# ความสัมพันธ์ระหว่างยีน FcγReceptor และยีน IL-10 กับการเกิดโรค SLE ในประชากรไทย

นางสาวแจนจุรีย์ เนตรสว่าง



วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
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# THE ASSOCIATION BETWEEN FcγRECEPTOR AND IL-10 GENE WITH SLE DISEASE IN THAI POPULATION

**Miss Janjuree Netsawang**

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| By | Miss Janjuree Netsawang |
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| Thesis Advisor | Assistant Professor Nattiya Hirankarn, M.D. Ph. D. |
| Thesis Co-advisor | Associate Professor Orrawadee Hanvivatvong |
| | Associate Professor Somchai Akkasilpa, M.D. |

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Accepted by the Graduate School, Chulalongkorn University in partial Fulfillment of the Requirements for the Master's Degree

…….. Dean of the Graduate School
(Assistant Professor M. R. Kalaya Tingsabadh, Ph.D. )

THESIS COMMITTEE

…… Chairman
(Associate professor Somatat Wongsawang, Dr. med. Vet )

……. Thesis Advisor
(Assistant Professor Nattiya Hirankarn, M.D. Ph. D.)

……... Thesis Co-Advisor
(Associate Professor Orrawadee Hanvivatvong)

……... Thesis Co-Advisor
(Associate Professor Somchai Akkasilpa , M.D.)

…… Member
( Panjong Hanvivatkul, M.D)

แจนจุรีย์ เนตรสว่าง : ความสัมพันธ์ระหว่างยีน FcγReceptor และยีน IL-10 กับการเกิดโรค SLE ในประชากรไทย (The Association Between IL-10 gene and FcγRIIa gene with SLE disease in Thai population )
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โรค SLE เป็นต้นแบบของโรคภูมิต้านทานเนื้อเยื่อต้นเอง ซึ่งสาเหตุของโรคนั้นยังไม่ปรากฏแน่ชัด แต่จากหลักฐานบอกว่า SLE เป็นโรคที่มีความซับซ้อน ซึ่งประกอบด้วยปัจจัยทางพันธุกรรมจากยีนหลายๆชนิด และปัจจัยทางสิ่งแวดล้อม การศึกษาทางพันธุกรรมทั้งในหนู และในคนสนับสนุนถึงความสำคัญของโครโมโซมที่ 1 ซึ่งมียีนที่น่าสนใจอยู่หลายชนิด เช่น FcγRIIa ที่มีบทบาทในการกำจัด immune complex และ IL-10 ก็มีบทบาทส่งเสริม B cell ทำงานมากผิดปกติ ทำให้มีการสร้าง autoantibody เพิ่มขึ้น ความหลากหลายของยีน FcγRIIa และ IL-10 อาจมีผลต่อความสามารถในการทำลาย immune complex และปริมาณไซโตไคน์ ตามลำดับ งานวิจัยนี้มีวัตถุประสงค์เพื่อศึกษาความหลากหลายของยีน FcγRIIa และ IL-10 ระหว่างผู้ป่วยโรค SLE เปรียบเทียบกับคนปกติ และศึกษาความสัมพันธ์กับการเกิด และ/หรือความรุนแรงของโรค SLE ในคนไทย โดยใช้การศึกษาแบบ population-based case-control รวบรวมผู้ป่วย 150 คน และคนปกติ 150 คน ซึ่งมีเชื้อสาย และถิ่นกำเนิดเดียวกัน ใช้วิธี PCR-SSP และ PCR-RFLP หารูปแบบความหลากหลายของยีน FcγRIIa และ IL-10 ตามลำดับ ผลการศึกษาพบรูปแบบของ R131 allele ของยีน FcγRIIa และ ACC haplotype ของยีน IL-10 ในผู้ป่วย SLE มากกว่าคนปกติอย่างมีนัยสำคัญทางสถิติ (OR= 1.57, 95%CI= 1.08-2.27, $p$= 0.01 และ OR= 1.57, 95%CI= 1.05-2.34, $p$= 0.02 ตามลำดับ) ที่สำคัญคือ การศึกษานี้เป็นการศึกษาแรกที่รายงานบทบาทที่เสริมกัน (Synergistic) ระหว่าง R131 allele และ ACC haplotype กับการเกิดโรค SLE (OR= 10.93, 95%CI= 1.29-242.64 , $p$= 0.006) นอกจากนี้ยังพบความสัมพันธ์ระหว่าง GCC haplotype กับอาการทางไต (OR =7.45, 95%CI= 1.04-154.1, $p$ = 0.02) และรูปแบบของยีนที่เป็น RR มีความสัมพันธ์กับการผลิต anticardiolipin antibody (OR=6.09, 95%CI= 1.38-30.54, $p$= 0.004) โดยสรุปคือ รูปแบบความหลากหลายที่เป็น ACC haplotype ในส่วน promoter ของ IL-10 และ R131 ของยีน FcγRIIa สามารถใช้เป็นเครื่องหมายของยีนที่กำหนดความเสี่ยงในการเกิดโรค SLE ในคนไทย โดยเฉพาะอย่างยิ่งคนที่มีทั้ง 2 รูปแบบ ส่วนความรุนแรงของโรคเช่น ไตอักเสบ อาจจะทำนายได้โดยรูปแบบ GCC haplotype ของยีน IL-10 และการผลิต anticardiolipin antibody ก็อาจจะทำนายได้โดยรูปแบบของยีน FcγRIIa ที่เป็น RR จากผู้ป่วย SLE

สาขาวิชา จุลชีววิทยาทางการแพทย์ ลายมือชื่อนิสิต.....
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KEYWORD : SYSTEMIC LUPUS ERYTHEMATOSUS/ IL-10 GENE POLYMORPHISM / FCγRIIA GENE POLYMORPHISM / SYNERGISTIC EFFECT AND LUPUS NEPHRITIS
JANJUREE NETSAWANG : THE ASSOCIATION BETWEEN FCγRECEPTOR AND IL-10 GENE WITH SLE DISEASE IN THAI POPULATION. THESIS ADVISOR : ASSIST.PROF.NATTIYA HIRANKARN, MD,Ph.D. THESIS CO-ADVISOR : ASSOC. PROF. ORRAWADEE HANVIVATVONG , ASSOC. PROF. SOMCHAI AKKASILPA.

Systemic lupus Erythematosus (SLE) is a prototype of systemic autoimmune disease. However, etiology of disease remains unclear. Consideral evidence suggests that SLE is a complex disease with a strong genetic basis, contributed from multiple genes as well as environmental factors. Genetic studies in both murine and human supported the importance of chromosome 1, in which several candidate genes were reported. For example, FcγRIIa plays a vital role in immune complex clearance and IL-10 promotes B cell hyperactivity leading to autoantibody production. Since polymorphisms in FcγRIIa and IL-10 gene may affect to the binding capacity and a quantity of cytokine production, respectively. The aim of this study was to identify the polymorphisms of FcγRIIA and IL-10 genes in patient with SLE compared with control group and to determine the association with disease susceptibility and/or severity of SLE in Thai population. This population-based case-control study included 150 SLE patients and 150 healthy control subjects with similar ethnic and genetic background. FcγRIIa and IL-10 gene polymorphisms were identified by PCR-sequence specific primer (SSP) and PCR-restriction fragment length polymorphism (RFLP) , respectively. The result of this study demonstrated that FcγRIIa R131 and ACC haplotype of IL-10 were found to be significantly increased in SLE patients compared to healthy controls (OR= 1.57, 95%CI= 1.08-2.27, *p*= 0.01 and OR= 1.57, 95%CI= 1.05-2.34, *p*= 0.02, respectively). Importantly, for the first time, synergistic between ACC haplotype and R allele in susceptible to SLE was observed (OR= 10.93, 95%CI= 1.29-242.64 , *p*= 0.006). In addition, there was an association between IL-10, GCC haplotype and renal involement (OR =7.45, 95%CI= 1.04-154.1, *p* = 0.02). FcγRIIa, RR homozygotes was also associated with anticardiolipin antibodies production (OR=6.09, 95%CI= 1.38-30.54, *p*= 0.004). In conclusion, ACC haplotype of IL-10 gene and FcγRIIa R131 polymorphism might be regarded as marker for genetic susceptibility to SLE in Thai population, particularly individuals carrying both of them . Severity of disease, such as lupus nephritis can be predicted by IL-10, GCC haplotype marker and anticardiolipin antibodies production can be predicted in FcγRIIa, RR homozygotes in SLE patient.

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Student's signature ……
Advisor's signature ……...
Co-advisor's signature……
Co-advisor's signature……

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# ABBREVIATIONS

| | |
|---|---|
| Ag | Antigen |
| Ab | Antibody |
| APCs | antigen-presenting cells |
| Bp | base pair |
| CD | Cluster of Differentiation |
| C | Complement |
| °C | degree Celsius |
| dsDNA | double–standed deoxyribonucleic acid |
| et al | et alii |
| ELISA | Enzyme-linked immunosorbent assay |
| FcγR | Fc gamma Receptor |
| HLA | Human Leukocyte Antigen |
| IgG | Immunoglobulin G |
| IL | Interleukin |
| KDa | Kilodalton |
| l | litter |
| λs | sibling risk ratio |
| LD | Linkage Disequilibriam |
| μl | microlitter |
| μg | microgram |
| ml | milliliter |
| mM | millimolar |
| MW | molecular weight |
| ng | nanogram |
| NK | Natural Killer |
| OD | Optimal Density |
| OR | Odds Ratio |
| PCR | Polymerase Chain Reaction |
| RFLP | Restriction Fragment Length Polymorphism |
| SSP | Sequence Specific Primer |
| SDS | Sodium Dodecyl Sulphate |

| | |
|---|---|
| SLE | Systemic Lupus Erythematosus |
| SNP | Single Nucleotide Polymorphism |
| TNF | Tumor Necrosis Factor |
| Th-1 | T helper –1 |
| U | Unit |

# CHAPTER I

# INTRODUCTION

Systemic Lupus Erythematosus (SLE), the prototype of human immune complex disease, is characterized by tissue deposition of circulating antigen-antibody complexes leading to release of inflammatory mediators and influx of inflammatory cells. SLE patients have symptoms that involve multi organ system with diverse clinical manifestations, including glomerulonephritis which is a common severe complication (Cochrane and Koffler 1973; Tsao 2002). SLE is a worldwide disease with a prevalence of approximately 1 in 2000 general US population but it varies among ethnic groups. SLE commonly presents in women (female:male = 9:1), and is 2- to 4-fold higher prevalence and severity in non-Caucasian as compared with Caucasian population (Hochberg 1997). Although, etiology of the disease remains unclear, various investigations indicate that both genetic and environmental factors contribute to disease susceptibility(Peterson K 2001). In particular, twin studies show an overall 10-fold greater risk for disease concordance in monozygotic twins compared with dizygotic twins (Deapen, Escalante et al. 1992). This data is consistent with the possibility of a strong genetic contribution to SLE, and suggest that a genetic approach to lupus may provide important insights into the disease etiology.

About 10 years ago, studies in lupus murine model have demonstrated an important of susceptible loci on chromosome 1 and polygenic complexity of SLE (Morel, Mohan et al. 1999). Similarity, the initial linkage studies in human have demonstrated significant linkages of at least 13 genomic intervals located in 5 autosomal chromosomes. The most consistent reports of disease susceptible genes in both murine and human SLE lie in the region 1q21-44 (Wakeland, Liu et al. 2001). Several candidate genes have been examined in the past including human leukocyte antigen(HLA) class II genes (Paisansinsup, Vallejo et al. 2001; Graham, Ortmann et al. 2002; Sirikong, Tsuchiya et al. 2002), complement genes (Arnett and Reveille 1992; Bowness, Davies et al. 1994), poly-ADP-ribosyl transferase (PARP) genes (Tsao, Cantor et al. 1999; Graham, Langefeld et al. 2001), tumor necrosis factor-α (TNF-α) genes (Hajeer, Worthington et al. 1997; Sullivan,

Wooten et al. 1997) and mannose-binding protein (MBP) genes (Davies, Snowden et al. 1995; Sullivan, Wooten et al. 1996) etc. Nevertheless, association studies from these genes are generally controversial.

Therefore, in this study, we concentrated on chromosome 1 as the result from strong evidence accumulated in both mice and human, especially in the region 1q21-44 (Wakeland, Liu et al. 2001). Several reports demonstrated that human receptor for IgG (FcγR) genes (Duits, Bootsma et al. 1995; Salmon 1996; Song, Han et al. 1998; Yun, Koh et al. 2001) and interleukin-10 (IL-10) (Lazarus, Hajeer et al. 1997; Mehrian, Quismorio et al. 1998; Mok, Lanchbury et al. 1998; Ou, Tsai et al. 1998) are the candidate SLE susceptible genes in this area. Fcγ receptor is instrumental in capture and elimination of IgG antibody and circulating IgG-containing immune complex (Salmon 1996). If the ability of FcγR in holding immune complex decreases, it might result in the deposition of these immune complex in particular organ, such as joint and kidney leading to arthritis and glomerulonephritis, respectively (Prada and Strife 1992; Wisnieski and Jones 1992). For IL-10, it is a major immunoregulatory cytokine and has number of immunomodulating effects on the immune system(Clerici, Wynn et al. 1994) . IL-10 was classified as the Th-2 cytokine which depress Th-1 cytokine but enhance B cell survival, proliferation, differentiation and antibodies production, and these effects appear to play a key role in autoimmune disease , including SLE (Emilie, Llorente et al. 1995; Lalani, Bhol et al. 1997).

In 2002, the meta-analysis, which consisted more than 1,000 patients, demonstrated the strong association between FcγRIIA-R131 and SLE, especially in African-American population (Karassa, Trikalinos et al. 2002), and current report in Vietnam population (Khoa, Sugiyama et al. 2003). Moreover, significant association studies between FcγRIIA-R131 with lupus nephritis were previously reported(Salmon 1996; Song, Han et al. 1998; Yun, Koh et al. 2001). International meta-analysis in 2003 indicated that FcγRIIA-R/H131 polymorphism is also an important determinant of predisposition to antiphospholipid syndrome (Karassa, Bijl et al. 2003). For IL-10, several reports demonstrated the association between IL-10 promoter polymorphism and severity of SLE, such as renal

involvement and anti-Ro antibodies production (Eskdale, Wordsworth et al. 1997; Lazarus, Hajeer et al. 1997; Ou, Tsai et al. 1998). However, the association of IL-10 haplotype with level of IL-10 is still controversy in each population (Lazarus, Hajeer et al. 1997; Mok, Lanchbury et al. 1998).

The aim of this study was to investigate the polymorphism of FcγRIIA and IL-10 genes in patient with SLE compared with control group and determine the association with SLE in Thai population. We are interested in the polymorphism of FcγRIIA and IL-10 genes that might influence disease susceptibility and/or severity, and act as marker for the disease. The genotyping method for FcγRIIA and IL-10 genes polymorphisms were done by PCR-sequence specific primer (PCR-SSP) and PCR-restriction fragment length polymorphism (PCR-RFLP), respectively. Then genotypes and allele frequencies were compared between patient and control subjects.

We hypothesized that the specific polymorphism of FcγRIIA and IL-10 genes that determine risk for development and/or severity of SLE in Thai population will be found. This study help contribute to the identification of SLE susceptible gene and might lead to development of new prognostic markers based on these genotypes, in order to predict disease activity. Furthermore, the knowledge from this research might lead to the better understanding of mechanism of SLE and development of new treatment and prevention. In addition, it will provide the frequency of FcγRIIA and IL-10 genes polymorphisms in Thai population which are basic knowledge for study these markers in other disease in the future.

# CHAPTER II

# OBJECTIVE

The objective of this study was:

To identify the polymorphisms of FcγRIIA and IL-10 genes 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)

SLE is a multi-organ autoimmune disease characterized by B cell hyperactivity leading to excessive autoantibody production. SLE patients have high levels of circulating autoantibodies against a number of nuclear antigens including dsDNA and ribonucleoproteins. Tissue damage is triggered by an inflammatory response to immune complex deposition. Clinical manifestations of SLE could be diverse, including glomerulonephritis, dermatitis, thombosis, vasculitis, seizures and arthritis. The primary cause of morbidity and mortality is glomerulonephritis which develops in about 60% of patients (Kotzin 1996).

## Epidemiology

SLE is found worldwide with a prevalent ranging from 25-250 per 100,000 individuals depending on racial and geographic background; it is second only to rheumatoid arthritis as the most common autoimmune disorder. It is more prevalent and severe in African-American, Asian and Hispanic populations as compared with Caucasian population. Although SLE can presents itself at nearly any age, women in their child-bearing years are primarily affected. The gender difference in SLE prevalence is striking, with a female to male ratio of ~9:1 (Kotzin 1996; Hochberg 1997).

**Etiology**

Although the factors, which are responsible for the initiation of SLE, are poorly understood, considerable evidence suggests that SLE is a complex disease with a strong genetic basis, contributed from multiple genes as well as environmental factors (Harley, Sheldon et al. 1994; Tsao, Cantor et al. 1998; Gray-McGuire, Moser et al. 2000). Environmental factors consist of bacterial or viral infection, hormone and drug-induced lupus. Strong evidence support the role of genetic. Firstly, familial prevalence ~10-12 % (Vyse and Kotzin 1996). Secondly, strong familial aggregation in SLE is documented by λs, estimates for the disease that are well in excess of 15, indicating that siblings of SLE patients have a much greater relative risk for the disease in comparison to the population as a whole (Lawrence, Martins et al. 1987; Hochberg 1997). Finally, analysis of monocygotic (MZ) and dicygotic twins suggests that concordance for SLE is ~24-50 % in MZ twins compared with 2-5 % in fraternal twins and siblings (Deapen, Escalante et al. 1992; Harley, Moser et al. 1998; Wakeland, Liu et al. 2001). These data are consistent with the possibility of a strong genetic contribution to the SLE diathesis, and suggest that genetic approach to lupus may provide important insights into the disease etiology.

**Classification of SLE**

The American Rheumatism Association (now the American College of Rheumatology, ACR) initially published classification criteria in 1971(Cohen 1971) , which were revise in 1982 (Tan, Cohen et al. 1982). A further revision has recently been proposed (Hochberg 1997). 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. The 1997 updated criteria are outlined in Table 1. For the purpose of identifying patients in clinical studies, it is determined that a patient has SLE when at least four of eleven criteria are present, serially or simultaneously, during any interval of observation (Hochberg 1997).

**Table 1**: Revised American Rheumatism Association criteria for classification of systemic lupus erythematosus (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

**Clinical symptoms**

The clinical expression of SLE is tremendously varied, but the most common finding include: fever, erythematosus rash, arthritis, serositis and nephritis. Photosensitivity, oral ulcers, hematologic disorders and neurological abnormalities are also seen.

**Skin Manifestations**

including the typical malar rash & the Discoid lupus erythematosus (DLE) lesions that are scarring and occur either alone or in association with SLE.

**Renal Manifestations**

Renal disease is common in most patients with SLE and can be asymptomatic until there's advanced disease. Until recently, renal disease has been the most common cause of death in patients with lupus .

- **The World Health Organization (WHO)** classification of lupus nephritis is the standard by which most lesions are classified and can prognosticate survival of the kidney.

  Class I : normal glomeruli – in 8% of the patients
  Class II: mesangial abnormalities - in 40% of the patients
  Class III : focal glomerulonephritis – in 15% of patients

  a. class IIIA: focal segmental glomerulonephritis
  b. class IIIB - focal proliferative glomerulonephritis

  Class IV: diffuse glomerulonephritis - in 25% of the patients
  Class V: diffuse membranous lesion - in less than 8 % of the patients
  Class VI: advanced sclerosing glomerulonephritis

**Musculoskeletal Manifestations**

common, including arthritis or arthralgia, typically involving the small joints of the hands, wrists, and knees, is usually episodic and symmetrical in distribution.

**Cardiac Manifestations**

Pericarditis is the most common cardiac (precordial chest pain and a pericardial rub), 20 to 30% in most large series. Myocarditis, endocarditis and coronary vasculitis are rare manifestation.

**PulmonaryManifestation**

include pleuritis, pulmonary alveolar hemorrhage, pneumonitis, pulmonary infiltrates, chronic interstitial lung disease, shrinking lung syndrome, pulmonary hypertension, and pulmonary embolism. Pleuritis and/or pleural effusion are the most common pulmonary manifestation.

**Neuropsychiatric Manifestations**

Diffuse manifestations are the most common CNS presentation in NP-SLE patients (60% of cases).

**Hematologic Manifestations**

Cytopenias, including anemia, leukopenia, lymphopenia, and thrombocytopenia, are frequent findings in SLE. .

**Antiphospholipid Antibodies**

A variety of clotting abnormalities, including the presence of the lupus anticoagulant, manifested as a prolonged activated partial thromboplastin time (APTT) that does not normalize with mixing studies. Patients with the lupus anticoagulant, a false-positive result on VDRL testing, or a high titer of **anticardiolipin antibodies** fall under the umbrella term of **"APL antibody-positive"** and are predisposed to thrombotic events. The APL antibody syndrome describes the association of these APL antibodies with arterial and venous thrombosis, recurrent fetal loss, and immune thrombocytopenia.

## The genetic study in SLE

- **Genetic study using lupus murine models**

**Congenic Mice**

Numerous initial linkage analyses to identify the SLE-susceptible genes are actually derived from the New Zealand inbred lupus mice or their relatives (NZBxNZW or BWF1, NZM2410). I would like to mention on linkage analysis from the NZM2410, which are the most studies lupus murine model.

By using *microsatellite* polymorphic markers, Morel L ,*et al.* have initially identified at least 4 SLE-susceptible genomic intervals and called them *Sle1*( on chromosome 1), *Sle2* (on chromosome 4), *Sle3* (on chromosome 7) and *Sle4* (on chromosome 17) (Morel, Rudofsky et al. 1994; Morel, Mohan et al. 1999). They subsequently developed multiple mouse strains of C57BL/6 (B6) mice *congenic* for these SLE-susceptible genomic intervals (by introducing these SLE-susceptible genomic interval into the nonautoimmune C57BL/6). These SLE-susceptible congenic strains allowed them to differentially evaluate the roles of these intervals to the clinical manifestation of lupus in these mice, suggesting a similar role in human disease. *Congenic* B6 *Sle1*mice develop a selective loss of B cell tolerance to chromatin and with a preferential targeting of H2A/H2B/DNA subnucleosome, reminiscent of drug-induced lupus. However, these mice develop no or late-onset minimal nephritis and have normal survival. *Congenic* B6 *Sle2* mice develop a distinctly significant B cell hyperactivity. There are increased number of CD5-expreesing peritoneal B-1 cells and elevated levels of polyreactive or polyclonal IgM. Hoever, *Sle2* insufficiant for generating IgG autoantibody or lupus nephritis. *Congenic* B6 *Sle3*mice have dysregulated T-cell function. Peripheral CD4+T cells in these mice are expanded . They also spontaneously produce low level of autoreactive antibodies and develop late-onset nephritis. In contrast, in *Congenic* B6 *Sle4*mice have normal immunophenotypes with normal survival.

It was not surprising that none of the monocongenic mice develop fully penetrant lethal glomerulonephritis like their original parents. The differential dissection of the roles of these genomic intervals using monocongenic emphasized the ***polygenic complexity of SLE***. Interestingly, the presence of bi- or tri-congenic SLE-susceptible genomic internals can reproduce the lethal glomerulonepritis similar to their parents (Bowness, Davies et al. 1994; Morel, Rudofsky et al. 1994; Morel, Mohan et al. 1999). For example, the *bicongenic* B6 *Sle1/Sle3* or *tricongenic* B6 *Sle1/Sle2/Sle3* contain the minimal set of genes sufficient to reconstitute a fully penetrant SLE lethal glomerulonephritis. Although the roles of *Sle1* and *Sle3* are clearly demonstrated, ***the presence of Sle1 is necessary for the production of nephritogenic antibodies and clinical glomerulonephritis in the bi/tricongenic recombinations.*** Fine mapping of *Sle1* revealed the presence of a cluster of functionally related loci (*Sle1a,Sle1b,and Sle1c*) that independently mediate the loss of tolerance to nuclear antigen. The fourth locus (*Sle1d)* affect end-organ susceptibility to autoimmune damage.

**Knock-out and Transgenic Mouse Models**

Recently, several transgenic and knock-out mouse models have identified molecular pathways that result in the lupus-like phenotype when disrupted (Gaffney, Moser et al. 2002). As shown in Table 2. For instance, genes that control immune cell apoptosis [ Fas, Fas ligand,Bcl-2] ,molecules that negatively regulate antigen receptor signaling in B and T cells [SHP-1, Lyn,CD22], components of the innate immune system involved in clearance of self –antigens from the circulation [C1q,C3, C4, Dnase 1, serum amyloid P component (Sap), the Fc receptors ], and certain cytokines (interleukin 10) have the potential contributed to SLE phenotype in the mouse.

**Table 2**. Candidate genes and pathways implicated in systemic lupus erythematosus (Gaffney, Moser et al. 2002)

| Proposed mechanism | Murine SLE | Human SLE |
|---|---|---|
| **Antigen/immune complex clearance** | C1q knock-out, C3/C4 knock-out, Sap knock-out, Dnase1 knock-out, Serum IgM knock-out, Fcγ common chain knock-out | C1q, C2, C3, C4, Mannose binding protein, FcγRIIA, FcγRIIB, FcγRIIIA |
| **Lymphoid Signaling** | SHP-1 knock-out, Lyn knock-out, Lyn/Fyn double knock-out, CD22 knock-out, Blys transgenic, PD-1 knock-out, IL-2 knock-out, CD45 E613R point mutation | T cell receptor δchain, TNF-alpha, IL-10 |
| **Apoptosis** | Fas knock-out, Fas-L knock-out, Bcl-2 transgenic, Pten heterogenous deficiency, p21 cyclin-dependent kinase knock-out | Poly-ADP ribose polymerase (PARP) |
| **Epitope modification** | α-mannosidase II knock-out | |

- **Genetic study in humans**

There are three approaches which are used in the search for susceptibility loci of SLE in human.

1. Linkage analysis

Linkage analysis assesses co-segregation of physically close genomic sequences among multiple affected members in families to establish strong relation between genotypes (shared genetic markers identical by descent) and phenotypes (shared disease status) (Lander and Schork 1994). The principle of

linkage analysis is based on the fact that if two genes or markers are close together on a chromosome, they will be co segregate because the likelihood that a recombination will occur between them during meiosis is low. Therefore, if a tested markers is close to a disease susceptibility gene, its allele will cosegregate with the disease in families (Tomer and Davies 2003). Linkage analysis can be performed as part of genome-wide screening, using di-,tri- or tetra-nucleotide repeated termed as microsatellite markers that generally scattered throughout the chromosome, varied in number of repeated among individuals and can be highly polymorphic with several allele at single locus (Allahabadia and Gough 1999). The property of genetic linkage has made it a method of choice to map susceptibility loci in the whole genome during the last decade. Hundreds of genetic marker tested in a genome scan raise the probability of false positive results. To address this concern, Lander and Kruglyak proposed threshold for significant linkage [a logarithm of odds (lod) score of 3.3 or 3.6, depending on the linkage method used], suggestive linkage[ a lod score of 1.9 or 2.2] and confirmed linkage [ significant linkage from one or a combination of initial study that has been confirmed with a *p* value of 0.01 in an independent sample] (Lander and Kruglyak 1995). Genetic linkage techniques are powerful tools for analyzing complex disease-related genes because they detect genes that have a major influence on the development of the disease (Greenberg 1993). However, linkage studies are less sensitive than association studies because they do not detect less influential genes. Therefore, it might be negative in the absence of major genes contributing to disease susceptibility.

These linkage results are encouraging, considering that confirmation of significant linkage of a locus offers the strongest evidence for the existence of putative susceptibility gene, and the next step is fine mapping by using linkage disequlibrium (LD) mapping to narrow the linked interval sufficiency to reduce the number of candidate genes. LD occurs when certain alleles of physically closely located loci [such as tested genetic polymorphism and the disease predisposing gene] appear to be jointly transmitted more frequently than expected from their respective frequencies (Lander and Schork 1994) .

2. Population-based case-control studies

Population-based case-control studies is used to assess whether a test candidate gene polymorphism is associated with susceptibility to the disease (Lander and Schork 1994). This method investigate association of marker allele with disease by comparing the frequency of the allele between a diseased population and a diseased-free population (Allahabadia and Gough 1999). The association is frequently expressed as odds ratio or relative risk that an individual may be develop the disorder ,if he or she carries the specific allele or marker compared to an individual who does not carry the allele or marker (Bidwell, Keen et al. 1999). The existence association might be explain that the associated allele itself is the genetic variant causing an increase risk for the disease, or itself is not causing the disease but rather a disease predisposing gene that closely located with it (gene in linkage disequlibrium) (Hodge 1994). There are a lot of advantages. Firstly, the identification and collection of samples from subjects is quicker and more resource-efficient than the collection from family samples. Secondly, it might be easier to recruit adequate numbers of subject in late-onset diseases rather than recruit index cases with additional family members, including parents who are less likely to be alive. Finally, this method is more sensitive than some of family-based studies. However, if cases and controls are not matched owing to population stratification, it could yield a false positive association.

3. Family-based transmission disequilibrium test (TDT)

There are new alternative for genetic study to avoid false positive from association study by using parents as internal control group from within each family (Spielman, McGinnis et al. 1993). This method is gaining recognition as a powerful alternative to classical linkage analysis in the search for susceptibility gene in complex disease (Allahabadia and Gough 1999) and evaluates the preferential transmission of the test allele (or a specific haplotype) from heterozygous parents to one or more affected offspring that provide evidence for association of the test allele (or haplotype) with disease susceptibility (Spielman, McGinnis et al. 1993). Sampling collection from the family for TDT analysis is easier than collecting affected sib pair for linkage analysis, as only one affected offspring is needed. Nevertheless, it may be used with even greater power

if two or more affected offspring are available. Therefore, TDT can be used in multiplex families. However, the major limitation of this technique is that it requires knowledge of candidate genes before the test can be performed, and a polymorphism within the gene, or one in strong disequlibrium with it, must be available (Allahabadia and Gough 1999).

- **Linkage study in SLE**

Evidence for linkage to SLE of 1q41-41 was first shown in a targeted genome screen of human chromosome 1 region corresponding to the identified murine SLE susceptibility loci (Tsao, Cantor et al. 1997). Subsequently, six genome wide linkage analyses and six targeted genome scans, using multiple cohorts, have mapped many chromosomal regions that are likely to contain SLE-susceptible genes. As shown in Table 3 and Table 4 summarized promising loci and their positional candidate genes. Cohorts used in these complete genome scans differ greatly in ethnic composition, sample size, family structure and geographic locations where families have been recruited. Results from these studies vary in their mapped loci, which might be attributed to genetic heterogeneity, disease heterogeneity, power difference in detecting linkage, chance finding of small numbers of samples and the method used in linkage analyses. Overall, the MHC-containing region is not the most prominent susceptibility loci detected in these genomes scan, which suggests that, the MHC contribution to SLE susceptibility is similar to that of other non-MHC loci, a feather different from other autoimmune disease including, ankylosing spondylitis, type I diabetes and rheumatoid arthritis (RA).

Collectively, the advents of human genome data and polymorphic markers have allowed several groups to further characterize the SLE susceptible genes. Thus, initial linkage analyzes in multiple murine SLE models have identified at least 31 susceptibility loci distributed among 21 non-overlapping genomic intervals. Similarity, the initial linkage studies in human have demonstrated significant linkage of at least 13 genomic intervals located in 5 autosomal chromosome. The most consistent reports of disease susceptible genes in both murine and human SLE lie in the region 1q21-44. These region contain multiple candidate genes involved in the SLE etiopathogenesis.

**Table 3**. Cohorts studies in whole genome scans for systemic lupus erythematosus susceptibility loci

| | **Study design** | **Number of families** | **Cohort ethnicity in %** | **Number of SLE patients** | **Number unaffected** | **Total subjects** | **Major loci** [e] | **Reference** |
|---|---|---|---|---|---|---|---|---|
| UMN[a]1 | Sibpairs | 105 | 80 Caucasian<br>8 hispanic<br>5African-American<br>3 Asian<br>4 other | 220 | 155 | 375 | 6p11-21<br>16q12<br>14q21<br>20p12 | (Gaffney, Kearns et al. 1998) |
| UMN 2 | Sibpairs | 82 | 78 Caucasian<br>6 hispanic<br>15African-American<br>1 other | 179 | 101 | 280 | 7p22<br>7q21 | (Gaffney, Ortmann et al. 2000) |
| UMN 1+2 | Sibpairs | 187 | 79 Caucasian<br>7 Hispanic<br>10African-American<br>2 Asian<br>2 Other | 399 | 256 | 655 | 10p13<br>7q36<br>6p11-12<br>16q12 | (Gaffney, Ortmann et al. 2000) |
| OMRF[b] | Extended pedigrees | 94 | 58 Caucasian<br>33African-merican<br>9 Other | 220 | 313 | 533 | 2p15<br>1q23<br>1q25<br>13q32<br>20q13 | (Moser, Neas et al. 1998) |
| OMRF | Extended pedigrees | 126 | 61 Caucasian<br>32African-american<br>7 Other | 295 | 449 | 744 | 4p16-15<br>1q22-24 | (Gray-McGuire, Moser et al. 2000) |
| USC[c] | Extended pedigrees | 80 | 46 Caucasian<br>54 Hispanic | 188 | 246 | 434 | 1q43[f] | (Shai, Quismorio et al. 1999) |
| Uppsala U[d] | Extended pedigrees | 17 | 100 Caucasian | 44 | 106 | 150 | 2q37<br>4p15-13<br>19p13<br>19q13 | (Lindqvist, Steinsson et al. 2000) |

[a]A study conducted at University of Minnesota

[b]A study conducted at Oklahoma Medical Research Foundation

[c]A study conducted at University of Southern California

[d]A study conducted at University of Uppsala

[e]olny loci with a lod score >2 using a complete cohort are listed here.

[f]Z score were converted to lod(logarithm of odds) score using $lod=Z^2/2\ln 10$

**Table 4.** Regions demonstrating 'Significant linkage'[1] in Human SLE[2] (Wakeland, Liu et al. 2001)

| Locus | Primary LOD score | Supporting LOD score | Candidate Genes | Murine model | Other autoimmune disease |
|---|---|---|---|---|---|
| 1q22-24 | OK3.45 (FcγR) | USC1.51[3] (D1S484) | FcγRIIa | *Sle1a&Sle1b* (NZM2410) | |
| 1q41-42 | OK 3.50 (D1s3462) | USC 2.40[3] (D1s2785) MN 1.92 (D1S235) | PARP | *Sle1d* (NZM2410) *Bsx3* (BXSB) | Type I diabetes, Multiple Sclerosis Rheumatoid Arthritis |
| 2q37 | SW 4.24 (D2S125) | OK 1.53 (D2S1363) | INPP5D | *Bxs1* (BXSB) | |
| 4p15-16 | OK 3.84 (D4S2366) | MN 1.50 (D4S2366) | CD38, BST1,ZNF36 | *Sle6* (NZM2410) | |
| 6p11-22 | MN 4.19 (D6S426) | OK 1.70 (D6S2439) SW 1.54 (D6S273) | HLA Class II genes, C4a ,TNF | *Sle1* (NZM2410) *Lbw1* (NZB/NZW) | Multiple autoimmune disease |
| 16q12-13 | MN 3.85 (D16S415) | USC 1.00[3] (D16S3136) | NOD2 | | Crohn'sDisease,Psoriasis, Type I diabetes |

[1]Recommended criteria for significant linkage in a genome-wide scan for a complex trait(LOD> 3.3 for complex pedigrees,LOD>3.6 for sibpairs

[2]Shown are LOD score(marker)meeting criteria for each interval .Supporting evidence(LOD>1.0)from an independent family collection is also shown if present.

[3]Z scores were converted to LOD scores by the equation :$LOD = Z^2/2\ln 10$

OK:Oklahoma Medical Research Foundation,USA ;USC: University of Southern California,USA;MN: University of Minnesota, USA;SW: University of Uppsala, Sweden .

- **Association study in SLE**

During the past decade, cumulative evidence supports a few gene variants conferring predisposition to SLE. These include the HLA region supported by study in lupus murine models (Tsao, Cantor et al. 1998). The HLA-DR2 and HLA-DR3 have been reported to be associated with SLE, mainly in Caucasian population (Arnett and Reveille 1992). Given the role of HLA class II molecules in the T-B cell interaction, it is not surprising that certain DR/DQ alleles are strongly associated with particular IgG autoantibodies profiles rather than disease itself (Arnett and Reveille 1992; Arnett, Reveille et al. 1997). Moreover some experiment demonstrated the important of HLA class II molecules in governing the repertoires, magnitude and the diversification of autoantibodies produced in SLE (Paisansinsup, Vallejo et al. 2001). More recently, the association of HLA-DRB1*1502-DQB1*0501 haplotype with susceptibility to SLE was described in Thai population (Sirikong, Tsuchiya et al. 2002), similar to report in Taiwan Chinese (Lu, Ding et al. 1997).

There are the association between deficiency of early components of the classical complement pathway ($C_1q$, $C_2$ and $C_4$) with SLE susceptibility (Pickering and Walport 2000). However, homozygous $C_1q$ deficiency is an extremely rare disorder manifested by early development of skin disease and severe glomerulonephritis beginning in the first or second decade of life (Bowness, Davies et al. 1994). $C_2$ and $C_4$ deficiency are also rare disorder ( 1:10,000 for $C_2$ and less than 1:10,000 for $C_4$) with limited clinical manifestation of skin and joint involvement (Arnett and Reveille 1992). Obviously, the association of early complement deficiencies and SLE susceptibility cannot be accounted for the majority of the SLE patients,which has a higher prevalence ( 12-64:100,000 in caucasian population).

Mannose-binding protein (MBP), a polymorphic protein act as opsonin, directly bind to the surface of bacteria and activating complement by both the alternative and classical pathways(Schweinle, Ezekowitz et al. 1989) .two studies of separate racial groups both show marginal statistical effects for an association of lupus with aspartic acid at amino acid position 54 of MBP (Davies, Snowden et al. 1995; Sullivan, Wooten et al. 1996). In studies of African-American(Davies, Snowden et al. 1995) and Spanish lupus patient (Davies, Teh et al. 1997), alleles or haplotypes producing low levels of serum mannose-binding protein were associated with increased lupus risk.

The association in certain cytokine gene were reported. For example, the association in the promoter polymorphism of TNF-α and HLA-DR3 with some clinical features of SLE in British lupus patients was described (Rudwaleit, Tikly et al. 1996; Hajeer, Worthington et al. 1997). Furthermore, some reports demonstrated the association of TNF-α -308 polymorphism with SLE and independent of HLA-DR3 (Rood, van Krugten et al. 2000). In contrast, report in Caucasian population demonstrated no association between TNF-α -308 and susceptibility to SLE (Chen, Yen et al. 1997). However, the association between TNF-α and SLE is controversial in each population.

Moreover, researcher have focused in the interval at 1q21-44 that shows evidence for linkage in nearly all of the SLE mapping studies reported to date. This region was initially identified by Tsao B, *et al* who proposed the poly-ADP-ribosyl transferase (PARP) (1q41-44) as a candidate gene ,based on strong transmission disequilibrium test (TdT) results with a polymorphic marker in the 5' region of the gene and its role in gene repairing process (Tsao, Cantor et al. 1999). However ,the data were not reproducible by other groups (Criswell, Moser et al. 2000) .Interestingly, this region is syntenic to *Sle1d* in NZM2410 gene mapping data (see Table 4).

Other genes, such as Fcγ receptor genes (1q21-23) and IL-10 genes (1q31-32), have strong supported evidences influencing SLE disease in various population as summarized in table 5 and 6.

**Fcγ receptor genes**

FcγR are encoded by members of the immunoglobulin superfamily of genes. In humans, 8 genes for FcγR are clustered on the long arm of chromosome 1 (1q21-23). Extensive structural diversity among FcγR family members lead to differences in binding capacity, distinct signal transduction pathways, and cell type-specific expansion patterns (Hulett and Hogarth 1994; Ravetch and Clynes 1998).

FcγR consists of 3 families ; FcγRI, FcγRII (IIa, IIb1, IIb2, IIc) and FcγRIII (IIIa, IIIb), as shown in Figure 1.

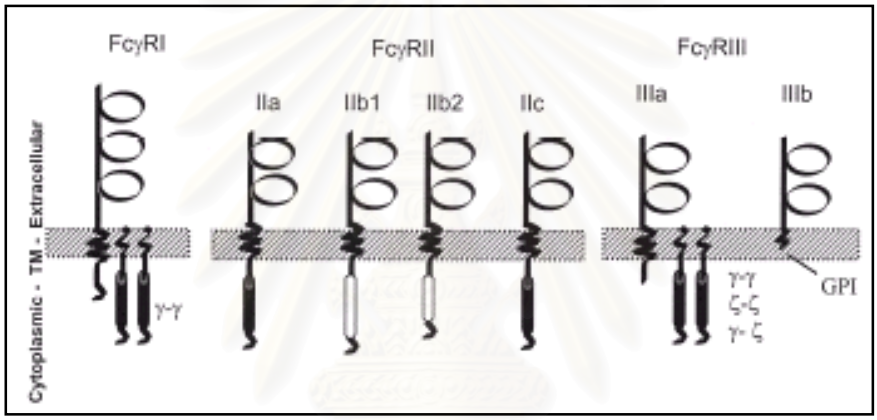


**Figure 1.** Schematic representation of the human FcγR family members (Salmon 2001)

FcγR are widely expressed on hematopoietic cell surface, such as polymorphonuclear leukocyte (PMN), monocyte and macrophage (Mϕ), dendritic cell (DC), mast cell (MC), B lymphocyte, platelet and natural killer cell (NK). In general, one or more isoforms of FcγR were expressed, often present as stimulatory and inhibitory pairs. Triggering stimulatory FcγR initiates phagocytosis, antibody-dependent cell-mediated cytotoxicity (ADCC), the release of inflammatory mediators, for example, cytokine, reactive oxidants, protease by phagocyte and so on. Conversely, coaggregation between stimulatory and inhibitory FcγR lead to inhibition of phagocytosis. Function of each FcγR family is different dependent on type of hematopoietic cells (Ravetch and Clynes 1998; Amigorena and Bonnerot 1999), as shown in Figure 2.

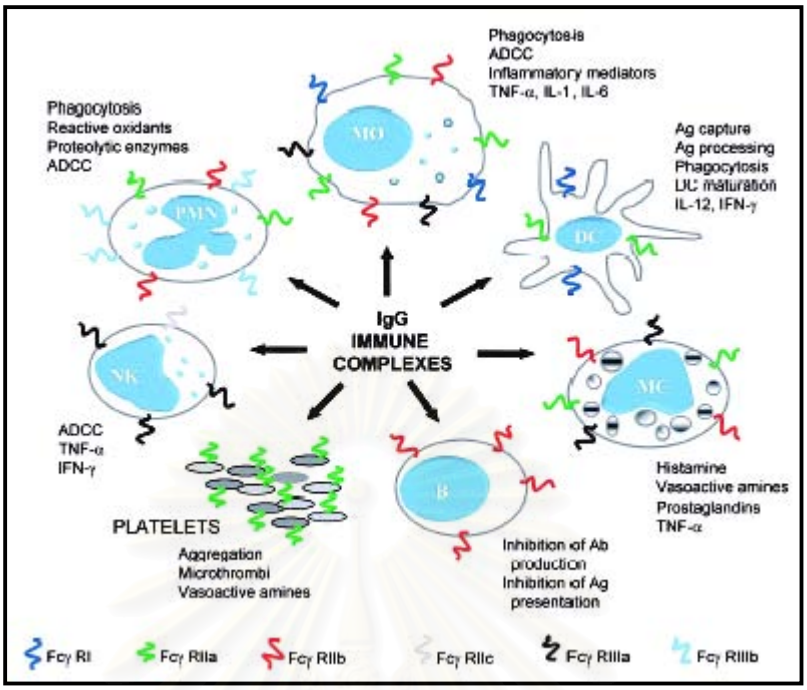


**Figure 2.** Cellular distribution and functions of FcγR(Salmon 2001)

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**Allelic variants of FcγR**

- **Functional consequences and implications for host defense**

Host susceptibility to autoimmune disease has acquired and inherited components. The alteration of FcγR expression is a dynamic process, and subtle changes in the relative representation of FcγR isoforms may compromise host defense. Genetically determined alteration in FcγR structure provide a basis for inherited predisposition to disease. Allelic variants of human FcγR profoundly influence phagocyte biologic activity. Single amino acid substitution within the extracellular domains of stimulatory FcγR alter the ability of the receptor to bind IgG and have been associated with risk for and phenotype of various autoimmune and infectious diseases (Salmon 2001).

- **Implications of FcγR allelic variants for the pathogenesis of autoimmune disease**

Allelic variants of FcγR are common within the population and are phenotypically benign in the normal host, but in certain environmental and genetic contexts they may influence susceptibility to, or outcome of, disease. In particular, FcγR polymorphism that alter IgG binding capacity provide a mechanism for heritable susceptibility to immune complex disease. SLE, the prototype human immune complex disease, is characterized by tissue deposition of circulating antigen-antibody complexes leading to release of inflammatory mediators and influx of inflammatory cells. These events are considered crucial for the development of lupus nephritis. The efficiency of the mononuclear phagocyte system in removing circulating immune complexes depends on FcγR and receptors for complement. It has been hypothesized that low-binding FcγR alleles are susceptibility factors for SLE. Proposed mechanism is that the low-binding FcγR might lead to the defective in clearance IgG autoantibody and IgG-containing immune complex, and then lead to deposition of these immune complex in particular organ (joint, kidney and vascular) generating arthritis , nephritis and vasculitis, respectively (Salmon 2001).

Allelic polymorphisms have been identified in 3 FcγR family members: FcγRIIa, FcγRIIIa, FcγRIIIb. As shown in Figure 3. Also, new family member, FcγRIIb, has been identified in recent year.

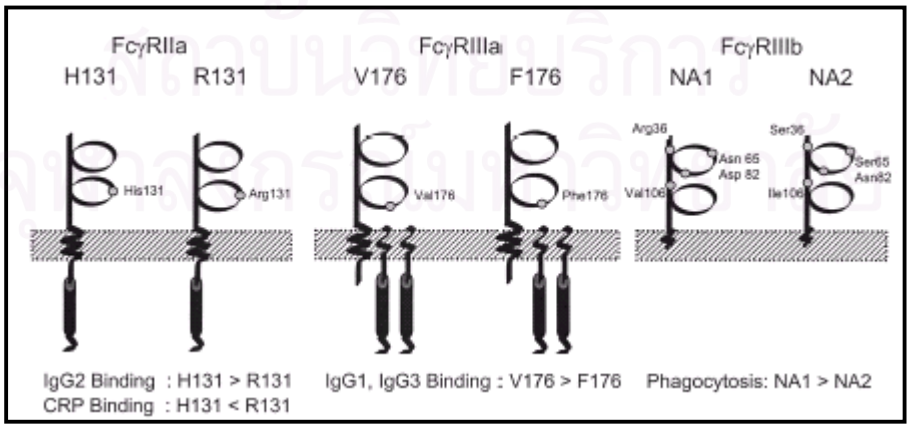



**Figure 3.** Allelic variants of human FcγR(Salmon 2001)

**FcγRIIa**

FcγRIIa expressed on mononuclear phagocyte, neutrophils and platelets, has two codominantly expressed alleles, H131 and R131, which differ at amino acid position 131 in the extracellular domain (histidine or arginine, respectively), an area which strongly influences ligand binding. The allelic variants differ substantially in their ability to bind human IgG2 (Clark, Stuart et al. 1991; Warmerdam, van de Winkel et al. 1991). H131 is the high-binding allele and R131 is low-binding ,while heterozygotes have intermediate function. Because IgG2 is a poor activator of the classical complement pathway, FcγRIIa-H131 is essential for handing IgG2 immune complexes. The genotype distribution of FcγRIIa in Caucasian and African-American population is ~25% homozygous for H131, ~50% heterozygous,and ~25% homozygous for R131. Among Asians, the frequency of R131 allele is much lower, and <10% of the population is homozygous for R131.

Previous reports demonstrated that the expression of R131 allele significantly increased in SLE patient, especially African-American, coupled with lower expression of H131 compared with control (Salmon 1996) as confirm by meta-analysis (Karassa, Trikalinos et al. 2002), similar to study in Korean population (Song, Han et al. 1998), and current report in Vietnam population (Khoa, Sugiyama et al. 2003). One previous association study of FcγRIIa in Thai popuation did not show a significant association ,which may be due to a limited sample size (Siriboonrit, Tsuchiya et al. 2003). More importantly, significant association studies between FcγRIIA-R131 with lupus nephritis are previously reported (Salmon 1996; Song, Han et al. 1998; Yun, Koh et al. 2001). In addition, from international meta-analysis in 2003 indicated that FcγRIIA-R/H131 polymorphism is an important determinant of predisposition to anticardiolipin syndrome (Karassa, Bijl et al. 2003). The previous positive association studies were shown in Table 5. Nevertheless, disparities among the studies did occur (Smyth, Snowden et al. 1997; Koene, Kleijer et al. 1998; Yap, Phipps et al. 1999).

**FcγRIIIa**

FcγRIIIa expressed on mononuclear phagocytes and NK cells, also displays codominantly expressed biallelic variants, F176 and V176, which differ in one amino acid at position 176 in the extracellular domain (phenylalanine or valine ,respectively) (Koene, Kleijer et al. 1997; Wu J 1997). FcγRIIIa alleles differ in IgG1 and IgG3 binding; V176 homozygotes bind IgG1 and IgG3 more avidity than F176 homozygotes. These differences in IgG binding have implications for antibody-mediated immune surveillance, ADCC, antibody-mediated host defense against pathogens, and autoimmune disease (Lehrnbecher, Foster et al. 2000). The distribution of genotypes of FcγRIIIa in disease-free Caucasian and African-American populations has been reported to be 40-50% homozygous for F176, 40-50% heterozygous , and 8-18% homozygous for V176.

From the previous reports, we found the association between FcγRIIIA and SLE, in particular nephritis in Caucasian population but not for non-Caucasian population (Wu J 1997; Seligman, Suarez et al. 2001).

**FcγRIIIb**

Two common allelic variants of FcγRIIIb, a receptor exclusively expressed on neutrophils, have been characterized and shown to alter neutrophil function. The allotypes, known as neutrophil antigen (NA1 and NA2), were identified as a consequence of their involvement in blood transfusion reactions and alloimmune neutropenias. They differ by 5 nucleotides, which results in substitution of 4 amino acid in the membrane distance first extracellular domain (Ory, Clark et al. 1989; Ory, Goldstein et al. 1989). Although binding of IgG does not seem to be affected, the NA1 and NA2 allelic forms do have different levels of quantitative function (Bredius, Fijen et al. 1994). Neutrophils obtains from NA1-homozygous donors have a more robust FcγR-mediated phagocytic response compared with cells from NA2 donors, despite equivalent receptor density (Bredius, Fijen et al. 1994).

Several reports revealed the positive associations between FcγRIIIb-NA2/NA2 polymorphism and SLE, particularly lupus nephritis in Japanease and Thai population (Hatta, Tsuchiya et al. 1999; Siriboonrit, Tsuchiya et al. 2003).

**FcγRIIb**

FcγRIIb is the only one gene among FcγR family that has the ability to transmit inhibitory signals in B cells and myelomonocytic cells (Ravetch and Lanier 2000). Deficiency for FcγRIIb in mice was recently shown to be associated with autoimmune disease, such as collagen-induced arthritis (Yuasa, Kubo et al. 1999) and Good pasture's syndrome (Nakamura, Yuasa et al. 2000). Moreover, spontaneous development of antinuclear antibodies and glomerulonephritis was observed when FcγRIIb deficiency was introduced into a C57BL/6 background (Bolland and Ravetch 2000).

FcγRIIb polymorphism has two codominantly expressed alleles, I232 and T232, when differ at amino acid position 232 (Isoleucine and Threonine, respectively), in transmembrane domain (Kyogoku, Tsuchiya et al. 2002). In 2002, first report for this gene indicated that significantly increased of T/T232 in Japanese population (Kyogoku, Dijstelbloem et al. 2002) as coupled with report in Thai population (Siriboonrit, Tsuchiya et al. 2003).

Collectively, FcγR were considered the first non-MHC gene associated with SLE. In this study, we would like to concentrated on FcγRIIa gene polymorphism that have been strongly proved to be functional polymorphism. In addition, we are particulary interested in the role of FcγRIIa polymorphism with various SLE clinical manifestation including the anticardiolipin antibody production in Thai population.

**IL-10**

IL-10 gene located on chromosome 1 region 1q31-32.

IL-10 has pleiotropic effects in immunoregulation and inflammation (Lalani, Bhol et al. 1997). As shown in Figure 4. It was initially identified as a type 2 T-helper cell cytokine and is produced by many cell types, including T lymphocyte, B lymphocyte, monocyte/macrophage, and mast cells (Mosmann 1994). IL-10 suppresses type 1 T-helper lymphocytes by decreasing IL-2 and interferon-γ production (Taga and Tosato 1992). It also inhibits certain functions of activated macrophage by down-regulating MHC classII and B7 expression (Ding, Linsley et al. 1993), and by inhibiting production of proinflammatory cytokines, such as TNF-α, IL-1, IL-6, IL-8 and IL-12 (Fiorentino, Bond et al. 1989). Contrary to its T cell and macrophage inhibitory actions, IL-10 has potent in vitro stimulatory effects on B lymphocytes leading to production of immunoglobulin, DNA replication and nitric oxide production from macrophage (Rousset, Garcia et al. 1992).

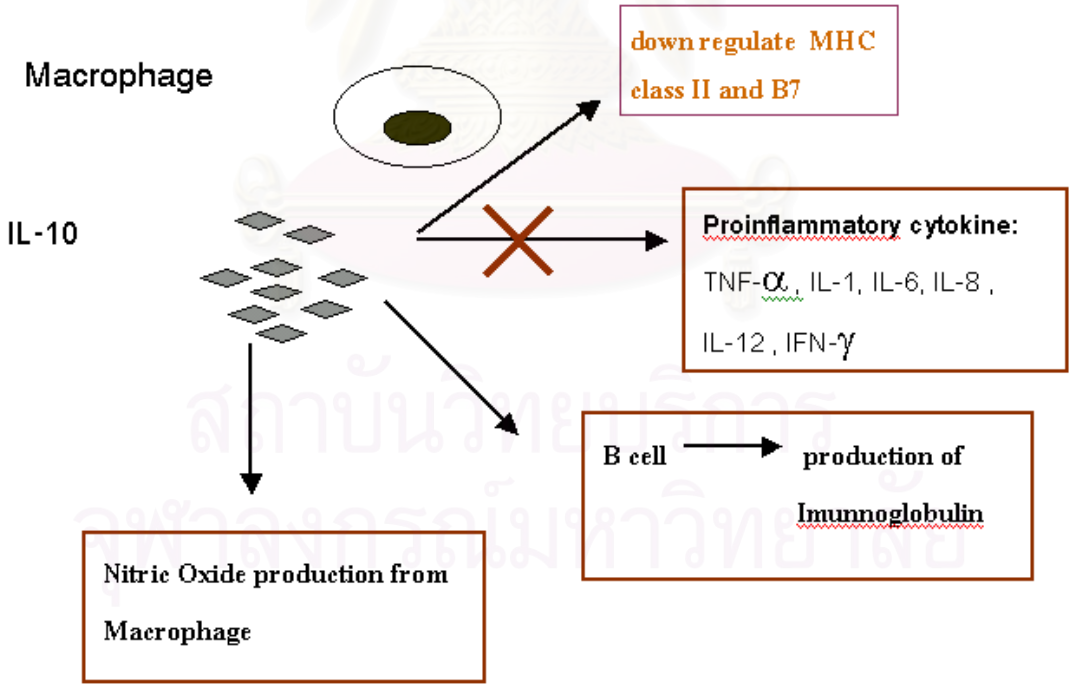



**Figure 4.** Biological functions of IL-10(Lalani, Bhol et al. 1997)

**Implication of IL-10 promoter polymorphism and SLE**

Because the production of cytokine is dependent on its promoter. If promoter has polymorphism, it may affect the binding activity of transcription factor to promoter region. We hypothesized that high production of IL-10 might be involved in pathogenesis of SLE by resulting in abnormal B cell activation that lead to B cell hyperactivity generating autoantibody production. As coupled with the study in lupus murine model, when continuous administration of neutralizing anti-IL-10, mices delayed the onset of autoimmunity. These finding indicated that IL-10 is an important candidate factor in the pathogenesis of SLE, and may play a role by damping down T cell responses and inducing B cell over activity (Ishida, Muchamuel et al. 1994).

The basis for heritable differences in IL-10 production remains unclear. The IL-10 gene promoter is highly polymorphic, and promoter reporter studies have identified several positive and negative regulatory promoter sequenced within the 1.3 kb. region upstream the transcription start site (Kube, Platzer et al. 1995; Kube, Laser et al. 1999) including two CA-repeated microsatellites, IL-10.R and IL-10.G, -4 kb. and –1.1 kb., respectively (Eskdale and Gallagher 1995; Eskdale, Wordsworth et al. 1997). However, the functional significance of these microsatellites in relation to the production of IL-10 is unclear. Three biallelic polymorphisms within the IL-10 promoter region, at position –1082 (G/A), -819 (C/T) and –592 (C/A) from the transcription initiation site, have also been identified (Turner, Williams et al. 1997) and proved by in vitro expression study suggesting that these position serve as functional polymorphism. In summary, various reports have revealed the haplotype of IL-10 promoter, GCC, ACC, and ATA, generated high, intermediate and low IL-10 production, respectively (Edwards-Smith, Jonsson et al. 1999).

According to previous reports, as shown in Table 6, represented the positive association between IL-10 gene promoter polymorphism and SLE (Eskdale, Wordsworth et al. 1997; D'Alfonso, Rampi et al. 2000). However, some controversy results were also reported. For example, reports from Southern Chinese show no significant association in frequency of allele or haplotype of IL-10 between SLE and control. Nevertheless, they found the association between ATA haplotype and clinical feature, particularly lupus nephritis (Mok, Lanchbury et al. 1998). Contrary to Mok CC, some report revealed that GCC haplotype associated with clinical feature, such as anti-Ro antibody and renal involvement (Lazarus, Hajeer et al. 1997).

**Table 5.** Positive association between FcγRIIa polymorphism and SLE

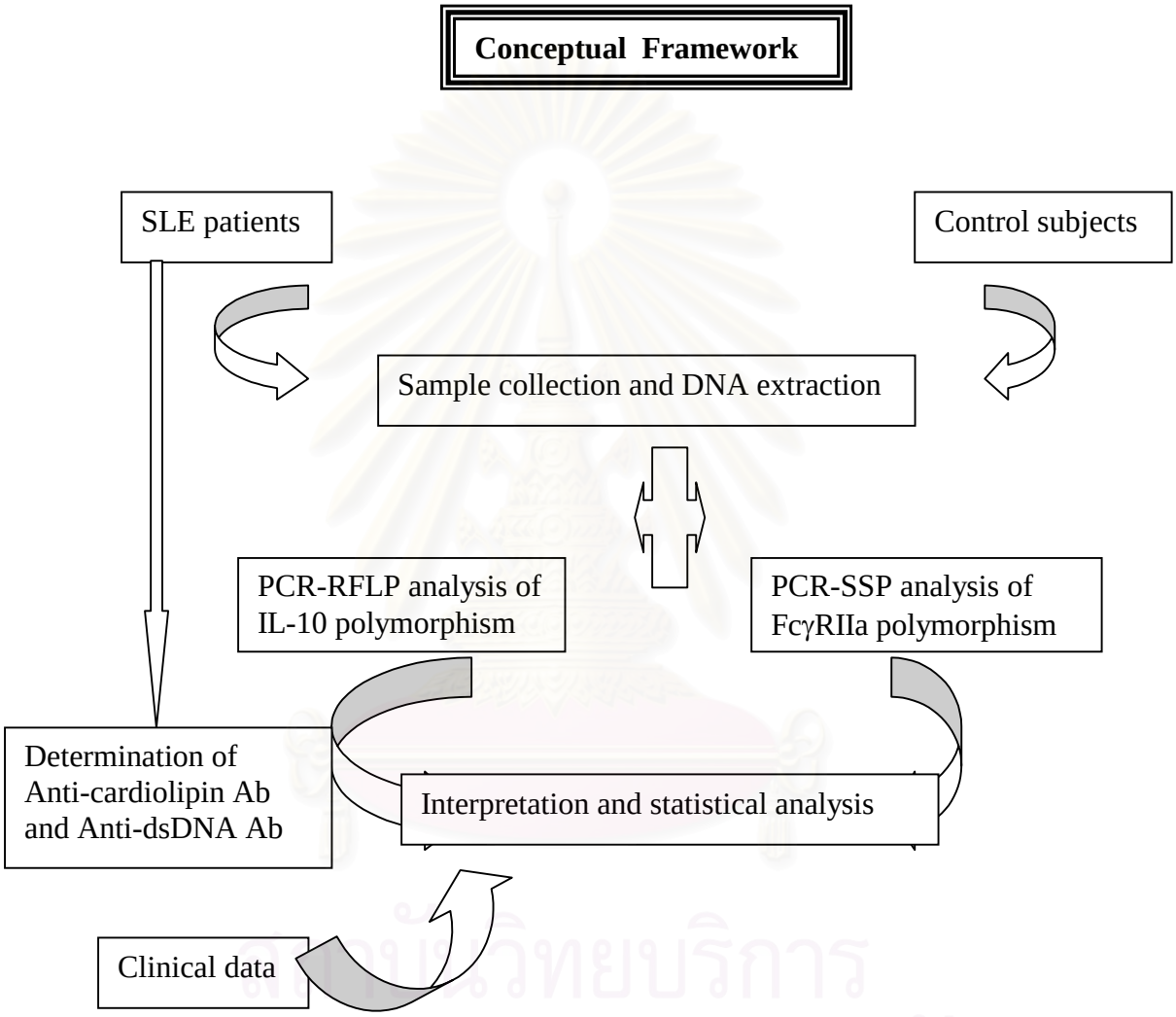
| Country/Ethnic | Cases/controls | Specificity | OR /RR | References |
|---|---|---|---|---|
| Netherands / European | 95/139 | Significant association between FcγRIIa-R/R131 and lupus nephritis | > 1 | (Duits, Bootsma et al. 1995) |
| US / African American | 257 /139 | Significant decrease FcγRIIa-H/H131 in SLE, particular in nephritis | < 1 | (Salmon 1996) |
| Korea / Korean | 73 / 64 | Significant decrease FcγRIIa-H/H131 in SLE ,particular in nephritis | < 1 | (Song, Han et al. 1998) |
| Germany / German | 108 / 187 | Significant association between FcγRIIa-R/R131 and clinical manifestation | > 1 | (Manger, Repp et al. 1998) |
| UK / Caucasian | 195 / 283 | Significant increase frequency of R131 allele in anti-C1q positive patient and in nephritis | > 1 | (Norsworthy, Theodoridis et al. 1999) |
| US / Mexican and central American | 67 / 53 | Significant increase FcγRIIa-R/R131in SLE with nephritis | > 1 | (Zuniga, Ng et al. 2001) |
| Korea / Korean | 300 / 197 | Significant association between FcγRIIa-R/R131 and lupus nephritis | > 1 | (Yun, Koh et al. 2001) |
| Meta-analysis | > 1000 | FcγRIIa-R/R131 polymorphism represent a significant risk factor for SLE | > 1 | (Karassa, Trikalinos et al. 2002) |
| Vietnam / vietnamese | 48 / 43 | Significant association between FcγRIIa-H/H131 polymorphism and SLE | < 1 | (Khoa, Sugiyama et al. 2003) |
| Meta-analysis | > 1000 | FcγRIIa polymorphism represent a significant risk factor in the anticardiolipin syndrome | > 1 | (Karassa, Bijl et al. 2003) |

**Table 6.** Positive association between IL-10 polymorphism and SLE

| Country/Ethnic | Cases/Controls | Specificity | OR/ RR | References |
|---|---|---|---|---|
| UK / Caucasian | 76 / 119 | -1082*G/-819*C/-592*C haplotype and relate with clinical feature of SLE (Anti-Ro antibody and SLE with renal involvement) | > 1 | (Lazarus, Hajeer et al. 1997) |
| UK / Caucasian | 56 / 102 | IL-10.G microsatellite associated with renal involvement | > 1 | (Eskdale, Wordsworth et al. 1997) |
| US / Mexican American | 158 / 223 | -IL-10 loci show significantly different alleic distribution between SLE patient and control<br>-Synergistic effect between IL-10 and bcl-2 in susceptible to SLE | > 1 | (Mehrian, Quismorio et al. 1998) |
| China / Chinese | 88 / 83 | -No significant different of allele or haplotype between SLE patient and control<br>-(-1082*A/-819*T/-592*A) haplotype associateed with renal involvement | > 1 | (Mok, Lanchbury et al. 1998) |
| Taiwan / Chinese | 100 / 103 | IL-10 promoter region allele strongly associated with progression of lupus nephritis | 82 | (Ou, Tsai et al. 1998) |
| Italy / Italian | 172 / 87 | IL-10G.microsatellite associated with SLE | 1.78 | (D'Alfonso, Rampi et al. 2000) |
| Netherland<br>-Caucasian<br>-African American | 52 128<br>60 64 | -Significant different between African American SLE and control at –2763 (C/A) | > 1 | (Gibson, Edberg et al. 2001) |

# 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 from the same geographic area. The ethics committee of the faculty of Medicine, Chulalongkorn University, Bangkok, Thailand approved the study and the subjects gave their informed consent. Demographic data of the subjects was summarized in Table 7.

**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 Interleukin-10**

We performed the Polymerase Chain Reaction-Restriction fragment Length Polymorphism (PCR-RFLP) analysis on the polymorphism of IL-10 at promoter position –1082 (G/A), -819 (C/T) and –592 (C/A) as previously described (Edwards-Smith, Jonsson et al. 1999; Santos, Suffys et al. 2002). The genomic DNA of 154 SLE patient and 154 healthy controls were amplified with the use of the IL-10 gene specific primers as shown in Table 8.

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 2 minutes, followed by 35 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 3 positions:

**1. At position –592 (A/C)**

PCR product size is 412 bp fragment and then , 10 μl of amplified DNA were digested with 5U of specific restriction enzyme *Rsa*I (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 40 minutes . If an A was present at this position, the enzyme would cut the 412 bp PCR product into two fragment; 176 and 236 bp. No digestion would occur if a C 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 –819(C/T)**

PCR product size is 209 bp fragments and then, 10 μl of amplified DNA were digested with 10U of specific restriction enzyme *Msl*I (New England Biolabs, Hitchin, UK) in 1X NEBuffer 2 in a total volume of 15 μl at 37°C for 14-16 hours, followed by 3% agarose gel electrophoresis at 100 volts for 40 minutes. If a C was present at this position, the enzyme would cut the 209 bp PCR product into two fragment; 125 and 84 bp. No digestion would occur if a T was present. A molecular ladder of 100-bp (Promega Madison WI, USA) was used to estimate the size of the PCR fragments.

**3. At position –1082(G/A)**

PCR product size is 139 bp fragment and then, 10 μl of amplified DNA were digested with 7U of specific restriction enzyme *Mnl*I (New England Biolabs, Hitchin, UK) in 1X NEBuffer 2 and 1X BSA 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 a G was present at this position, the enzyme would cut the 139 bp PCR product into two fragment; 106 and 33 bp. No digestion would occur if an A 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 3 SNPs site for IL-10 genotyping by DNA sequencing.

**Polymerase Chain Reaction-Sequence Specific Primer (PCR-SSP) Analysis of FcγRIIa polymorphism**

FcγRIIa R/H polymorphism at amino acid position 131 were identified by using the PCR-SSP method as previously described (Hatta, Tsuchiya et al. 1999). The genomic DNA of 154 SLE patient and 154 healthy controls were amplified with the use of the FcγRIIa gene specific primers as shown in Table 8.

The reaction volume for the amplification reaction was 20 μl, containing 100 ng of genomic DNA, 0.1 μl of 5.0 Taq polymerase (Promega Madison WI, USA), 2 μl of 10x PCR buffer (20mM Tris-HCL pH 8.0, 100 mM KCL), 1.2 μl of 25 mM $MgCl_2$, 0.4 μl of 10 mM dNTP and 1 μl(20 pmol) of each specific primers. Internal control primers were used to check for successful PCR amplification. These primers, which amplify a human growth hormone sequence were used with less than 10-fold of specific primers or 0.1 μl. Then, 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 2 minutes, followed by 30 cycles of denaturation (94 °C, 20 seconds), annealing (59 °C, 50 seconds) and extension (72 °C, 20 seconds) 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 .The positive results of FcγRIIa gene and human growth hormone gene showed band of 225 and 428 bp fragment, respectively. A molecular ladder of 100-bp (Promega Madison WI, USA) was used to estimate the size of the PCR fragments.

Additionally, the selected PCR products were analyzed to confirm the results of FcγRIIa genotyping by DNA sequencing. Specific primers for sequencing were shown in Table 8. The reaction volume for the amplification reaction was 50 μl, containing 400 ng of genomic DNA, 0.25 μl of 5.0 Taq polymerase (Promega Madison WI, USA), 5 μl of 10x PCR buffer (20mM Tris-HCL pH 8.0, 100

mM KCL), 3 μl of 25 mM $MgCl_2$, 1 μl of 10 mM deoxynucleotide triphosphates and 2.5 μl(20 pmol) of each specific primers. Then, 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 2 minutes, followed by 30 cycles of denaturation (94 °C, 20 seconds), annealing (59 °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. Successful amplification produces a 308 bp fragment. A molecular ladder of 100-bp (Promega Madison WI, USA) was used to estimate the size of the PCR fragments. After the process of sequencing, the sequences of each allele were perceived using CHROMAS program.

**DNA sequencing**

DNA sequencing were used to validate the results of FcγRIIa gene and IL-10 gene polymorphism by PCR-SSP and PCR-RFLP, respectively. 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.

**Determination of Anti-cardiolipin (aCL) antibodies**

The anti-phospholipid (aPL) antibodies can be divided into 3 subgroups: Lupus Anticoagulant (LAC), anticardiolipin antibodies and anti-β2 glycoprotein I antibodies. Anti- phospholipid antibodies have recently been included into ACR criteria for SLE. Since aPL interferes with coagulation mechanisms, it involves in a pro-thrombotic stage. Anti-phospholipid antibodies have been associated with the occurrence of thrombosis, thrombocytopenia, fetal wastage, cerebro-vascular insufficiency and myocardial infarction, so-called anti-phospholipid syndrome. Thirty to 40% of lupus patients were found to have aPL antibodies. In addition, the higher titers of aPL antibodies are generally found in the phase of disease activity. However, serum titer of aPL antibodies is not particularly useful for monitoring disease activity.

Anti-cardiolipin antibodies were assessed by solid-phase enzyme immunoassay (ETI-Cardiolipin Screen Kit) or ELISA, as previously described (Tincani, Balestrieri et al. 1998).

Microtitre wells are coated with cardiolipin and β2 glycoprotein I (β2-GPI, apolopoprotein H), the latter being cardiolipin co-factor, which forms complexes with cardiolipin. Consequently, the presence of cardiolipin and β2 glycoprotein I complexes allow specific cardiolipin antibodies to bind solid phase.

One hundred microliters of positive control, negative control, and 1:101 patient sera diluted in diluent were dispensed into the wells and incubated for 30-35 minutes at room temperature. Afterthat well contents were discarded by aspirating with a vacuum device and filled with 300 µl diluted wash buffer per well. Immediately, 100 µl of enzyme tracer was added into all wells and incubated for 30-35 minutes at room temperature. Then, 100 µl of chromogen were immediately pipetted into all wells and incubated for 10 minutes at room temperature, away from light. Finally, 50 µl of stop solution was dispensed in each well and the color will change from blue to yellow. Absorbance of each well were measured at 450/620-690 nm within 30 minutes of completing the assay.

The results were calculated by comparation with the calculated cut-off.

$$\text{Signal-to-cut-off ratio} = \frac{\text{Sample absorbance}}{\text{Cut-off absorbance}}$$

Note: Cut-off value = Calibrator absorbance x Specific factor

The interpretation of results were classified in 3 groups; samples with signal-to-cut-off ratios > 1.4 are considerate positive, samples with signal-to-cut-off ratios between 1.1and 1.4 should be considered borderline, and samples with signal-to-cut-off ratios $\leq$ 1.0 are considered negative.

**Determination of Anti-dsDNA antibodies**

Anti-dsDNA antibodies, found primarily in SLE patients, have been shown to be pathogenetic in tissue injury in SLE and have long been established as a disease and activity marker, especially for active renal disease. Clinically, the presence of high anti-DNA antibodies titers usually suggests the diagnosis of SLE, but lower titer anti-DNA antibodies occur in liver disease, juvenile rheumatoid arthritis, and in some normal population. SLE patients without anti-DNA antibodies tend to have milder disease and less nephritis than those with these antibodies. Only 70% of SLE patients with SLE ever make anti-DNA antibodies. Prognostically, the idea that an anti-DNA antibody is associated with a worse prognosis in SLE has been challenged. Both the nature of DNA as an antigen and the immunochemical and idiotypic characteristics of the anti-DNA antibodies are important in renal damage and disease activity.

Anti-DNA antibodies were determined by *Crithidia* indirect immunofluorescence test, as previously described (Aarden, de Groot et al. 1975) .

*Crithidia luciliae* kinetoplast is a source of purified dsDNA; therefore this source does not have any antigen and its freedom from contamination with single-standed DNA. *Crithidia* were coated in each well on slide. Twenty-five microliters of positive control, negative control, and 1:10 patient sera diluted in PBS were added into wells on slide and incubated for 30 minutes at room temperature in moist chamber. Then, each wells were washed by PBS solution in order to remove excess uncapture-antibodies and soaked in PBS solution for 5 minutes, repeat this step again. Conjugated anti human IgG antibodies were filled in each wells and incubated for 30 minutes at room temperature in moist chamber. Proceeding with discarded excess conjugated antibodies. The last step is a preservation of *Crithidia* cells by mount with mounting media (buffered glycerol), following detection by fluorescence microscope.

The positive results will show the shining of green color fluorescence in kinetoplast and/or nucleus, but not in basal body. Samples, which are positive in screening test, were examined at more dilution (1:20,1:40, 1:80,1:160,1:320, 1; 640, and 1:1280) in order to find titer that might be correlated with disease activity.

**Statistical Analysis**

Allele and genotype frequencies were compared between groups using the Chi-square ($\chi^2$) 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). Furthermore, the mode of inheritance analysis was also included.

**Haplotype analysis by PHASE program**

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).

**Table 7.** Characteristics of healthy controls and patients with SLE

| Characteristics | Healthy controls | SLE |
|---|---|---|
| Number of patients | 150 | 150 |
| Females/males | 96/54 | 146/4 |
| Mean age $\pm$ SD yr | 23$\pm$ 12.3 | 36.34$\pm$ 11.9 |

**Table 8.** Primers used for analysis of the FcγRIIa gene and IL-10 gene polymorphism

| Gene polymorphism and primer | Sequence of primers (5'→ 3') | Reference |
|---|---|---|
| FcγRIIa<br>-F1(Forward)of H allele<br>-F2(Forward)of R allele<br>-R(Reverse)<br>Internal control primer<br>-Forward<br>-Reverse<br>Sequencing primer<br>-Forward<br>-Reverse | <br>AAT CCCAGAAATTCTCCCA<br>AATCCCAGAAATTCTCCCG<br>TCTCAGACCTCCATGTAG<br><br>GCCTTCCCAACCATTCCCTTA<br>TCACGGATTTCTGTTGTGTTTC<br><br>CCAGGAGGGAGAAACCATCA<br>TCTCAGACCTCCATGTAG | (Hatta, Tsuchiya et al. 1999) |
| IL-10(-592A/C)<br>-Forward<br>-Reverse | <br>GGTGAGCACTACCTGACTAGC<br>CCTAGGTCACAGTGACGTGG | (Edwards-Smith, Jonsson et al. 1999) |
| IL-10(-819C/T)<br>-Forward<br>-Reverse | <br>TCATTCTATGTGCTGGAGATGG<br>TGGGGGAAGTGGGTAAGAGT | (Edwards-Smith, Jonsson et al. 1999; Santos, Suffys et al. 2002) |
| IL-10 (-1082G/A)<br>-Forward<br>-Reverse | <br>CTCGCTGCAACCCAACTGGC<br>TCTTACCTATCCCTACTTCC | (Edwards-Smith, Jonsson et al. 1999) |

# CHAPTER V

# RESULTS

## 1. Polymerase Chain Reaction-Restriction Fragment Length Polymorphism (PCR-RFLP) analysis of IL-10

### 1.1 PCR-RFLP analysis of IL-10 promoter at position -592

Polymorphism at –592A/C in the promoter region of the IL-10 were identified by the PCR-RFLP method. If an A was present at this position, the *Rsa*I restriction enzyme would cut the 412 bp PCR product into two fragment; 176 and 236 bp . No digestion would occur if a C was present. (Edwards-Smith, Jonsson et al. 1999) (Figure 5).

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

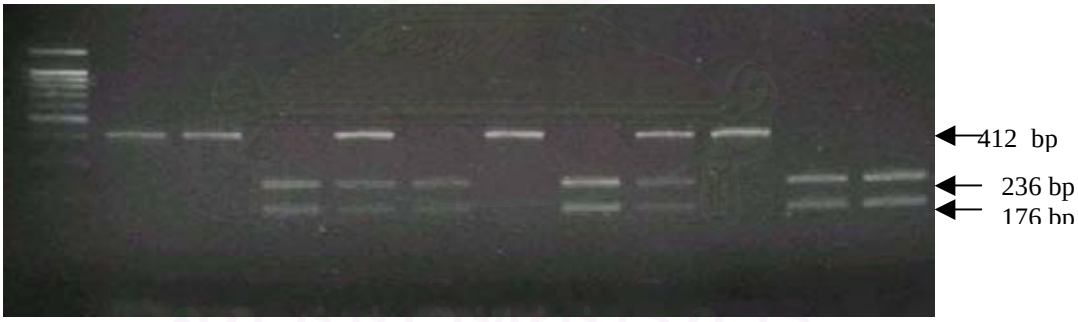



**Figure 5.** The representative of PCR-RFLP results from samples with homozygous of –592C, homozygous of –592A and heterozygous –592A/C.

Lane 1 is 100 bp molecular markers

Lane 3,7,10 are homozygous of –592C.

Lane 4,6,8,11,12 are homozygous of –592A.

Lane 5,9 are heterozygous of –592A/C.

U = not add restriction enzyme, C = add restriction enzyme

**1.2 Sequencing confirmation of the PCR-RFLP results of polymorphisms at -592A/C of the IL-10 gene**

DNA sequencing confirm the results of polymorphisms at –592A/C of the IL-10 gene from PCR-RFLP method as shown in Figure 6.

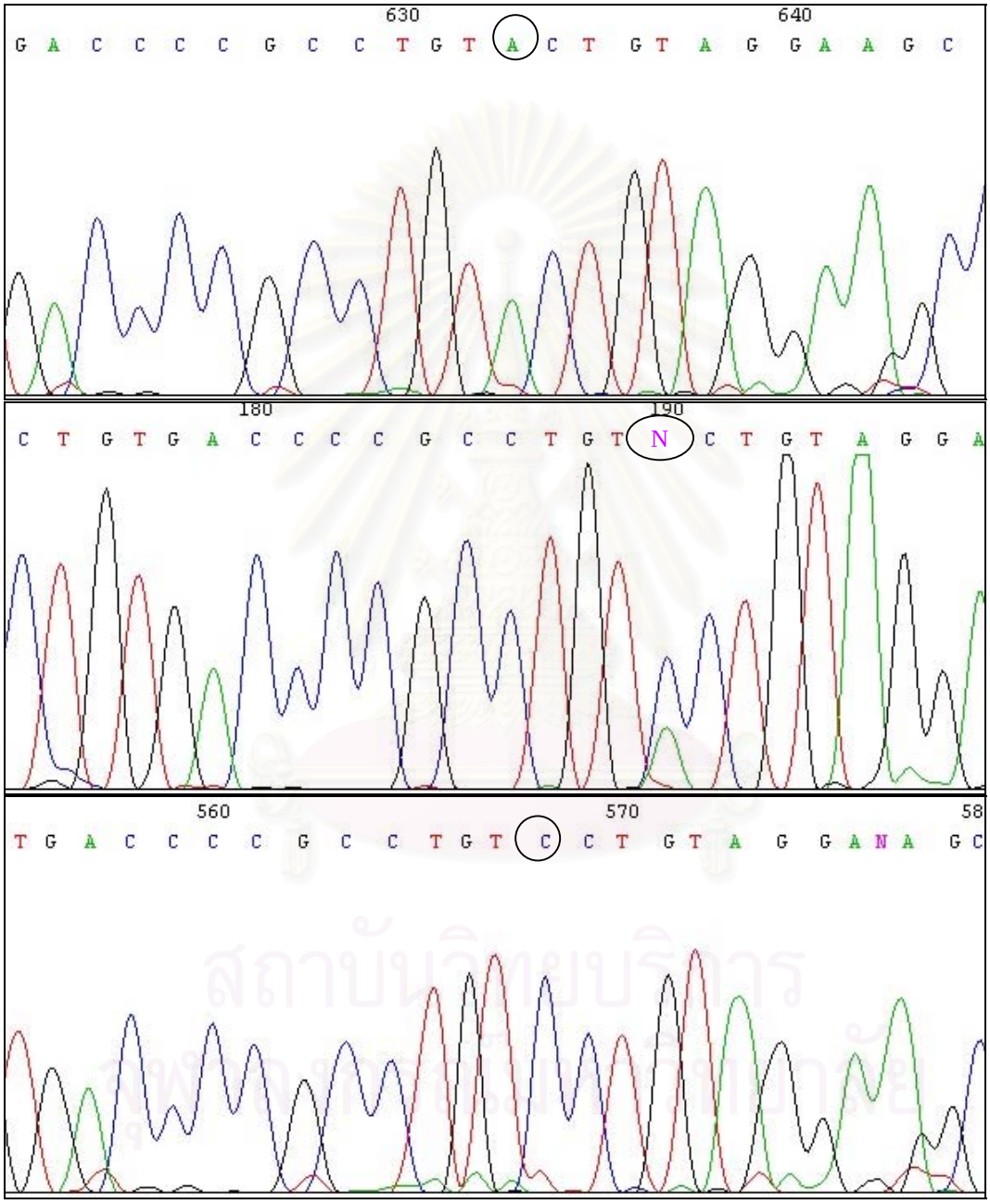



**Figure 6.** Chromatogram of DNA sequences in homozygous of –592A (A), heterozygous of –592A/C (B) and homozygous of –592C (C).

**1.3 PCR-RFLP analysis of IL-10 promoter at position –819**

Polymorphism at –819C/T in the promoter region of the IL-10 were identified by the PCR-RFLP method. If a C was present at this position, the *Msl*I restriction enzyme would cut the 209 bp PCR product into two fragment; 125 and 84 bp. No digestion would occur if a T was present. (Santos, Suffys et al. 2002) (Figure 7)

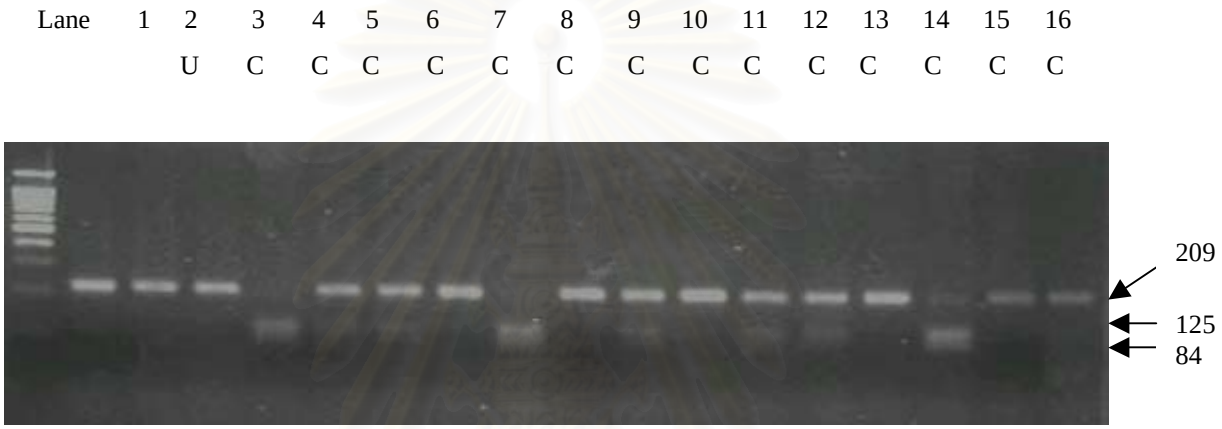



**Figure 7.** The representative of PCR-RFLP results from samples with homozygous of –819C, homozygous of –819T and heterozygous –819C/T.

Lane 1 is 100 bp molecular marker.

Lane 5,9,16 are homozygous of –819C .

Lane 3,4,8,10,12,15 are homozygous of –819T .

Lane 6,7,11,13,14 are heterozygous of –819C/T .

U = not add restriction enzyme, C = add restriction enzyme

**1.4 Sequencing confirmation of the PCR-RFLP results of polymorphisms at -819C/T of the IL-10 gene**

DNA sequencing confirm the results of polymorphisms at –819C/T of the IL-10 gene from PCR-RFLP method as shown in Figure 8 .

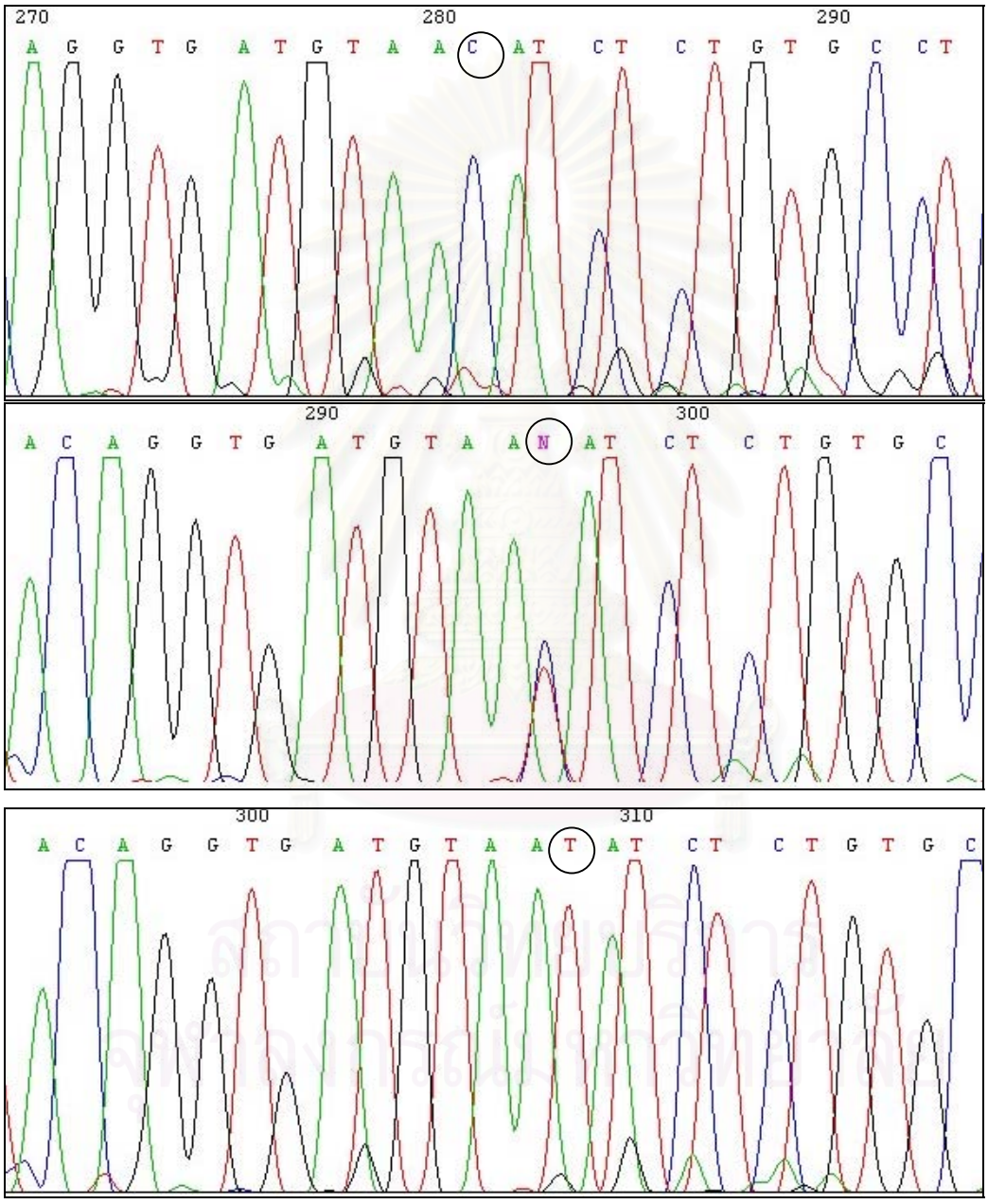



**Figure 8.** Chromatogram of DNA sequences in homozygous of –819C (A), heterozygous of –819C/T (B) and homozygous of –819T (C).

**1.5 PCR-RFLP analysis of IL-10 promoter at position –1082**

Polymorphism at –1082G/A in the promoter region of the IL-10 were identified by the PCR-RFLP method. If a G was present at this position, the *Mnl*I restriction enzyme would cut the 139 bp PCR product into two fragment; 106 and 33 bp. No digestion would occur if an A was present. (Edwards-Smith, Jonsson et al. 1999) (Figure 9).

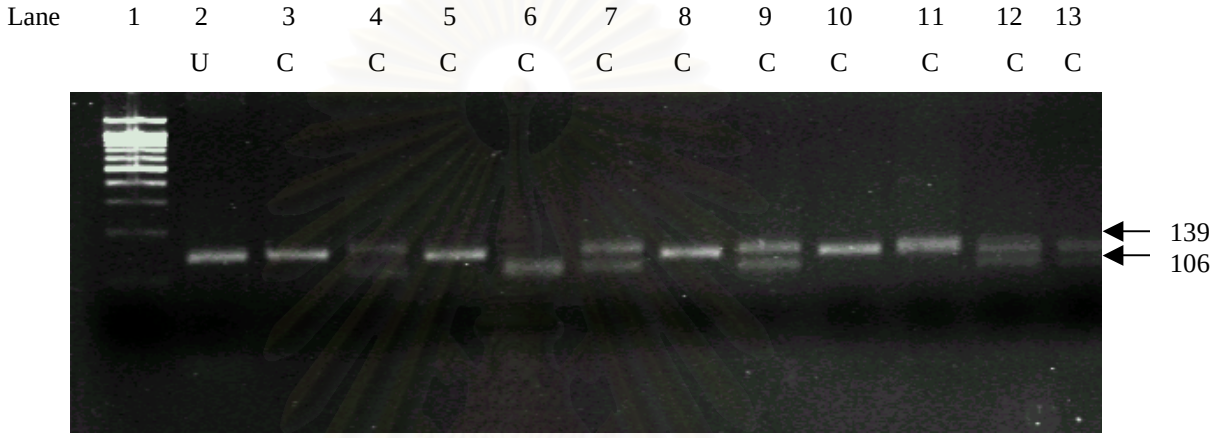



**Figure 9.** The representative of PCR-RFLP results from samples with homozygous of –1082G, homozygous of –1082A and heterozygous –1082G/A.

Lane 1 is 100 bp molecular marker.

Lane 6 is homozygous of –1082G.

Lane 3,5,8,10,11 are homozygous of –1082A .

Lane 4,7,9,12,13 are heterozygous of –1082G/A.

Under these electrophoresis condition the 33 bp product is not visible.

U = not add restriction enzyme, C = add restriction enzyme

**1.6 Sequencing confirmation of the PCR-RFLP results of polymorphisms at -1082G/A of the IL-10 gene**

DNA sequencing confirm the results of polymorphisms at –1082G/A of the IL-10 gene from PCR-RFLP method as shown in Figure 10 .

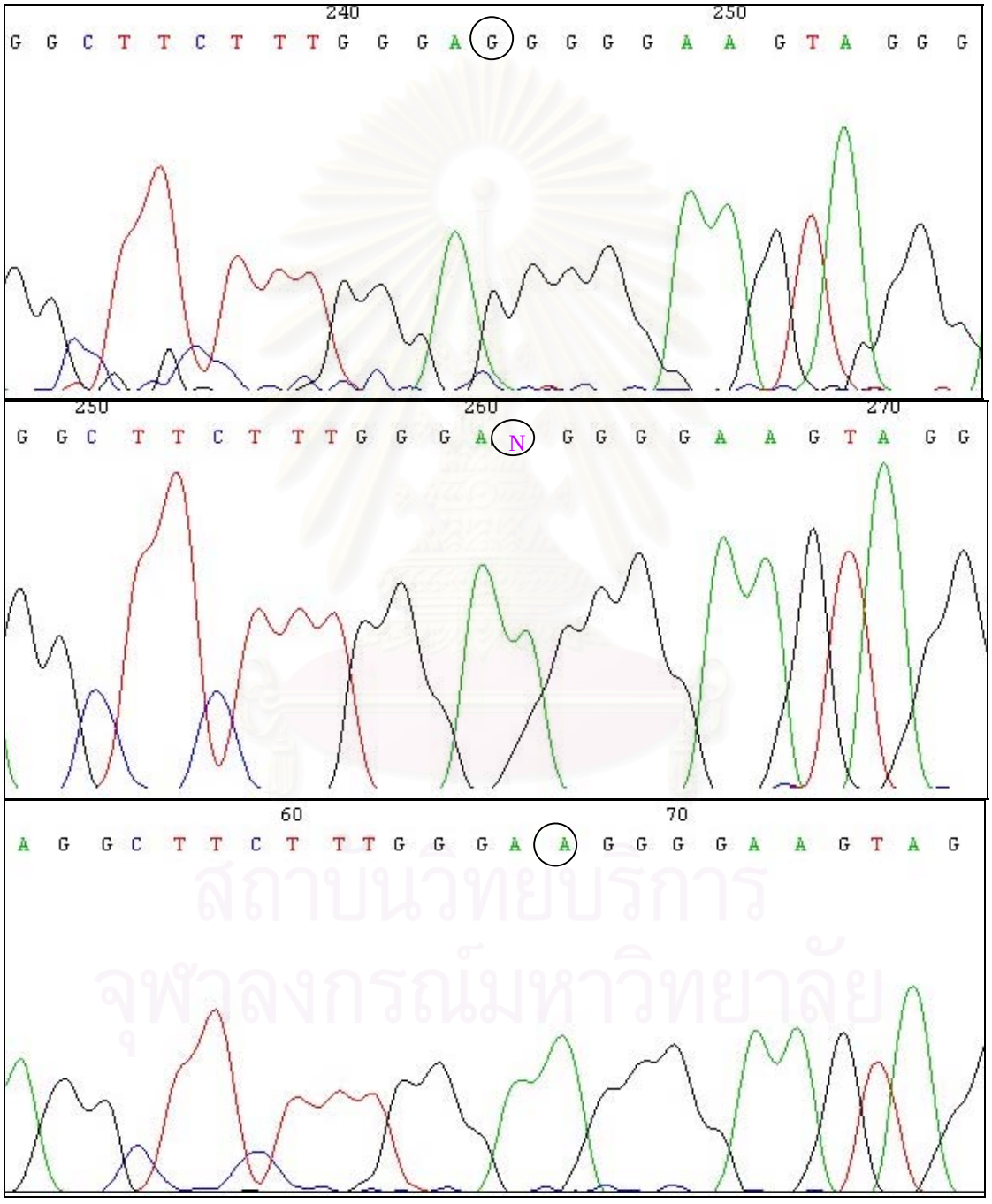



**Figure 10.** Chromatogram of DNA sequences in homozygous of –1082G (A), heterozygous of –1082G/A (B) and homozygous of –1082A (C).

**2. Polymerase Chain Reaction-Sequence Specific Primer (PCR-SSP) Analysis of FcγRIIa**

FcγRIIa R/H polymorphism at amino acid position 131 were identified by using the PCR-SSP method. The positive results of FcγRIIa gene and human growth hormone gene (internal control) showed band of 225 and 428 bp fragment, respectively. (Hatta, Tsuchiya et al. 1999) (Figure 11).

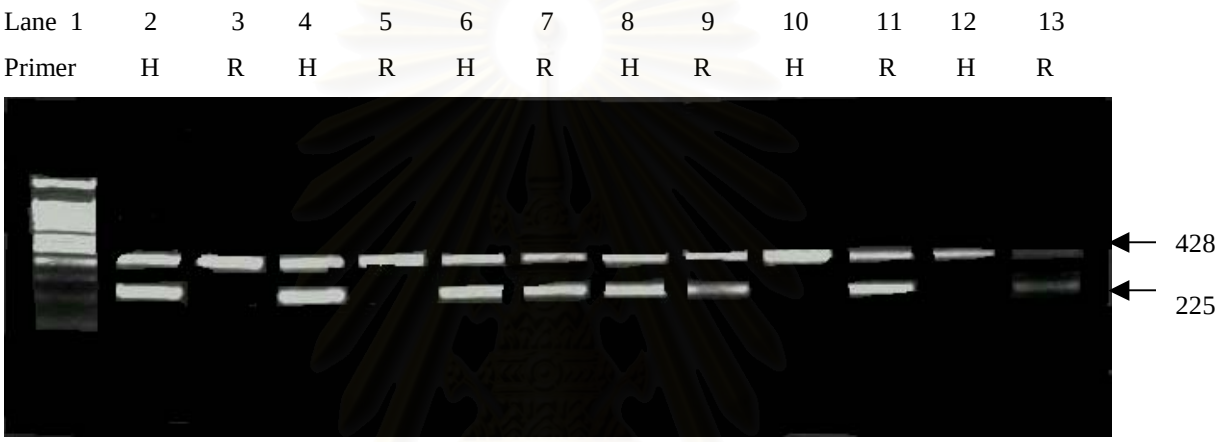



**Figure 11.** The representative of PCR-SSP results from samples with homozygous of H131, heterozygous R/H131 and homozygous of R131.

Lane 1 is 100 bp molecular marker.

Lane 2-5 are homozygous of H131.

Lane 6-9 are heterozygous of R/H131.

Lane 10-13 are homozygous of R131.

**2.1 Sequencing confirmation of the PCR-SSP results of polymorphisms at R/H131 of FcγRIIa gene .**

DNA sequencing confirm the results of polymorphisms at R/H131 of FcγRIIa gene from PCR-SSP method as shown in Figure 12 .

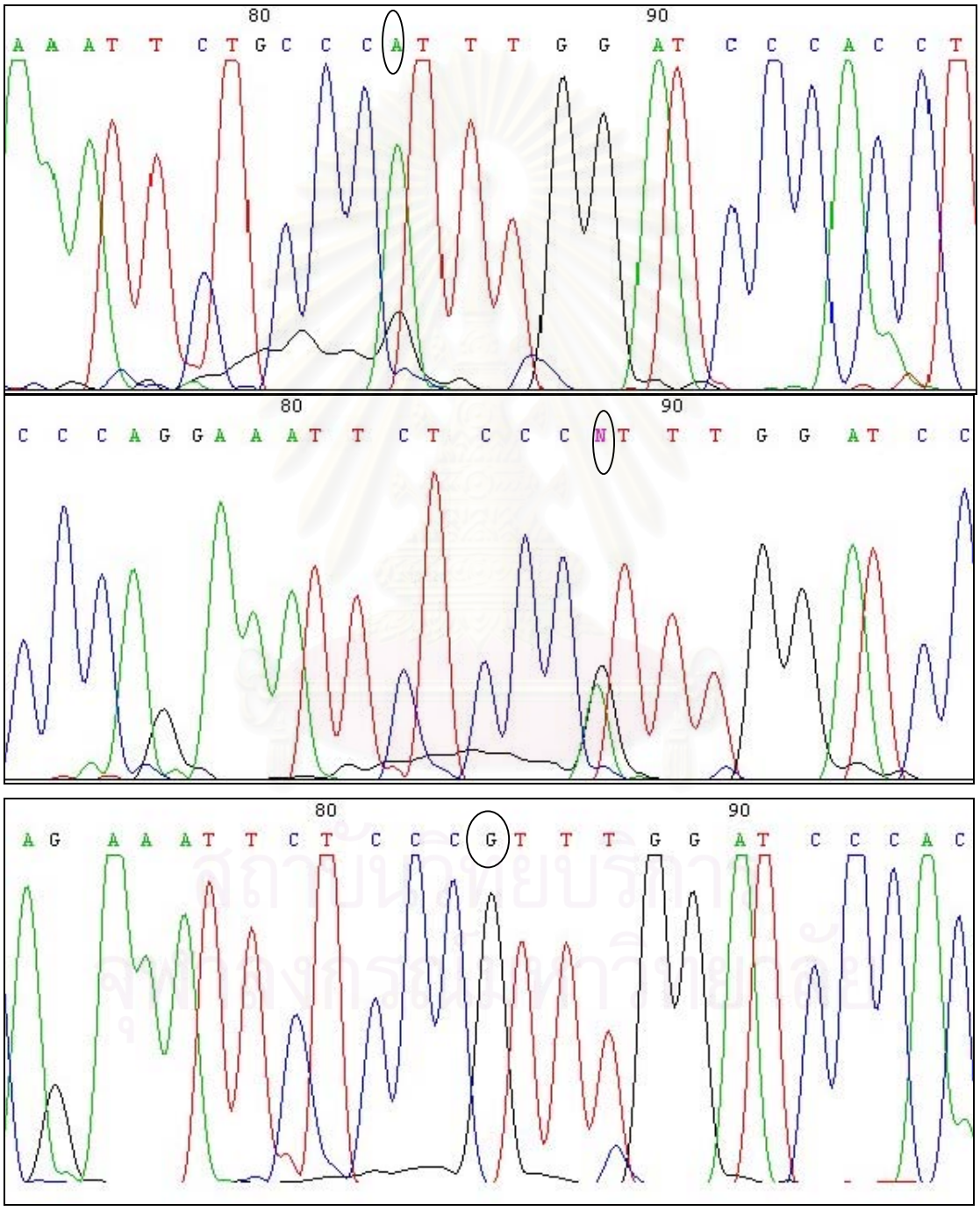



**Figure12.** Chromatogram of DNA sequences in homozygous of H131 (A), heterozygous of R/H131 (B) and homozygous of R131(C).

**3. The association results of IL-10 and FcγRIIa gene polymorphisms with SLE**

**3.1 IL-10 gene polymorphism**

**3.1.1 IL-10 gene promoter polymorphism at position –592(A/C)**

Genotype and allele frequencies for –592 at the promoter of IL-10 gene in healthy controls and SLE patients were shown in table 9 and 10. Eighty of 150 healthy controls (53.3%) were homozygous for A/A genotype, 62(41.3%) were heterozygous and 8(5.4%) were homozygous for the C/C genotype. The allele frequencies were 74% for A allele and 26% for C allele. In comparison, 64 of 150 SLE patients (42.7%) were homozygous for A/A genotype, 71(47.3%) were heterozygous and 15(10%) were homozygous for the C/C genotype. The allele frequencies were 66.3% for A allele and 33.7% for C allele. The –592*C allele was found to be significantly increased in SLE patients compared with healthy controls (OR= 1.44, 95%CI= 1.00-2.09, $p$= 0.04).

**3.1.2 IL-10 gene promoter polymorphism at position –819 (C/T)**

Genotype and allele frequencies for –819 at the promoter of IL-10 gene in healthy controls and SLE patients were shown in table 11 and 12. Seven of 150 healthy controls (4.6 %) were homozygous for C/C genotype, 64(42.7%)were heterozygous and 79(52.7%)were homozygous for the T/T genotype. The allele frequencies were 26% for C allele and 74 % for T allele. In comparison, 15 of 150 SLE patients (10%) were homozygous for C/C genotype, 71(47.3%) were heterozygous and 64(42.7%) were homozygous for the T/T genotype. The allele frequencies were 33.7% for C allele and 66.3% for T allele. The –819*C allele was found to be significantly increased in SLE patients compared with healthy controls (OR= 1.44, 95%CI= 1.00-2.09, $p$= 0.04).

**Table 9.** Genotype and allele frequencies for IL-10 promoter polymorphism at position –592 in healthy controls and SLE patients.

| | SLE patients n = 150 | Healthy controls n =150 |
|---|---|---|
| Genotype frequencies | | |
| A/A | 64(42.7%) | 80(53.3%) |
| A/C | 71(47.3%) | 62(41.3%) |
| C/C | 15(10%) | 8(5.4%) |
| Allele frequencies | | |
| A | 199(66.3%) | 222(74%) |
| C | 101(33.7%)[a] | 78(26%) |

[a] $p$ =0.04, OR = 1.44, 95%CI = 1.00-2.09

**Table 10.** Risk of SLE associated with IL-10 (-592A/C) genotype according to different models of inheritance.

| | SLE patients n = 150 | Healthy controls n =150 |
|---|---|---|
| C dominance, A wild type | | |
| C/C or A/C | 86(57.3%) [a] | 70(46.7%) |
| A/A | 64(42.7%) | 80(53.3%) |
| C recessive, A wild type | | |
| C/C | 15(10%) [b] | 8(5.3%) |
| A/A or A/C | 135(90%) | 142(94.7%) |

[a] $p$ =0.08

[b] $p$ = 0.1

**Table 11.** Genotype and allele frequencies for IL-10 promoter polymorphism at position –819 in healthy controls and SLE patients.

| | SLE patients n = 150 | Healthy controls n =150 |
|---|---|---|
| Genotype frequencies | | |
| C/C | 15(10%) | 7(4.6%) |
| C/T | 71(47.3%) | 64(42.7%) |
| T/T | 64(42.7%) | 79(52.7%) |
| Allele frequencies | | |
| C allele | 101(33.7%)[a] | 78(26%) |
| T allele | 199(66.3%) | 222(74%) |

[a] $p$ =0.04, OR = 1.44, 95%CI = 1.00-2.09

**Table 12.** Risk of SLE associated with IL-10 (-819C/T) genotype according to different models of inheritance .

| | SLE patients n = 150 | Healthy controls n =150 |
|---|---|---|
| C dominance, T wild type | | |
| C/C or C/T | 86(57.3%)[a] | 71(47.3%) |
| T/T | 64(42.7%) | 79(52.7%) |
| C recessive, T wild type | | |
| C/C | 15(10%)[b] | 7(4.7%) |
| C/T or T/T | 135(90%) | 143(95.3%) |

[a] $p$ =0.1

[b] $p$ = 0.1

### 3.1.3 IL-10 gene promoter polymorphism at position –1082(G/A)

Genotype and allele frequencies for –1082 at the promoter of IL-10 gene in healthy controls and SLE patients were shown in table 13 and 14. One hundred-thirty three of 150 healthy controls (88.7%) were homozygous for A/A genotype, 17(11.3%) were heterozygous and no one were homozygous for the G/G genotype. The allele frequencies were 5.7% for G allele and 94.3% for A allele. In comparison, 133 of 150 SLE patients (88.7%) were homozygous for A/A genotype, 16(10.7%) were heterozygous and 1(0.6%) was homozygous for the G/G genotype. The allele frequencies were 6 % for G allele and 94 % for A allele. There were no significance differences in allele frequency of –1082(G/A) promoter polymorphism of IL-10 gene between patients with SLE and healthy controls.

### 3.1.4 Haplotype analysis of IL-10 promoter polymorphism at position (-592A/C, -819C/T, -1082G/A)

The haplotype frequencies of the IL-10 promoter polymorphism were also determined by PHASE program .The genotype frequencies of the haplotype and haplotype frequencies in SLE patients and healthy controls were shown in table 15 and 16. In haplotype analysis, we found 6 haplotypes; ATA, ACC, GCC, ATC, and ACA in SLE patients and healthy controls. ACC haplotype was found to be significantly increased in SLE patients (27.3%), as compared with healthy controls (19.3%) (OR= 1.57, 95%CI= 1.05-2.34, $p$= 0.02). In contrast, ATA haplotype was decreased in SLE patients (66%) compared with healthy controls (73.3%), but did not reach statistical significance. However, no significant differences in haplotype frequencies of GCC, ATC, and ACA could be demonstrated between SLE patients and healthy controls.

**Table 13.** Genotype and allele frequencies for IL-10 promoter polymorphism at position –1082 in healthy controls and SLE patients.

| | SLE patients n = 150 | Healthy controls n =150 |
|---|---|---|
| Genotype frequencies | | |
| G/G | 1(0.6%) | 0 |
| G/A | 16(10.7%) | 17(11.3%) |
| A/A | 133(88.7%) | 133(88.7%) |
| Allele frequencies | | |
| G allele | 18(6%) [a] | 17(5.7%) |
| A allele | 282(94%) | 283(94.3%) |

[a] $p$ =0.8

**Table 14.** Risk of SLE associated with IL-10(-1082G/A) genotype according to different models of inheritance .

| | SLE patients n = 150 | Healthy controls n =150 |
|---|---|---|
| G dominance, A wild type | | |
| G/G or G/A | 17(11.3%) [a] | 17(11.3%) |
| A/A | 133(88.7%) | 133(88.7%) |
| G recessive, A wild type | | |
| G/G | 1(0.6%) [b] | 0(0%) |
| G/A or A/A | 153(99.4%) | 154(100%) |

[a] $p$ =1

[b] $p$ = 1

**Table 15.** Genotype frequencies of haplotype for IL-10 promoter polymorphism at position (-592A/C, -819C/T, -1082G/A) between healthy controls and SLE patients .

| | SLE patients n = 150 | Healthy controls n =150 |
|---|---|---|
| Genotype frequencies of haplotype | | |
| GCC/GCC | 1 | 0 |
| GCC/ACC | 3 | 3 |
| GCC/ATA | 13 | 15 |
| ACC/ACC | 11 | 3 |
| ACC/ATA | 57 | 47 |
| ACC/ATC | 0 | 2 |
| ATA/ATA | 63 | 79 |
| ATA/ATC | 1 | 0 |
| ATA/ACA | 1 | 0 |
| ACA/ACA | 0 | 1 |

**Table 16.** Haplotype frequencies of IL-10 promoter polymorphism at position (-592A/C, -819C/T, -1082G/A) between healthy controls and SLE patients .

| | SLE patients *n* = 300 | Healthy controls *n* =300 |
|---|---|---|
| Haplotype frequencies | | |
| ATA | 198(66%) [a] | 220(73.3%) |
| other haplotype | 102(34%) | 80(26.7%) |
| ACC | 82(27.3%) [b] | 58(19.3%) |
| other haplotype | 218(72.7%) | 242(80.7%) |
| GCC | 18(6%) [c] | 18(6 %) |
| other haplotype | 282(94%) | 282(94%) |
| ATC | 1(0.3%) [d] | 2(0.7%) |
| other haplotype | 299(99.7%) | 298 (99.3%) |
| ACA | 1(0.3%) [e] | 2(0.7%) |
| other haplotype | 299(99.7%) | 298(99.3%) |

[a] $p$ =0.06

[b] OR= 1.57, 95%CI= 1.05-2.34, $p$= 0.02

[c] $p$ =1

[d] $p$ =1

[e] $p$ =1

**3.2 FcγRIIa gene polymorphisms**

Genotype and allele frequencies for FcγRIIa R/H polymorphism at amino acid position 131 in healthy controls and SLE patients were demonstrated in table 17 and 18. Thirteen of 150 healthy controls (8.6%) were homozygous for R/R genotype, 49(32.7%) were heterozygous and 88(58.7%) were homozygous for H/H genotype. The allele frequencies were 25% for R allele and 75% for H allele. In comparison, 14 of 150 SLE patients (9.3%) were homozygous for R/R genotype, 75(50%) were heterozygous and 61(40.7%) were homozygous for H/H genotype. The allele frequencies were 34.3% for R allele and 65.7% for H allele. The R131 allele was found to be significantly increased in SLE patients compared with healthy controls (OR= 1.57, 95%CI= 1.08-2.27, $p$= 0.01).

The effect of R131 allele of FcγRIIa gene was similar to autosomal dominance mode of inheritance. The presence of one R allele (RR or RH) conferred the significant OR of 2.07 (95%CI=1.27-3.37, $p$= 0.001).

**Table 17.** Genotype and allele frequencies for FcγRIIa R/H polymorphism at amino acid position 131 in healthy controls and SLE patients .

| | SLE patients n = 150 | Healthy controls n =150 |
|---|---|---|
| Genotype frequencies | | |
| R/R | 14(9.3%) | 13(8.6%) |
| R/H | 75(50%) | 49(32.7%) |
| H/H | 61(40.7%) | 88(58.7%) |
| Allele frequencies | | |
| R allele | 103(34.3%) [a] | 75(25%) |
| H allele | 197(65.7%) | 225(75%) |

[a] OR= 1.57, 95%CI= 1.08-2.27, *p*= 0.01

**Table 18.** Risk of SLE associated with FcγRIIa R/H131 genotype according to different models of inheritance.

| | SLE patients n = 150 | Healthy controls n =150 |
|---|---|---|
| R dominance, H wild type | | |
| R/R or R/H | 89(59.3%) [a] | 62(41.3%) |
| H/H | 61(40.7%) | 88(58.7%) |
| R recessive, H wild type | | |
| R/R | 14(9.3%) [b] | 13(8.7%) |
| R/H or H/H | 136(90.7%) | 137(91.3%) |

[a] OR= 2.07, 95%CI=1.27-3.37, *p*= 0.001

[b] *p* =0.84

**3.3 Synergistic effect between IL-10 and FcγRIIa gene polymorphisms in susceptible to SLE**

Since both IL-10 and FcγRIIa gene were located on chromosome 1, we hypothesized that individuals carrying both specific genotype of IL-10 and FcγRIIa were at higher risk of developing of SLE than those carrying only one genotype.

Then, we analyzed by comparing 3 groups using chi-square test, and the odds ratios were calculated, as shown in table 19;

1. Individuals carrying both specific genotype of IL-10 (ACC haplotype) and FcγRIIa (R allele) compared with individuals, who did not carry specific genotype of IL-10 (ACC haplotype) and FcγRIIa (R allele).
2. Individuals carrying only specific genotype of IL-10 (ACC haplotype) but not carrying FcγRIIa (R allele) compared with individuals, who did not carry specific genotype of IL-10 (ACC haplotype) and FcγRIIa (R allele).
3. Individuals carrying only specific genotype of FcγRIIa (R allele) but not carrying IL-10 (ACC haplotype) compared with individuals, who did not carry specific genotype of IL-10 (ACC haplotype) and FcγRIIa (R allele).

**Table 19**. Synergistic effect between IL-10 and FcγRIIa gene polymorphisms in susceptible to SLE .

| Group | SLE | Control | OR |
|---|---|---|---|
| 1 | 7 | 1 | 10.93 [a] |
| | 57 | 89 | |
| 2 | 4 | 3 | 2.08 [b] |
| | 57 | 89 | |
| 3 | 86 | 61 | 2.20 [c] |
| | 57 | 89 | |

[a] $p$= 0.006, 95%CI= 1.29-242.64

[b] $p$= 0.3

[c] $p$= 0.0008, 95%CI= 1.34-3.61

According to table 19, the synergistic effect between IL-10 (ACC/ACC) and FcγRIIa (R/R or R/H) gene as observed since the OR from group 1 is more than the multiplicity of OR from group 2 and group 3.

**4. The association results of IL-10 and FcγRIIa gene polymorphisms with clinical manifestation SLE**

**4.1 Clinical manifestation of SLE patients**

The clinical expression of SLE is tremendously varied among individuals .In this study, we obtained clinical data of 115 patients, as shown in table 20.

**Table 20.** Clinical manifestation and certain autoantibodies production of SLE patients in this study

| Clinical manifestation | No. of SLE patients |
|---|---|
| 1.Musculoskeletal | 95 (82.6%) |
| 2.Mucocutaneous | 96 (83.5%) |
| 3. Raynaud phenomenon | 27 (23.5%) |
| 4. Hematologic | 93 (80.9%) |
| 5. Cardiovascular | 5 (4.3%) |
| 6. Neuropsychiatric | 12 (10.4%) |
| 7. Pulmonary | 4 (3.5%) |
| 8. Gastrointestinal | 7 (6.1%) |
| 9. Renal | 82 (71.3%) |
| Autoantibodies production<br>1. Anti-cardiolipin antibodies | 43 (37.3%) |
| 2. Anti –dsDNA antibodies | 58 (50.4%) |

### 4.2 IL-10 promoter polymorphisms and clinical presentation of SLE

The clinical manifestation and autoantibodies profiles of our SLE patients with various haplotypes of the IL-10 promoter were compared using chi-square test, and the odds ratios were calculated. No significances in clinical features between patients with various haplotypes could be demonstrated, except for the association between ATA haplotype and renal disorder. Table 21 shows the frequencies of various clinical manifestation and autoantibodies profiles in SLE patients with and without the ATA, ACC, and GCC haplotype .The GCC haplotype was significantly associated with renal involvement (OR =7.45, 95%CI= 1.04-154.1, $p$ = 0.02).

### 4.3 FcγRIIa gene polymorphisms and clinical presentation of SLE

The clinical manifestation and autoantibodies profiles of our SLE patients with allele frequencies and genotype of FcγRIIa gene were compared and shown in table 22 and 23, respectively. No significances association of clinical features and genotype or allele frequencies of FcγRIIa gene could be demonstrated, except for the R/R genotype and frequencies of R allele with anti-cardiolipin antibodies production. The R/R genotype was found to be significantly increased in SLE patient with positive for anti-cardiolipin antibodies production, compared with healthy controls (OR=2.79, 95%CI= 1.00-7.72, $p$= 0.02). Interestingly, R/R genotype was significantly higher in SLE patient with positive for anti-cardiolipin antibodies production compared with SLE controls, who did not produce anti-cardiolipin antibodies (OR=6.09, 95%CI= 1.38-30.54, $p$= 0.004). Similarity, the frequencies of R allele were significantly associated with anti-cardiolipin antibodies production (OR=1.82,95%CI=1.00-3.31,$p$=0.03).

**Table 21**. The frequencies of various clinical manifestation and autoantibodies profiles in SLE patients with and without the ATA, ACC, and GCC haplotype.

| Haplotype / Clinical features | ATA (*n*=151) (%) | Non-ATA (*n*=79) (%) | *p* | ACC (*n*=61) (%) | Non-ACC (*n*=169) (%) | *p* | GCC (*n*=16) (%) | Non-GCC (*n*=214) (%) | *p* |
|---|---|---|---|---|---|---|---|---|---|
| -Musculoskeletal | 128(85) | 62(79) | NS | 50(82) | 140(83) | NS | 10(63) | 180(84) | NS |
| -Mucocutaneous | 125(83) | 67(85) | NS | 52(85) | 140(83) | NS | 13(82) | 179(83) | NS |
| -Raynaud phenomenon | 32(21) | 22(28) | NS | 19(31) | 35(21) | NS | 2(13) | 52(24) | NS |
| - Hematologic | 125(83) | 61(77) | NS | 47(77) | 139(82) | NS | 14(88) | 172(80) | NS |
| -Cardiovascular | 13(9) | 11(14) | NS | 9(15) | 15(9) | NS | 2(13) | 22(10) | NS |
| -Neuropsychiatric | 6(4) | 4(5) | NS | 4(7) | 6(4) | NS | 0 | 10(5) | NS |
| -Pulmonary | 7(5) | 1(1) | NS | 1(2) | 7(4) | NS | 0 | 8(4) | NS |
| -Gastrointestinal | 9(6) | 5(6) | NS | 4(7) | 10(6) | NS | 1(7) | 13(6) | NS |
| Renal | 104(69) | 60(76) | NS | 44(72) | 120(71) | NS | 15(94) | 143(67) | 0.02* |
| Autoantibodies profiles<br>-Anti-cardiolipin antibodies | 55(36) | 31(39) | NS | 24(39) | 62(37) | NS | 7(44) | 79(37) | NS |
| -Anti –dsDNA antibodies | 76(50) | 40(51) | NS | 31(51) | 85(50) | NS | 8(50) | 108(50) | NS |

* The *p* value was lower than 0.05, NS = Not Significant

**Table 22**. The frequencies of various clinical manifestation and autoantibodies profiles in SLE patients with FcγRIIa gene polymorphisms.

| Allele / Clinical features | R allele (*n*=75) (%) | H allele (*n*=155) (%) | *p* |
|---|---|---|---|
| -Musculoskeletal | 62(83) | 128(82) | NS |
| -Mucocutaneous | 63(84) | 129(83) | NS |
| -Raynaud phenomenon | 17(23) | 37(24) | NS |
| -Hematologic | 59(79) | 127(82) | NS |
| -Cardiovascular | 6(8) | 18(12) | NS |
| -Neuropsychiatric | 3(4) | 7(5) | NS |
| -Pulmonary | 2(3) | 6(4) | NS |
| -Gastrointestinal | 5(7) | 9(6) | NS |
| -Renal | 56(75) | 108(70) | NS |
| Autoantibodies profiles<br>-Anti-cardiolipin antibodies | 35(47) | 52(34) | 0.03* |
| -Anti –dsDNA antibodies | 39(52) | 77(50) | NS |

* The *p* value was lower than 0.05, NS = Not Significant

**Table 23**. Genotype of FcγRIIa R/H polymorphism in SLE patient with positive for anti-cardiolipin antibodies production, SLE controls, and healthy controls.

| Genotype | SLE patient with positive for anti-cardiolipin antibodies production (N=43) (%) | SLE controls (N=72) (%) | Healthy controls (N=150) (%) |
|---|---|---|---|
| R/R | 9(21)[a,b] | 3(4) | 13(9) |
| Others | 34(79) | 69(96) | 137((91) |
| R/H | 16(37) | 35(49) | 49(33) |
| Others | 27(63) | 37(51) | 101(67) |
| H/H | 18(42) | 34(47) | 88(59) |
| Others | 25(58) | 38(53) | 62(41) |

[a] *p*=0.004, 95%CI= 1.38-30.54, OR = 6.09, when compared with SLE controls

[b] *p*=0.02, 95%CI= 1.00-7.72, OR = 2.79, when compared with healthy controls

**5. Pattern of IL-10 and FcγRIIa gene polymorphisms in various populations**

This study will provide the basic knowledge of allele distribution for IL-10 and FcγRIIa gene polymorphisms in healthy Thais individuals, as compared with other populations from previous reports.

**5.1 Pattern of IL-10 gene polymorphism**

Allele and haplotype frequencies of IL-10 gene polymorphism comparing between study group and previous report groups, that consist of Caucasians, African-American, Hispanic, and Asians population, are reflected in Table 24 and Table 25. The frequencies of the –1082*A,-819*T, and –592*A alleles were significantly higher, and –1082*G, -819*C, and –592*C alleles were lower in Thais population ,compared with Caucasians, Hispanic, and African-American (see Table 24) . Also, the haplotype frequencies, the ATA haplotype was significantly higher in Thais than Caucasians, Hispanic, and African-American. In contrast, the frequency of the GCC haplotype was significantly lower in Thais compared with those populations , except for Hispanic .The ATC and ACA haplotypes ,which were rare haplotypes, however , its were found in this study as same as in Chinese Han people. By the way , the frequency of GTA haplotype was not observed in this study identical to other populations, with the exception of Chinese population (see Table 25).

**5.2 Pattern of FcγRIIa gene polymorphisms**

Allele and genotype frequencies of FcγRIIa gene polymorphisms were analyzed and shown in Table 26. There were significant differences in allele and genotype frequencies in Thai population compared with Caucasian, Korean, Vietnamese, African-American. Contrary, there was no significant difference between Thais and Japanese population.

**Table 24.** Allele frequencies of the IL-10 promoter polymorphisms in healthy Thais individuals compared with other groups

| Genotype position | This study (Thais) n=300 (%) | Korean n=622 (%) (Pyo, Hur et al. 2003) | Chinese n=152 (%) (Mok, Lanchbury et al. 1998) | Japanese n=104 (%) (Miyazoe, Hamasaki et al. 2002) | Italian n=144 (%) (Matsushita, Tanaka et al. 2002) | Greek n=200 (%) (Costeas, Koumas et al. 2003) | Caucasians n=660 (%) (Perrey, Pravica et al. 1998) | African-American n=428 (%) (Hoffmann, Stanley et al. 2002) | Hispanic n=50 (%) (Hoffman, Stanley et al. 2002) |
|---|---|---|---|---|---|---|---|---|---|
| -1082 | | | | | | | | | |
| G allele | 18(6) | 46(7.4) | 9(6.0) | 4(3.8) | 48(33)* | 76(38)* | 323(49.0)* | 198(46.3)* | 11(22.0)* |
| A allele | 282(94) | 576(92.6) | 143(94.0) | 100(96.2) | 96(67)* | 124(62)* | 337(51.0)* | 230(53.7)* | 76(66.7)* |
| -819 | | | | | | | | | |
| C allele | 101(33.7) | 200(32.2) | 50(33.0) | 32(30.8) | 104(72)* | 153(76.5)* | 508(77.0)* | 330(77.1)* | 30(60.0)* |
| T allele | 199(66.3) | 422(67.8) | 102(67.0) | 72(69.2) | 40(28)* | 47(23.5)* | 152(23.0)* | 98(22.9)* | 20(40.0)* |
| -592 | | | | | | | | | |
| C allele | 101(33.7) | 200(32.2) | 50(33.0) | 32(30.8) | 104(72)* | 153(76.5)* | 508(77.0)* | 330(77.1)* | 30(60.0)* |
| A allele | 199(66.3) | 422(67.8) | 102(67.0) | 72(69.2) | 40(28)* | 47(23.5)* | 152(23.0)* | 98(22.9)* | 20(40.0)* |

* The *p* value was lower than 0.05 when compared with Thais .

**Table 25.** Haplotype frequencies of the IL-10 promoter polymorphisms in healthy Thais individuals compared with other groups

| Haplotype | This study (Thais) n=300 (%) | Korean n=622 (%) (Pyo, Hur et al. 2003) | Chinese n=152 (%) (Mok, Lanchbury et al. 1998) | Japanese n=104 (%) (Miyazoe, Hamasaki et al. 2002) | Italian n=144 (%) (Matsushita, Tanaka et al. 2002) | Greek n=200 (%) (Costeas, Koumas et al. 2003) | Caucasians n=660 (%) (Perrey, Pravica et al. 1998) | African-American n=428 (%) (Hoffmann, Stanley et al. 2002) | Hispanic n=50 (%) (Hoffmann, Stanley et al. 2002) |
|---|---|---|---|---|---|---|---|---|---|
| GCC | 18(6) | 46(7.4) | 3(2.0) | 4(4) | 47(33)* | 70(35)* | 326(49.4)* | 173(46.7)* | 9(18.8) |
| ACC | 58(19.3) | 154(24.8) | 46(30.0) | 28(27) | 56(39) | 83(41.5) | 190(28.8) | 115(31.1) | 19(39.6) |
| ATA | 220(73.3) | 422(67.8) | 97(64.0) | 72(69) | 41(28)* | 47(23.5)* | 144(21.8)* | 82(22.2)* | 20(41.6)* |
| ATC | 2(0.7) | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| ACA | 2(0.7) | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| GTA | 0 | 0 | 6(4.0) | 0 | 0 | 0 | 0 | 0 | 0 |

* The *p* value was lower than 0.05 when compared with Thais .

**Table 26.** Genotype and allele frequencies of FcγRIIa-R/H131 polymorphisms in healthy Thais individuals compared with other groups

| | This study (Thais) N=150 | Vietnamese N=43 (Khoa, Sugiyama et al. 2003) | Japanese N= 217 (Hatta, Tsuchiya et al. 1999) | Korean N=64 (Song, Han et al. 1998) | Caucasian N=187 (Manger, Repp et al. 1998) | African-American N=100 (Salmon 1996) |
|---|---|---|---|---|---|---|
| Genotype frequencies | | | | | | |
| R/R | 13(8.6%) | 4(9.3%)* | 7(3.2%) | 5(7.8%)* | 50(26.7%)* | 23* |
| R/H | 49(32.7%) | 23(53.5%)* | 72(33.2%) | 37(57.8%)* | 85(44.9%)* | 50* |
| H/H | 88(58.7%) | 16(37.2%)* | 138(63.6%) | 22(34.4%)* | 52(28.4%)* | 27* |
| Allele frequencies | | | | | | |
| R allele | 75(25%) | 31(36%)* | 86(20%) | 47(36.7%)* | 185(49.5%)* | 96(48%)* |
| H allele | 225(75%) | 55(64%)* | 350(80%) | 81(63.3%)* | 189(50.5%)* | 104(52%)* |

* The *p* value was lower than 0.05 when compared with Thais .

# CHAPTER VI

# DISCUSSION

The present study revealed that ACC haplotype of IL-10 gene and FcγRIIa R131 polymorphism might be regarded as marker for genetic susceptibility to SLE in Thai population. This finding is consistent with the proposed biology of selected SNPs. The function of each genotypes related to pathogenesis of SLE are discussed below.

In 2002, Karassa and co-workers demonstrated the result from meta-analysis, which indicated that the homozygosity of R131, the low-binding allele of FcγRIIa gene, was a greater risk for the development of SLE compared with other genotypes. The effect of R131 allele on susceptibility to SLE has been found to have a dose-response character (autosomal dominant) (Karassa, Trikalinos et al. 2002). In this study, we found the association consistent to the meta-analysis. As mention indetail in the introductuion part, R131 allele does not capture efficiently with immune complex, whereas H131 allele can. Thus, optimal IgG2-mediated immune complex clearance occurs in homozygous H131 individuals, R/H131 heterozygotes have intermediate phagocytic capacity and R/R131 homozygotes have poor phagocytosis (Salmon 2001).

In addition, we analyzed the association between FcγRIIa genotype with clinical manifestation and the production of certain autoantibodies. The R131 allele seemed to confer risk for anticardiolipin (aCL) antibodies production under a recessive model. The anti phospholipid syndrome (APS) is characterized by recurrent vascular thromboses involving the venous, arterial, and placental circulation. The APS can be categorized into primary or secondary to other autoimmune disease (Levine, Branch et al. 2002). From international meta-analysis in 2003, the positive association occurred between R/R131 homozygous in secondary APS (mainly SLE) compared with disease-free controls under a recessive mode of inheritance (Karassa, Bijl et al. 2003), identical to this study. In this study, we reported that RR conferred a risk for aCL antibodies production and supported by a hypothesis below. Apoptotic cells are a major source of autoantigens, and an impairment of their physiologic

clearance may promote the development of autoimmunuty. Anionic phospholipid redistribute from inner leaflet to the alter leaflet of cell membrane during apoptosis. This systemic exposure could enable the binding of phospholipid- binding proteins such as β-glycoprotein I ($\beta_2$-GPI) to apoptotic cell membranes and may also trigger the production to aPL. Phospholipid-$\beta_2$-GPI complexes on the surface of membrane blebs are recognized by aPL, which leads to opsonization of apoptotic cells that are then phagocytosed by FcR-positive macrophage. Considering that aPL antibodies, especially those with reactivity to $\beta_2$-GPI, show IgG2-dominant distribution, such antibodies would be predicted to be poor opsonins in RR homozygous subjects. Defective clearance of aPL-opsonized apoptotic particles by macrophage may led to inflammatory removal pathways, favoring an autoimmune, rather than an anti-inflammatory, response to apoptotic cells. Thus antigen-processing and presentation by antigen presenting cells provide an antigenic stimulus for specific T and B clones, leading to further aPL antibodies production that may exert procoagulant effects. Moreover, persistently circulating apoptotic cells could express procoagulant properties, thus supporting thrombotic events (Levine, Branch et al. 2002). Interestingly, the comparison between secondary APS and SLE controls revealed the importance of H/H131 homozygotes for the development of APS. The hypothesis for HH in APS pathogenesis, aPL antibodies binding to protein-phopholipid complexes on platlets, endotherial cells, or other cell may result in their activation via crosslinking of high-binding FcγRIIa. Finally, This activation may induce a prothrombotic phenotype (Arnout 1996). However, in the present study, we did not analyze clinical data for APS patients. Hence, we cannot display the role of HH for predisposition to APS.

Some reports indicated that R131 allele is associated with a risk for lupus nephritis (Duits, Bootsma et al. 1995; Zuniga, Ng et al. 2001). In contrast, Karassa and co-workers demonstrated the result from meta-analysis, which revealed the negative association between R131 allele and lupus nephritis (Karassa, Trikalinos et al. 2002). Although, no significant association was observed in this study, the tendency of increased R131 allele in lupus nephritis can be seen. Seventy-five percent of R131 allele was observed in lupus nephritis compared with 70% in non-

lupus nephritis patients. However, this negative association suggests that R131 might not be a main risk for lupus nephritis.

Three biallelic polymorphisms within the IL-10 promoter region, at position -1082 (G or A), -819 (C or T), and –592 (A or C), have been identified (Turner, Williams et al. 1997). Linkage disequilibrium (LD) occurred between position –592 and –819, which meant that individuals with –592*A also had –819*T, whereas those with –592*C also had –819*C (Mok, Lanchbury et al. 1998; Matsushita, Tanaka et al. 2002). Functional studies of each positions have been reported as summarized next. At position –592, it has been indicated that C allele was associated with high IL-10 production by EMSA (Shin, Winkler et al. 2000). No direct evidence was reported for position –819, however, linkage disequilibrium between these positions may confer high IL-10 production for –819*C allele. The controversy results were demonstrated at position –1082. For example, G allele was associated with high IL-10 production by invitro stimulation assay with concanavalin A (conA) (Turner, Williams et al. 1997). On the other hand, A was also associated with high IL-10 production by conA, EMSA, and luciferase reporter gene assay (Rees, Wood et al. 2002). The level of expression is more consistent with haplotype rather than single SNP position. In Thai population, there are three common haplotypes; GCC, ACC, ATA and rare GTA haplotype. Functional studies of these haplotypes suggested that GCC, ACC, ATA are associated with high, intermediate, and low IL-10 production, respectively (Crawley, Kay et al. 1999; Edwards-Smith, Jonsson et al. 1999).

Although, negative association between IL-10 and SLE was reported in two previous reports (Lazarus, Hajeer et al. 1997; Mok, Lanchbury et al. 1998). In this study, we found the positive association between ACC haplotype (intermediate production) and SLE supporting the hypothesis that increase IL-10 production might be involved in SLE pathogenesis by resulting in abnormal B cell activation that lead to B cell hyperactivity generating autoantibody production. We hypothesize that the low frequency of G allele in Thai population resulted in the negative association between the high production GCC hapolotype and SLE. Interestingly, we discovered the association between GCC haplotype and renal involvement, similar to previous report in UK population (Lazarus, Hajeer et al. 1997). Although, GCC haplotype frequency was low in Thai population as mention

above, the positive association between GCC haplotype and lupus nephritis observed in this study might help emphasize the role high IL-10 production in the pathogenesis of lupus nephritis.

Most interestingly, the fact that we found the synergistic effect between FcγRIIa and IL-10 gene as susceptibility gene with SLE. We hypothesize that the synergistic effect help emphasize the important role of multiple genes, which in this case not only located at the same region but also work together in the pathogenesis of SLE. This result might help explain the consistent finding that chromosome 1 (1q21-44) has been regarded as a major susceptibility loci for SLE.

# CHAPTER VII

# CONCLUSION

The present study revealed that ACC haplotype of IL-10 gene and FcγRIIa R131 polymorphism might be regarded as marker for genetic susceptibility to SLE in Thai population. This finding is consistent with the proposed biology of selected SNPs. For example, the low-binding FcγRIIa R allele lead to defective in IgG autoantibody and IgG-containing immune complex clearance. Several studies demonstrated that ACC haplotype of IL-10 gene is associated with intermediate-high IL-10 production. IL-10 is proposed to play a role in pathogenesis of SLE by activating autoreactive B cell leading to hyperactive autoantibody production. The synergistic between IL-10, ACC haplotype and FcγRIIa, R allele in susceptible to SLE indicated that individuals carrying both specific genotype of IL-10 and FcγRIIa were at higher risk of developing of SLE than those carrying only IL-10 or FcγRIIa. Additionally, the strong positive association were discovered between GCC haplotype of IL-10 gene and renal involvement and implied that high Il-10 production correlate with severity of disease. Aside from the association with lupus nephritis, anticardiolipin antibodies production provided the association with R131 allele of FcγRIIa under a recessive mode of inheritance. These finding help explain both the basic biological events and indicate to clinicians ways of predicting, preventing or managing harmful situations in disease with immunological components.

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# APPENDICES

# APPENDIX I

## 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 II

## 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 III

## Reagent for indirect immunofluorescence assay

### 1. 0.15 M Phosphate Buffer Saline (PBS), pH 7.4

| | | |
|---|---|---|
| KCl | 0.2 | g |
| $KH_2PO_4$ | 0.2 | g |
| NaCl | 8.0 | g |
| $Na_2HPO_4$ or | 11.44 | g |
| $Na_2HPO_4.7H_2O$ | 2.16 | g |

Adjust volume to 1 liter with distilled water . Adjust pH to 7.4 with 1 N HCL. Store this solution at room temperature .

# APPENDIX IV

## Reagent for solid phase enzyme immunoassay

**1. Coated strips**

12 x 8 coated microtitrer test strips, prefixed in the frame . Removable microtitrer wells are coated with bovine heart cardiolipin and human $\beta_2$-glycoprotein I ($\beta_2$-GPI), the latter being cardiolipin co-factor .

**2. Enzyme tracer**

Anti-human IgM and IgG(rabit) conjugated with horseradish peroxidase, PBS buffer, BSA and an inert green dye .

**3. Negative control**

Normal human serum/ plasma, PBS buffer, BSA and preservatives .

**4. Positive control/ calibrator**

Normal human serum/ plasma containing IgM and IgG antibodies to cardiolipin, PBS buffer, BSA and preservatives .

**5. Specimen diluent**

5x PBS buffer concentrate containing BSA, preservatives and an inert ochre dye .

**6. Wash buffer**

20x PBS buffer concentrate and preservatives .

**7. Chromogen**

Buffered substrate (hydrogen peroxide) and chromogen (3,3',5,5'-tetramethylbenzidine) .

**8. Stop solution**

1 N sulphuric acid

# APPENDIX V

## 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/.

### 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).

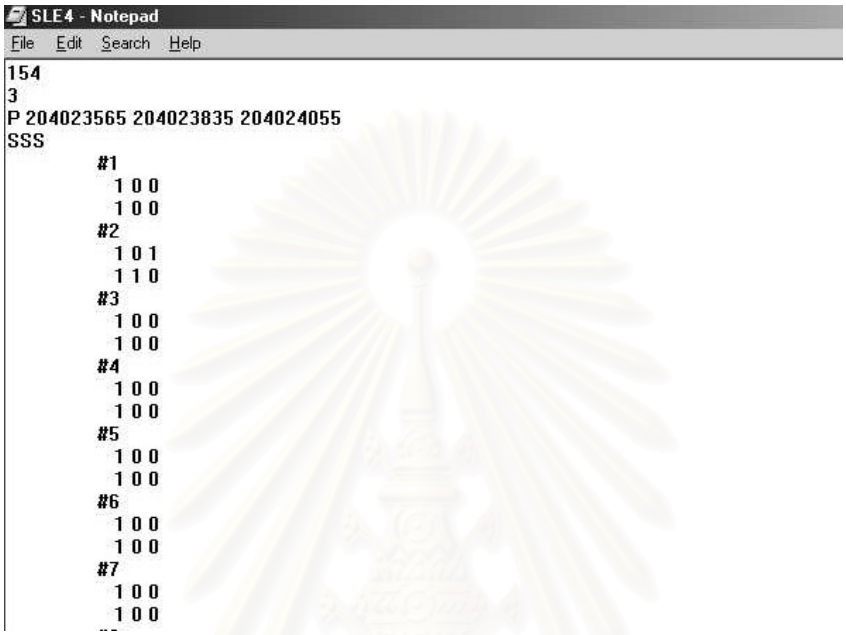
-LocusType (i) A letter indicating the type of locus i. The options are (a) S for a biallelic (SNP) locus, or biallelic site in sequence data. (b) m for microsatellite, or other multi-allelic locus (eg trallelic SNP, or HLA allele). The default assumption is that this denotes a microsatellite locus with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

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

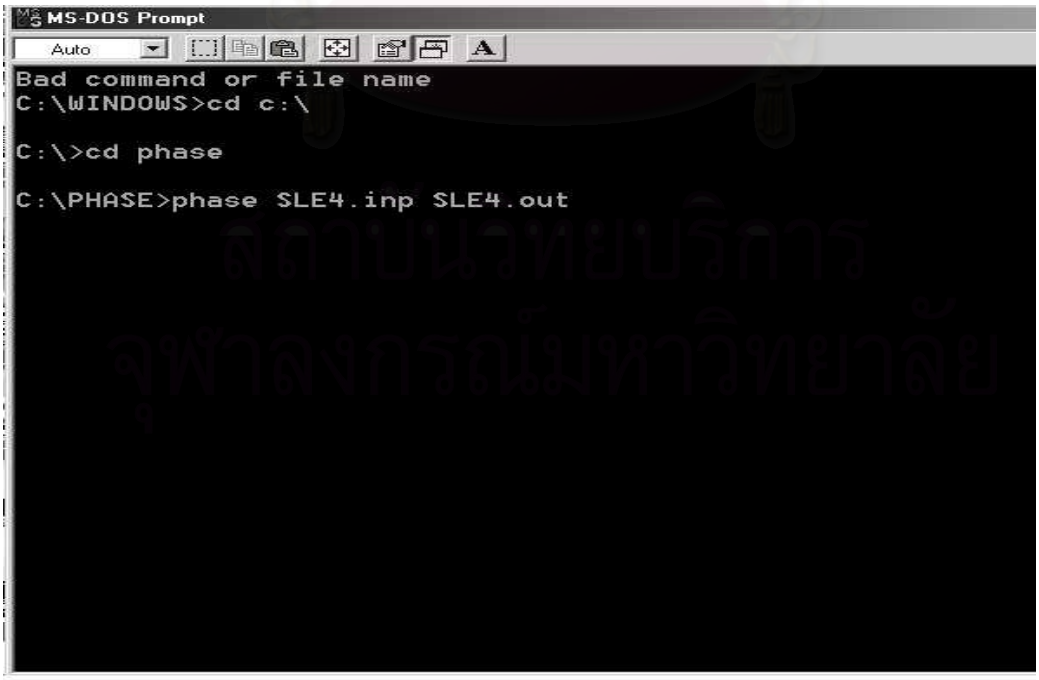
-Genotype (i) The genotypes for the ith individual. This is given on two consecutive rows. At each locus, one allele is entered on the first row, and one on the second row. It does not matter which allele is entered on each row. For biallelic loci, any two characters (e.g. A/C, G/T, 0/1) can be used 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 .



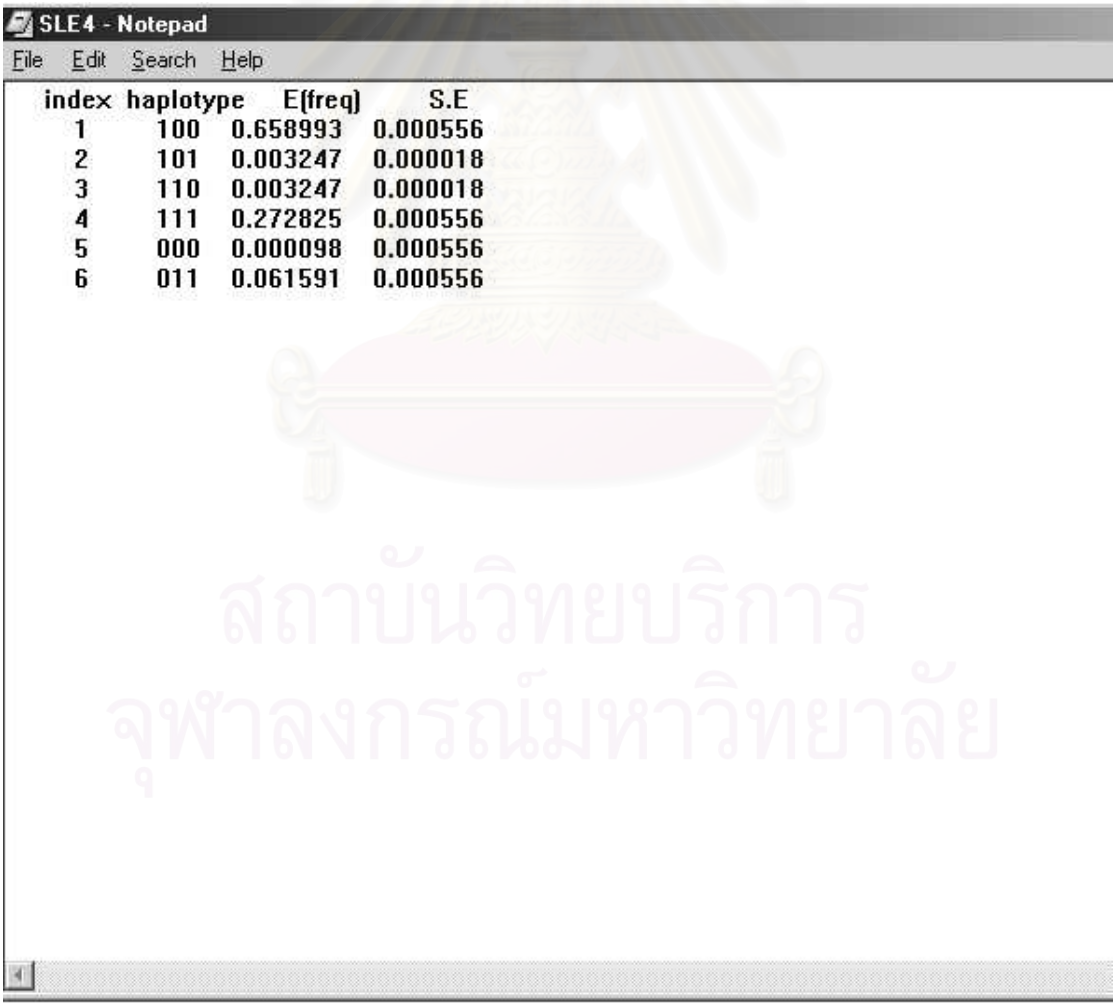
**Output file**

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 .

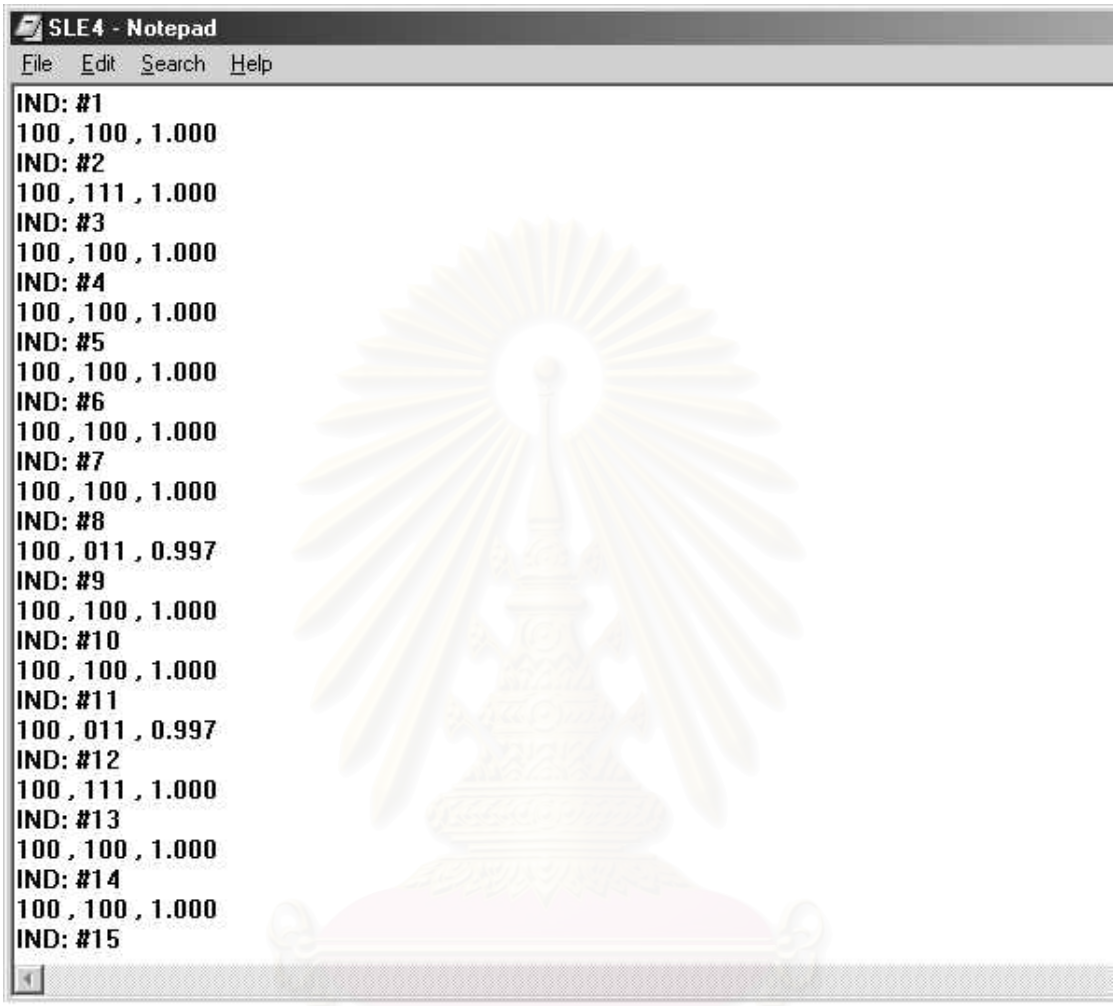
1. Output frequencies : defined as how many haplotype in this study group and used the number (frequencies ) to estimate how many the people ,who have these haplotypes .

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



2. Output pairs : defined as the individuals haplotype

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



# BIOGRAPHY

Miss Janjuree Netsawang was born on January 20,1981 in Bangkok ,Thailand . She graduated Bachelor of Science (Medical Technology) ,Faculty of Medical Technology, Chulalongkorn University in 2002 and attended to particulate in medical Microbiology program, Graduate School, Chulalongkorn University for her Master's Degree .