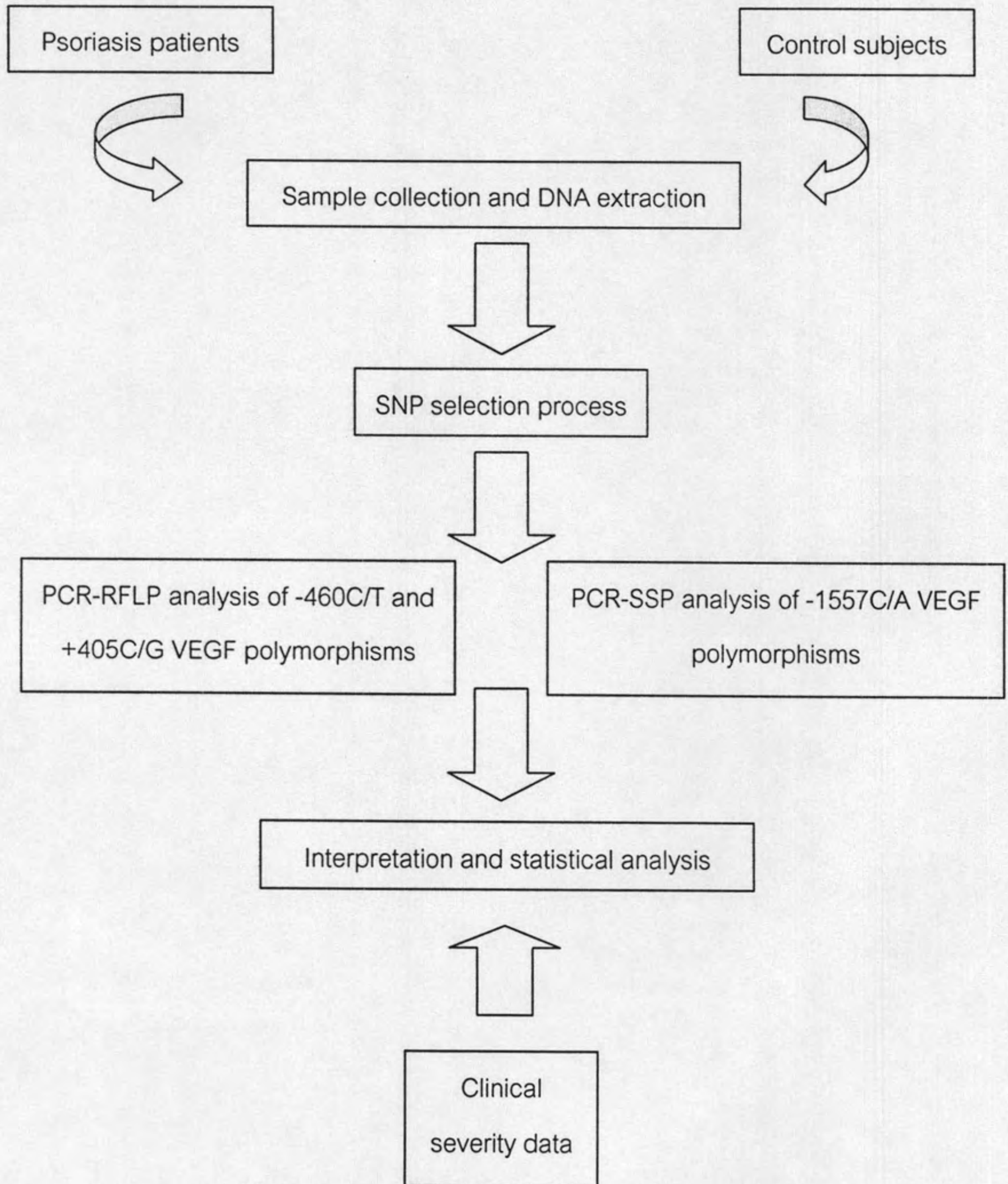
CHAPTER IV

MATERIALS AND METHODS



Subjects

One hundred and fifty-four Thai patients with chronic plaque type psoriasis from outpatients and inpatients service of King Chulalongkorn Memorial hospital were included in the study. Healthy subjects group contained two hundred and thirty-four subjects were recruited from volunteer donors with the same ethnic and background geographic from Thai red cross for the population based case-control association study. The study was approved by the ethics committee of the King Chulalongkorn University and the subjects gave their informed consent. Demographic data of the subjects was summarized in table 9.

Sample sizes

We calculated a sample sizes by this formula for case-control study (Unmatched).

$$n/group = (Z_{\alpha/2} \sqrt{2PQ} + Z_{\beta} \sqrt{(P_1Q_1+P_0Q_0)})^2 / (P_1-P_0)^2$$

P_0 = Risk probability of control groups, 0.04

R = Odds Ratio, 9.62 (Ku, Wan et al. 2005)

$P_1 = P_0R / (1+ P_0(R-1)) = 0.04 \times 9.62 / (1+ 0.04(9.62 - 1)) = 0.2861$

$P = (P_1+P_0)/2 = (0.2861 + 0.04) / 2 = 0.1631$

$Q = 1-P = 1 - 0.1631 = 0.8369$

$Q_0 = 1-P_0 = 1 - 0.04 = 0.96$

$Q_1 = 1-P_1 = 1 - 0.2861 = 0.7139$

$Z_{\alpha/2} = 1.96$

$Z_{\beta} = 1.28$

$$n/group = \frac{(1.96 \sqrt{2 \times 0.1631 \times 0.8369} + 1.28 \sqrt{0.2861 \times 0.7139 + 0.04 \times 0.96})^2}{(0.2861 - 0.04)^2}$$

$= 45.2025 \sim 45$ samples/group

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 microfuge. 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 the 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 incubated at 65°C until completely resuspended.

Selecting SNPs for analysis

First step : we would like to select SNPs that have frequency greater than 5% in Asian population in order to obtain enough power to detect positive association within

sample size of 150. So we searched public databases (http://ncbi.nlm.nih.gov/SNP/; http://snpper.chip.org) for SNP distribution and frequency data as shown in table 6.

Second step: we analyzed each SNP from table 6 for potential of functional SNP that might 1.) Affect the function of the gene product. 2.) Affect the expression of the gene. However, there are no SNP that cause non-synnonymous change in the exon. But even so, some previous SNP data in the Exon of VEGF gene showed that they also found significantly enhanced VEGF serum levels. Then, we are focusing in the SNP within the promoter instead using bioinformatics tools, we analyzed those SNP using TFSEARCH program for searching transcription factor binding sites at http://www.cbrc.jp/research/db/TFSEARCH.html. We found some SNP within VEGF promoter was predicted to affect the binding of transcription factor.

Please note that, we would like to detect 3 SNPs of VEGF gene (the data was shown in table 8) that have frequency greater than 5% from 3 SNPs in SNPper report in order to obtain enough power to detect positive association within sample size of 154.

Table 6. SNPs with frequency data.

VEGF	No. of SNP	Validated SNP	SNP with frequency data
VEGF (VEGF-A)	136		19
- 5' UTR	23	11	6
- Exon	3	2	1
- Intron	80	74	11
- 3' UTR	30	20	1

Table 7. SNPs with allele frequencies data in Asian population.

VEGF	Position / Role[a]	Allele	Minor allele frequency
VEGF	-2595 (-1557) C/A promoter (rs699947)	C/A	Cau (C) = 0.00 Afr (C) = 0.01 Chn (A) = 0.28 Jap (A) = 0.32
	-1498 (-460) C/T promoter (rs833061)	C/T	-
	-634 (+405) C/G Exon (rs2010963)	C/G	Cau (C) = 0.31 Asi (C) = 0.29

NOTE---Alleles and position are given according to SNPper (CHIP Bioinformatics Tools)

[a] Nucleotide position is as counted from translation initiation (ATG) site.

Cau = Caucasian population

Afr = African population

Chn = Chinese population

Jap = Japan

Asi = Asian

Table 8. Summary of SNPs used in this study.

Cadidate gene	Position [a]	rs number	Allele frequency in Asian	Transcription factor
VEGF	-2595 (TI) -1557 (Tc)	rs699947	C = 0.04 A = 0.96	GATA-2 Absent
VEGF	-1498 (TI) -460 (Tc)	rs833061	C = - T = -	Absent Absent
VEGF	+634 (TI) +405 (Tc)	rs2010963	C = 0.29 G = 0.71	Absent Absent

NOTE---Alleles and position are given according to SNPer (CHIP Bioinformatics Tools)

[a] Nucleotide position is as counted from translation initiation (ATG) site.

TI = Translational start site

Tc = Transcriptional sart site

Genotyping methodology

Polymerase Chain Reaction-Sequence Specific Primer (PCR-SSP)

Analysis of VEGF -1557 (C/A) polymorphism

We performed the polymerase Chain Reaction-Sequence Specific Primer (PCR-SSP) analysis of -1557 C/A VEGF as previously described(Papazoglou, Galazios et al. 2004). The genomic DNA of 154 chronic plaque psoriasis patients were amplified with the use of the -1557 C/A VEGF (-1557CF, -1557AF) specific primers as shown in table 10.

The reaction volume for the amplification reaction was 10 μl, containing 150 ng/μl genomic DNA, 0.05 μl of 5.0 U Flexi Taq polymerase (Promega Madison WI, USA), 2 μl of 5x Flexi PCR buffer (20mM Tris-HCl pH 8.0, 100 mM KCl), 2 μl of 5x Flexi Green buffer, 1.1 μl of 25 mM $MgCl_2$, 0.2 μl of 10 mM deoxynucletide triphosphates,

0.5 μl (20 pmol) of each specific primers and 0.05 μl (25 pmol) of internal control primers. Internal control primers were used to check for successful PCR amplification. These primers amplify a Interferon-Alpha (IFN-α) sequence (table 10). Amplification was performed in Perkin Elmer/GeneAmp PCR system 2400 or Applied Biosystems/ GeneAmp PCR system 9600. The PCR cycling parameters of -1557 C and A VEGF allele consisted of an initial denaturation at 94°C for 5 minutes, followed by 35 cycles of denaturation (94°C, 40 seconds), annealing (59°C, 1 minute) and extension (72°C, 40 seconds) final extension at 72°C for 5 minutes(Papazoglou, Galazios et al. 2004). The resulting products were further analyzed by electrophoresis in 3 % Tris-acetate agarose gel containing 50 μg/ml ethidium bromide, in Tris-acetate buffer at 100 volts for 50 minutes and visualized under UV light by Camera Gel Doc™ MZL (BIO-LAD). Negative controls without DNA template were included in each experiment. The positive results of -1557 C VEGF allele, -1557 A VEGF allele and IFN-α gene showed band of 77, 95 and 274 bp fragment, respectively. A Molecular ladder of 100-bp (Promega) was used to estimate the size of the PCR fragments.

Polymerase Chain Reaction-Restriction fragment Length Polymorphism

Analysis of -460 (C/T) and +405 (C/G) VEGF

We performed the Polymerase Chain Reaction-Restriction fragment Length Polymorphism (PCR-RFLP) analysis of VEGF at promoter position -460 (C/T) and +405 (C/G) as previously described (Watson, Webb et al. 2000; Lin, Wu et al. 2003; Bhanoori, Arvind Babu et al. 2005). The genomic DNA of 154 chronic plaque psoriasis patients were amplified with the use of the VEGF gene specific primers as shown in table 10.

The reaction volume for the amplification reaction at position -460 (C/T) was 20 μl, containing 100 ng of genomic DNA, 1.6 U Taq polymerase (Eppendorf Hamburg, Germany), 10 μl of Eppendorf MasterMix (2.5x), 2.0 mM $MgCl_2$, 0.25 mM dNTP and 0.2 μl (20 pmol) of each oligonucleotide primer. The reaction volume for the amplification reaction at position +405 (C/G) was 20 μl, containing 100 ng of genomic DNA, 0.1 μl of 5.0 U Taq polymerase (Promega or Gibco), 2 μl of 10x PCR buffer (20nM Tris-HCL pH

8.0, 100 mM KCL), 3.2 μl of 25 mM $MgCl_2$, 1.5 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 Biosystem/ GeneAmp PCR system 9600 under specific PCR condition. At position -460 (C/T), the PCR protocol consisted of an initiation denateration at 94 ^{0}C for 5 minutes, followed by 35 cycles of denateration (94 ^{0}C, 30 seconds), annealing (59 ^{0}C, 45 seconds) and extension (72 ^{0}C, 30 seconds) and final extension at 72 ^{0}C, 7 minutes. At position +405 (C/G), the PCR protocal consisted of an initiation denateration at 94 ^{0}C for 3 minutes, followed by 35 cycles of denateration (94 ^{0}C, 45 seconds), annealing (58 ^{0}C, 45 seconds) and extension (72 ^{0}C, 45 seconds) and final extension at 72 ^{0}C, 5 minutes. The resulting products were further analyzed by electrophoresis in 2.5% 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). Negative controls without DNA template were included in each experiment. A molecular ladder of 100-bp (Promega) was used to estimate the size of the PCR fragments. Successful amplification PCR products for 2 positions:

1. At position -460 (C/T)

PCR product size is 175 bp fragment and then, 10 μl of amplified DNA were digested with 1U of specific restriction enzyme *BstUI* (New England Biolabs, Hitchin, UK) in 1X NEBuffer 2 in a total volume of 15 μl at 60 ^{0}C for 14-16 hours, followed by 3% agarose gel electrophoresis at 100 volts for 50 minutes. If an C was present at this position, the enzyme would cut the 175 bp PCR product into two fragment; 155 and 20 bp. No digestion would occur if a T was present. A molecular ladder of 100-bp (promega) was used to estimated the size of the PCR fragment.

2. At position +405 (C/G)

PCR product size is 304 -bp fragment and then, 10 μl of amplified DNA were digested with 5U of specific restriction enzyme *BsmFI* (New England Biolabs, Hitchin, UK) in 1X NEBuffer 4 in a total volume of 15 μl at 65 ^{0}C for 14-16 hours, followed by 3% agarose gel electrophoesis at 100 volts for 50 minutes. If an G was present at this

position, the enzyme would cut the 304 bp PCR product into two fragment; 193 and 111 bp. No digestion would occer if a C was present. A molecular ladder of 100-bp (Promega) was used to estimated the size of the PCR fragment.

DNA sequencing

DNA sequencing were used to validate the results of -1557 (C/A), -460 (C/T) and +405 (C/G) VEGF gene polymorphism by PCR-SSP and PCR-RFLP methods. For direct cycle sequencing, 40 μl of the PCR products were purified by the QIAquick PCR Purification Kit (QIAGEN Inc.) to obtain clean double-standed DNA amplificates. 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) was used for sequencing. Thus, each sequencing reaction mixture of 10 μl final volume contained 1 μl of 5 pmol primer, 3 μl of template and 3 μl of the 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. The condition of cycle sequencing reaction consisting of denaturation at 96°C for 30 seconds, annealing at 55°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, pH4.6) 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 the ABI 310 a sequencing system.

Determination of VEGF plasma levels: Quantitative sandwich enzyme immunoassay technique (Sandwich-ELISA) by Quantikine® Human VEGF Immunoassay

Commercial quantitative enzyme immunoassay kit was used to perform the expression of vascular endothelial growth factor in plasma of chronic plaque psoriasis patients associated with genetic polymorphisms. This assay occupies the quantitative sandwich enzyme immunoassay technique. A monoclonal antibody specific for VEGF had been pre-coated onto a micro plate. Standards and samples pipettes into the wells and any VEGF present was bound by the immobilized antibody. After washing away any unbound substances, an enzyme-linked polyclonal antibody specific for VEGF was added to the wells. Following a wash to remove any unbound antibody-enzyme reagent, a substrate solution was added to the wells and color develops in proportion to the amount of VEGF bound in the initial step. The color development was stopped and the intensity of the color was measured (http://www.RnDSystems.com).

VEGF plasma levels were assessed in 12 psoriasis patients. Patients had stopped systemic treatments at least 1 month before plasma collection. Plasma of psoriasis patients were classified using the genotype of haplotype such as CTG/CTG, CTG/Other and non-CTG. Moreover, the early-onset and mild severity were included. Results were expressed as picograms per millititer and VEGF was measured in all samples in a single run. The sensitivity of the assay was <9.0 pg/ml. Samples were analyzed in duplicate.

Furthermore, VEGF plasma levels were assessed in 27 psoriasis patients. Patients had stopped systemic treatments at least 1 month before plasma collection. Plasma of psoriasis patients were classified using the genotype of haplotype including haplotype containing +405G and haplotype containing +405C. Results were expressed as picograms per millititer and VEGF was measured in all samples in a single run. The sensitivity of the assay was <9.0 pg/ml. Samples were analyzed in duplicate.

Statistical Analysis

We compared the allele and genotype frequencies between chronic plaque psoriasis and normal controls both susceptibility of disease and severity. In addition, we compared the haplotype distribution to the expression of VEGF in plasma of psoriasis patients. The statistical significance of the difference was tested by chi-square (χ^2) method. Fisher's exact tests were applied if the expected frequency was less than 5. A *P* value of < 0.05 was considered to be significant. Odd ratio (OR) with 95% confidence interval (CI) were calculated using the statistical program Epi Info version 6 (Centers for Disease Control and Prevention [CDC], 1994). The genotype frequencies were checked by consistency among normal controls with those expected from Hardy-Weinberg equilibrium. The goodness of fit to Hardy-Weinberg equilibrium, calculating the expected frequencies of each genotype and comparing them with the observed values, was performed using a chi-square test. The mode of inheritance analysis was also included. Furthermore, when one element in the 2x2 table (Chi-square (χ^2) test) was zero, OR was calculated with the Haldane' s modified formula [RR_h= (2a+1)(2d+1)/(2b+1)(2c+1)]. Haldane' s correction for the OR was used when either all patients were positive or all controls were negative for a particular specificity or allele (Haldane 1956).

For statistical analysis of VEGF-ELISA, The statistical significance of the difference of VEGF concentration in each groups were tested by 2 independent samples tests (Wilcoxon Mann-Whitney test). SPSS program was applied. A *P* value of < 0.05 was considered to be significant.

Haplotype analysis by PHASE program (Stephens and Donnelly 2003) and linkage disequilibrium analysis

The program PHASE were used to reconstructing haplotypes from population genotype data (Stephens and Donnelly., 2003). The software can deal with SNP, microsattellite, and other multi-allelic loci, in any combination and missing data are allowed. The remaining ambiguous sites are assigned by PHASE, and the uncertainly

associated with each PHASE assignment is calculated (see Appendix C). In addition, The values of linkage disequilibrium was calculated using the LDPlotter Tool (http://innateimmunity.net/IIPGA2/Bioinformatics/index_html) (see Appendix D).

Table 9. Characteristics of healthy controls and patients with psoriasis.

Characteristics	Healthy controls	Chronic plaque psoriasis
Number of patients	234	154
Females/males	170/64	64/90
Mean age ± SD yr	26.02 ± 10.93	45.32 ± 14.26

Table 10. Primers used for analysis of the VEGF gene polymorphism.

Gene polymorphisms and primers	Sequence of primers (5'→3')	References
-1557 (C/A) VEGF		
Specific primers		(Papazoglou, Galazios et al. 2004)
-1557C forward	TAG GCC AGA CCC TGG CAC	
-1557A forward	TAG GCC AGA CCC TGG CAA	
-1557 reverse	TGC CCC AGG GAA CAA AGT	
Internal control primer		
IFN-α forward	TCC ATG AGA TGA TCC AGC AG	
IFN-α reverse	ATT TCT GCT CTG ACA ACC TCC C	
-460 (C/T) VEGF		
-460 forward	TGT GCG TGT GGG GTT GAG CG	(Watson, Webb et al. 2000; Lin, Wu et al. 2003)
-460 reverse	TAC GTG CGG ACA GGG CCT GA	

+405 (C/G) VEGF		
+405 forward	ATT TAT TTT TGC TTG CCA TT	(Watson, Webb et al. 2000; Bhanoori, Arvind Babu et al. 2005)
+405 reverse	GTC TGT CTG TCT GTC CGT CA	