CHAPTER IV

MATERIALS AND METHODS

Subjects

One hundred and fifty-three Thai patients with SLE attending at 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 thirty-three 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 (Characteristics of healthy controls and patients with SLE).

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 ^{o}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 ^{o}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 ^{o}C for 15 minutes. Use gentle vortexing to resuspend. If clums of undissolved DNA are present, it will be incubated at 65 ^{o}C until completely resuspended.

Selecting SNPs for analysis

First step: we would like to select TGFß2 SNPs in Thai population. So we searched public databases (http://ThaiSNP) for SNP distribution and frequencies. Data as shown in table 5.

Second step: we found 7 TGFß2 SNPs from ThaiSNP reported, so we analyzed each SNP from table 5 by SNP haplotype tagging from DNA pools of two individuals by Pools2 package. Pools2 package program estimates haplotype frequencies in pools of DNA from two individuals each. The DNA pooling is a technique to reduce genotyping effort while incurring only minor losses in accuracy of allele frequency estimates for single nucleotide polymorphism (SNP) markers. The following programs are distributed under the terms of the GNU General Public License (GPL). The software is available online at http://linkage.rockefeller.edu/register/ (Hoh J, 2003).

Please note that, we would like to detect 7 haplotypes of TGFß2 so we selected 4 SNPs (the data was shown in appendixD) that have frequency greater than 5% from 7 SNPs in ThaiSNP report in order to obtain enough power to detect positive association within sample size of 153. Data as shown in table 6.

Table5. Transforming growth factor beta2 (TGFß2) allele frequencies for ThaiSNP Report

Position/Role	rs number	Allele	Allele frequencies in Thai populaton
+71_72 5' UTR	__	__/ACAA (insertion)	ACAA = 0.453 __ = 0.547
+720 Intron 1	rs1106569	T/G	G = 0.062 T = 0.938
+89835 Intron 5	rs6684205	A/G	A = 0.266 G = 0.734
+91052 Intron 6	rs10482823	T/C	C = 0.016 T = 0.984
+94400_94401 Intron 6	New SNP (Only found on Thai population)	__/A (insertion)	A = 0.141 __ = 0.859
+94973 Exon 7	New SNP (Only found on Thai population)	G/A	A = 0.031 G = 0.969
+95038 Exon 7	rs900	A/T	A = 0.281 T = 0.719

Table6. Summary of SNPs used in this study

Position/Role	Rs number	Allele	Allele frequencies in Thai population
+71_72 5' UTR	__	__/ACAA (insertion)	ACAA = 0.453 __ = 0.547
+720 Intron 1	rs1106569	T/G	G = 0.062 T = 0.938
+89835 Intron 5	rs6684205	A/G	A = 0.266 G = 0.734
+94400_94401 Intron 6	New SNP (Only found on Thai population)	__/A (insertion)	A = 0.141 __ = 0.859

Genotyping methodology

1. 5' – end labeling primer (kinase reaction) of TGF-β2 gene at position 71_72insACAA and +94400_94401insA

We performed the 5' – end labelling analysis on the polymorphism of TGF-β2 at promoter region 5' untranslated region (5' UTR) upstreams 109 bp far from initiation codon of a 4-bp insertion (ACAA) and 1-bp insertion (A) in intron 6. The genomic DNA of 153 SLE patients and 133 healthy controls were amplified with the use of the TGF-β2 gene specific primers as shown in table 8.

The reaction volume for the amplification reaction was 10 μl, containing 75 ng of genomic DNA, 0.05 μl of 5.0 Taq polymerase (Promea Madison WI, USA), 1 μl of 10X PCR buffer (20 mM Tris-HCL pH 8.0, 100 mM KCL), 0.6 μl of 25 mM $MgCl_2$, 0.2 μl of 10 mM dNTP, 0.25 μl (10 pmol) of reverse oligonucleotide primer and 0.50 μl (10 pmol) of forward oligonucleotide primer which labeled by γ- P^{32} ATP. 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 95°C for 5 minutes, followed by 30 cycles of denaturation (95 °C, 1 minute), annealing (59 °C, 1 minute) and extension (72 °C, 1 minute) and final extension at 72 °C for 7 minutes. The resulting products were futher analyzed by DNA sequencing in 6% denaturing polyacrylamide gel containing urea 25.2 g, 40% polyacrylamide: Bis 19:1 (ml) 9 ml, 10X TBE 6 ml, H_2O 26 ml, 10% Ammoniumpersulphate 396 μl and TEMED 34.8 μl, in Tris- Boric acid EDTA(TBE) buffer at 70 watte for 5 hours and 30 minutes for a 4-bp insertion (ACAA) and 4 hours for 1-bp insertion (A) then visualized under phosphoimmature. Successful amplification PCR products for 2 positions.

1.1 At position 71_17insACAA in 5' untranslated region (5' UTR)

PCR product size is 223 bp fragment which 5' – end forward primer labeled by γ- P^{32} and then, running on DNA sequencing in 6% denaturing polyacrylamide gel and then, transfer on membrane molecular dynamics for 6 hours at lease or over night and visualized under phosphoimmature scanner control and ImageQuant 5.2. If a 4-bp insertion ACAA was present at this position, homozygous insertion ACAA was present one band at upper DNA ladder when compare with positive control with heterozygous (two bands). Heterozygous a 4-bp insertion ACAA was present two bands between upper and lower DNA ladder and the homozygous none a 4 bp insertion ACAA was present one band at lower DNA ladder when compare with positive control with heterozygous.

1.2 At position + 94400_94401insA in intron 6

PCR product size is 150 bp fragment which 5' – end forward primer labeled by γ- P^{32} and then, running on DNA sequencing in 6% denaturing polyacrylamide gel and then, transfer on membrane molecular dynamics for 6 hours at lease or over night and visualized under phosphoimmature scanner control and ImageQuant 5.2. If a 1-bp insertion A was present at this position, homozygous insertion A was present two bands at upper DNA ladder when compare with positive control with heterozygous (three bands). Heterozygous a 1-bp insertion A was present three bands between upper and lower DNA ladder and the homozygous none of a 1 bp insertion A was present two bands at lower DNA ladder when compare with positive control with heterozygous.

2. Polymerase Chain Reaction - Restriction Fragment Length Polymorphism analysis of TGF-β2 gene

We performed the Polymerase Chain Reaction-Restriction Fragment Length Polymorphism (PCR-RFLP) analysis of the polymorphism of TGF-β2 gene at position + 720 (G/T) and + 89835 (A/G). The genomic DNA of 153 SLE patients and 133 healthy controls were amplified with the use of the TGF-β2 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 (Fermentus), 2 µl of 10X PCR buffer (20 mM Tris-HCL pH 8.0, 100 mM KCL), 1.6 µl of 25 mM $MgCl_2$, 0.4 µl of 10 mM dNTP and 0.5 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 95 °C for 5 minutes, followed by 35 cycles of denaturation (95 °C, 1 minute), annealing (59 °C, 1 minute) and extension (72 °C, 1 minute) and final extension at 72°C for 5 minutes. The resulting products were futher analyzed by electrophoresis in 1.5% Tris-acetate agarose gel containing 50 µg/ml ethidiumbromide, in Tris-acetate buffer at 100 volts for 40 minutes and visualized under UV light by Camera Gel Doc™ MZL (BIO-LAD, USA). 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 frangments. Successful amplification PCR products for 2 positions.

1. At position + 720(T/G) in intron 1

PCR product size is 281 bp fragment and then, 10 µl of amplified DNA were digested with 5U of specific restriction enzyme TaaI (Tsp4CI), (Fermentus) in 1X Buffer Tango in a total volume of 15 µl at 65 °C for 14-16 hours, followed by 3% agarose gel electrophoresis at 100 volts for 40 minutes. If a G was present at this position, the enzyme would cut the 281 bp PCR product into two fragment; 185 and 96 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.

2. At position + 89835(A/G) in intron 5

PCR product size is 311 bp fragment and then, 10 µl of amplified DNA were digested with 7U of specific restriction enzyme FspBI (MeaI), (Fermentas) in 1X Buffer Tango 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 G was present at this position, the enzyme would cut the 311 bp PCR product into two fragment; 277 and 84 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 4 SNPs site for TGF-β2 genotyping by DNA sequencing.

DNA sequencing

DNA sequencing were used to validate the results of TGF-β2 gene polymorphism by 5' – end labeling primer (kinase reaction) 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, 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 at 55 °C for 10

seconds and extension 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 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 vided with performed using the software package provided with ABI 310 a sequencing system.

Statistcal Analysis

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

Haplotype, linkage disequilibrium analysis and the goodness of fit to Hardy-Weinberg equilibrium

The PHASE program was 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 appendixE)

The values of linkage disequilibrium (LD) were determined using LDPlotter (http://innateimmunity.net.gov/SNP/.) (see AppendixF).

The goodness of fit to Hardy-Weinberg equilibrium, calculating the expected frequencies of each genotype and comparing them with the observed values (see appendixG).

Table7. Characteristics of healthy controls and patients with SLE

Characteristics	Healthy controls	SLE
Number of patients	133	153
Females/males	77/56	151/2
Mean age ± SD yr	26±13.8	36.34±11.9

Table8. Primers used for analysis of the TGF-β2 gene polymorphism

Gene polymorphism and primer	Sequence of primers (5'⟶3')
+71_72 insACAA	
-Forward	TTTTGGAACTACTGGCCTTTTC
-Reverse	ACTACTGTGTGCTGAGCGCT
+ 720T/G T/G	
-Forward	TTTGACTTCCATCCCCTGAG
-Reverse	AGTTCTTTAGTCGGCCACCA
+ 89835A/G	
-Forward	GCGGCCTATTGGTTTAGGTA
-Reverse	GCTCTCTGCTCCTCAGAACAA
+ 94400_94401 insA	
-Forward	ATGTATGTGCAGCCAAGCAT
-Reverse	GTTCCGTGTGGGAAAATAATGA