CHAPTER IV

RESULTS

1. Study population

For the purposes of our analyses case and control were limited to Thais, to reduce the ethnic variation between groups. Of 108 case mothers, 68 were from Rachanukul hospital, and the other additional 40 cases from King Chulalongkorn Memorial Hospital. All children with Down syndrome were karyotypically confirmed to have trisomy 21. The 187 controls were pregnant women that recruited through the antenatal care clinic at King Chulalongkorn Memorial Hospital. All children of the control group were unfortunately followed up. One of them had Down syndrome so we moved her to the case group. After adjustment, we have 109 case mothers and 186 controls in this study. In addition, there were no significant differences between groups in term of mean age at conception as show in table 7

Table 7. Characteristic of responding age-matched control mothers and mothers of children with Down syndrome

	Control mother (n=186)	Case mother (n=109)	χ^2	P value
Age at conception ($\bar{X}\pm$SD)	32.4$\pm$ 5.12	33.9$\pm$5.81	0.034	0.85

2. Genotyping

1.1 *MTHFR* 677C->T

To determine the *MTHFR* 677C->T polymorphism, restriction enzyme analysis with *Hinf*I was performed and electrophoreses on 3 % agarose gel (figure 2) The 198 bp fragment is obtained after PCR amplification. In case of homozygous 677CC, an undigested PCR product of 198 bp is the only fragment presented. Where as heterozygote 677 CT reveals the 198 and 176 bp fragment, due to 677T allele created *Hinf* I restriction site. Thus, the homozygous variant which contains two allele of 677T, were totally cut and presented only the fragment of 176 bp.

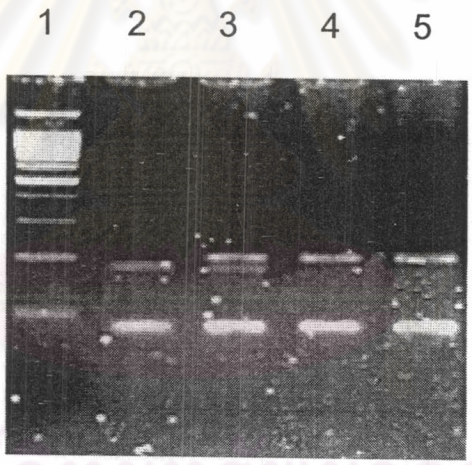

Figure 2. RFLP patterns of *MTHFR*677C->T. Lane 1 is 100 bp DNA marker. Lane 2 is 677TT genotype. Lane 3 is heterozygous 677CT genotype. Lane 4 and 5 are homozygous, wild type, 677CC genotype.

1.2 *MTHFR* 1298A->C

Genotyping of 1298A->C was performed in all specimens genotyped for 677C->T. To determine the *MTHFR* 1298A->C polymorphism, restriction enzyme analysis with *Mbo* II was performed and electrophoreses on 3 % agarose gel (figure 3) The 241 bp fragment is obtained after PCR amplification. In case of homozygous 1298AA, were totally cut and presented only the fragment of 204 bp. Where as heterozygote 1298AC reveals the 241 and 204 bp fragment, due to 1298A allele created *Mbo* II restriction site. Thus, the homozygous variant which contains two allele of 1298CC, were uncut and presented only the fragment of 241 bp.

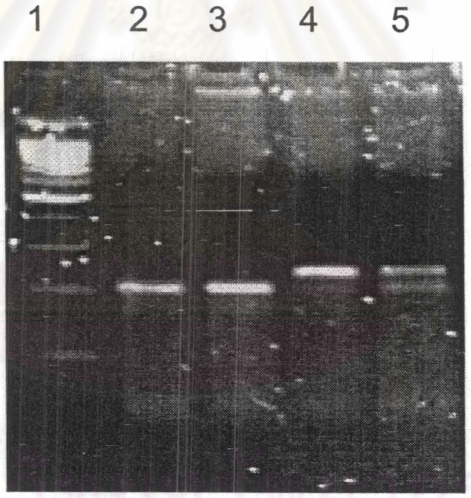

Figure 3. RFLP patterns of MTHFR1298A->C. Lane 1 is 100 bp DNA marker. Lane 2 and 3 are homozygous, wild type, 1298AA genotype. Lane 4 is homozygous 1298CC genotype. Lane 5 is heterozygous 1298AC genotype.

1.3 *MTRR* 66A->G

Genotyping of 66A->G was performed in same specimens genotyped for 677C->T. To determine the *MTRR* 66A->G polymorphism, restriction enzyme analysis with *Nde* I was performed and electrophoreses on 3 % agarose gel (figure 4) The 325 bp fragment is obtained after PCR amplification. In case of homozygous 66AA, were totally cut and presented the fragment of 282 and 43 bp . Where as heterozygote 66AG reveals the 325, 282 and 43 bp fragment, due to 66A allele created *Nde* I restriction site. Thus, the homozygous variant which contains two allele of 66GG, were uncut and presented the fragment of 325 bp.

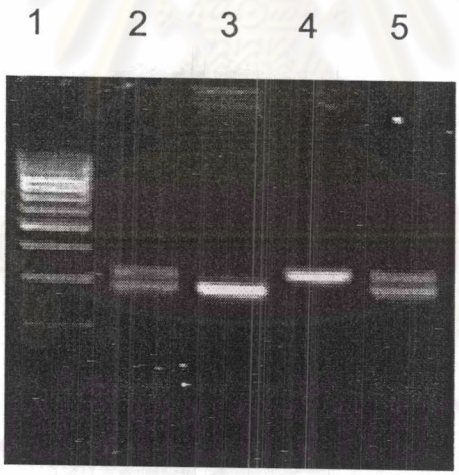

Figure 4 RFLP patterns of MTRRA->G . Lane 1 is 100 bp DNA marker. Lane 2 and 5 are heterozygous 66AG genotype homozygous. Lane 3 is homozygous, wild type, 66AA genotype. Lane 4 is homozygous 66GG genotype.

1.4 *MTR* 2756A->G

Genotyping of 2756A->G was performed in same specimens genotyped for 677C->T. The 341 bp fragment is obtained after PCR amplification. The PCR fragment of 341 bp remained uncut in the presence of A allele, but was digested in to fragment 198 and 143 bp in the presence of the G allele, due to G allele created *Hae* III restriction site. Thus heterozygote 2756AG reveals three fragment the 341,198 and 143 bp fragment. To determine the *MTR* 2756A->G polymorphism, restriction enzyme analysis with *Hae* III was performed and electrophoreses on 3 % agarose gel (figure 5)

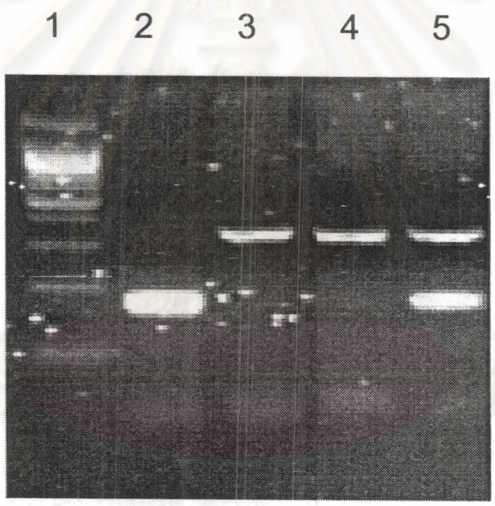

Figure 5 RFLP patterns of MTRA->G . Lane 1 is 100 bp DNA marker. Lane 2 is homozygous 2756GG.Lane 3 and 4 are homozygous, wild type, 2756AA genotype and Lane 5 is heterozygous 2756 AG genotype.

3. Allele frequency

Table 8 shows the distribution of the *MTHFR, MTRR* and *MTR* genotypes in the control population was found to be in Hardy-Weinberg equilibrium. There are no significant differences in mothers who having children with Down syndrome compared to age-matched control mothers.

Table 8. Allele frequency of polymorphisms from *MTHFR, MTR* and *MTRR* in mothers having children with Down syndrome and Age-matched control mothers

		Case	Control	χ^2	*P*
Genotype	Allele	Number (%)	Number (%)		
MTHFR 677 C->T	C	190 (0.87)	318 (0.85)	0.321	0.571
	T	28 (0.13)	54 (0.15)		
MTHFR 1298 A->C	A	151 (0.69)	266 (0.72)	0.333	0.564
	C	67 (0.31)	106 (0.28)		
MTR 2756 A->G	A	182 (0.83)	317 (0.85)	0.315	0.575
	G	36 (0.17)	55 (0.15)		
MTRR 66 A->G	A	154 (0.71)	267 (0.72)	0.086	0.769
	G	64 (0.29)	105 (0.28)		

MTHFR, Methylenetatrahydrofolate reductase; *MTR*, methionine synthase; *MTRR*, methionine synthase reductase

4. Association between MTHFR polymorphisms and Down syndrome

4.1 *MTHFR* 677C->T

Regarding the nucleotide 677 of the 186 controls responding for Thai population, the distribution of the CC, CT and TT genotypes were 73%(136/186), 25%(46/186) and 2%(4/186) respectively. The corresponding frequencies among all of mother who having a child with Down syndrome were found to be 77%(84/109), 20%(22/109) and 3%(3/109) respectively.

Odd Ratios (OR) calculation were performed to determine genotype associated risk of mother who having a child with Down syndrome (table 9)

Table 9. Frequency of *MTHFR* 677C->T polymorphisms in mothers having children with Down syndrome and Age-match control mothers

MTHFR	Case	Control	Odd ratio (95%CI)
677 Genotype	n = 109 (%)	n = 186 (%)	
CC	84 (77)	136 (73)	Ref.
CT	22 (20)	46 (25)	0.77 (0.42-1.42)
TT	3 (3)	4 (2)	1.29 (0.22-6.96*)
CT+TT	25 (23)	50 (25)	0.81 (0.45-1.45)

Ref. = Reference category, * Calculated by Yates' extract.

4.2 *MTHFR* 1298A->C

The composite data in table 5 show that the frequencies of the nucleotide 1298 of the 186 controls responding for Thai population, the distribution of the AA, AC and CC genotypes were 50% (93/186), 43% (80/186) and 7% (13/186) respectively. The corresponding frequencies among all of mother who having a child with Down syndrome were found to be 49%(53/109), 43%(47/109) and 8%(9/109) respectively.

Odd Ratios (OR) calculation were performed to determine genotype associate risk of mother who having a child with Down syndrome (table 10)

Table 10. Frequency of *MTHFR* 1298 A->C polymorphisms in mothers having children with Down syndrome and Age-match control mothers

MTHFR 1298 Genotype	Case n = 109 (%)	Control n = 186 (%)	Odd ratio (95%CI)
AA	53 (50)	93 (49)	Ref.
AC	47 (43)	80 (43)	1.00 (0.61-1.67)
CC	9 (7)	13 (8)	1.20 (0.45-3.13)
AC+CC	56 (50)	93 (51)	1.06 (0.67-1.74)

Ref. = Reference category

4.3 *MTHFR* 677C->T in combination with 1298A->C genotype and mother who having a child with Down syndrome

To investigate the joint effects of the two polymorphisms, analysis of the combined genotype distribution of the 677C->T and 1298A->C polymorphism in 109 case mothers and 186 control mothers, were performed. The prevalence and calculated OR of the combined genotypes are shown in Table 11. In controls showed no individuals with 677TT genotype. Calculated ORs for case mothers revealed no statistical significance in all genotypes.

When haplotype distributions were considered, EH program was used to estimate distribution of haplotype frequencies. Four possible haplotypes were observed and suggested in table 12. Data did not show significant differences neither in haplotype distributions among cases, mothers and controls nor in the prevalence of each haplotypes compared with controls.

The distributions of the haplotype combination were also observed (table 13). Except for the individuals with 677CT/1298AC genotype, individuals with an other genotypes can be easily identified as haplotype. By using haplotype frequencies (f) from EH program reported previously in table 12, we could estimate numbers of individuals with cis (C-A/T-C) or trans (C-C/T-A) for the 677CT/1298AC genotype. The result showed that only probability of having cis haplotype were found in this study. Consequently, chi-

square test was performed to test for differences of distribution in each combined haplotype in case compared with control group. The results did not show significant differences among them.

Table 11. Frequency polymorphisms from *MTHFR* in mothers having children with Down syndrome and Age-matched control mothers

MTHFR	Case	Control	Odd ratio (95%CI)
677/1298 Genotype	n = 109(%)	n = 186 (%)	
CC/AA	37 (34)	59 (32)	Ref.
CC/AC	38 (35)	64 (34)	1.02 (0.60-1.73)
CC/CC	9 (8)	13 (7)	1.20 (0.45-3.13)
CT/AA	13 (12)	30 (16)	0.70 (0.33-1.49)
CT/AC	9 (8)	16 (9)	0.96 (0.37-2.40)
CT/CC	No	No	ND
TT/AA	3 (3)	4 (2)	1.26 (0.22-6.96*)
TT/AC	No	No	ND
TT/CC	No	No	ND

MTHFR, Methylenetatrahydrofolate reductase

Ref. = Reference category. NO = not observed. ND = not determined. * Calculated by Yates' extract.

Table 12. Distribution of the haplotypes over the groups of case mothers and control.

Group	No. case	No. allele	Haplotype frequencies (nt677-nt1298) C-A	C-C	T-A	T-C	X^2_{EH}	P value$_{EH}$
Controls	186	372	f=0.569929 n=212 (56.99%) Ref.	f=0.284910 n=106 (28.49%) Ref.	f=0.145125 n=54 (14.52%) Ref.	f=0.000036 n=0 (0%) Ref.	Ref.	Ref.
DS mothers	109	218	f=0.573429 n=125 (57.34%) X^2=0.007 P=0.933	f=0.278065 n=65 (28.90%) X^2=0.011 P=0.916	f=0.128406 n=28 (12.84%) X^2=0.32 P=0.571	f=0.000035 n=0 (0%)	0.36	0.948

f = haplotype frequencies calculated by EH program, n = observed number of cases, P=P value.

X^2 = Pearson's chi-square if n>5 or Yates' correction if n<5 which were used to compared number of haplotype in each group with that in controls.

X^2_{EH}= Chi-square calculated based on EH program which was conducted to compare haplotype distribution between Case and control with P value$_{EH}$

Table 13. The distribution of *MTHFR* haplotype combination in DS mothers and control

	Haplotype distribution									
genotype	677CC / 1298AA	677CC / 1298AC	677CC / 1298CC	677CT / 1298AA	677CT / 1298AC		677CT / 1298CC	677TT / 1298AA	677TT / 1298AC	677TT / 1298CC
haplotype	C A / C A	C A / C C	C C / C C	C A / T A	C A / T C	C C / T A	C C / T C	T A / T A	T A / T C	T C / T C
Controls n=186	n=59 Ref.	n=64 Ref.	n=13 Ref.	n=30 Ref.	n=0	n=16 Ref.	n=0 Ref.	n=4 Ref.	n=0	n=0
DS Mothers n=109	n=37 X^2=0.155 P=0.694	n=38 X^2=0.006 P=0.938	n=9 X^2=0.16 P=0.689	n=13 X^2=0.975 P=0.323	n=0	n=9 X^2=0.011 P=0.916	n=0	n=3 X^2=0.107 P=0.744	n=0	n=0

C A, C C, T A, and T C implied 677C-1298A, 677C-1298C, 677T-1298A, and 677T-1298C haplotypes respectively. n= estimated number of cases which were calculated based on probability of haplotype frequencies after EH calculation. P= P value

X^2 = Pearson's chi-square if n>5 or Yates' correction if n<5 which were used to compared number of combined haplotypes in each groups with that in controls

5. Association between *MTRR* polymorphisms and Down syndrome

MTRR 66A->G

As show in Table 14, The frequencies of the MTRR genotypes (AA, AG, GG) among Age-match control mothers were 49%(92/186), 45%(83/186) and 6%(11/186) respectively. The corresponding frequencies among mothers having children with Down syndrome were 49%(53/109), 43%(47/109) and 8%(9/109) respectively.

Odd Ratios (OR) calculation were performed to determine genotype associate risk of mother who having a child with Down syndrome.

Table 14. Frequency of *MTRR* 66 A->G polymorphisms in mothers having children with Down syndrome and Age-match control mothers

MTRR 66 Genotype	Case n = 109 (%)	Control n = 186 (%)	Odd ratio (95%CI)
AA	53 (49)	92 (49)	Ref.
AG	47 (43)	83 (45)	0.94 (0.57-1.56)
GG	9 (8)	11 (6)	1.61 (0.61-4.24)
AG+GG	57 (51)	94 (51)	1.07 (0.65-1.77)

MTRR, methionine synthase reductase. Ref. = Reference category

For analysis of gene-gene interaction, the MTRR homozygous AA genotype and the heterozygous AG genotypes were combined because neither genotype was associated with increased risk of neural tube defects[37]. For *MTHFR*, the heterozygous CT and homozygous TT genotypes were combined for the gene-gene interaction analysis, because both have been associated with an increased risk of neural tube defects.[115]

6. Association between *MTHFR* and *MTRR* polymorphisms and Down syndrome

Table 15. Interaction between *MTHFR* and *MTRR* in mothers having children with Down syndrome and Age-matched control mothers

		Case	Control	Odd ratio (95%CI)
MTHFR	*MTRR*	n = 109 (%)	n = 186 (%)	
CC	AA+AG	76 (70)	127 (68)	Ref.
CT +TT	AA+AG	24 (22)	48 (26)	0.81 (0.45-1.47)
CC	GG	4 (4)	8 (5)	0.75 (0.19-2.75)
CT+TT	GG	5 (4)	3 (1)	2.93 (0.6-15.83*)

Ref. = Reference category. * Calculated by Yates' extract.

7. Association between *MTR* polymorphisms and Down syndrome

MTR 2756 A->G

Regarding the nucleotide 2756 of the 186 controls responding for Thai population, the frequencies of the MTRR genotypes AA, AG and GG among Age-match control mothers were 72%(133/186), 27%(51/186) and 1%(2/186) respectively. The corresponding frequencies among mothers having children with Down syndrome were 73%(80/109), 24%(26/109) and 3%(3/109) respectively. Odd Ratios (OR) calculation were performed to determine genotype associate risk of mother who having a child with Down syndrome (table 16)

Table 16. Frequency of MTR 2756 A->G polymorphisms in mothers having children with Down syndrome and Age-match control mothers

MTR	Case	Control	Odd ratio (95%CI)
2756 Genotype	n = 109 (%)	n = 186 (%)	
AA	80 (73)	133 (72)	Ref.
AG	26 (24)	51 (27)	0.83 (0.46-1.48)
GG	3 (3)	2 (1)	2.60 (0.35-22.64*)
AG+GG	29 (27)	53 (28)	0.91 (0.53-1.60)

MTR, methionine synthase. Ref. = Reference category. * Calculated by Yates' extract.

8. Association between *MTHFR* and *MTR* in mothers having children with Down syndrome and Age-matched control mothers

Table 17. Interaction between *MTHFR* and *MTR* in mothers having children with Down syndrome and Age-match control mothers

		Case	Control	Odd ratio (95%CI)
MTHFR	*MTR*	n = 109 (%)	n = 186 (%)	
CC	AA	62 (56.88)	95 (51.07)	Ref.
CC	AG	20 (18.35)	39 (20.97)	0.85 (0.44-1.60)
CC	GG	2 (1.83)	2 (1.08)	1.72 (0.17-17.34*)
CT	AA	16 (14.68)	35 (18.82)	0.74 (0.37-1.48)
CT	AG	6 (5.51)	11 (5.91)	0.93 (0.29-2.81*)
CT	GG	No	No	ND
TT	AA	3 (2.75)	3 (1.61)	1.73 (0.27-10.92*)
TT	AG	No	1 (0.54)	ND
TT	GG	No	No	ND

Ref. = Reference category. * Calculated by Yates' extract.

NO = not observed. ND = not determined.

9. Association between *MTRR* and *MTR* in mothers having children with Down syndrome and Age-match control mothers

Table 18. Interaction between *MTRR* and *MTR* in mothers having children with Down syndrome and Age-match control mothers

		Case	Control	Odd ratio (95%CI)
MTRR	*MTR*	n = 109 (%)	n = 186 (%)	
AA	AA	42 (38.53)	67 (36.02)	Ref.
AA	AG	11 (10.09)	24 (12.90)	0.76 (0.33-1.70)
AA	GG	1 (0.92)	1 (0.54)	1.71 (0.06-63.31*)
AG	AA	32 (29.36)	59 (31.72)	0.89 (0.52-1.54)
AG	AG	13 (11.93)	23 (12.37)	0.96 (0.44-2.09)
AG	GG	1 (0.92)	1 (0.54)	1.71 (0.06-63.31*)
GG	AA	7 (6.42)	7 (3.76)	1.75 (0.54-15.75)
GG	AG	2 (1.83)	4 (2.15)	0.85 (0.11-5.5*)
GG	GG	No	No	ND

Ref. = Reference category. * Calculated by Yates' extract.

NO = not observed. ND = not determined.

10. Association between four polymorphisms of *MTHFR*, *MTR* and *MTRR* and Down syndrome

All the distribution of *MTHFR, MTR and MTRR* genotypes were in Hardy-Wienberg equilibrium in the groups. We compared the genotype frequencies of the polymorphisms of *MTHFR, MTR* and *MTRR* genes and find no significant difference in mothers who having children with Down syndrome compared to age-matched control mothers.

Table 19. Interaction between *MTHFR*, *MTRR* and *MTR* genotype in mothers having children with Down syndrome and Age-match control mothers

MTHFR 677C->T	MTHFR 1298A->C	MTRR 66A->G	MTR 2756A->G	No. (%) Of cases (n=109)	No. (%) Of controls (n=186)	Odds ratio	95% CI
CC	AA	AA	AA	16	16	Ref	Ref
CC	AA	AA	AG	2	12	0.27	0.04-1.31*
CC	AA	AA	GG	1	No	ND	ND
CC	AA	AG	AA	11	23	0.80	0.35-1.8
CC	AA	AG	AG	4	4	ND	ND
CC	AA	AG	GG	No	No	ND	ND
CC	AA	GG	AA	2	3	ND	ND
CC	AA	GG	AG	No	No	ND	ND
CC	AA	GG	GG	No	No	ND	ND
CC	AC	AA	AA	13	25	0.87	0.4-1.88
CC	AC	AA	AG	5	7	1.23	0.33-4.45*
CC	AC	AA	GG	1	No	ND	ND
CC	AC	AG	AA	11	18	1.05	0.44-2.45
CC	AC	AG	AG	6	10	2.03	0.32-3.18*
CC	AC	AG	GG	1	No	ND	ND
CC	AC	GG	AA	2	2	1.72	0.17-17.34*
CC	AC	GG	AG	No	2	ND	ND
CC	AC	GG	GG	No	No	ND	ND
CC	CC	AA	AA	4	5	1.38	0.30-6.07*
CC	CC	AA	AG	2	2	1.72	0.17-17.34*
CC	CC	AA	GG	No	No	ND	ND
CC	CC	AG	AA	2	2	1.72	0.17-17.34*
CC	CC	AG	AG	1	2	ND	ND
CC	CC	AG	GG	No	No	ND	ND
CC	CC	GG	AA	No	1	ND	ND
CC	CC	GG	AG	No	1	ND	ND

CC	CC	GG	GG	No	No	ND	ND
CT	AA	AA	AA	3	14	0.35	0.08-1.33*
CT	AA	AA	AG	1	2	ND	ND
CT	AA	AA	GG	No	No	ND	ND
CT	AA	AG	AA	7	9	1.35	0.44-4.10
CT	AA	AG	AG	1	4	0.42	0.02-4.06*
CT	AA	AG	GG	No	No	ND	ND
CT	AA	GG	AA	1	1	1.71	0.0*-31.31*
CT	AA	GG	AG	No	1	ND	ND
CT	AA	GG	GG	No	No	ND	ND
CT	AC	AA	AA	1	7	0.24	0.01-1.95*
CT	AC	AA	AG	1	2	ND	ND
CT	AC	AA	GG	No	No	ND	ND
CT	AC	AG	AA	3	5	1.02	0.19-5.04*
CT	AC	AG	AG	1	2	ND	ND
CT	AC	AG	GG	No	No	ND	ND
CT	AC	GG	AA	1	No	ND	ND
CT	AC	GG	AG	2	No	ND	ND
CT	AC	GG	GG	No	No	ND	ND
CT	CC	AA	AA	No	No	ND	ND
CT	CC	AA	AG	No	No	ND	ND
CT	CC	AA	GG	No	No	ND	ND
CT	CC	AG	AA	No	No	ND	ND
CT	CC	AG	AG	No	No	ND	ND
CT	CC	AG	GG	No	No	ND	ND
CT	CC	GG	AA	No	No	ND	ND
CT	CC	GG	AG	No	No	ND	ND
CT	CC	GG	GG	No	No	ND	ND
TT	AA	AA	AA	1	No	ND	ND
TT	AA	AA	AG	No	No	ND	ND
TT	AA	AA	GG	1	No	ND	ND
TT	AA	AG	AA	No	No	ND	ND

TT	AA	AG	AG	1	No	ND	ND
TT	AA	AG	GG	No	No	ND	ND
TT	AA	GG	AA	No	No	ND	ND
TT	AA	GG	AG	No	No	ND	ND
TT	AA	GG	GG	No	No	ND	ND
TT	AC	AA	AA	No	1	ND	ND
TT	AC	AA	AG	No	No	ND	ND
TT	AC	AA	GG	No	2	ND	ND
TT	AC	AG	AA	No	1	ND	ND
TT	AC	AG	AG	No	No	ND	ND
TT	AC	AG	GG	No	No	ND	ND
TT	AC	GG	AA	No	No	ND	ND
TT	AC	GG	AG	No	No	ND	ND
TT	AC	GG	GG	No	No	ND	ND
TT	CC	AA	AA	No	No	ND	ND
TT	CC	AA	AG	No	No	ND	ND
TT	CC	AA	GG	No	No	ND	ND
TT	CC	AG	AA	No	No	ND	ND
TT	CC	AG	AG	No	No	ND	ND
TT	CC	AG	GG	No	No	ND	ND
TT	CC	GG	AA	No	No	ND	ND
TT	CC	GG	AG	No	No	ND	ND
TT	CC	GG	GG	No	No	ND	ND

Ref. = Reference category. * Calculated by Yates' extract.

NO = not observed. ND = not determined.

11. Association between *MTHFR, MTRR* and *MTR* polymorphism and Down syndrome in mother aged 30 years old or less.

11.1 Study population

Of our 109 case mothers, 32 were ≤30 years old when their Down syndrome children were born. 64 out of 186 control mothers were ≤30 years old. In addition, there were no significant differences between groups in term of mean age at conception as show in table 20.

Table 20. Characteristic of responding age-matched control mothers and mothers of children with Down syndrome (≤ 30 years old)

	Control mother (n=64)	Case mother (n=32)	χ^2	P value
Age at conception ($\overline{X}\pm SD$)	26.97± 2.92	27.06± 3.09	0	1.00

11.2 Allele frequency

Table 21 shows the distribution of the *MTHFR, MTRR* and *MTR* genotypes in the control population was found to be in Hardy-Weinberg equilibrium. There are no significant differences in mothers aged 30 years old or less to have children with Down syndrome compared to age-matched control mothers.

Table 21. Allele frequency of polymorphisms from *MTHFR*, *MTR* and *MTRR* in mothers aged 30 years old or less to have children with Down syndrome and Age-matched control mothers

Genotype	Allele	Case	Control	χ^2	*P*
		Number (%)	Number (%)		
MTHFR 677 C->T	C	54 (0.83)	106 (0.83)	0.075	0.784
	T	10 (0.17)	22 (0.17)		
MTHFR 1298 A->C	A	40 (0.63)	89 (0.70)	0.957	0.328
	C	24 (0.37)	39 (0.30)		
MTR 2756 A->G	A	58 (0.91)	107 (0.84)	1.745	0.187
	G	6 (0.09)	21 (0.16)		
MTRR 66 A->G	A	46 (0.72)	94 (0.73)	0.053	0.818
	G	18 (0.28)	34 (0.27)		

MTHFR, Methylenetatrahydrofolate reductase; *MTR*, methionine synthase; *MTRR*, methionine synthase reductase

11.3 *MTHFR* 677C->T

Regarding the nucleotide 677 of the 64 controls responding for Thai population, the distribution of the CC, CT and TT genotypes were 68% (43/64), 31% (20/64) and 1% (1/64) respectively. The corresponding frequencies among all of mother who having a child with Down syndrome were found to be 72% (23/32), 25% (8/32) and 3% (1/32) respectively.

Odd Ratios (OR) calculation were performed to determine genotype associated risk of mother who having a child with Down syndrome (table 22)

Table 22. Frequency of *MTHFR* 677C->T polymorphisms in mothers aged 30 years old or less to have children with Down syndrome and Age-match control mothers

MTHFR 677 Genotype	Case n = 32 (%)	Control n = 64 (%)	Odd ratio (95%CI)
CC	23 (72)	43 (69)	Ref.
CT	8 (25)	20 (31)	0.73 (0.25-2.10)
TT	1 (3)	1 (1)	2.03 (0.00*-77.44*)
CT+TT	9 (28)	21 (32)	0.80 (0.28-2.23)

Ref. = Reference category, * Calculated by Yates' extract.

11.4 *MTHFR* 1298A->C

The composite data in table 15 show that the frequencies of the nucleotide 1298 of the 64 controls responding for Thai population, the distribution of the AA, AC and CC genotypes were 48.4% (31/64), 42.2% (27/64) and 9.4% (6/64) respectively. The corresponding frequencies among all of mother who having a child with Down syndrome were found to be 37.5%(12/32), 50%(16/32) and 12.5%(5/32) respectively.

Odd Ratios (OR) calculation were performed to determine genotype associated risk of mother who having a child with Down syndrome (table 23)

Table 23. Frequency of *MTHFR* 1298 A->C polymorphisms in mothers aged 30 years old or less to have children with Down syndrome and Age-match control mothers

MTHFR 1298 Genotype	Case n = 32 (%)	Control n = 64 (%)	Odd ratio (95%CI)
AA	12 (37.5)	31 (48.4)	Ref.
AC	16 (50)	27 (42.2)	1.37 (0.54-3.51)
CC	4 (12.5)	6 (9.4)	1.79 (0.43-7.45*)
AC+CC	21 (62.5)	33 (51.6)	1.79 (0.68-4.75)

Ref. = Reference category

11.5 *MTHFR* 677C->T in combination with 1298A->C genotype and mother who having a child with Down syndrome

To investigate the joint effects of the two polymorphisms, analysis of the combined genotype distribution of the 677C->T and 1298A->C polymorphism in 32 case mothers and 64 control mothers, were performed. The prevalence and calculated OR of the combined genotypes are shown in Table 24. In controls showed no individuals with 677TT genotype. Calculated ORs for case mothers revealed no statistical significance in all genotypes.

When haplotype distributions were considered, EH program was used to estimate distribution of haplotype frequencies. Four possible haplotypes were observed and suggested in table 25. Data did not show significant differences neither in haplotype distributions among cases, mothers and controls nor in the prevalence of each haplotypes compared with controls.

The distributions of the haplotype combination were also observed (table 26). Except for the individuals with 677CT/1298AC genotype, individuals with an other genotypes can be easily identified as haplotype. By using haplotype frequencies (f) from EH program reported previously in table 25, we could estimate numbers of individuals with cis (C-A/T-C) or trans (C-C/T-A) for the 677CT/1298AC genotype. The result showed that only probability of having cis haplotype were found in this study. Consequently, chi-square test was performed to test for differences of distribution in each combined haplotype in case compared with control group. The results did not show significant differences among them.

Table 24. Frequency polymorphisms from *MTHFR* in mothers aged 30 years old or less to have children with Down syndrome and Age-matched control mothers

MTHFR 677/1298 Genotype	Case n = 32(%)	Control n = 64 (%)	Odd ratio (95%CI)
CC/AA	6 (19)	19 (30)	Ref.
CC/AC	13 (41)	18 (28)	1.75 (0.60-1.73)
CC/CC	4 (12)	6 (9)	1.38 (0.3-6.17*)
CT/AA	5 (16)	11 (17)	0.89 (0.24-3.18*)
CT/AC	3 (9)	9 (14)	0.63 (0.12-2.55*)
CT/CC	No	No	ND
TT/AA	1 (3)	1 (2)	2.03 (0.0*-77.44*)
TT/AC	No	No	ND
TT/CC	No	No	ND

MTHFR, Methylenetatrahydrofolate reductase

Ref. = Reference category. NO = not observed. ND = not determined. * Calculated by Yates' extract.

Table 25. Distribution of the haplotypes over the groups of case mothers and control.

Group	No. case	No. allele	Haplotype frequencies (nt677-nt1298) C-A	C-C	T-A	T-C	X^2_{EH}	P value$_{EH}$
Controls	64	128	f=0.523482 n=67 (52.34%) Ref.	f=0.171831 n=39 (30.47%) Ref.	f=0.304643 n=22 (17.19%) Ref.	f=0.000044 n=0 (0%) Ref.	Ref.	Ref.
DS mothers	32	64	f=0.468760 n=30 (46.87%) X^2=0.51 P=0.315	f=0.156240 n=24 (37.5%) X^2=0.957 P=0.327	f=0.374990 n=10 (15.63%) X^2=0.32 P=0.571	f=0.00001 n=0 (0%)	0.47	0.989

f = haplotype frequencies calculated by EH program, n = observed number of cases, P=P value.

X^2 = Pearson's chi-square if n>5 or Yates' correction if n<5 which were used to compared number of haplotype in each group with that in controls.

X^2_{EH}= Chi-square calculated based on EH program which was conducted to compare haplotype distribution between case and control with P value$_{EH}$

Table 26. The distribution of *MTHFR* haplotype combination in DS mothers and control

	Haplotype distribution									
genotype	677CC / 1298AA	677CC / 1298AC	677CC / 1298CC	677CT / 1298AA	677CT / 1298AC		677CT / 1298CC	677TT / 1298AA	677TT / 1298AC	677TT / 1298CC
haplotype	C A / C A	C A / C C	C C / C C	C A / T A	C A / T C	C C / T A	C C / T C	T A / T A	T A / T C	T C / T C
Controls n=64	n=19 Ref.	n=18 Ref.	n=6 Ref.	n=11 Ref.	n=0	n=9 Ref.	n=0 Ref.	n=1 Ref.	n=0	n=0
DS Mothers n=32	n=6 X^2=0.296 P=0.586	n=13 X^2=1.25 P=0.217	n=4 X^2=0.107 P=0.744	n=5 X^2=0.038 P=0.845	n=0	n=3 X^2=0.429 P=0.512	n=0	n=1 X^2=0.064 P=0.8	n=0	n=0

C A, C C, T A, and T C implied 677C-1298A, 677C-1298C, 677T-1298A, and 677T-1298C haplotypes respectively. n=estimated number of cases which were calculated based on probability of haplotype frequencies after EH calculation. P= P value

X^2 = Pearson's chi-square if n>5 or Yates' correction if n<5 which were used to compared number of combined haplotypes in each groups with that in controls

11.6 Association between *MTRR* polymorphisms and Down syndrome

MTRR 66A->G

As show in Table 27,The frequencies of the MTRR genotypes (AA, AG, GG) among Age-match control mothers were 53%(34/64), 41%(26/64) and 6%(4/64) respectively. The corresponding frequencies among mothers having children with Down syndrome were 47%(15/32), 50%(16/32) and 3%(1/32) respectively.

Odd Ratios (OR) calculation were performed to determine genotype associated risk of mother who having a child with Down syndrome.

Table 27. Frequency of *MTRR* 66 A->G polymorphisms in mothers aged 30 years old or less to have children with Down syndrome and Age-match control mothers

MTRR 66 Genotype	Case n = 32 (%)	Control n = 64 (%)	Odd ratio (95%CI)
AA	15 (47)	34 (53)	Ref.
AG	16 (50)	26 (41)	1.46 (0.57-3.75)
GG	1 (3)	4 (6)	0.48 (0.01-4.95*)
AG+GG	17 (53)	30 (47)	1.28 (0.5-3.28)

MTRR, methionine synthase reductase. Ref. = Reference category

11.7 Association between *MTHFR* and *MTRR* polymorphisms and Down syndrome

Table 28. Interaction between *MTHFR* and *MTRR* in mothers aged 30 years old or less to have children with Down syndrome and Age-match control mothers

MTHFR	*MTRR*	Case n = 32 (%)	Control n = 64 (%)	Odd ratio (95%CI)
CC	AA+AG	23 (71.875)	41 (64.0.625)	Ref.
CT +TT	AA+AG	8 (25)	19 (29.6875)	0.79 (0.27-2.27)
CC	GG	0 (0)	2 (3.125)	ND
CT+TT	GG	1 (3.125)	2 (31.25)	1.00 (0.00*-14.92*)

Ref. = Reference category. * Calculated by Yates' extract.

11.8Association between *MTR* polymorphisms and Down syndrome

MTR 2756 A->G

Regarding the nucleotide 2756 of the 186 controls responding for Thai population, the frequencies of the MTRR genotypes AA, AG and GG among Age-match control mothers were 69%(44/64), 30%(19/64) and 1%(1/64) respectively. The corresponding frequencies among mothers having children with Down syndrome were 81%(26/32), 19%(6/32) and 0%(0/32) respectively.

Table 29. Frequency of MTR 2756 A->G polymorphisms in mothers aged 30 years old or less to have children with Down syndrome and Age-match control mothers

MTR 2756 Genotype	Case n = 32 (%)	Control n = 64 (%)	Odd ratio (95%CI)
AA	26 (81)	44 (69)	Ref.
AG	6 (19)	19 (30)	0.55 (0.17-1.70*)
GG	0 (0)	1 (1)	ND
AG+GG	6 (19)	20 (31)	0.51 (0.16-1.57*)

MTR, methionine synthase. Ref. = Reference category. * Calculated by Yates' extract.

11.9 Association between *MTHFR* and *MTR* in mothers aged 30 years old or less to have children with Down syndrome and Age-matched control mothers

Table 30. Interaction between *MTHFR* and *MTR* in mothers aged 30 years old or less to have children with Down syndrome and Age-match control mothers

MTHFR	*MTR*	Case n = 32 (%)	Control n = 64 (%)	Odd ratio (95%CI)
CC	AA	18 (57)	25 (39)	Ref.
CC	AG	5 (16)	16 (25)	0.56 (0.16-1.87*)
CC	GG	No	2 (3)	ND
CT	AA	7 (21)	10 (16)	1.51 (0.45-4.9)
CT	AG	1 (3)	8 (13)	0.23 (0.01-1.93*)
CT	GG	No	2 (3)	ND
TT	AA	1 (3)	No	ND
TT	AG	No	1 (1)	ND
TT	GG	No	No	ND

Ref. = Reference category. * Calculated by Yates' extract.

NO = not observed. ND = not determined.

11.10 Association between *MTRR* and *MTR* in mothers aged 30 years old or less to have children with Down syndrome and Age-matched control mothers

Table 31. Interaction between MTRR and MTR in mothers aged 30 years old or less to have children with Down syndrome and Age-match control mothers

		Case	Control	Odd ratio (95%CI)
MTRR	*MTR*	n = 32 (%)	n = 64 (%)	
AA	AA	11 (34)	26 (40)	Ref.
AA	AG	4 (13)	9 (14)	0.87 (0.2-3.5*)
AA	GG	No	No	ND
AG	AA	15 (47)	16 (25)	2.65 (0.99-7.16)
AG	AG	1 (3)	8 (13)	0.23 (0.01-1.93*)
AG	GG	NO	1 (1)	ND
GG	AA	NO	2 (3)	ND
GG	AG	1 (3)	2 (3)	1.00 (0.0*-14.92*)
GG	GG	No	No	ND

Ref. = Reference category. * Calculated by Yates' extract.

NO = not observed. ND = not determined.