A STUDY OF *MTHFR* GENE POLYMORPHISMS IN PATIENTS WITH NONSYNDROMIC OROFACIAL CLEFTS AND THEIR PARENTS

by

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List of Abbreviations

^{o}C	Degree celcius
µl	microlitre
A260/A280	ratio of 260 absorbance over 280 absorbance
bp	base pair
Buffer AL	Lysis buffer
Buffer AW1	Wash buffer 1
Buffer AW2	Wash buffer 2
Buffer AE	Elution buffer
CL/P	Cleft lip with or without cleft palate
CL	Cleft lip alone
CLP	Cleft lip with palate
CP	Cleft palate alone
DHFR	Dihydro folate reductase
DFE	Dietary folate equivalents
FH4/THF	Tetra hydro folate
FH2/DHF	Dihydro folate
mM	milli Molar
MTHFR	Methylene tetrahydro folate reductase
NTD	Neural tube defect
PCR	Polymerase Chain Reaction
RBC	Red Blood Cells
RDA	Recommended dietary allowance
RE	Restriction enzyme
RFLP	Restriction fragment length polymorphism
TDT	Transmission Disequilibrium Test

KAJIAN MENGENAI POLIMORFISME GEN *MTHFR* PADA PESAKIT REKAHAN OROFASIAL TANPA SINDROM SERTA IBU BAPA MEREKA

Abstrak

Tesis ini menggambarkan perkaitan antara polimorfisme nukleotida tunggal (SNP) gen *MTHFR* 677C→T dan 1298A→C dengan rekahan orofacial dalam populasi Malayu. Dua kaedah kajian, kes kawalan dan ujian ketakseimbangan penularan (TDT) telah digunakan. Subjek terdiri dari 53 pesakit Malayu yang mengalami orofacial rekah, 53 ibu, 49 bapa dan 49 kawalan untuk kajian kes kawalan. 38 triad kesuluruhan ibu-bapa dan kanak-kanak telah dikaji untuk alel-alel varian daripada ibubapa kepada anak-anak dengan menggunakan TDT. Klasifikasi genotip dilakukan menggunakan PCR dan diikuti oleh RFLP serta gel elektroforesis. Frekuensi *MTHFR* SNP 677CT pada kumpulan kawalan telah didapati lebih rendah (14.3%) jika dibandingkan dengan populasi am Kaukasia 30% pada sesetengah kajian. Walau bagaimanapun, prevalens 1298AC pada kumpulan kawalan (28.5%) adalah sama dengan populasi lain (27-36%). Prevalens *MTHFR* 677 CT heterozygus genotip pada populasi ibu (n=53), didapati semasa penentuan genotip ibu pada lokus 677 dijalankan, adalah lebih rendah (5.6%) jika dibandingkan dengan kumpulan yang dikawal tanpa orofasial rekah (14.3%). Tetapi perbezaan ini adalah secara statistiknya tidak signifikan (P=0.340). Frefuensi alel T pada ibu (4.7%) juga adalah lebih rendah daripada yang dikawal (9.2%). TDT juga tidak menunjukkan penularan yang rendah secara signifiken aleh T dari ibu kepada anak (P=0.38), tetapi risiko penularan aleh T dari ibu yang membawa alel ini adalah rendah dibandingkan dengan alel liar jenis C (OR=0.59, 95% CI=0.187-1.921). Hal ini menunjukkan terdapat kemungkinan alel T ibu memainkan peranan dalam melindungi daripada

rekahan orofacial. Tiada perkaitan dijumpai antara genotip *MTHFR* 677CT anak atau bapa dengan rekahan orofasial. Tiada perkaitan dilihat antara genotip *MTHFR* 1298AC dari sebarang kumpulan (ibu, bapa atau anak) dengan risiko rakahan orofasial. Analisis empat haplotip (677C/1298A, 677C/1298C, 677T/1298A, 677T/1298C) yang diambil dari genotip gabungan/ sebatian menunjukkan pengedaran asimetri haplotip yang menandakan perkaitan ketakseimbungaan antara kedua-dua loci (*MTHFR* 677CT and 1298AC), konsisten dengan dapatan kajian-kajian lain. Ujian penularan, TDT menunjukkan penularan haplotip yang rendah secara signifikan 677T/1298A dari ibubapa kepada anak (P=0.018), dan hal ini menunjukkan perkaitan songsang haplotip ini dengan rekahan orofasial. Purata serum folat adalah tinggi pada kajian kumpulan ibu dan juga kumpulan ibu yang dikawal. Sejarah pengambilan multivitamin yang mengandungi folat semasa mengandung didapati pada 83% ibu. Dapatan ini menolak kemungkinan status folat rendah pada ibu mengandung sebagai faktor risiko untuk rekahan orofasial. Kajian yang lebih besar untuk populasi Malayu perlu dijalankan untuk mengesahkan dapatkan kajian ini.

A STUDY OF *MTHFR* GENE POLYMORPHISMS IN PATIENTS WITH NONSYNDROMIC OROFACIAL CLEFTS AND THEIR PARENTS

Abstract

This thesis describes the association of two important *MTHFR* gene single nucleotide polymorphisms 677C→T and 1298A→C with orofacial clefts in the Malay population. Two study methods, Case control and Transmission disequilibrium test (TDT) were employed. The subjects included 53 Malay patients with orofacial clefts, 53 mothers, 49 fathers and 49 controls for the case control study. Thirty eight complete triads of mother-father-child were studied for transmission of variant alleles from parents to offspring by using TDT. The genotyping was done by Polymerase Chain Reaction (PCR) followed by restriction fragment length polymorphism (RFLP) and gel electrophoresis. The frequency of *MTHFR* SNP 677CT in the control group was found to be lower (14.3%) than that in Caucasian general populations (30% in some studies). However, the prevalence of 1298AC in the control group (28.5%) was similar to that of other populations (27-36%). The prevalence of *MTHFR* 677 heterozygous CT genotype in the maternal population (n=53), was found to be lower (5.6%) compared to that in controls without orofacial clefts (14.3%) but the difference was not statistically significant (P=0.340). The TDT did not show a significantly low transmission of T allele from mothers to offspring either (P=0.38), but the risk of transmitting the T allele from mothers carrying this allele was lower compared to that of the wild type C allele (OR=0.59, 95% CI=0.187-1.921). This indicated a possible protective role of maternal T allele in orofacial clefts in the present study. No association was found between *MTHFR*

677CT genotypes of child or father with orofacial clefts. No association was seen between *MTHFR* 1298AC genotypes of any group (mother, father or child) with the risk of orofacial clefts. Analysis of the four haplotypes (677C/1298A, 677C/1298C, 677T/1298A, 677T/1298C) derived from compound genotypes, showed an asymmetric distribution of haplotypes indicating linkage disequilibrium between these 2 loci (*MTHFR* 677CT and 1298AC), consistent with previous study findings. The test of transmission, TDT showed a significantly low transmission of haplotype 677T/1298A from parents to offspring (P=0.018) indicating an inverse association of this haplotype with orofacial clefts. The average serum folate was high in the study group mothers as well as in control mothers. A history of multivitamin containing folate intake during pregnancy was found in 83% of mothers. These findings ruled out the possibility of low maternal folate status as a risk factor for orofacial clefts. Larger studies in the Malay population are needed to confirm the present findings.

CHAPTER ONE

INTRODUCTION

Orofacial clefts are a common malformation involving the face. Non syndromic orofacial clefts are best understood as a multifactorial disorder meaning that genes and environmental factors are both responsible for it**.** A study of the genetic factors in orofacial clefts showed that many genes have a role in causation of nonsyndromic orofacial clefts and no single gene can be pinpointed. Methylene tetrahydrofolate reductase (*MTHFR*) is one of the genes and folic acid deficiency is one of the environmental factors thought to be important in the causation of this defect. While folic acid is clearly beneficial in reducing the risk of neural tube defects, its role in prevention of orofacial clefts is less definite. Though controversy exists, a recent meta- analysis of 17 studies supported the protective role of folic acid supplementation in pregnancy in cleft lip with or without cleft palate and more so in cleft palate alone . Genetic polymorphisms in the enzymes of folic acid and one carbon metabolic pathway could be associated with risk of orofacial clefts, possibly interacting with low maternal folic acid intake. The *MTHFR* gene, the other genes and environmental factors in orofacial clefts are discussed further in the review of literature (Chapter 2, Sections 2.3).

1.1 Orofacial clefts

Orofacial clefts are one of the most common birth defects in humans. The prevalence of orofacial clefts worldwide is 2 per thousand on an average (Murray,

2002a). Transcription factors, signalling molecules and proteins are involved in formation of palate and lip. Orofacial clefts can result from breakdown of any of the mechanisms which control the cascade of events involving these factors or molecules. Based on embryological origin and inheritance patterns, cleft lip with or without cleft palate (CL/P) and cleft palate (CP) are the main types of orofacial clefts (Christensen and Fogh-Andersen, 1993). The development of the lip and palate are closely linked thereby making cleft lip with cleft palate (CLP) more common than any of the defects in isolation (Owens *et al.*, 1985). Males are more commonly affected than females with cleft lip with cleft palate (CLP) whereas the female incidence is possibly higher for cleft palate alone (CP) (WHO collaborative study, 2002). There are various types of orofacial clefts and some cases fit into syndromes (defined as a group of several different malformations as primary events arising from the same underlying cause) while others are isolated defects (nonsyndromic).

Cleft lip is an obvious physical defect while cleft palate has to be looked for by examining the newborn. Orofacial clefts can be a cosmetic defect alone or may be extensive enough to cause speech and feeding difficulties in childhood. Treatment often consumes a lot of time and resources for staged surgeries, speech therapy, dental reconstruction and psychological support of the families are often needed. Surgery for cleft lip may be done as early as 6 weeks, while cleft palate (CP) is repaired 6 months later when the tissues have grown enough (Sandberg *et al.*, 2002). Earlier repair of CP has chances of severe orodental deformities while a greater delay may lead to poorer speech. Hence, the timing for repair of cleft palate remains a controversy (Patel *et al*., 2006). Feeding difficulties can interfere with the nutritional status of an infant and must be dealt with early. Speech defects require speech therapy and psychological problems due to cosmetic defect need competent

management. Thus, the management of the patients of orofacial clefts is a process that starts in infancy and continues into adulthood and often there are no simple solutions for approaching the problems associated with orofacial clefts (Thornton *et al.*, 1996).

1.1.1 Folic acid deficiency in orofacial clefts

Role of folate deficiency as a risk factor for orofacial clefts is important because it is a modifiable environmental factor. Recurrent cleft lip and palate has been seen in siblings of a patient with malabsorption syndrome, probably because of folate and B12 deficiency resulting from malabsorbtion (Faron *et al.*, 2001). An increased risk of orofacial clefts with maternal use of antiepileptic drugs like phenytoin and phenobarbitone is also thought to be due to disturbed folic acid metabolism (Dansky *et al.*, 1987). Though there is no conclusive evidence linking orofacial clefts and the use of any vitamin, folic acid supplementation in pregnancy has been shown to reduce its risk (Shaw *et al.*, 2006, Wilcox *et al.*, 2007).

Based on the observation that intake of folic acid reduces the risk of orofacial clefts in some populations, it was suggested that variant genes involved in the folic acid metabolism could also be associated with the risk of oral clefts and as folic acid is needed for DNA and RNA synthesis, and embryonic tissues especially the developing palate has a high requirement of DNA production, any event which reduce the supply of DNA or enhanced DNA damage can theoretically result in orofacial clefts (Jugessur *et al.*, 2003).

1.1.2 Methylenetetrahydrofolate reductase (*MTHFR*) gene in orofacial clefts

MTHFR gene coding for the MTHFR enzyme of folate metabolic pathway has been studied in orofacial clefts and maternal *MTHFR* polymorphisms are possibly more important risk factors for developing this defect rather than the child's

own genotype. Moreover, the involvement of *MTHFR* gene is indirect because its polymorphic variants influencing the maternal folate status are a risk factor for orofacial clefts rather than the gene itself being directly responsible. Historically, the possibility of involvement of this gene in orofacial clefts has been investigated because of its association with other congenital malformations. Of all the variants of *MTHFR* gene, two of the *MTHFR* polymorphic variants 677CT and 1298AC, are the commonest and very important in the population. Other *MTHFR* SNPs reported are at bp 1059, bp 1289, bp 1317, and bp 1793 but are of unknown significance (Trembath *et al.,* 1999; Rady *et al.,* 2002).

MTHFR 677CT and 1298AC polymorphisms are known risk factors for congenital malformations like neural tube defect (Shields *et al.,* 1999, Christensen *et al.,* 1999) and possibly Down syndrome (Hobbs *et al.,* 2000, James *et al.*, 1999). Several authors have noted an association of maternal *MTHFR* 677CT and possibly maternal *MTHFR* 1298AC polymorphisms rather than the child's genotype in the risk for developing orofacial clefts (Shaw *et al.*, 1998, Jugessur *et al.*, 2003, Martinelli *et al.*, 2001, Pezzetti *et al.*, 2004). Possibly, maternal *MTHFR* polymorphisms and folate deficiency act together to increase risk of orofacial clefts (Brouns *et al.*, 2008). However, the association of *MTHFR* polymorphisms 677CT and 1298AC with orofacial clefts remains controversial, as several reports have found no association (Boyles *et al.*, 2008, Blanton *et al.*, 2000, Vieira *et al.*, 2005). *MTHFR* gene polymorphisms in orofacial clefts have been reviewed further to answer the question regarding its association or lack of association of these SNPs with this malformation in the section on literature review (Chapter 2, Section 2.5.3).

1.1.3 Mechanism for *MTHFR* polymorphisms and folate deficiency causing orofacial clefts

MTHFR 677CT and 1298AC polymorphisms reduce the *MTHFR* enzyme activity in the folate metabolic pathway known as the 'methyl cycle'. It has been postulated that reduced enzyme activity reduces DNA methylation or supply of purines or cause hyperhomocysteinemia which may be harmful to the developing lip and palate. On the other hand, some authors have found that some of these *MTHFR* variants are inversely associated with the risk of orofacial clefts the explanations for which has been discussed in the Chapter 2 (Literature Review, Section 2.5.3). Overall, the majority of studies imply the involvement of the methyl cycle of folate in the risk for orofacial clefts. *MTHFR* 677CT and 1298AC polymorphisms imply a C→T and A→C single nucleotide change at position 677 and 1298 respectively of cDNA of the gene.

1.2 SNPs and Study of SNPs

Alterations in the genome sequence which occur commonly and do not result in major phenotypic alterations are known as polymorphisms. A Single Nucleotide Polymorphism, or SNP (pronounced "snip"), is a small genetic change, or variation occurring due to variation in the genetic code specified by the four nucleotide "letters" A (Adenine), C (Cytosine), T (Thymine), and G (Guanine). SNP variation occurs when a single nucleotide, such as an A, replaces one of the other three nucleotide letters C, G, or T. SNP commonly occur in more than 1% of the population. Because SNPs occur frequently throughout the genome and tend to be relatively stable genetically, they serve as 'biological markers'. As genetic markers, SNPs can be used to follow the inheritance patterns of chromosomal regions from generation to generation and are powerful tools in the study of genetic factors

associated with human diseases (Risch, 2000). SNPs are useful in population studies and genome wide scans for complex diseases and eventually physicians will be able to screen individuals for susceptibility to a disease just by analyzing their DNA samples for specific SNP patterns. SNP detection technologies have evolved from labor intensive, time consuming, and expensive processes to some of the most highly automated, efficient, and relatively inexpensive methods. However, genotyping with Polymerase Chain Reaction (PCR) followed by Restriction Fragment Length Polymorphism (RFLP) are useful for screening small number of samples and studies involving few SNPs.

Polymorphisms can result in variations in the protein expression. However, because only about 3 to 5 percent of a person's DNA sequence codes for the production of proteins, most SNPs are found outside of "coding sequences". SNPs found within a coding sequence are of particular interest to researchers because they are more likely to alter the biological function of a protein.

An "asociation study", can detect differences between the SNP patterns of the two groups, thereby indicating which pattern is most likely associated with the disease-causing gene. Another application of SNP technology is in pharmacogenetics, a branch of genetics studying the variable responses to the same drug in different individuals (Kalow, 1997).

1.2.1 Methods of SNP detection

The general strategy of polymorphism detection is by Polymerase Chain Reaction (PCR) which amplifies a small segment of the genome millions of times and its detection by various methods like Restriction Fragment Length Polymorphism (RFLP) followed by gel electrophoresis, denaturing High Performance Liquid Chromatography (dHPLC), sequencing and Single Stranded

Confirmation Polymorphism (SSCP). Analysis by SSCP is one of the most widely used methods for mutation detection and is based on the principle that the electrophoretic mobility of a molecule within the gel matrix is sensitive to the size and shape of the molecule. In SSCP, DNA is first amplified by PCR, and then single stranded DNA is generated by denaturing of the PCR product and separated on a nondenaturing polyacramide gel. A difference in single nucleotide between two sequences is sufficient to alter the folded structure of one relative to the other. This conformational change will migrate differently from the wild-type DNA.

Another method that can be applied to detect unknown mutation is Denaturing Gradient Gel Electrophoresis (DGGE). DGGE is based on DNA heteroduplexes differing by a single base pair having different melting characteristics. The PCR products are resolved in a polyacramide gel with an increasing denaturing gradient of formamide and urea under careful temperature control. Heteroduplex DNA fragments with a single mismatched base pair are revealed by the differences of migration compared to the homoduplexes. However, the disadvantage of DGGE is the difficulty in optimization of the method.

There are several methods available for identification of known mutations. These methods are much simpler than those of detecting unknown mutation detections but the choice is not easy (Cotton, 2000). For example, commonly used methods include gel-electrophoresis techniques such as PCR coupled with RFLP. RFLP is based on variation in the distance separating the two restriction sites; there will be a difference in the size of fragment and resultant differences of the position of the detected band. The PCR products are then digested with appropriate restriction enzymes and visualized by staining the gel after electrophoresis. The difference in size of the DNA fragments helps to detect the SNPs. However, major limitation of

this method is the requirement that the polymorphisms alter a restriction enzyme cutting site (Shi *et al.*, 1999).

Other detection method which is based on the selective extension of primers or hybridization with specific oligonucleotide probe are allele-specific PCR (ASP) or amplification refractory mutation system (ARMS) and oligonucleotide ligation assay (OLA). Usually, ARMS assay comprises of two PCRs which are conducted using the same substrate DNA, and it relies on one primer of a pair of PCR primers being specific for one allele. The specific primer for the other allelic variant is used in a second PCR reaction. The specificity of primer is determined by the 3' nucleotide of the primer, which complements to one allele but not to the other. Oligonucleotide ligation assay (OLA) relies on hybridization with specific oligonucleotide probes that effectively discriminate between the wild type and variant sequences. The gene fragment containing the polymorphic site is amplified by PCR and incubated with probes. Ligation of the fluorescent-labeled probe to allele-specific probe occurs with the PCR product in the presence of thermally stable DNA ligase. The ligation products are separated by electrophoresis which allows the recognition of the wild-type genotypes, variants, heterozygotes and unligated probes. If there is highly GC-rich DNA region, it makes the allele-specific ligation step in OLA difficult to optimize and multiplex (Baron *et al.*, 1996). Recently, non-gel based high-throughput genotyping technologies are rapidly evolving to become the dominant genotyping platforms especially in large scale pharmacogenetic studies. Considering the number of gene mutations that have now been reported as more than 1000 different human genes that cause a disease there arise a need for cost effective and high throughput method to identify mutation or polymorphisms in bolstering the progress of the analysis and diagnosis of diseases through characterization of the

underlying genes. One of the recent and versatile technologies for the analysis of genetic unknown variations is dHPLC.

1.2.2 Use of RFLP in detection of SNPs in *MTHFR* gene

Despite available modern technology, PCR followed by RFLP remains the standard technique used for detection of *MTHFR* gene SNPs. The other methods of detection like Amplification Refractory Mutation System (ARMS) used by some authors has not replaced the standard RFLP technique. Amplification Refractory Mutation System (ARMS) PCR has been used for detecting *MTHFR* C677T mutation (Hessner *et al.*, 1999) as well as for *MTHFR* 1298 AC genotyping (McCarthy *et al.*, 2004). No difference in genotyping between RFLP and sequencing for *MTHFR* 1298AC was found (de Alvarenga *et al.*, 2008). The only drawback of RFLP according to these authors is that, genotyping by analysis of electrophoresis gel is dependent on the observer and on the laboratory conditions (de Alvarenga *et al.*, 2008). Most authors studying *MTHFR* 677CT have used RFLP by replication of methods described earlier (Frosst *et al.*, 1995). This method is laborious; however, it is accurate and suitable for doing genotyping of limited sample size.

1.3 Detection of folate deficiency

Folate deficiency in the body can be reliably detected by doing both serum folate and red cell folate levels but these tests are not done routinely. Serum folate and B12 levels are done for patients with megaloblastic anemia or doubtful iron deficiency anemia. Low serum levels of folate or low RBC folate indicates that either dietary folate is low or absorption is poor, but does not indicate a poor tissue folate status. Thus serum folate estimation should be interpreted with caution as the low levels do not have diagnostic value. A low red cell folate has more diagnostic value but requires two steps in detection and is more expensive than serum folate alone

(www.labcorp.com/datasets/labcorp/html/chapter/mono/ri004400.htm). In order to institute treatment with folic acid for deficiency states, both serum folate and RBC folate need to be low and for thoroughness, the serum vitamin B_{12} level should also be determined. This is because more than half of the patients of low folate have B_{12} deficiency. Serum folate can possibly be a screening tool for low folate and some people who have low folate can be further investigated with RBC folate levels (Bauer, 1982).

1.4 Method of studying association: Linkage analysis and transmission disequilibrium test (TDT)

Sometimes transmission disequilibrium test (TDT) is used in a genetic study to test for association of locus and alleles with disease. The transmission disequilibrium test (TDT) was proposed as a family-based association test for the presence of genetic linkage between a genetic marker and a trait, and it is an application of a statistical test called Mc Nemar's test (Spielman *et al.*, 1993).

Usually the sample consists of a set of trios - affected case with their parents. In essence, it tests whether there are an unusually large or small number of transmissions of an allele, under the null hypothesis. Iles (2002) has explained TDT by an example which states that the probability of observing a particular allele at one locus is independent of the alleles observed at another locus. However, this is not the case when two alleles are 'associated.' If the frequency of allele 1 at locus 1 is p_1 and the frequency of allele 2 at locus 2 is p_2 and the two alleles are not associated, then the frequency with which they appear together is p_1p_2. If their joint frequency is greater than p_1p_2 then the two alleles are said to be positively associated. If their joint frequency is less than p_1p_2, the two alleles are said to be negatively associated.

Association is the non-independence of allele frequencies at different loci (Iles, 2002).

If a parent is heterozygous for a marker, the chances of them transmitting either marker allele to an affected case will be equal unless the marker is linked with the disease gene and unless the marker and disease are associated. A sample of cases and their parents is genotyped and deviations from the expected 50-50 transmission are observed i.e. we look for symmetrical transmission. If an allele is transmitted to unrelated cases more often than would be expected by chance, this implies that it is linked and associated with the disease mutation. If the sample contains cases related to each other, coming from the same pedigree, then the TDT can become a test only of linkage rather than association (Iles, 2002).

1.5 Gap statement/ Justification for the study

This study can be justified because of the following reasons: (1) Clarify association: Maternal *MTHFR* 677CT and 1298AC gene polymorphisms have been found to be risk factor for orofacial clefts by some authors while others have found no or reverse association. The review of literature highlights the various reports on *MTHFR* and orofacial clefts. From the review of literature one can see that there is a need for study of these SNPs in orofacial clefts for clarifying a firm association. As orofacial clefts are an important disorder in Malaysia, studies are needed to determine the association of folate and the related genes with the risk of this disease in the Malays. (2) Rarity of combined genetic and environment studies on orofacial clefts: Gene environment interaction studies are a recent phenomenon. Some populations and ethnic groups have a higher prevalence of orofacial clefts compared to others. The prevalence of orofacial clefts in Malaysians has been found to be 1.9 per 1000 which is relatively high (Boo and Arshad, 1990). Malaysia has a

heterogeneous population from numerous ethnic groups and different prevalence have been reported from various parts of Malaysia with some areas having a higher than average prevalence (Ghee, 2001). (3) Clarify role of maternal folate status: Periconceptional folic acid supplementation has been shown to reduce the risk of orofacial clefts in countries like the USA where it has been found that serum folate level of non pregnant women is low (O'Keefe *et al.*, 1995). Depending on the existing folate status of Malay women, recommendations about supplementation in pregnancy can be made. The folate status is dependent however, not only on dietary intake and folate supplementation in pregnancy, but also on the women's genotype of folate metabolic genes like *MTHFR* (Yates *et al.*, 1987). Thus there is a need to know the *MTHFR* 677CT and 1298AC variant status of the Malay population as well as the the folate status of the Malay women. In the present study, both genotype and nutritional status (maternal serum folate) has been studied, focussing mainly on the genetic aspects, to examine the role of folate metabolism in the risk for orofacial clefts. (4) Detect prevalence of these SNPs of *MTHFR* in the Malay population: *MTHFR* 677CT polymorphism had been studied widely in the Caucasians. The prevalence of *MTHFR* 677TT genotype has been found to be common in northern China (20%), southern Italy (26%), and Mexico (32%) (Wilcken *et al.*, 2003). No studies on *MTHFR* prevalence are available from the Malay population. Thus, knowing the prevalence of *MTHFR* 677CT and 1298AC in the Malays would be useful database for future reference.

1.6 Objectives

1.6.1 Main objective

To study the Single Nucleotide Polymorphisms (SNPs) of the *MTHFR* 677C→T and 1298A→C in the Malay patients with orofacial clefts.

1.6.2 Specific objectives

1) To determine the association of the *MTHFR* polymorphisms 677C→T and 1298A→C in patients and their parents with the risk of orofacial clefts.

2) To determine the role of individual alleles and different haplotypes derived from these two SNPs in the risk for orofacial clefts and to do linkage analysis.

3) To determine the prevalence of these two SNPs in the Malay healthy control population.

4) To ascertain the folate status of mothers of patients of orofacial clefts by doing maternal serum folate assay and from history of folic acid containing multivitamin intake during pregnancy.

CHAPTER TWO

LITERATURE REVIEW

2.1 Prevalence of orofacial clefts

Orofacial clefts (OMIM 119530) are the commonest facial malformation worldwide (Cooper, 1979). On an average, about 1 in every 500-750 live births results in a cleft lip and/ or palate (Peterson-Falzone, 2001). An estimated 700 children with orofacial clefts are born every day (Tolarova *et al.*, 2002). The prevalence of this developmental defect differs in different populations. Orofacial clefts have a birth prevalence ranging from 1/1000 to 2.69/1000 amongst different parts of the world (McLeod *et al.*, 2004). This malformation is more common in certain Asian races compared to their western counterparts (Marazita *et al.*, 1986b).

2.1.1 Caucasian vs. Asian prevalence

Ross and Johnson (1972) noted a difference among racial groups from a review of selected surveys. In this study, African-Americans had the lowest incidence with a range of 0.21 to 0.41 per 1,000 live births; Japanese, who were the most extensively studied among Orientals, had the highest incidence of clefts with a range from 1.14 to 2.13 per 1,000 live births, and in the United States and Western Europe whites had an incidence between 0.77 and 1.40 per 1,000 live births (Ross and Johnston, 1972). Certain tribes and races have a higher incidence of orofacial clefts (Tretsven, 1963). In Caucasian populations, the incidence of clefts lip/palate ranges from 1.0 to 2.21 per 1000 live births as per hospital registries (Gorlin, 1971).

In a study in the Chinese, done among 4,489,692 births, the prevalence of facial cleft at birth was reported to be 14.0/10,000 (Dai *et al.*, 2003). The rate of occurrence ranges in various Chinese populations from 1.09 to 4.04 per 1000 births (Wong *et al.,* 1997). In a study done in Pakistani population the incidence for cleft lip and/or cleft palate was found to be 1.91 per 1000 births (one per 523 births). In this study, Cleft lip alone was noted more frequently than isolated cleft palate and combined cleft lip and palate deformities (Elahi *et al.,* 2004). From these data the exact prevalence cannot be ascertained as complete ascertainment of orofacial clefts is impossible from birth registries as many of the defects are subtle and not recognizable at birth; moreover, the reports include both syndromic and non syndromic clefts (Kozelj, 1996). The difficulty in a uniform assessment of burden of the disease was because many data also included cases of still birth where a higher prevalence of malformations is expected (Wyszynski and Wu, 2002).

The prevalence of orofacial clefts among Caucasian and Asian populations has been compared in Table 2.1 below. The Caucasian incidence of orofacial clefts was described by Magdalenic–mestrovic (2005), while the Asian prevalence data has been adapted from a study by Wong (1997) and others.

2.1.2 Prevalence of orofacial clefts in Malaysia

It is well known that certain populations consistently show higher incidence of orofacial clefts as compared to others and therefore the racial differences are important in studies on orofacial clefts (Vanderas, 1987, Croen *et al.*, 1998). Norway, for example, has one of the highest rates of orofacial clefts in the world (Abyholm, 1978).

Table 2.1 Data comparing the prevalence of orofacial clefts among the Caucasians versus Asians**.** Table adapted from: (Magdalenic-Mestrovic and Bagatin, 2005).

Countries	*Incidence per 1000 live birth*	*Reference*
Caucasian populations		
Finland	1.74	(Rintala, 1986)
Sweden	1.72	(Milerad *et al.*, 1997)
Denmark	1.89	(Jensen *et al.*, 1988)
Denmark	1.0-1.4	(Christensen *et al.*, 1999)
The Netherlands	1.47	(Owens *et al.*, 1985)
Germany	1.48	(Derijke *et al.*, 1996)
Poland	2.0-2.11	(Hillig, 1991)
North Italy	1.33	(Shaw *et al.*, 1995)
Asian populations		
Taiwan	1.92	(Emanuel *et al.*, 1973)
Singapore	1.7	(Tan, 1988)
Malaysia	1.2	(Boo and Arshad, 1990)
China	1.4	(Dai *et al.*, 2003)
Korea	1.8	(Kim *et al.*, 2002)
Pakistan	1.91	(Elahi *et al.*, 2004)

The exact prevalence of orofacial clefts among the Malay population is difficult to state as Malaysian data consists of heterogeneous populations and ethnic races. The Malaysian incidence of orofacial clefts was found to be 1.24 per 1000 live births (nearly 1 in 600) in a large hospital based study in Kuala Lumpur in 1987 (Boo and Arshad, 1990). In this data, Chinese babies had the highest incidence (1.9 per 1000) while Malays had the lowest (0.98 per1000) and the most common type of orofacial cleft was a complete unilateral cleft palate. This study was based on mixed racial groups, which compromised the quality of the final data. The dental survey of Ministry of Health have estimated prevalence of cleft lip as 1 in 700 while that of cleft palate to be 1 in 600 in Peninsular Malaysia in 1970 and this prevalence possibly reduced to 1 in 738 for cleft lip and 1 in 1230 for cleft palate in 1988 (Ghee, 2001). Some authors found orofacial clefts in Malaysia to be 1 in 700 (Rahoma, 2002). Malaysian data are currently based on surgical or orofacial experiences and epidemiological studies are not available.

2.2 Classification of orofacial clefts

Several classifications for oral clefts have been introduced over the years. The more detailed classifications describe the extent, symmetry and structures involved in the cleft (Coleman and Sykes, 2001, Smith *et al.*, 1998). The severity of cleft lip can range from a slight notch in the upper lip to a complete cleft involving the nostrils (Merritt, 2005). Anatomically, orofacial clefts can simply be classified as cleft palate alone (CP) or cleft lip with or without cleft palate (CL/P). Cleft palate (CP) may involve soft and hard palates, or just the soft palate, but very rarely is the hard palate affected in isolation. The International Statistical Classification of diseases and health problems divides orofacial clefts according to the affected structures and location of defect, into eight cleft types / groups (Kernahan, 1971,

Wei, 1988). They include, bilateral total cleft lip, alveolar ridge and palate, unilateral total cleft lip, alveolar ridge and palate (left or right side), unilateral partially cleft lip and palate (left or right side), unilateral cleft of lip and alveolar ridge (left or right side), bilateral cleft of lip and alveolar ridge, bilateral cleft lip, unilateral cleft of lip (left or right side), and cleft of palate (soft or hard).

2.2.1 Contemporary anatomical classification of orofacial clefts

While anatomical alveolar ridge was the significant landmark in the division of oral clefts in earlier classifications, the incisive foramen, an embryological landmark, which marks the boundary between the primary palate and the secondary palate, was the point of demarcation in later classifications. The area anterior to the incisive foramen makes up the primary palate, which includes the lip and alveolus. Cleft lip includes those cases where the cleft is of the primary palate. The secondary palate is posterior to the incisive foramen and includes the hard and soft palate. Orofacial clefts signify clefting of primary or secondary palate or both (Edwards, 1980, Veau, 1931). Some of the anatomical types of orofacial clefts are shown in Appendix I.

2.2.2 Genetic classification of orofacial clefts

A widely used and accepted classification divides oral clefts into isolated malformations (nonsyndromic orofacial clefts) and associated with other malformations (syndromic) (Murray, 2002a). For our study purpose, we have taken cases of nonsyndromic orofacial clefts using the contemporary anatomical classification system describe above. Although they are usually isolated, 10% of all infants with orofacial clefts also have an associated syndrome. Thirty percent of patients with CL alone and 50% of cases of CP alone are syndromic (Wantia and Rettinger, 2002). Syndromic clefts are further subdivided as that due to chromosomal

disorders, known teratogen exposures, and uncategorized syndromes which has been described later. Nonsyndromic clefts occur in infants with no other physical or developmental anomalies except the CL and/or CP and with no known teratogen exposure (Bender, 2000). Orofacial clefts can be classified based on the genetic pattern of inheritance into the following types: (1) *syndromic*, which includes monogenic (single-gene disorders), chromosomal, and environmental etiologies (1 to 8% of CL/P), (2) *familial* where 2 or more family members including cousins have the same malformation which accounts for 12 to 25% of isolated CL/P; and (3) *isolated* or *nonfamilial*, which includes all kindred with only the proband (i.e. the first person in a pedigree found to have the defect) affected in first, second and third degree relatives (Bixler, 1981).

2.3 Embryological origin of the orofacial clefts

The facial structures begin to develop around the end of the fourth week of human development (Moore, 1977, Sadler, 1990). Five facial prominences appear around the stomadeum or primitive mouth. These facial prominences are the unpaired frontonasal prominence, paired maxillary prominences, and paired mandibular prominences (Sadler, 1990). These structures are diagrammatically shown in Figure 2.1.

CLP consists of both CL and CP. However, Cleft lip (CL) and cleft palate (CP) have different embryologic origins. Cleft lip (CL) results from a failed union of the maxillary and medial nasal elevations on one or both sides. Cleft palate results from failure of the palatine processes to meet and fuse with each other. Cleft palate is more frequently associated with a syndrome, whereas CL is most often an isolated defect (Wong and Hagg, 2004).

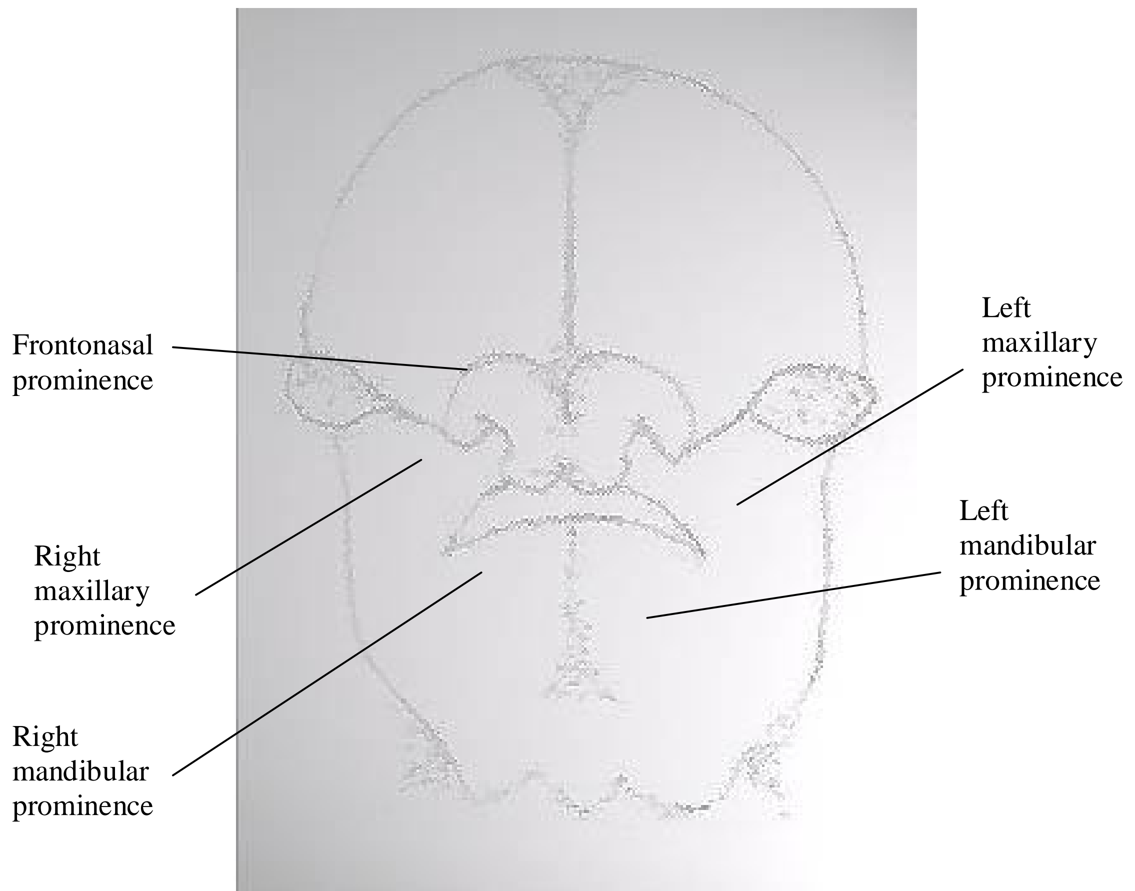

Figure 2.1 Facial development at 45 intrauterine days [adapted from (Bender, 2000)]

2.3.1 Embryology of cleft lip

During early embryonic craniofacial development, the 2 olfactory placodes invaginate to form nasal grooves that form the lateral boundaries of the frontonasal process. The placodes then begin to curl outward around the edges to give rise to the lateral and medial nasal processes, both of which grow and eventually fuse with the maxillary process to form much of the upper lip and primary palate. Fusion of these regions giving rise to the lip and primary palate (area in front of incisive foramen) takes place between 4th to 6th weeks of development. Any abnormality in the fusion process gives rise to cleft lip which may be unilateral or bilateral depending on anatomically whether the nasal process of one side or both sides fail to fuse with the maxillary process. Cleft lip can extend from gum margin up to incisive foramina (Kerrigan *et al*., 2000).

2.3.2 Embryology of cleft palate

Formation of the soft and hard palate (area behind the incisive foramen) occurs between 7th to 13th weeks. The periods from 6th to 9th weeks are the most crucial. It is a process which occurs after lip formation (Bender, 2000). From the two sides the 2 maxillary processes give rise to ridges called the palatine shelves (Thornton *et al*., 1996). The palatine shelves grow horizontally and come to lie above the tongue. Later the shelves fuse forming the hard and soft palate which makes up the secondary palate. Next, the primary palate fuses with the secondary palate. The incisive foramen represents the midline landmark between the primary and secondary palates. The palate is thought to form strictly by midline fusion of the palatine shelves. Any defect in this process results in cleft palate. By 12 weeks, the fusion is complete and bone extends from the maxillae and palatine bones (Kerrigan *et al.*, 2000).

2.4 Aetiology of orofacial clefts

Orofacial clefts are a complex disorder of facial development. There are different factors involved in origin of this disorder. The syndromic orofacial clefts have clear genetic basis while the nonsyndromic disorder has many etiological factors as described in the following sections. Several genes and loci which have been implicated in the causation of this disorder are discussed.

2.4.1 Aetiology of syndromic orofacial cleft

A syndrome is a collection of findings occurring together in an individual (a syndrome means running together in Greek). Causes of syndromic orofacial clefts are often easily recognizable. They can be (a) monogenic disorders/ single gene disorders for example Van der Woude syndrome, Apert syndrome and Seckel syndrome. They follow the Mendelian mode of inheritance. Example of single gene syndromes are, Meckel syndrome, which is an autosomal recessive disorder and is associated with polydactyly, polycystic kidneys, encephalocoele, cardiac anomalies, and other abnormalities (Chung *et al.*, 1986). Van der Woude syndrome, an autosomal dominant disorder is characterized by the presence of lip pits in lower lip (Cervenka *et al.*, 1967). (b) Chromosomal disorders like Patau syndrome (trisomy 13), trisomy 18, Turner syndrome, Down syndrome, Cri-du chat syndrome and Wolf-Hirschhorn syndrome (Taylor *et al.*, 1970, Smith, 1982, Gorlin, 1971). (c) Unknown cause. In syndromes like Pierre Robin syndrome associated with orofacial clefts, no single aetiology can be pin pointed.

2.4.2 Aetiology of nonsyndromic orofacial clefts

In isolated or nonsyndromic orofacial clefts, the aetiology is difficult to pin point. An interaction between genetic and environmental factors during a critical stage of development is thought to be responsible for these cases (Murray, 2002a,

Stanier and Moore, 2004). Genes play the more significant role in nonsyndromic orofacial clefts as there is a higher concordance rate for orofacial clefts in monozygotic twins (60%) than among dizygotic twins (10%) (Christensen *et al.*, 1999). This difference implies that genes have a major role to play in nonsyndromic orofacial clefts. However 40% discordance among monozygotic twins implies that environment influences are also important. Among the genes involved in the risk of orofacial clefts methylene tetrahydrofolate reductase (*MTHFR*) gene is important possibly because of its effect on environmental factor of folic acid deficiency. This gene and folate have been described in later sections. The environmental factors are postulated below.

2.4.2a Environmental factors in aetiology of nonsyndromic orofacial clefts

The factors for the aetiology of nonsyndromic orofacial clefts are (a) Nutritional deficiency: An environmental component to clefting was recognized when Warkany (1943), associated nutritional deficiencies with cleft palate (Warkany *et al.*, 1943). Folate, B6 and B12 deficiencies have been studied with variable findings (Lettieri, 1993). The role of folate supplementation in pregnancy in prevention of orofacial clefts has been elaborated in Section 2.7.3. (b) Infections: Infections during pregnancy can produce birth defects. These are considered environmentally induced defects. During pregnancy it is important to be cautious about which medications are taken and cautious about being exposed to viral infections, such as the cytomegalovirus or the rubella virus (Thornton *et al*., 1996). (c) Drugs: There are many factors which influence the development of birth defects when a pregnant mother is exposed to a teratogen. Some of the factors which determine the impact of a teratogen include the genotype of the mother and the child

and the timing and the dose of the drugs (Isreal, 1987). A drug, for example, cannot affect the development of a cleft lip or palate if it is taken by the mother after the closure of the lip and the palate in utero. Recognized teratogens that cause clefts include rare exposures, such as phenytoin, valproic acid and thalidomide; and also common environmental exposures, such as maternal alcohol or herbicides such as dioxin (Garcia *et al.*, 1999). Apart from anti-epileptic medication, other drugs have been implicated in cleft palate. Corticosteroid exposure in the first trimester of pregnancy increases the risk of orofacial clefts in offspring (Rodriguez-Pinilla and Martinez-Frias, 1998, Carmichael and Shaw, 1999). Prednisolone increases the risk of orofacial clefts by 3-4 folds (Park-Wyllie *et al.*, 2000). (d) Cigarette smoking: Maternal smoking has been associated with increased risk for oral clefts especially during first trimester of pregnancy as shown by (Wyszynski and Beaty, 1996). Of all other congenital malformations orofacial clefts is possibly most definitely associated with smoking and there is enough evidence showing the positive association (Chung *et al.*, 2000, Lieff *et al.*, 1999). The mechanism by which cigarette smoke detrimentally affects pregnancy outcome is not well understood but some authors suggest a gene environment interaction by polymorphisms involving biotransformation genes resulted in congenital malformation due to cigarette smoke (van Rooij *et al.*, 2001).

2.4.2b Genes and loci in the etiology of nonsyndromic orofacial clefts

Fogh-Andersen first defined genetic factors involved in orofacial clefting, which were later confirmed by other authors (Marazita *et al.*, 1986b, Fogh-Andersen, 1942). To date no single gene has been identified as a universal explanation for all cases of isolated orofacial clefts (Kerrigan *et al.*, 2000). Possibly different genes are