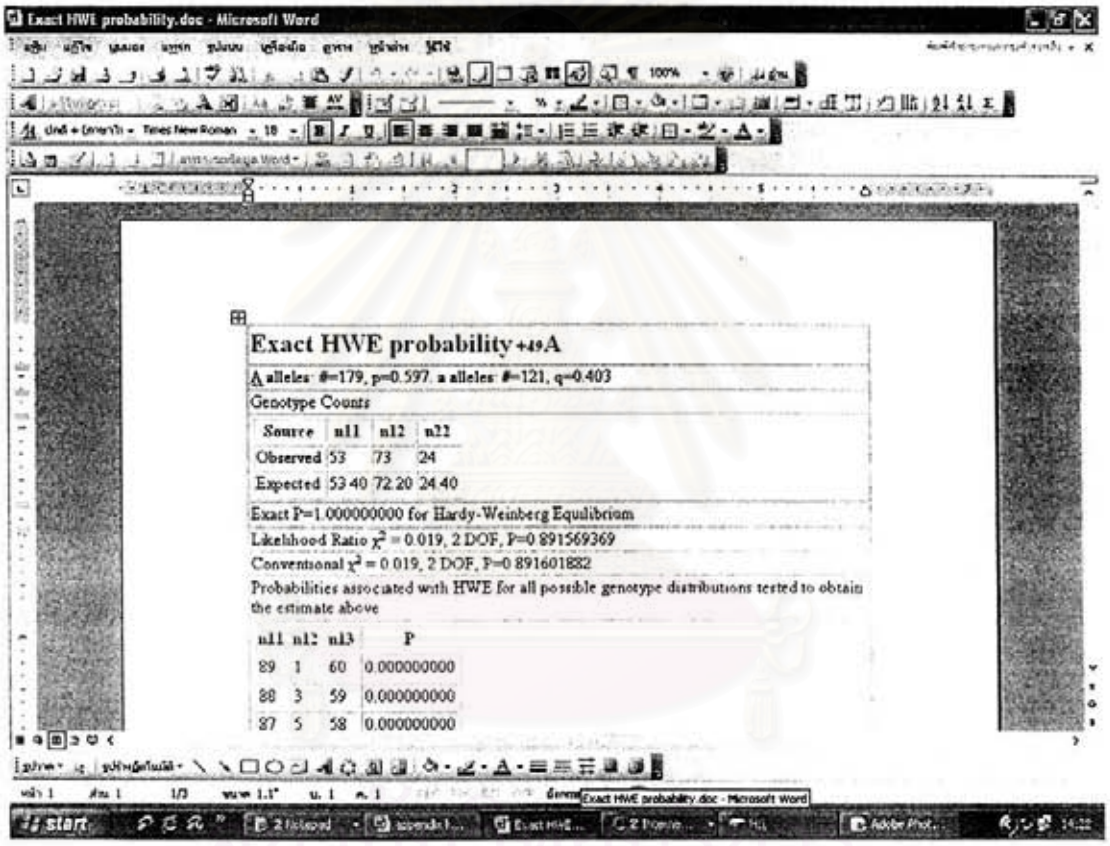


The genotype counts of CTLA-4 (CT60) was shown



When run, the program will estimate the statistical significance of a test that the genotype counts are in Hardy-Weinberg equilibrium. The output file of CTL-4 (+49A/G and CT60) was shown below.

The output file of CTLA-4 (+49A/G) was shown below.



Exact HWE probability.doc - Microsoft Word

Exact HWE probability +49A

A alleles: #=179, p=0.597. a alleles: #=121, q=0.403

Genotype Counts

Source	n11	n12	n22
Observed	53	73	24
Expected	53.40	72.20	24.40

Exact P=1.000000000 for Hardy-Weinberg Equilibrium

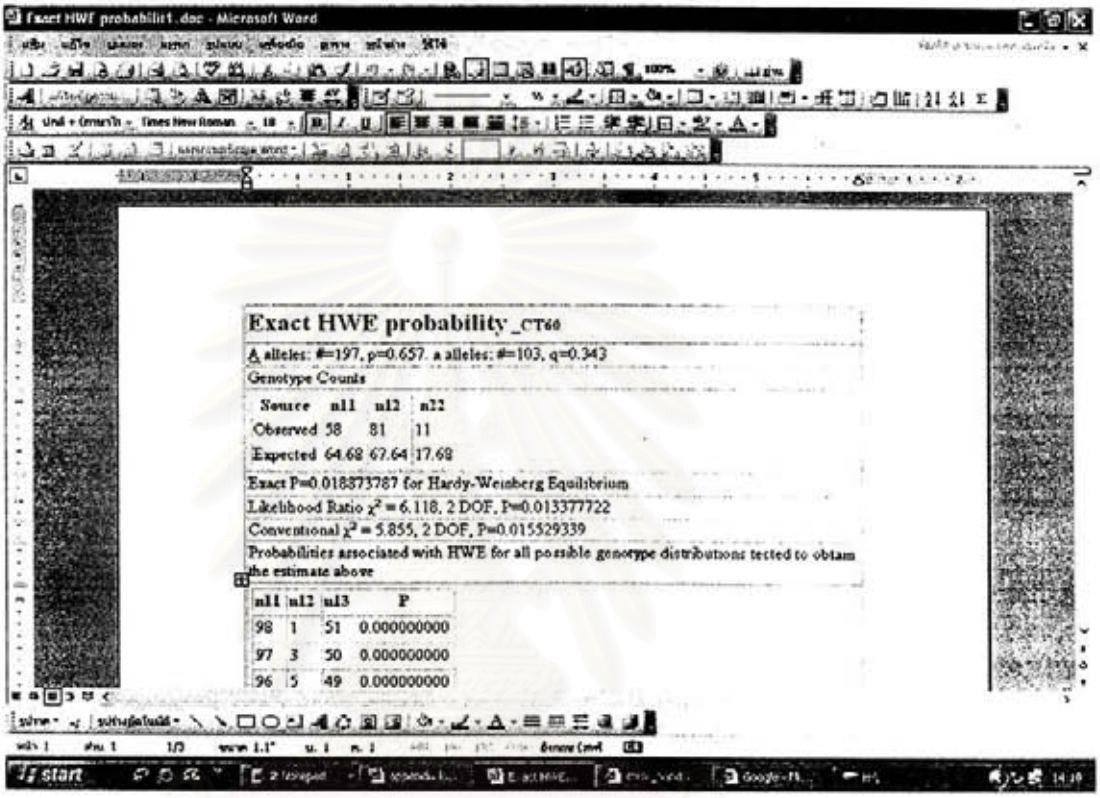
Likelihood Ratio χ^2 = 0.019, 2 DOF, P=0.891569369

Conventional χ^2 = 0.019, 2 DOF, P=0.891601882

Probabilities associated with HWE for all possible genotype distributions tested to obtain the estimate above

n11	n12	n13	P
89	1	60	0.000000000
88	3	59	0.000000000
87	5	58	0.000000000

The output file of CTLA-4 (CT60) was shown below.



Exact HWE probability _CT60

A alleles: #=197, p=0.657. a alleles: #=103, q=0.343

Genotype Counts

Source	n11	n12	n22
Observed	58	81	11
Expected	64.68	67.64	17.68

Exact P=0.018873787 for Hardy-Weinberg Equilibrium

Likelihood Ratio $\chi^2 = 6.118$, 2 DOF, P=0.013377722

Conventional $\chi^2 = 5.855$, 2 DOF, P=0.015529339

Probabilities associated with HWE for all possible genotype distributions tested to obtain the estimate above

n11	n12	n13	P
98	1	51	0.000000000
97	3	50	0.000000000
96	5	49	0.000000000

BIOGRAPHY

Miss Siriwalee Sae-Ngow was born on April 24, 1979 in Nakorn Srithammarath, Thailand. She graduated with the Bachelor degree of Doctor of Veterinary Medicine from Chulalongkorn University in 2003, and then attended to particulate in Medical Microbiology program, Graduate School, Chulalongkorn University for her master degree.