CHAPTER V

DISCUSSION

As the human genome is sequenced, the mapping of the human genome has now presented the opportunity to identify the common genetic polymorphisms such as the single nucleotide polymorphisms (SNPs). The challenge will be to determine wheter they result in the increased risk of particular disease. Recently, a number of studies have demonstrated that the two common polymorphisms, 677C→T and 1298A→C, of the gene encoding 5,10-methylenetetrahydrofolate reductase enzyme (MTHFR) are associated with NTD and other congenital anomalies such as CL/P. Most of the reports analyzed on the interaction of the 677C→T, with fewer documented on 1298A→C, and some studies investigated these two polymorphisms simultaneously. Two previous studies have documented on association between 677C→T and 1298A→C and NTD[28,118]. However, these reports were considered in other types of NTD not FEEM. Moreover, there have been no studies on an association between the two *MTHFR* polymorphisms and a risk of CL/P although some previous studies analyzed the 677C→T. The present study examines the genotype distribution of each locus (nucleotide677 and nucleotide1298) separately and also examines the haplotype distribution. In addition, this study evaluates the association between genotypes of each polymorphisms and risks of FEEM and CL/P. Especially, the association between *MTHFR* haplotypes and risk of diseases were also included in this study. To the best of our knowledge, this is the first study to examine interaction of *MTHFR* polymorphisms and FEEM and is the only report on association between CL/P and *MTHFR* haplotypes.

Regarding MTHFR genotype distribution, the previous reports on 677C→T genotype distribution appear in high variance among each ethnic group. In this study, 202 control individuals were found to have the 677T allele frequency of 0.15 which is consistent with that of 0.142 reported in the previous report in the Thai population[119] and 0.164 in the Indonesian population.[120] However, the frequency in Southeast Asia is relatively low when compared with the 677T allele frequencies of 0.41-0.48 among Hispanic,[121,122] 0.24-0.43 in Europe, 0.34 among European American, 0.352 in Japan

and 0.38 in China.[123,124] However, the T allele ferquency of 0.06 among African is lower than ours.

We further analyzed the *MTHFR* 1298A→C polymorphism among the same samples. The frequency of variant C allele in Thais was found to be 0.25 which consistent with that of 0.20 reported among Chinese population in Taiwan.[125] In Europe, it is responsible for 0.35 in Natherland,[28] 0.31 in Austria [126] and 0.33 in USA.[127] Similar to the variant of 677T allele frequency, 1298C allele frequency among Thais was found to be lower than those found in Caucasians. These suggest that frequencies of variant allele of these two *MTHFR* polymorphisms are depended on population ethnic. The reason for the variation among many population is still unclear, the possible explanation of the low frequencies of 677T allele and 1298C allele in the Thai population are caused by founder effect. Moreover, as the possible explanation of selective adventage, the 677C in the Thai population may play a preventive role against lumbosacral and occipital encephalocele. Whereas the high frequency of the 677T allele in Caucasians may have a selective adventage againts the development of FEEM. However, additional studies should be conducted to evaluated the reason of variation in distribution of a common *MTHFR* polymorphism in different ethnic groups.

We next investigated the distribution of the joint effects of these two polymorphisms in our control subjects. Here we found the compound heterozygote genotype 677CT/1298AC accounts for 3 percent of controls (6 of 202 individuals) which is relatively low when compare with 20 percent in the Netherland,[28] 17 percent in the United States[57] and 15 percent in Canada.[87] The lower prevalence report here may be according to the low prevalence of the 677CT and 677TT among Thais which can effect on the prevalence of the combined *MTHFR* genotype. It was found to be consistent with previous studies [28,126] that all control subjects who were homozygous for one polymorphism show the wide-type sequence of the other polymorphism and *vice versa*. According to estimation of the haplotype frequencies by EH program, we observed that the two *MTHFR* polymorphisms were never present on the same allele in all groups. Van der Put (1998)[28] suggested that the allele of 677T and 1298A polymorphisms evolved on different allele and cross over has not occurred. It is possible that if both mutation would occurred in *cis* C-A/T-C this could result in selection against these individuals because

of severe clinical phenotype. Similar result was presented in CL/P patients and groups of their parents, whereas both patients with FEEM and their mothers have no individual with 677TT/1298AA genotype. This may be due to rare presence of 677TT among Thais and small sample sizes effect leads to unobserved of 677TT genotype.

As to hypothesis that *MTHFR* polymorphisms are associated with disease risks similar to many studies previously considered, an association between these two *MTHFR* polymorphisms and another subtype of NTD, FEEM,was studied. Single locus analysis examine each locus separately did not show association between neither genotypes of 677C→T nor genotypes of 1298A→C and risk of FEEM. No a statistical significance was found neither in patients nor group of their mothers. After combined genotype was analyzed, no association between combined genotypes and risk of FEEM in patients was found. The protective effect was presented in mothers with 677CC/1298AC with significant odd ratio at 0.41 (95%CI:0.17-0.94). However, in case of individuals with combined wildtype 677CC and 1298AC genotypes, the significant OR for the combined 677CC/1298AC genotype seem to be the spurious result , because of no statistical significant OR was found for 1298AC genotype in analysis of single locus for 1298A→C. Consistently, chi-square test shows no statistical significance was found for their C-C/C-A haplotype compared to controls (p=0.156; table 14). The difference between group of mothers of CL/P patients and controls, is likely presented due to samling effect.. Thus, the requirement of larger population size would be suggested for further studies to find whether maternal 677CC/1298AC genotypes are still associated with an decreased risk of having child with FEEM. Additionally, TDT must be carried out to test for association, simultaneously with traditional case-control studies. It will be excluded some spurious association by using parents of patients as the internal control which can give us more confidential results. Data of nuclear family must be included to perform TDT analysis. Without paternal genotypes due to no the unavailable of paternal DNA, TDT analysis could not be performed in FEEM.

An association between 677C→T and 1298A→C *MTHFR* polymorphisms and CL/P was documented in 162 patients and their parents from many parts of Thailand. Similar to strategies performed in the study of FEEM, we first analyzed in single locus separately and combined genotype and haplotype. Associations between genotype in

each locus, 677C→T and 1298A→C, were not observed in neither patients nor their parents. Regarding combined genotype associated with CL/P risk, no statistic significance in any categories of combined genotypes, therefore no evidence of association was found in patients. These suggested that *MTHFR* polymorphism in patients may not play an important role for CL/P development. Consistently, TDT analysis showed no deviation from the theoretical parental alleles, which were equally transmitted to their offsprings. In other words,there have been neither allele of 677C→T nor 1298A→C favor to be transmitted. In addition, alleles of combined polymorphism also showed transmission with in equilibrium.(table 22,23) However, the statistical significance revealed in CL/P mothers who are double heterozygote (677CT/1298AC). With more than 3.5 fold of increased risk (OR=3.67; 95%CI=1.58-8.59) and $p<0.001$, this genotype seems to be a genetic risk factor for this developmental anomaly. Consistently, the C-C/T-A haplotype of 677CT/1298AC observed by EH program, showed the statistical significant difference compared with that of controls ($p<0.01$, table 21). It is very likely that *MTHFR* polymorphisms, mothers may associate with risk of CL/P in their offspring. The observed odd ratio between heterozygotes of each polymorphism (677CT or 1298AC) and CL/P did not show statistical significance, the significant increased risk of mothers with combined 677CT and 1298AC genotypes can be explained by the concept of additionally reduction of an enzyme activity. The significant reduced *MTHFR* specific activities of individuals with the combined heterozygosity for the 677C→T and 1298A→C polymorphisms were previously reported[28] this possible explanation is probable for our result. Individuals with 677CT/1298AC genotype were found to reduce enzyme activity into 47.71% compared to Individuals with the wildtype 677CC/1298AA genotypes whom are assumed to produce normal enzyme activity (100%) (table24). The alteration of MTHFR enzyme activity in those mothers may cause error in folate-homocysteine metabolism leading to lower levels of circulating folate, lower availability of methionine, and high level of homocysteine which may cause defect in their babies. Inaddition, individuals with 677TT/1298AA genotypes were found to produce the lowest of enzyme activity of 24.81%. In this study, mothers with 677TT/1298AA show 2.20 fold of increased risk of having child with CL/P compared to controls. However, It was found to be non statistical

significance with 95%CI = 0.41-10.59 which may be due to effect of low distribution of 677TT genotype presented in our population. The relation of maternal 677TT/1298AA genotype and risk of having children with CL/P requires further studies with a larger sample size.

Table 24 – Relationship between *MTHFR* genotype and enzyme activity[28]

Genotype	1298AA	1298AC	1298CC
677CC	100.00%	83.21%	61.07%
677CT	66.79%	47.71%	-
677TT	24.81%	-	-

Concerning practical benefit , the results from this study enable us to convince role of folic supplementation and CL/P development in the Thai population. The interaction of folic supplementation and correction of the lower MTHFR activity is still unclear. In any case the supplementation may reduce CL/P incidence as has been shown in NTD.[37] During pregnancy, mothers with variant alleles of *MTHFR* polymorphisms were hypothesized to have a lower MTHFR enzyme activity, resultin reduced plasma folate. The folate deficiency has been considered as a potential cause of defects. Impaired MTHFR enzyme change 5,10-methylenetetrahydrofolate into 5-methyltetrahydrofolate inadequately (figure 3). This lead to accumulation of amino acid homocysteine which may be responsible for CL/P development as reported on NTD.[81] Unlike NTDs that is now firmly established that woman can reduce risk of giving birth to a baby with NTD by supplementing with folic acid in early of pregnancy and during pregnancy, [92,93,95] the role in CL/P is still less unclear. However, there have been reports suggested that women can also markedly reduce their risk of giving birth to a child with CL/P by supplementing daily with a multivitamin containing 0.4 to 0.8 mg of folic acid.[23] Although only 677CT/1298AC mothers confer risk of giving birth to CL/P child. We can conclude that approximately 3 percent (calculated by multiplied frequencies of C-C haplotype with frequencies of T-A haplotype : 0.267324 x 0.123759 = 0.0330837 ~ 3%) of Thai mothers could benefit from this inexpensive strategy urge us to recommend a folate supplementation prior and during pregnancy.

In conclusion, although there is no evidence of association between *MTHFR* polymorphisms and risk of FEEM, we can not entirely exclude the interaction of these *MTHFR* polymorphisms and development of FEEM among the Thai population due to study of small sample size in this study. We still need to clarify the association between MTHFR polymorphisms and FEEM in larger sample groups. Additionally, the family-based analysis, such as TDT, should be employed, given the samples from patients and their parents are available. Additionally, not only impairment of MTHFR enzyme which is responsible for elevated homocysteine levels, the other four enzymes in foltate-homocysteine metabolism such as cystathionine β–synthase (CβS), betaine methyltransferase, s-adenosyl homocystaine hydrolase, and methionine synthase, should be documented to their susceptibilities for FEEM and CL/P. In addition to previous studies suggesting of possibilities of folate supplementation widely used to reduce risk of NTD and other congenital anomalies, the recent study suggested the possible strong preventive effect of other supplementation such as inositol during pregnancy which can reduce recurrence risk of folate resistance NTD.[128] This idea suggested that other susceptible genes may be responsible for development of congenital anomalies in addition to the effects from genes in folate-homocysteine metabolism. These encourage the need for further studies to find other related to FEEM and CL/P.