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)

18. pH meter (Eutech Cybernatics)

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

20. Balance (Precisa, Switzerland)

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

22. Microcentrifuge (Eppendorf, Germany)

23. Mastercycler personal (Eppendorf, Germany)

24. Thermal cycler (Touch Down, Hybraid USA)

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

26. Power poc 3000 (Bio-RAD)

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

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

29. Heat block (Bockel)

30. Incubator (Memmert)

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

32. Spectronic spectrophotometers (Genesys5, Milon Roy USA)

33. UV Transilluminator (Fotodyne USA)

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

35. Gel doc 1000 (Bio-RAD)

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

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

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

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

40. 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 *Hinf I* (Biolabs)

3.2 *Mbo II* (Biolabs)

3.3 *Nde I* (Biolabs)

3.4 *Hae III* (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 300 pregnant women coming for antenatal care at King Chulalongkorn Memorial Hospital. Selection criteria of control population, they were Thai (They described themselves as "Thai" and could speak Thai clearly and fluently). In addition, they had to had experience no miscarriages, other abnormal pregnancies or had previous children with possible genetic factor related to *MTHFR, MTRR* and *MTR* SNPs, i.e. cleft lip with or without cleft palate (CL/P) and neural tube defect (NTD). The 187 control populations were used in this thesis.

The study population consisted of 112 women who have children with Down syndrome presenting to Rachanukul hospital or King Chulalongkorn Memorail Hospital, Bangkok. Selection criteria of study population, their children with Down syndrome had to have karyotypically confirmed full trisomy 21. The 108 women who have children with Down syndrome were selected.

2.DNA Extraction

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

1. 3 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. Genotyping

Since DNA extracted from whole blood was obtained, All SNPs was performed by PCR amplification and Restriction Fragment Length Polymorphism (RFLP) analysis.

3.1 PCR amplification

The PCR primer sets for genotyping *MTHFR* 677C->T, *MTHFR* 1298A->C, *MTR* 2756A->G and *MTRR* 66A->G were designed using the genomic sequence of MTHFR (AF107885), MTRR (AH008763), and MTR (AC026443) from NCBI database. As for *MTRR* 66A->G, based on site-directed mutagenesis, the reverse mutagenesis primer increased a restriction site in C allele. For the last two SNPs, *MTHFR* 677C->T, *MTHFR* 1298A->C were designed form the published sequence. [73,74,113] All primers sequence, PCR components and PCR condition showed in table 1,table 2 and table 3 respectively.

Table 3. Primers and PCR products

PCR amplification		Sequence 5'->3'	Product (bp)
MTHFR	677C->T	Forward: 5' AGA AGG TGT CTG CGG GAG C 3'	198
		Reverse: 5' CAC TTT GTG ACC ATT CCG GTT TG 3'	
MTHFR	1298A->C	Forward: 5' CTT TGG GGA GCT GAA GGA CTA CTA C 3'	241
		Reverse: 5' CAC TTT GTG ACC ATT CCG GTT TG 3'	
MTRR	66 A->G	Forward: 5' GCA AAG GCC ATC GCA GAA GAC AT 3'	325
		Reverse: 5' GTG AAG ATC TGC AGA AAA TCC ATG TA 3'	
MTR	2756 A->G	Forward: 5' CTG AAG GAG GTG TTA TCA GC 3'	341
		Reverse: 5' CAC TGA AGA CCT CTG ATT TG 3'	

Table 4. Mixture of PCR reaction for detection

Component	Volume per reaction* (μl)			
	MTHFR 677C->T	MTHFR 1298A->C	MTRR 66A->G	MTR 2756A->G
1.10X PCR buffer	2.0 (1X)	2.0 (1X)	2.0 (1X)	2.0 (1X)
2.25mM $MgCl_2$	1.6 (1.5mM)	1.6 (2mM)	2.0 (2.5mM)	1.2 (1.5mM)
3.10mM dNTP	0.4 (0.2mM)	0.4 (0.2mM)	0.4 (0.2mM)	0.4 (0.2mM)
4.10μM Forward primer	0.1 (0.2μM)	0.2 (0.1μM)	0.4 (0.2μM)	0.4 (0.2μM)
5.10μM Reverse primer	0.1 (0.2μM)	0.2 (0.1μM)	0.4 (0.2μM)	0.4 (0.2μM)
6 5U/μl Taq polymerase	0.08 (0.025U)	0.08 (0.025U)	0.1 (0.025U)	0.1 (0.025U)
7.Distilled water	13.72	12.52	12.7	13.5
8.50ng/μl Genomic DNA	2 (50 ng/μl)	3.0 (50 ng/μl)	2.0 (50ng/μl)	2.0 (50ng/μl)
Total volume (μl)	20	20	20	20

*Final concentration per reaction in each PCR reaction

Table 5. PCR cycle

Step	Temperature and Incubation time			
	MTHFR 677C->T	MTHFR 1298A->C	MTRR 66A->G	MTR 2756A->G
1.Initial denaturation	94°C/ 4 min	94°C/ 5 min	94°C/ 5 min	94°C/ 5 min
2.40 cycles of				
Denature	94°C/ 30 sec	94°C/ 1 min	94°C/ 45 sec	94°C/ 45sec
Annealing	64°C/ 30 sec	60°C/ 1 min	55°C/ 30 sec	54°C/ 30 sec
Extension	72°C/ 45sec	72°C/ 1 min	72°C/ 30sec	72°C/ 30sec
3. Final extension	72°C/ 7 min	72°C/ 7 min	72°C/ 7 min	72°C/ 7 min

3.2 Digested by restriction enzymes

Each SNPs had specific restriction site with among restriction enzymes. There were *Hinf* I, *Mbo* II, *Nde* I and *Hae* III for *MTHFR* 677C->T, *MTHFR* 1298A->C, *MTRR* 66A->G and *MTR* 2756A->G respectively. According to the digestion procedure, for all SNPs, 10 μl of PCR products incubated with various units of

specific restriction enzyme. For *Hinf* I and *Mbo* II, used 5 units of enzyme and incubated overnight at 37 ^{0}C. Additionally, *Nde* I and *Hae* III, 4 units were required for complete cut at 37 ^{0}C overnight in order to completely digest the DNA.

3.3 Agarose gel eletrophoresis

The genotypes of entire SNPs were defined by the different of RFLP pattern on agarose gel electrophoresis, all digested product were separated on 3 percent of agarose gel.

4. Statistics analysis

4.1 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).

Eventuality table analysis with significance calculated by chi square test, The test statistic is usually symbolized χ^2. Chi square was employed to test for 1) difference between observed genotype distribution of each population and their expected distribution if population were in Hardy weinberg equilibrium, 2) allelic frequency of cases and controls, 3) genotype frequency of cases and controls.

Chi square test was performed according to data entered into table shown below;

Table 6. Eventuality table showing combination of Groups and Conditions for chi square (χ^2) test

Condition	Group				
	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_{i_1}	n_{i_2}	...	n_{ij}	$n_{i.}$
Total	$n_{.1}$	$n_{.1}$	...	$n_{.j}$	N

According to the table 4, G were comparative groups, observed and expected (calculated from observed group) groups, while C were conditions, which were intact genotypes. N was total number of individuals, whereas n_{ij} was

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

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:

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. In case of Yates' chi-sqaure was required, we performed with Spss program version 11.5.

4.2 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 and candidate SNPs.

4.3 Estimate of Haplotype frequency

In addition, EH program which was previously described by Zhao et al (2000), was employed to estimate haplotype frequencies in the population. Number of cases with double heterozygous genotypes (677CT with 1298AC) in with the C-A/T-C and C-C/T-A haplotype are included, we can determine the number of each haplotype by using formulation bases 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-C),f(T-C), f(C-C), and f(T-A) were the haplotype alleles of 677C->T and 1298->C estimated from EH program. The difference of haplotype between groups was estimated by T (χ^2 /2) = ln(L,group1)+ln(L,group2)-ln (L,group1+group2) as previous described by Zhao et al., 2000.[114]