CHAPTER III

MATERIALS AND METHODS

Research Instruments

1. Pipette tip : 10 µl, 1,000 µl (Elkay, USA)
2. Microcentrifuge tube : 0.2 ml, 0.5 ml, 1.5 ml (Bio-RAD, Elkay, USA)
3. Polypropylene conical tube : 15 ml (Elkay, USA)
4. Beaker : 50 ml, 100 ml, 200ml, 500 ml, 1,000 ml (Pyrex)
5. Flask : 250 ml, 500 ml, 1,000 ml (Pyrex)
6. Reagent bottle : 100 ml, 250 ml, 500 ml, 1,000 ml (Duran, USA)
7. Cylinder : 25 ml, 50 ml, 100 ml, 250 ml, 500 ml, 1,000 ml (Witeg, Germany)
8. Glass pipette : 5 ml, 10 ml (Witeg, Germany)
9. Pipette rack (Autopack, USA)
10. Thermometer (Precision, Germany)
11. Parafilm (American National Can, USA)
12. Plastic wrap
13. Stirring-magnetic bar
14. Combs
15. Automatic adjustable micropipette : P2 (0.1-2 µl), P10 (0.5-10 µl), P20 (5-20 µl), P100 (20-100 µl), P1000 (0.1-1 ml) (Gilson, France)
16. Pipette boy (Tecnomara, Switzerland)

17. Vortex (Scientific Industry, USA)

19. pH meter (Eutech Cybernatics)

20. Stirring hot plate (Bamstead/Thermolyne, USA)

21. Balance (Precisa, Switzerland)

22. Centrifuge (J.P.Selecta, Span)

23. Microcentrifuge (Eppendorf, Germany)

24. Mastercycler personal (Eppendorf, Germany)

25. Thermal cycler (Touch Down, Hybraid USA)

26. Power supply model 250 (Gibco BRL, Scothland)

27. Power poc 3000 (Bio-RAD)

28. Horizon 11-14 (Gibco BRL, Scothland)

29. Sequi-gen sequencing cell (Bio-RAD)

30. Heat block (Bockel)

31. Incubator (Memmert)

32. Thermostat shaking-water bath (Heto, Denmark)

33. Spectronic spectrophotometers (Genesys5, Milon Roy USA)

34. UV Transilluminator (Fotodyne USA)

35. UV-absorbing face shield (Spectronic, USA)

36. Gel doc 1000 (Bio-RAD)

37. Refrigerator 4 ^{0}C (Misubishi, Japan)

38. Deep freeze -20 ^{0}C, -80 ^{0}C (Revco)

39. Water purification equipment (Water pro Ps, Labconco USA)

40. Water bath (J.P.Selecta, Span)

41. Storm 840 and ImageQuaNT solfware (Molecular dynamics)

Reagents

1.General reagents

1.1 Absolute ethanol (Merck)

1.2 Agarose, molecular glade (Promega)

1.3 Ammonium acetate (Merck)

1.4 Boric acid (Merck)

1.5 Bromphenol blue (Pharmacia)

1.6 Disodium ethylenediamine tetracetic acid : EDTA (Merck)

1.7 Ethidium bromide (Gibco BRL)

1.8 Ficoll 400 (Pharmacia)

1.9 Hydrochloric acid (Merck)

1.10 Mineral oil (Sigma)

1.11 Phenol (Sigma)

1.12 Chloroform (Merck)

1.13 Isoamyl alcohol (Merck)

1.14 Sodium chloride (Merck)

1.15 Sodium dodecyl sulfate (Sigma)

1.16 Sodium hydroxide (Merck)

1.17 Sucrose (BDH)

1.18 Tris base (USB)

1.19 Triton X-100 (Pharmacia)

1.20 100 base pair DNA ladder (Biolabs)

1.21 40%acrylamide/bis solution 19:1 (Bio-RAD)

1.22 GelStar (Camberx)

2.Reagents of PCR

2.1 10X PCR buffer (500 mM KCl, 200 mM Tris-HCl pH 8.4) (Promega)

2.2 10X PCR buffer (500 mM KCl, 100 mM Tris-HCl pH 8.8, 0.8% Nonidet P40) (Fermentas)

2.3 Magnesium chloride (Promega)

2.4 Magnesium chloride (Fermentas)

2.5 Deoxynucleotide triphosphates (dNTPs) (Promega)

2.6 Deoxynucleotide triphosphates (dNTPs) (Fermentas)

2.7 Oligonucleotide primers (BSU)

2.8 Oligonucleotide primers (Biogenomed)

2.9 *Taq* DNA polymerase (Promega)

2.10 *Taq* DNA polymerase (Fermentas)

2.11 100% DMSO

2.12 Genomic DNA sample

3.Restriction enzyme

3.1 *Mea*II (Biolabs)

3.2 *BstN*I (Biolabs)

3.3 *Dpn*II (Biolabs)

3.4 *Nci*I (Biolabs)

3.5 *Mwo*I (Biolabs)

3.6 *Msp*I (Biolabs)

Procedure

1. Subjects and Sample collection

After clinical diagnosis and informed consents were received, 3 ml of peripheral blood was obtained from the different groups. The control populations were 310 healthy individuals nonrandomly collected from the Thai Red Cross of Nakorn Ratchasima, Kalasin, Nong Khia and Bangkok. The study population consisted of patients with CL/P, CPO, FEEM, and their patent groups (Table 3). The patients with CL/P and CPO, plus their parents (trio, non-trio) recruited from Mae Hongson, Nan, Uthai Thani, Prachin Buri, Nakorn Ratchasima, Sa Kaeo, Kalsin, Nongkhia , Maha Saracham, and Trang. The children with FEEM and their parents were collected from Uthaithain and King Chulalongkorn Memorail Hospital, Bangkok.

Table 3 Number of the study population

	CL/P	CPO	FEEM
Patients	213	54	89
Mothers	134	37	54
Fathers	82	17	23

Controls (n = 295)

2.DNA Extraction

The extraction of DNA from peripheral blood leukocyte was performed as follow:

1) 5-10 ml. of whole blood is centrifuged for 10 minutes at 3,000 rpm.

2) Remove supernatant and collect buffy coat to a new polypropylene tube. Then add 10 volumes of cold lysis buffer1 (or 10 ml.), mix thoroughly and incubate at –20°C for 5 minutes.

3) Centrifuge for 8 minutes at 1,000 g, then remove supernatant.

4) Add 3 ml. Cold lysis buffer1, mix thoroughly and centrifuge for 8 minute at 1,000g,

5) Discard supernatant afterward add 900µl lysis buffer2, 10µl

6) Proteinase K solution (20 mg Proteinase K in 1.0 ml. Of 1% SDS-

7) 2mM EDTA, should be prepare 30 min before use.), and 10% SDS 50µl. Mix vigorously for 15 seconds.

8) Incubate the tube(s) in 37°C shaking waterbath overnight for complete digestion.

9) Add 1 ml. Phenol-chloroform-isoamyl alcohol shake vigorously for 15 seconds and centrifuge at 6,000 rpm for 5 minutes.

10) Transfer the supernatant from each tube (contains DNA) to a new microcentrifuge tube.

11) Add 0.5 volumes of 7.5 M CH_3COONH_4 and 1 volume of 100%ethanol mix by inversion. The DNA should immediately form a stringy precipitate. Recover the DNA by centrifugation at 14,000 rpm for 15 minutes. Then remove supernatant.

12) Rinse the pellet with 70% ethanol. Decant the ethanol and air-dried the pellet. (It is important to rinse well to remove any residual salt and phenol.)

13) Resuspend the digested DNA in 20-300µl of the double distilled water at 37°C until dissolved.

3. Genotyping

Genotying of all SNPs was performed by PCR-RFLP (Restriction Fragment Length Polymorphism) analysis.

3.1 PCR amplification

The PCR primer sets for genotyping *TGFβ-3* 179C->T, *SKI* 185C->G and *SKI* 1163C->T were designed using the genomic sequence of TGFβ-3 (AF107885), SKI (AY331180), and SKI (AH013034) from NCBI database. As for *TGFβ-3* 179C->T, based on site-directed mutagenesis, the reverse mutagenesis primer increased a restriction site in C allele. For the last three SNPs, *TGFβ-3* 383A->G, *IRF6* 820G->A, and *MTHFD1* 1958G->A, were designed form the published sequence.[22,116,113] All primers sequence, PCR components and PCR condition were shown in table 4 to 6.

Table 4 Primers and PCR products

PCR amplification	Sequences 5"->3"	PCR product (bp)
1.TGFβ-3 179C->T	TGFB3E1F : TAC AAG GCA CAC AG CCG CTT CTT CGT C	426
	TGFB3E1R : GAC CTG ATA GGG GAT GTG GGT CAT CAA C	
2. TGFβ-3 383A->G	TGFB3E2F : AGC ACA CTG TTC CTG CAT	275
	TGFB3E2R : AAG GAA CCA GCT TTC CC	
3. IRF6 820G->A	IRF6E7F : AGT GGC CTT CCT GAA TGC TG	647
	IRF6E7R : CTT GAC CTC CTC CAG ACT AA	
4.SKI 185C->G	SKIE1F : AGA AGA CGC TGG AGC AGT TC	435
	SKIE1F : CTT TGA GGA TCT CCA GCT GG	
5. SKI 1163C->T	SKIE3F : CCG GCT CTT CCA ATA AGG TG	350
	SKIE3R : CGT GAT CAG AAA CAC CTG TG	
6. MTHFD1 1958G->A	MTHFD1F : CAC TCC AGT GTT TGT CCA TG	330
	MTHFD1R : GCA TCT TGA GAG CCC TGA C	

Table 5 PCR components

Component	Volume per reaction* (µl)					
	TGFβ-3 179C->T	TGFβ-3 383A->G	IRF6 820G->A	SKI 185C->G	SKI 1163C->T	MTHFD1 1958G->A
1.10X PCR buffer	2.0 (1X)	2.0 (1X)	2.0 (1X)	2.0 (1X)	2.0 (1X)	2.0 (1X)
2.25mM $MgCl_2$	1.2 (1.5mM)	1.2 (1.5mM)	1.2 (1.5mM)	1.6 (2.0mM)	1.2 (1.5mM)	1.2 (1.5mM)
3.10mM dNTP	0.4 (0.2mM)	0.4 (0.2mM)	0.4 (0.2mM)	0.4 (0.2mM)	0.4 (0.2mM)	0.4 (0.2mM)
4.10µM Forward primer	0.2 (0.1µM)	0.2 (0.1µM)	0.2 (0.1µM)	0.4 (0.2µM)	0.2 (0.1µM)	0.2 (0.1µM)
5.10µM Reverse primer	0.2 (0.1µM)	0.2 (0.1µM)	0.2 (0.1µM)	0.4 (0.2µM)	0.2 (0.1µM)	0.2 (0.1µM)
6 5U/µl Taq polymerase	0.1 (0.5U)	0.1 (0.5U)	0.1 (0.5U)	0.1 (0.5U)	0.1 (0.5U)	0.1 (0.5U)
7.Distilled water	14.9	15.4	14.9	11.1	14.9	15.4
8.100%DMSO	-	-	-	2.0 (10%)	-	-
9.50ng/µl Genomic DNA	1.0 (50 ng/µl)	0.5 (25 ng/µl)	1.0 (50 ng/µl)	2.0 (100ng/µl)	1.0 (50 ng/µl)	0.5 (25 ng/µl)
Total volume (µl)	20	20	20	20	20	20

*Final concentration per reaction.

Table 6 PCR cycle

Step	Temperature and Incubation time					
	TGFβ-3 179C->T	TGFβ-3 383A->G	IRF6 820G->A	SKI 185C->G	SKI 1163C->T	MTHFD1 1958G->A
1.Initial denaturation	94 °C / 5 min	94 °C/5 min	94 °C / 5 min	94 °C / 10 min	94 °C / 5 min	94 °C / 5 min
2.35 cycles of						
Denaturation	94 °C / 30 sec	94 °C / 1 min	94 °C / 1 min	94 °C / 1 min	94 °C / 30 sec	94 °C / 45 sec
Annealing	62 °C / 30 sec	54 °C / 30 sec	56 °C / 30 sec	58 °C / 1 min	58 °C / 30 sec	60 °C / 30 sec
Extension	72 °C / 30 sec	72 °C / 45 sec	72 °C / 1 min	72 °C / 30 sec	72 °C / 30 sec	72 °C / 30 sec
3.Final extension	72 °C / 10 min	72 °C / 10 min	72 °C / 10 min	72 °C / 10 min	72 °C / 10 min	72 °C / 10 min

3.2 Digested by restriction enzymes

Each SNPs had specific restriction site analyzed by unique restriction enzymes digestion. There were *Mea*II, *BstN*I, *Dpn*II, *Nci*I, *Mwo*I, and *Msp*I for *TGFβ*-3 179C->T, *TGFβ-3* 383A->G, *IRF6* 820G->A, *SKI* 185C->G, *SKI* 1163C->T, *MTHFD1* 1958G->A, respectively. According to the digestion procedure, for each SNPs, 20 μl aliquots of PCR product were incubated with various amounts of specific restriction enzyme. For *Mea*II, *BstN*I, and *Dpn*II, 5 units of enzyme was incubated overnight at 37 ^{0}C. Whereas, 10 units of *Nci*I and *Msp*I and 2.5 units of *Mwo*I were required for the same condition.

3.3 Agarose gel eletrophoresis

The genotypes of entire SNPs were defined by the differences of RFLP patterns on agarose gel electrophoresis (figure 6), all digested product were separated on 2% agarose gel. Note that if *TGFβ-3* 179C->T was analyzed based on site-directed mutagenesis, the result of 2% agarose gel electrophoresis was confirmed again by 12% nondenaturing acrylamide gel electrophorsis.

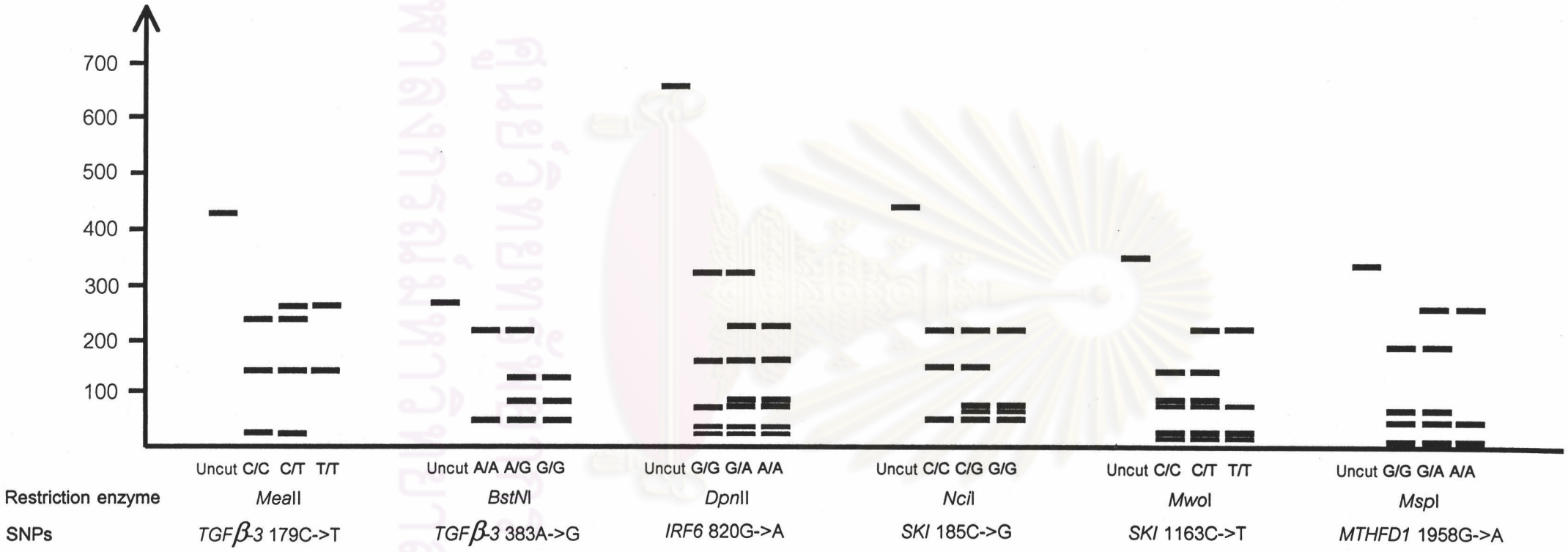

Figure The schematic of RFLP profile for genotyping

4. Data analysis

4.1 The difference of genotype and allele frequencies among control group, patient and parent groups.

Chi square test was employed to test for difference of genotype and allele frequencies between case groups (patients and their parents) and controls. As for calculation, except comparative groups, entered controls and each case group in the eventuality table.

Eventuality table (Table 7) analysis with significance calculated by chi square test (χ^2) was employed to test for difference between observed genotype distribution of each population and their expected distribution. Chi square test was performed according to data entered into table shown below;

Table 7 Eventuality table showing combination of Groups and Conditions for chi square (χ^2) test

	Group				
Condition	G_1	G_2	...	G_j	Total
C_1	n_{11}	n_{12}	...	n_{1j}	$n_{1.}$
C_2	n_{21}	n_{22}	...	n_{2j}	$n_{2.}$
...	...	...	...	...	
C_i	n_{i1}	n_{i2}	...	n_{ij}	$n_{i.}$
Total	$n_{.1}$	$n_{.1}$	...	$n_{.j}$	N

According to table 7, G were comparative groups, control groups and study groups, while C were conditions, which were intact genotypes. N was total number of individuals, whereas n_{ij} was number of individuals with condition i in group j. The symbols "$n_{i.}$" and "$n_{.j}$" indicate total number of all groups in condition i (total of row i) and the total number of all condition in group j (total of column j), respectively. Chi square test was calculate according to the following formulation:

$$\chi^2 = \sum_i \sum_j \frac{(O_{ij} - E_{ij})^2}{E_{ij}}$$

From the formulation, E_{ij} was expected number calculated in accordance with formulation: $E_{ij} = (n_{i.} \times n_{.j})/N$. Furthermore, web page "**http://www.unc.edu/~preacher/chisq/chisq.htm**" in which the test for maximum 10 conditions and 10 groups was offered, help us for chi square calculation and so is p value. This program generally result in Pearsons' chi square except in case of expected frequency was less than 5 in more than 20% of entered cells will be corrected with Yates' correction. Conclusion of difference was allowed when p value is less than 0.05

4.2 Hardy weinberg equilibrium (HWE)

The Hardy-Weinberg model defines and predicts genotype and allele frequencies in a non-developing population. This principal consist of five basic assumptions: 1) the population is large (i.e., there is no genetic drift); 2) there is no gene flow between populations, from migration or transfer of gametes; 3) mutations are negligible; 4) individuals are mating randomly; and 5) natural selection is not operating on the population. Given these assumptions, a population's genotype and allele frequencies will remain unchanged over successive generations, and the population is said to be in Hardy-Weinberg equilibrium. The Hardy-Weinberg model can also be applied to the genotype frequency of a single gene. If observe genotype frequencies differ from expect, it can assume that one or more of the principal's assumptions are being violated, and attempt to determine which one(s).

The usual test for goodness of fit of observed data to HWE is a chi square test. The test statistic is usually symbolized χ^2, and under the hypothesis of HWE the χ^2 has approximately a chi square distribution. The *p* and *q* were calculated from the observed genotype, then the number of expected genotype were determined by these formulae:

Expected genotype of p^2 = total number * $(p)^2$

Expected genotype of 2pq = total number * $(2pq)$

Expected genotype of q^2 = total number * $(q)^2$

After that, the comparison was thus between the observed and expected number. The value of χ^2 was calculated as the below formula.

$$\chi^2 = \sum_{i=1}^{k} \frac{(O-E)^2}{E}$$

Note that, the probability value (P-value) was determined by corresponded with one degree of freedom.

4.3 Odds ratio

Moreover, odds ratio (OR) and 95 percent confidence interval (95%CI) by utilizing Epi info version 6 program, were carried out to test for correlation between diseases (CL/P, CPO, and FEEM) and candidate SNPs.

4.4 Transmission disequilibrium test (TDT)

The Transmission Disequilibrium Test (TDT),[98] biallelic marker, different from association study which use unrelated controls, TDT apply parents as internal control. This will not prone to exhibit false positive results in the presence of population stratification. TDT statistic is effective for detecting of disequilibrium of parental allele transmitted to their effected offspring. It includes all parents of patient who are heterozygous for a specific marker allele. Then it compares how often that specific marker allele is passed to their affected offspring from such heterozygous parents. If there is no linkage, then 50 percent of the time that M1 allele would be transmitted and 50 percent of the time the other allele (M2) would be transmitted. If there is linkage and association, then the marker M allele would mostly be on the chromosome with disease allele and they would be inherited together more than 50 percent of the time. This statistic is calculated from the below equation.

$$\chi^2_{TD} = (M1-M2)^2/(M1+M2),\ df = 1$$

According to this equation, M1 is the number of events, that M1M2 parents transmits M1 to affected offspring, besides M2 is the number of times that M1M2 parents transmits M2 to affected offspring. The results from this equation are denoting as particular chi-square for TDT (χ^2_{TD}).

In this study, TDT was used to test for disequilibrium transmission of alleles of candidate SNPs from the heterozygous parents to their affected offspring. In case of lost one parental genotype, the remaining were discarded. Only alleles of heterozygous parents, which can be distinguished as being transmitted to their offspring, are considered as informative and then were scored (Table 8).

Table 8 informative trios with scoring of transmission alleles.

genotype			Time of transmitted	
offspring's	Parent1	Parent2	M1	M2
M1M1	M1M1	M1M2	1	-
M1M1	M1M2	M1M1	1	-
M1M1	M1M2	M1M2	2	-
M1M2	M1M1	M1M2	-	1
M1M2	M1M2	M1M1	-	1
M1M2	M1M2	M2M2	1	-
M1M2	M2M2	M1M2	1	-
M2M2	M1M2	M1M2	-	2
M2M2	M1M2	M2M2	-	1
M2M2	M2M2	M1M2	-	1

Time of transmitted was scored for only allele which was transmitted from heterozygous parents (shaded)