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. Electrophoresis chamber set
16. Mini-protein 3 electrophoresis

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)

18. Pipette boy (Tecnomara, Switzerland)

19. Vortex (Scientific Industry, USA)

20. pH meter (Eutech Cybernatics)

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

22. Balance (Precisa, Switzerland)

23. Microcentrifuge (Fotodyne, USA)

24. DNA Thermal cycler 480 (Perkin Elmer, Cetus USA)

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. Beta shield (C.B.S scientific. Co.)

31. Heat block (Bockel)

32. Incubator (Memmert)

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

34. Spectronic spectrophotometers (Genesys5, Milon Roy USA)

35. UV Transilluminator (Fotodyne USA)

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

37. Gel doc 1000 (Bio-RAD)

38. Mitsubishi Video copy processor

39. Thermal paper

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

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

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

43. Water bath

44. 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)

2.Reagents of PCR

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

2.2 Magnesium chloride (Promega)

2.3 Deoxynucleotide triphosphates (dNTPs) (Promega)

2.4 Oligonucleotide primers (BSU)

2.5 *Taq* DNA polymerase (Promega)

2.6 Genomic DNA sample

3.Restriction enzyme

3.1 *Hinf*I and 10XNE buffer 1(Biolabs)

3.2 *Mbo*II and 10XNE buffer 2 (Biolabs)

Procedure

1. Subjects and Sample collection

After clinical diagnosis and informed consents were obtained, blood samples for genetic analysis were collected from the different groups.

The control population comprised 202 health individuals nonrandomly collected from the Thai Red Cross of Nakornratchasima and Bangkok. Forty eight individuals born with FEEM phenotype were diagnosed and enrolled to the study. Only 23 blood samples of their mothers were available for genotyping. Additionally, blood samples were also collected from 162 individuals diagnosed with CL/P, 97 of maternal samples and 65 of paternal samples were also obtained.

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

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

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

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

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

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

9. 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.

10. 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.)

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

3. Mutation analysis

Since DNA extracted from whole blood was obtained, MTHFR 677C→T and 1298A→C polymorphisms were analysis by PCR amplification and restriction enzyme analysis.

3.1 detection of 677C→T polymorphism

The presence of the *MTHFR* 677C→T polymorphism was firstly described by Frosst et al.[27]. This polymorphism reflects on chamging of an alanine into a valine residue (A222V), creating a *Hinf*I restriction site. Ten μl of PCR product was performed according to PCR condition and PCR parameter which are shown in table 1 and 2 respectively. The PCR products were 5 μl aliqouted and visualized for the presence of exact 198 bp fragment. The remainder 5 μl of PCR products then were digested by adding 0.3 μl (1.5 units) of *Hinf*I and incubated overnight at 37 °C in order to

completely digest the DNA. The digested fragments were then separated on 3 percent agarose gel electrophoresis.

Table 1 – Mixture of PCR reaction for 677C→T detection

Contents		Final concentration		Concentration per reaction	
25mM	$MgCl_2$	2.0	mM	0.8	µl
10X	PCR buffer	1	X	1.0	µl
10mM	dNTP	0.2	mM	0.2	µl
10µM	MTHFR-F1 primer	0.025	µM	0.05	µl
10µM	MTHFR-R1 primer	0.025	µM	0.05	µl
5 U/µl	*Taq* polymerase	0.2	U	0.04	µl
50ng/µl	Genomic DNA	150	ng	2.0	µl
	Distilled water			5.86	µl
			total	10.0	µl

MTHFR-F1 primer 5' TGG AGG AGA AGG TGT CTG CGG GA 3' and MTHFR-R1 primer 5' AGG ACG GTG CGG TGA GAG TG 3' were proposed by Frosst et al. (1995)[27]

Table 2 – PCR parameter for 677C→T detection

Step		Temperature	Time
Initial denaturation		94 °C	4 min
35 cycles of	Denaturation	94 °C	30 sec
	Primer annealing	64 °C	30 sec
	Primer extension	72 °C	45 sec
Final extention		72 °C	7 min

3.2 Detection of 1298A→C polymorphism

The second common polymorphism of *MTHFR*, 1298A→C, change a glutamine into alanine residue at position 649 (Q649A). The nucleotide substitution in which A is substited by C, abolish a *Mbo*II restriction site. Detection of 1298A→C was performed by PCR amplification using primers which were proposed by Van de Putt

and Blom (2000)[116] and then digest amplified product with *Mbo*II. The mixture of PCR reaction was indecated in table 3 in accordance with PCR parameter shown in table 4. After that, the total volume of 20 µl of PCR product was separated equally into 2 parts. The first 10 µl was visualized by 1 percent of agarose gel electrophoeresis. Whereas the remainder 10 µl was digested by adding 0.2 µl (1 units) of *Mbo*II followed by incubation at 37 °C overnight to ensure complete digestion. Finally, the digested PCR fragment was electrophoresed on a 2 percent of agarose gel.

Table 3 – Mixture of PCR reaction for 1298A→C detection

Contents		Final concentration		Concentration per reaction	
25mM	$MgCl_2$	2.0	mM	1.6	µl
10X	PCR buffer	1	X	2.0	µl
10mM	dNTP	0.2	mM	0.4	µl
10µM	F-N1298 primer	0.1	µM	0.2	µl
10µM	R-N1298 primer	0.1	µM	0.2	µl
5 U/µl	*Taq* polymerase	0.4	U	0.08	µl
50ng/µl	Genomic DNA	150	ng	3.0	µl
	Distilled water			12.52	µl
			total	20.0	µl

F-N 1298 primer 5' ATG TGG GGG GAG GAG CTG AC 3' and R-N1298 primer 5' TCC CAA CTT ACC CTT CTC CC 3' were proposed by Van de Putt et al[116].

Table 4 – PCR parameter for 1298A→C detection

Step		Temperature	Time
Initial denaturation		95 °C	5 min
35 cycles of	Denaturation	95 °C	1 min
	Primer annealing	60 °C	1 min
	Primer extension	72 °C	1 min
Final extention		72 °C	7 min

4. Statistic analysis.

4.1 Chi square test (χ^2)

Contingency table analysis with significance calculated by chi aquare test (χ^2) was employed to test for independence between 1) observed genotype distribution and expected distribution if population were in Hardy weinberg equilibrium, 2) allelic frequency of cases and controls, 3) genotype frequency of cases and controls, and 4) haplotype freqyency of cases and controls. Chi square test was performed according to data entered into table shown below ;

Table 5 – Contingency 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

As shown in table 5, G imply groups and C are conditions in which alleles, genotypes, or haplotypes used in this study were represented. N is total number of indiduals, whereas n_{ij} is observed number of individuals with condition i and group j. The symbols "$n_{i.}$" and "$n_{.j}$" imply total number of condition i (total of row i) and the total number of group j (total of column j) respectively. Chi square test was calculate according to the following formulation :

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

Note that E_{ij} imply expected number calculated in accordance with formulation : $E_{ij} = (n_{i.} \times n_{.j})/N$. Web page **"http://www.quantrm2.psy.ohio-state.edu/kris/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 frequencies less than 5 will be corrected with Yates' correction. Conclusion of diffence was allowed when p value is less than 0.05.

4.2 Odd ratio

In addition, odd ratio (OR) and 95 percent confidence interval (95%CI) by using Epi info version 6 program, was carried out to test for interaction between diseases (FEEM or CL/P) and *MTHFR* genotypes. Furthermore, EH program which was previously described by Zhao et al. (2000)[117], was employed to estimate haplotype frequencies in the population. Regarding number of cases with double heterozygous genotypes (677CT with 1298AC) in which the C-A/T-C and C-C/T-A haplotype are included, we can determine the numbers of each haplytype by using formulation based on probability.

Number of individuals with C-A/T-C = Nx[f(C-A)xf(T-C)]/[f(C-A)xf(T-C)+f(C-C)xf(T-A)] whereas number of individuals with C-C/T-A = Nx[f(C-C)xf(T-A)]/[f(C-A)xf(T-C)+ f(C-C)xf(T-A)]. N is a total number of cases with heterozygous genotype and f(C-A), f(T-C), f(C-C), and f(T-A) were the haplotype alleles of 677C→T and 1298A→C estimated from EH program. The difference of haplotypes between groups was estimated by $T(\chi^2/2)$ = ln(L,group1)+ln(L,group2)-ln(L,group1+group2) as previously described (Zhao, 2000)[117].

4.3 Transmission disequilibrium test (TDT)

4.3.1 TDT for biallelic marker

The transmission disequilibrium test[113], unlike association study which used unrelated controls, TDT use parents as internal control. This will not prone to produce false positive results in the presence of population stratification. TDT statistic is useful for detecting of disequilibrium of parental allele transmitted to their effected offspring. It look at all parents of affected children who are heterozygous for a specific maeker 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 equaltion $\chi^2_{TDT} = (M1-M2)^2/(M1+M2)$ at degree of freedom was 1. From this equation, M1 is the number of times an M1M2 parents transmits M1 to affected offspring, M2 is the number of times an M1M2 parents transmits M2 to affected offspring .The results from this equation is represent as particular chi square for TDT (χ^2_{TDT}).

In this study, TDT was used to test for disequilibrium transmission of alleles of the *MTHFR* polymorphisms (677C→T and 1298A→C) from the heterozygous parents to their affected offspring. **If one parental genotype is missing the remaining parent-child pair were discarded**. Only alleles of heterozygous parents which can be distinguished as being transmitted to their offspring, are concidered as informative and then were scored (table 6). In our study, *MTHFR* polymorphisms were hypothesized as the marker which associate with FEEM or CL/P. C allele and T allele of the 677C→T polymorphism were considered as M1 and M2 marker allele respectively. Similarly, A allele of the 1298A→C was considered as M1 whereas C allele was concidered as M2.

Table 6 – Informative families with scoring of transmission alleles.

genotype			Time of transmitted	
offsprings	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 wastransmitted from heterozygous parents (shaded)

4.3.2 TDT for multiallelic marker

Furthermore, TDT test was also used according to the possible four alleles of C-A, C-C, T-A, and T-C (allele of nt677-nt1298), were hypothesized as the multiallelic marker. The study included nuclear families in which genotypes of parents and child were available. If one parental genotype is missing the remaining parent-child pair were discarded. Regrding combination of allele as haplotype, 10 possible haplotype was shown below

C-A/C-A

C-A/C-C C-C/C-C

C-A/T-A C-C/T-A T-A/T-A

C-A/T-C C-C/T-C T-A/T-C T-C/T-C

Parents with these expected haplotypes were proposed mating and produced offspring. **Allele from heterozygous parents will be concidered as informative if such allele can be distinguished as allele transmitted or untarnsmitted to their offsping**. For example;

Family 1 - if father's haplotype is C-A/C-C, mother's is C-A/T-A and their affected child is C-A/T-A, we can conclude that child inherited T-A from mother and C-A from father. Thus C-A (from father) and T-A (from mother) are scored as one time transmitted whereas C-C (from father) and C-A (from mother) are scored as one time untransmitted.

Family 2 - if both father and mother are C-A/C-C and their child is C-A/C-A, we can conclude that their child inherited two alleles of C-A from parents whose C-C alleles were clearly defined as allele not transmitted to their child. Two times of C-A transmitted and two times of C-C untransmitted were scored (table 7)

Table 7 – Example for scoring number of time allele transmitted and untransmitted.

family	haplotype			Transmitted score				Untransmitted score			
	Parent 1	Parent 2	Child	C-A	C-C	T-A	T-C	C-A	C-C	T-A	T-C
1	C-A/C-C	C-A/T-A	C-A/T-A	1	-	1	-	1	1	-	-
2	C-A/C-C	C-A/C-C	C-A/C-A	2	-	-	-	-	2	-	-
...	...	...	...	...	...	...	...	...	...	...	...
Total				T_{C-A}	T_{C-C}	T_{T-A}	T_{T-C}	U_{C-A}	U_{C-C}	U_{T-A}	U_{T-C}

More than these two conditions, there are several conditions for scoring transmitted and untransmitted allele, these based on the principle of informative mentioned before. The TDT data for mulltiallelic marker were analyzed by the recently proposed TDT statistic using a (k-1)/k correction[115] in accordance with level of degree of freedom (df) = (k-1)(g-1). Where k is the number of alleles and g is the number of groups. In this study, k = 4 alleles and g = 2 groups (transmitted and untransmitted group). The equation of TDT for multiallelic marker is according to the formulation : χ^2_{TDT} for 4 multiallelic marker = $[3/4]x[(T_{C-A} - U_{C-A})^2/(T_{C-A} + U_{C-A}) + (T_{C-C} - U_{C-C})^2/(T_{C-C} + U_{C-C}) + (T_{T-A} - U_{T-A})^2/(T_{T-A} + U_{T-A}) + (T_{T-C} - U_{T-C})^2/(T_{T-C} + U_{T-C})]$ at level of df = 3. The letter T represented the total number of each allele transmission, U implied the total number of each allele untransmitted, and 3/4 represented (k-1)/k correction. Similar to χ^2_{TDT} for 2 marker alleles, χ^2_{TDT} for multiallelic marker were used to define deviation of allele transmission from equilibrium when association is presented.