CHAPTER IV

RESULTS

1. Genotyping

1.1 *MTHFR* 677C→T

The *MTHFR* polymorphisms were genotyped in 202 controls, 48 patients with FEEM and 162 patients with nonsyndromic CL/P. In addition, 23mothers of FEEM patients, 97 mothers of CL/P patients and 65 fathers of CL/P patients were also genotyped. To determine the *MTHFR* 677C→T polymorphism, restriction enzyme analysis with *Hinf*I was performed and electrophoresed on 3% agarose gel (figure 4). In case of homozygous 677CC, an undigested PCR product of 193 bp is the only fragment presented. Whereas heterozygote (677CT) reveals the 198 and 176 bp fragments, due to 677T allele created *Hinf*I restriction site. Thus, the homozygous variant which contains two alleles of 677T, were totally cut and presented only the fragment of 176 bp.

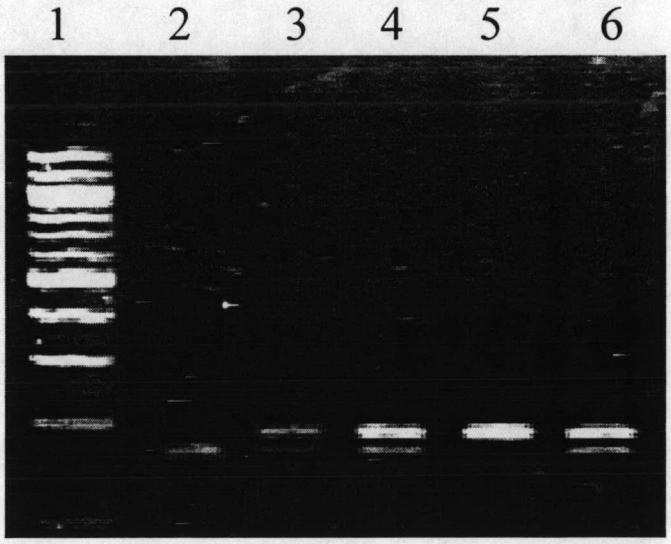

Figure 4 RFLP patterns of *MTHFR*677C→T. Lane 1 is 100 bp DNA marker. Lane 2 is 677TT genotype. Lane 3, 4 and 6 are heterozygous 677CT genotypes. Lane 5 is homozygous, wild type, 677CC genotype

1.2 MTHFR 1298A→C

Genotyping of 1298A→C was performed in all specimens genotyped for 677C→T. The 241 bp fragment is obtained after PCR amplification. In contrast the restriction enzyme analysis of 677C→T for which the variant alleles create restriction site of *Hinf*I, variant allele of 1298A→C (1298C allele) abolish the restriction site of *Mbo*II. If the wildtype genotype (1298AA) is presented, then the *Mbo*II RFLP results in two fragments – 204 and 37 bp. For the homozygous variant genotype (1298CC), only the 241 bp fragment is obtained and for the heterozygous genotype (1298AC), all three fragments are obtained which can easily be differentiated by 2% agarose gel electrophoresis. (figure 5)

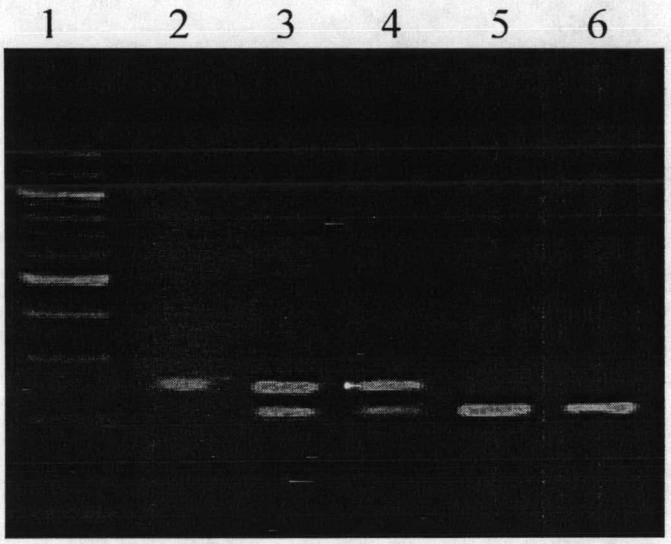

Figure 5 RFLP patterns of MTHFR1298A→C. Lane 1 is 100 bp DNA marker. Lane 2 is 1298CC genotype. Lane 3 and 4 are heterozygous 1298AC genotypes. Lane 5 and 6 are homozygous, wild type, 1298AA genotypes.

2. Association between MTHFR polymorphisms and FEEM

Forty eight individuals diagnosed with FEEM and 23 of their mothers were genotyping to examine prevalences of the two *MTHFR* polymorphisms compared with these in 202 controls.

2.1 *MTHFR* 677C→T and FEEM

After genotyping, data of genotypic and allellic distribution of *MTHFR* polymorphisms in FEEMs and their mothers are available. Regarding the nucleotide 677 of the 202 controls representing for Thai population, the distribution of the CC,CT and TT genotypes were 156, 44 and 2 respectively. The distribution is in Hardy Weinberg equilibrium (HWE). Thirty nine of FEEMs and 18 of their mothers were found to be 677CC. Whereas 9 of the patients and 5 of their mothers were 677CT. No TT genotype was observed in patients nor the mother group. No statistical significant differences for the allelic distribution of 677C→T among cases and controls were presented (*P* value = 0.488 and 0.840) (table 8).

Odd Ratios (OR) calculation were performed to determine genotype associated risk of FEEM (table 9). Since no TT genotype was found in patients and their mothers, OR were analysed to estimate the susceptible risk only in CT genotype as compare to normal CC. The OR of patients with CT genotype was 0.77 (95% CI:0.42-1.40) whereas OR among mother of patients was 0.93 (95% CI:0.41-2.04).

Table 8 – Allelic distribution of the *MTHFR* 677C→T in groups of patients with FEEM and their mothers.

Subject type	No. of subjects	Genotype			No. of chromosomes of allele type		Allele frequency		x^2	p value (df=1)
		CC	CT	TT	C	T	C	T		
Controls	202	156	44	2	356	48	0.88	0.12	Ref.	Ref.
FEEMs	48	39	9	0	87	9	0.91	0.09	0.482	0.488
FEEM mothers	23	18	5	0	41	5	0.89	0.11	0.041	0.840

Ref. = Reference category

Table 9 – Genotype distribution and calculated OR showing association between patients with FEEM their mother and the *MTHFR* 677C→T polymorphism.

Groups	Genotypes	No.	OR	95% CI
Controls	CC	156		
(n=202)	CT	44		
	TT	2		
FEEMs	CC	39	1.00	
(n=48)	CT	9	0.77	0.42-1.40
	TT	0	0.00	0.00
FEEM mothers	CC	18	1.00	
(n=23)	CT	5	0.93	0.41-2.04
	TT	0	0.00	0.00

2.2 *MTHFR* 1298A→C and FEEM

We further analyzed the MTHFR 1298A→C polymorphism in all of the samples. Genotyping revealed CC in 108 controls, 23 of patients with FEEM and 14 of mothers. Eighty controls, 20 patients and 7 of mothers were heterozygotes, 1298AC. Whereas 14 controls, 5 patients and 2 of mothers were 1298CC. In all groups they were in Hardy Weinberg equilibrium. However, allelic distribution of 1298A→C revealed no significant difference neither between controls and patients nor controls and mothers with P=0.373 and 0.681 respectively (table 10).

Genotype distribution of 1298A→C was also investigated to find risk associated with AC or CC as compare to normal AA genotype by OR calculation shown in table 11. In case of FEEM patients, OR were 1.17 (95% CI:0.71-1.93) for AC genotype and 1.68 (95% CI:0.47-5.68) for CC genotype without statistical significance. In addition, no substantial susceptibility of FEEM were found in mothers with AC and CC genotypes reflect on ORs of 0.68 (95% CI:0.33-1.38) and 1.10 (95% CI:0.00-5.97).

Table 10 - Allelic distribution of the *MTHFR* 1298A→C in groups of patients with FEEM and their mothers.

Subject type	No. of subjects	Genotype			No. of chromosomes of allele type		Allele frequency		x^2	p value (df=1)
		AA	AC	CC	A	C	A	C		
Controls	202	108	80	14	296	108	0.75	0.25	Ref.	Ref.
FEEMs	48	23	20	5	66	30	0.69	0.31	0.792	0.373
FEEM mothers	23	14	7	2	35	11	0.76	0.24	0.169	0.681

Ref.= Reference category

Table 11 – Genotype distribution and calculated OR showing association between patients with FEEM, their mothers and the 1298A→C *MTHFR* polymorphism.

Groups	Genotypes	No.	OR	95% CI
Controls	AA	108		
(n=202)	AC	80		
	CC	14		
FEEMs	AA	23	1.00	
(n=48)	AC	20	1.17	0.71-1.93
	CC	5	1.68	0.47-5.68
FEEM mothers	AA	14	1.00	
(n=23)	AC	7	0.68	0.33-1.38
	CC	2	1.10	0.00-5.97

2.3 *MTHFR* 677C→T in combination with 1298A→C genotype and FEEM

To investigate the joint effects of the two polymorphisms, analysis of the combined genotype distribution of the 677C→T and 1298A→C polymorphism in 48 patients and 23 of their mothers, were performed. The prevalences and calculated OR of the combined genotypes are shown in Table 12. In controls, all individuals who were homozygous for one polymorphism revealed the wildtype sequence of the other polymorphism and *vice versa*. Whereas in groups of FEEM patients and their mothers showed no individuals with 677TT genotype. Calculated ORs for patients revealed no statistical significance in all genotypes. Whereas mothers with 677CC/1298AC interestingly revealed the protective effect of decreased risk more than 2 fold of having children with FEEM (OR 0.41 : 95% CI:0.17-0.94 and P=0.034).

When haplotype distributions were considered, EH program was used to estimate distribution of haplotype fequencies. Four possible haplotypes were observed and suggested in table 13. Data did not show significant differences neither in haplotype distributions among cases, mothers and controls nor in the prevalences of each haplotypes compared with controls.

The distributions of the haplotype combination were also observed (table 14). 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 13, 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 patients and their mothers compared with control group. The results did not show significant diferrences among them.

Table 12 – Prevalence and calculated OR with 95% CI of the 677C→T in combination with 1298A→C *MTHFR* polymorphism among FEEMs, their parents and controls

Groups	677C→T	1298A→C		
		AA	AC	CC
Controls	CC	66	74	14
(n=202)	CT	40	6	-
	TT	2	-	-
FEEMs	CC	18	17	4
(n=48)		OR:1.00 (0.57-1.74)	OR:0.84 (0.48-1.47)	OR:1.05 (0.40-2.67)
	CT	5	3	-
		OR:0.46 (0.61-1.50)	OR:1.83 (0.57-5.75)	
	TT	-	-	-
	CC	11	5	2
FEEM mothers		OR:1.00 (0.50-1.98)	OR:0.41 (0.17-0.94)	OR:0.86 (0.23-2.92)
(n=23)			P=0.034	
	CT	9	2	-
		OR:0.77(0.42-1.40)	OR:2.00 (0.49-7.57)	
	TT	-	-	-

Table 13 – Distribution of the haplotypes over the groups of patients with FEEM and their mothers

Group	No. case	No. allele	Haplotype frequencies (nt677-nt1298) C-A	C-C	T-A	T-C	X^2_{EH}	P value$_{EH}$
Controls	202	404	f=0.608914	f=0.267324	f=0.123759	f=0.000003	Ref.	Ref.
			n=246	n=108	n=50	n=0		
			(60.9%)	(26.7%)	(12.4%)	(0%)		
			Ref.	Ref.	Ref.	Ref.		
FEEMs	48	96	f=0.617105	f=0.297789	f=0.085023	f=0.000083	1.320	0.796
			n=58	n=29	n=8	n=1		
			(60.4%)	(30.2%)	(8.3%)	(1.1%)		
			X^2=0.007	X^2=0.471	X^2=1.236	X^2=0.613		
			P=0.933	P=0.493	P=0.266	P=0.434		
FEEM mothers	23	46	f=0.660928	f=0.230377	f=0.099942	f=0.008754	0.380	0.944
			n=30	n=11	n=5	n=0		
			(65.2%)	(23.9%)	(10.9%)	(0%)		
			X^2=0.326	X^2=0.169	X^2=0.087			
			P=0.568	P=0.681	P=0.768			

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 cases and control with P value$_{EH}$

Ref. = Reference category

Table 14 – The distribution of *MTHFR* haplotype combination in patients with FEEM and their mothers.

	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=202	n=66 Ref.	n=74 Ref.	n=14 Ref.	n=40 Ref.	n=0	n=6 Ref.	n=0 Ref.	n=2 Ref.	n=0	n=0
FEEMs n=48	n=18 X^2=0.377 P=0.539	n=17 X^2=0.025 P=0.874	n=4 X^2=0.001 P=0.975	n=5 X^2=2.315 P=0.128	n=0	n=3 X^2=0.443 P=0.506	n=1 X^2=0.614 P=0.433	n=0 X^2=0.044 P=0.833	n=0	n=0
FEEM Mothers n=23	n=11 X^2=2.056 P=0.152	n=5 X^2=2.011 P=0.156	n=2 X^2=0.013 P=0.909	n=3 X^2=0.251 P=0.616	n=0	n=2 X^2=0.567 P=0.418	n=0	n=0 X^2=0.48 P=0.488	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

3. Association between *MTHFR* polymorphism and CL/P

3.1 *MTHFR* 677C→T and CL/P

The *MTHFR* 677C→T prevalances of 162 CL/P patients and their parents (97 mothers and 65 fathers) were investigated. There was no difference between the expected and observed genotype prevalences of both polymorphisms according to Hardy Weinberg Principle within FEEM patients, their parents and controls. As allelic distributions were observed, result showed no differences between them (table 15).

Moreover, Odd Ratios reflecting the risk of CL/P, were not substantially different between CL/P patients and control regarding 677CT and TT genotype. Also, ORs of their parents did not exhibit any significant differences neither in CT nor TT genotype (table 16). Although the OR for 677TT genotype of CL/P fathers was 4.28 which indicated tendency of increased risk, it was still in term of no significant differences with 95% CI:0.13-42.60.

Table 15 – Allelic distribution of the *MTHFR* 677C→T in groups of patients with CL/P and their parents.

Subject type	No. of subjects	Genotype			No. of chromosomes of allele type		Allele frequency		x^2	p value (df=1)
		CC	CT	TT	C	T	C	T		
Controls	202	156	44	2	356	48	0.88	0.12	Ref.	Ref.
CL/Ps	162	123	38	1	284	40	0.88	0.12	0.037	0.847
CL/P mothers	97	73	22	2	168	26	0.87	0.13	0.280	0.597
CL/P fathers	65	48	14	3	110	20	0.85	0.15	1.086	0.297

Ref. = Referenc category

Table 16- Genotype distribution and calculated OR showing association between patients with CL/P their parents and the 677C→T *MTHFR* polymorphism.

Groups	Genotypes	No.	OR	95% CI
Controls	CC	156		
(n=202)	CT	44		
	TT	2		
CL/Ps	CC	123	1.00	
(n=162)	CT	38	1.03	0.72-1.49
	TT	1	0.63	0.08-4.00
CL/P mothers	CC	73	1.00	
(n=97)	CT	22	1.01	0.66-1.55
	TT	2	1.03	0.21-21.44
CL/P fathers	CC	48	1.00	
(n=65)	CT	14	0.98	0.59-1.62
	TT	3	4.81	0.63-42.60

3.2 *MTHFR* 1298A→C and CL/P

The second common polymorphism of MTHFR, 1298A→C, was also hypothesized whether it confer risk of CL/P. The data shown in table 17 indicate that patients with CL/P, their parents, and controls are also in HWE. Case-control study was performed and subsequently showed no association were found between patients with AC and CC genotypes and CL/P with OR 1.13 (95% CI:0.82-1.56) and 1.45 (95% CI:0.80-2.61) (table18). In case of the fathers of CL/P individuals, there were also no

relation between heterozygotes (AC) and homozygous CC and the involvement of CL/P. However, interestingly, mothers of CL/Ps revealed a tend toward increased risk more than 1.5 fold of having affected offspring with OR 1.44 ranging in 95% CI :0.99-2.99.

Table 17 - Comparison of the *MTHFR* 1298A→C polymorphism allele frequencies CL/Ps, their parents and controls.

Subject type	No. of subjects	Genotype			No. of chromosomes of allele type		Allele frequency		x^2	p value (df=1)
		AA	AC	CC	A	C	A	C		
Controls	202	108	80	14	296	108	0.75	0.25	Ref.	Ref.
CL/Ps	162	80	67	15	227	97	0.70	0.30	0.91	0.339
CL/P mothers	97	44	47	6	135	59	0.70	0.30	0.88	0.348
CL/P fathers	65	39	22	4	100	30	0.77	0.23	0.69	0.408

Ref. = Reference category

Table 18 - Genotype distribution and calculated OR showing association between Patients with CL/P and the 1298A→C *MTHFR* polymorphism.

Groups	Genotypes	No.	OR	95% CI
Controls	AA	108		
(n=202)	AC	80		
	CC	14		
CL/Ps	AA	80	1.00	
(n=162)	AC	67	1.13	0.82-1.56
	CC	15	1.45	0.80-2.61
CL/P mothers	AA	44	1.00	
(n=97)	AC	47	1.44	0.99-2.09
	CC	6	1.05	0.48-2.27
CL/P fathers	AA	39	1.00	
(n=65)	AC	22	0.76	0.49-1.34
	CC	4	0.79	0.21-2.79

3.3 *MTHFR* 677C→T in combination with1298A→C genotype and CL/P

Combined genotype between 677C→T and 1298A→C *MTHFR* polymorphisms was investigated in order to examine genotypic distribution among CL/P patients and their parents compared with control group. Note that data of the combined genotype of

202 controls are the same as the one presented previously in table 12. The genotypic distribution of 162 CL/P patients, 97 of their mothers, and 65 of their fathers with the calculated ORs for all groups were shown in table 11. In all groups, homozygous variants of one polymorphism are always accompanied with the homozygous normal of the other polymorphism. Therefore, the 677TT/1298 AC and 677TT/1298CC were not observed in this study. In case of their fathers, they didn't show significant ORs in all genotypes which were observed in this study. Similarly, patients with CL/P did not show any statistical significant in ORs when compared with controls. The 677CT/1298AC genotypes, however, have a tendency towards increased risk of CL/P genesis in patients with OR 1.98 (95% CI:0.86-4.61).

Interestingly, statistical significant OR for the 677CT/1298AC mother was dramatically shown to be more than 3.5 fold to contribute risk of having CL/P offsprings (OR 3.67 with 95% CI 1.58-8.59). No other genotypes of CL/P mothers confer risk of CL/P.

Regarding haplotypes, EH program was conducted to estimate haplotype frequencies. Distribution of four possible haplotypes did not show significant differences between them and controls with P $value_{EH}$ >0.05 (table 20). Also, chi square test of differences between haplotype in each group and control shows that there are no difference between haplotype frequencies. However, as combinded haplotype was considered, chi square of C-C/T-A mothers compared with control was significant different (table 21). This confirms the result shown in table 19 that 677CT/1298AC mothers were at increased risk of having babies with CL/P.

Table 19 – Prevalence and calculated OR with 95% CI of the 677C→T in combination with 1298A→C *MTHFR* polymorphism among CL/Ps, their parents and controls

		1298A→C		
Groups	677C→T	AA	AC	CC
Controls	CC	66	74	14
(n=202)	CT	40	6	-
	TT	2	-	-
CL/Ps	CC	50	58	15
(n=162)		OR:1.00 (0.68-1.47)	OR:1.03 (0.71-1.50)	OR:1.41 (0.76-2.62)
	CT	29	9	-
		OR:0.96 (0.61-1.50)	OR:1.98 (0.86-4.61)	
	TT	1	-	-
		OR:0.66 (0.08-4.29)		
CL/P mothers	CC	30	37	6
(n=97)		OR:1.00 (0.63-1.58)	OR:1.10 (0.71-1.70)	OR:0.94 (0.42-2.09)
	CT	12	10	-
		OR:0.66 (0.37-1.18)	OR:3.67 (1.58-8.59)	
			P<0.001	
	TT	2	-	-
		OR:2.20(0.44-10.91)		
CL/P fathers	CC	25	19	4
(n=65)		OR:1.00 (0.61-1.63)	OR:0.68 (0.41-1.13)	OR:0.75 (0.29-1.88)
	CT	11	3	-
		OR:0.73 (0.39-1.34)	OR:1.32 (0.41-4.06)	
	TT	3	-	-
		OR:3.96 (0.50-36.48)		

Table 20 – Estimated haplotype frequencies of CL/Ps and their parents compared with controls

Group	No. case	No. allele	Haplotype frequencies (nt677-nt1298)[a] C-A	C-C	T-A	T-C	X^2_{EH}	P value$_{EH}$
Controls	202	404	f=0.608914	f=0.267324	f=0.123759	f=0.000003	Ref.	Ref.
			n=246	n=108	n=50	n=0		
			(60.9%)	(26.7%)	(12.4%)	(0%)		
			Ref.	Ref.	Ref.	Ref.		
CL/Ps	162	324	f=0.577177	f=0.299366	f=0.123441	f=0.000016	0.96	0.84
			n=187	n=97	n=40	n=0		
			(57.7%)	(30.0%)	(12.3%)	(0%)		
			X^2=0.752	X^2=0.913	X^2=0.00			
			P=0.386	P=0.339	P=1.000			
CL/P mothers	97	194	f=0.561903	f=0.304077	f=0.133974	f=0.000047	1.24	0.81
			n=109	n=59	n=26	n=0		
			(56.2%)	(30.4%)	(13.4%)	(0%)		
			X^2=1.203	X^2=0.882	X^2=0.124			
			P=0.273	P=0.348	P=0.725			
CL/P fathers	65	130	f=0.615391	f=0.230762	f=0.153839	f=0.000007	1.20	0.81
			n=80	n=30	n=20	n=0		
			(61.5%)	(23.1%)	(15.4%)	(0%)		
			X^2=0.017	X^2=0.686	X^2=0.781			
			P=0.896	P=0.408	P=0.377			

f = haplotype frequencies calculated by EH program, n = observed number,

X^2 = Pearson's chi-square if n>5 or Yates' correction if n<5

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

Ref. = Reference category

Table 21 – The distribution of *MTHFR* haplotype combination in patients with CL/P and their parents

	Haplotype distribution									
genotype	677CC / 1298AA	677CC / 1298AC	677CC / 1298CC	677CT / 1298AA	677CT / 1298AC[a]		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
Control n=202	n=66 Ref.	n=74 Ref.	n=14 Ref.	n=40 Ref.	n=0	n=6 Ref.	n=0	n=2 Ref.	n=0	n=0
CL/Ps n=162	n=50 X^2=0.136 P=0.712	n=58 X^2=0.027 P=0.869	N=15 X^2=0.665 P=0.415	n=29 X^2=0.211 P=0.646	n=0	n=9 X^2=1.521 P=0.937	n=0	n=1 X^2=0.037 P=0.847	n=0	n=0
CL/P Mothers n=97	n=30 X^2=0.092 P=0.762	n=37 X^2=0.064 P=0.800	n=6 X^2=0.058 P=0.810	n=12 X^2=2.519 P=0.112	n=0	n=10 X^2=6.969 P=0.008	n=0	n=2 X^2=0.047 P=0.828	n=0	n=0
CL/P Fathers n=65	n=25 X^2=0.733 P=0.392	n=19 X^2=1.187 P=0.276	n=4 X^2=0.005 P=0.944	n=11 X^2=0.264 P=0.607	n=0	n=3 X^2=0.409 P=0.522	n=0	n=3 X^2=1.821 P=0.177	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 each of haplotype between cases and controls.

Ref.= Reference categoty

3.4 Transmission disequilibrium test

In order to avoid population stratification which may cause spurious association, transmission disequilibrium test (TDT) was carried out into the study. However, TDT can only be used in cases of patients whose paternal and maternal genotype are available and informative. Thus, twenty one of patients with CL/P with which both parental genotypes are available, were analyzed for the TDT in order to find whether there is statistical difference between the transmtitted and the untransmitted allele.

First, TDT of 677C→T and 1298A→C were analysed independently and the results were shown in table 22. The 677C→T did not revealed disequilibrium for allele transmitted, both two alleles (C and T) were transmitted to the offsping equally (X^2=2.13,P = 0.27). Similarly, the TDT result of 1298A→C was not deviated from equilibrium . The transmitted A allele and C from parents were passed as equaly as C allele (X^2=0.12, P = 0.87). Note that difference of the total number of allele transmitted in analysis of locus 677C→T and 1298A→C is due to the diferrence of available informative families in each locus.

Secondly, TDT was also performed in 34 informative CL/P patients to observed the disequilibrium of transmission of the combined *MTHFR* haplotypes. Forty two transmitted alleles were observed and compared with the same amount of allele untransmitted for 4 possible haplotypes. As the result, non-significant difference was found between the transmitted and the untransmitted haplotypes with respect to X^2=4.32 and P = 0.38 regarding degree of freedom at level 3 (table 23).

Table 22 – Transmission disequibrium test (TDT) of *MTHFR* polymorphisms in CL/P patients.

Polymorphisms	Transmitted allele		X^2_{TDT}	P (df=1)
677C→T	C=15	T=8	2.13	0.27
1298A→C	A=16	C=18	0.12	0.87

X^2_{TDT} implied to chi-square calculated base on TDT analysis, P=P value, df = degree of freedom

Table 23 – Transmission disequibrium test (TDT) of *MTHFR* haplotype from heterozygous parent to the CL/P offsprings.

Haplotype (nt677-nt1298)	Transmitted	Untransmitted	X^2_{TDT}	P (df=3)
C-A	22	14		
C-C	12	18		
T-A	7	9		
T-C	1	1	4.29	0.38

X^2_{TDT} implied to chi-square calculated base on TDT analysis, in this case, were corrected by multiplication with a (k-1)/k correction : k is the number of alleles

P=P value, df = degree of freedom