An Analysis of the MTHFR Gene and Clinical Phenotypes in Familial Non-Syndromic Cleft Palate

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Abstract

Non-syndromic Cleft Lip and/or Palate (NSCL/P) constitutes the most common form of Orofacial Cleft (OFC). The methylene tetra hydrofolate reductase (MTHFR) encoding gene has been reported as playing a vital role in the pathogenesis of NSCL/P. Allele-specific Oligonucleotide (ASO) PCR analysis of A1298C and C677T of MTHFR gene polymorphism was performed using DNA from both the mother and father, in addition to circulating fetal cell-free DNA followed by electrophoresis. Homozygote mutation of A1298C was identified in the circulating fetal cell-free DNA, while heterozygote mutation was found to be present in both parents. Surprisingly, the affected infant possessed normal allele of C677T, despite both parents being heterozygote-mutated. Following birth, the infant presented cleft palate defect, even though both parents were phonetically normal. The mutation of both C677T and 1298C genes can cause fusion failure during oral and maxillofacial development. The latter may occur without following typical Mendelian hereditary patterns, despite the fact that NSCL/P is inherited in numerous cases. The irregular hereditary pattern is probably due to an interaction between genetic susceptibility and environmental stimulation.

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Introduction

Orofacial Cleft (OFC) is defined as facial deformity occurring during early gestation with Cleft Lip and/or Palate (CLP) the most common form.[1] It is reported that the frequency of CL/P may fluctuate due to geographical variations. Individuals suffering from CL/P are predominantly Asians and Native Americans with a prevalence of 0.7 per 1000 live births worldwide and remains on the increase, while Caucasians and Africans tend to be at lower risk of developing the condition.[2] CL/P patients may experience problems related to feeding, phonetics, hearing, dental conditions, recurrent ear infections and cognitive functioning. Due to the fact that CL/P may affect physical, psychological and social aspects, it is necessary for those afflicted with the condition to undergo continuous medical treatment from their first year of life. This places the affected families under financial and emotional pressure.[1,2] The etiology of CL/P is complex and heterogeneous since it is influenced by a combination of genetic and environmental factors. The nutritional deficiency, smoking habits and alcohol consumption of the mother, together with external teratogenic exposures in early pregnancy were reported as environmental risk factors contributing to the pathogenesis of CL/P.[3] While numerous studies have sought to reveal the mechanism of related genes in influencing the risk of CL/P, the result remains unclear and inconsistent. However, strong familial aggregation indicates that genetics play a significant role in its etiology. Close relatives of cleft cases have a marked increased risk of being

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similarly afflicted compared to those relatives at greater genetic distance.[4,5] Genetics have been recognized as contributing to the development of CLP due to the high incidence rate of recurrence within families. Individuals who are first-degree relatives are expected to be at higher risk compared to those with no family history of CLP.[6] The strong genetic factor influencing CLP is indicated by a higher concordance rate in monozygotic (MZ) twins (40-60%) compared to their dizygotic (DZ) counterparts (3-5%).[7] However, the fact that CLP follows non-Mendelian hereditary patterns suggests that genetics may not represent its only associated risk factor. It is evident that CLP demonstrates etiological heterogeneity between genetic and environmental factors that predispose certain individuals to orofacial clefts.[8]

The candidate genes that play a causal role in NSCL/P include: PVRL1 (Poliovirus receptor-like 1), MSX1 (muscle segment homeobox 1), TGF-β (transforming growth factor-beta 3), IRF6 (interferon regulatory factor-6), RUNX2 (runt-related transcription factor 2), BCL3 (B-cell leukemia/lymphoma 3), TBX22 (T-box transcription factor *22*) and MTHFR (methylenetetrahydrofolate reductase).[9,10] The MTHFR gene, located in chromosome 1 (1p36.3), encodes enzymes for folic acid, vitamins B6, B12 and homocysteine metabolism. MTHFR provides methyl to transform homocysteine into methionine and purines, converting 5, 10-metylenetetrahydrofolate into 5-metyltetrahydrofolate.[11] Any defects present in this pathway may result in methionine deficiency and an accumulation of homocysteine potentially resulting in teratogenicity during embryogenesis.[12]

The most extensively investigated polymorphisms of MTHFR gene are C677T (rs 1801133) and A1298C (rs1801131). The variant inhibits 20-70% of MTHFR gene activity, induces the accumulation of homocyteine and reduces the concentration of folate in plasma and serum.[13] The alanine located in codon 222 of 677T allele is replaced with valine (A222V) leading to the production of thermolabile enzyme with 70% reduction during specific catalytic activity. In addition, glutamic acid is substituted for alanine at codon 429 (G429A) due to 1298C allele that reduces 40% of MTHFR activity.[14]

Due to the vital role of MTHFR in folate metabolism, its variants may disrupt orofacial structure development during early pregnancy. Various studies have elucidated the mutation of MTHFR in NSCL/P patients, but the results remain inconsistent across populations. This variability in results may be explained by differences in ethnicity.[15] Pan *et al.,* (2015) demonstrated that a Chinese population whose members possess C677T genotype are at higher risk of having NSCL/P offspring, while maternal polymorphism of A1298C demonstrates no association with NSCL/P.[14] Nevertheless, a similar study by Rochmah *et al*. (2018) of the Indonesia-based Sasak tribe revealed that only maternal polymorphism A1298C is associated with NSCL/P.[16] With respect to ethnicity variations, this study aims to assess the relationship between variants of MTHFR A1298C and C677T, as well as the development of NSCL/P in the Indonesian population of the island of Madura.

Materials and methods

The study consisted of 50 NSCL/P offspring-parent triads within the ethnically Madurese population. All the research subjects provided written informed consent for their participation. The DNA was obtained from the peripheral blood of each subject by means of a salting out method. The polymorphism of MTHFR C677T and A1298C was analyzed through PCR using Alelle Specific Oligonucleotide-PCR (ASO-PCR). Samples were subjected to electrophoresis with agarose gel 2% analysis to confirm mutation. This investigation was conducted under ethical approval issued by the Faculty of Dental Medicine, Airlangga University with approval number 156/HRECC.FODM/VI/2020.

Subjects were classified into a mutant (MT) group and a wild-type (WT) heterozygote group. A chi-square analysis to confirm the presence of polymorphism and a risk factor analysis with a 95% confidence interval were both performed. Details of the primer and PCR condition performed in this study are described in Tables 1.

Mutation MTHFR	Forward Primer	Reverse Primer	PCR Product (bp)
C677T	5-TGC TGT TGG AAG GTG CAA GAT-3	RW 5-GCG TGA TGA TGA AAT CGG-3 RM 5-GCG TGA TGA TGA AAT CGA-3	226 226
A1298C	5-CCTTTGGGGAGCTGAAGGACACTAC-3	RW 5-CAAAGGACTTCAAAGACAGTC-3 RM 5-GGTAAAGAACAAAGACTCAAAGACACTGTG-3	120 127

Table 1. Primers and PCR condition.

Results

Of the 50 participating subjects (Table 2), one family produced female offspring with Cleft Palates and mutated genotype with A1298C homozygote. However, there was no mutation in C677T which was, therefore, normal. The affected parents possessed both heterozygote genotypes and normal phenotypes.

Subjects	Genotype	N	%
Father	AA	7	14
	AC	43	86
	CC	-	-
Mother	AA	3	6
	AC	47	94
	CC	-	-
Offspring	AA	37	74
	AC	12	24
	CC	1	2

Table 2 : Frequency of familial genotype with A1298C.

Offspring homozygote mutation in A1298C (Table 3). This mutation has been checked by ASO-PCR (Allele Specific Oligonucleitide - Polymerase Chain Reaction).

Offspring			Mother			Father		
N	M	Type	N	M	Type	N	M	Type
-	+	Homozygote	+	+	Heterozygote	+	+	Heterozygote

Table 3: Mutation of A1298C in affected subjects.

Offspring			Mother			Father		
N	M	Type	N	M	Type	N	M	Type
+	-	Normal	+	+	Heterozygote	+	+	Heterozygote

Table 4 : Mutation of C677C in affected subject.

Discussion

It has long been established that CLP is an inherited condition. A study of genetic pedigrees by Shah *et al.* (2016) stated that 33.3% cases of CLP were autosomal dominant, while 66.7% were autosomal recessive, while Nouri *et al.* (2012) suggest that NSCLP follow a X-linked recessive inheritance pattern (as shown in Figure 1,2,3).[17,18] The term 'autosomal dominant' is defined as a condition when parents transmit the allele disease to their offspring with the result that the disease is likely to recur in every successive generation.

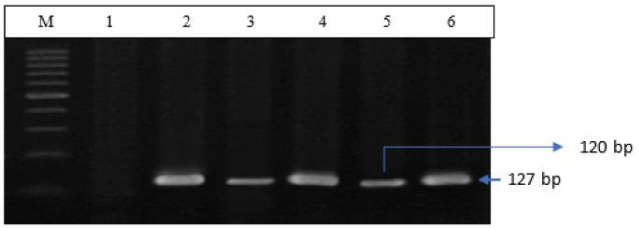

Figure 1: The electrophoresis image of affected subject against A1298C (Mutant = 127 bp; Normal = 120 bp). The symbols on the figure 1. (the number 1 and 2 is offspring, 3 and 4 is his mother, and 5 and 6 is his father) (as shown on the table 3).

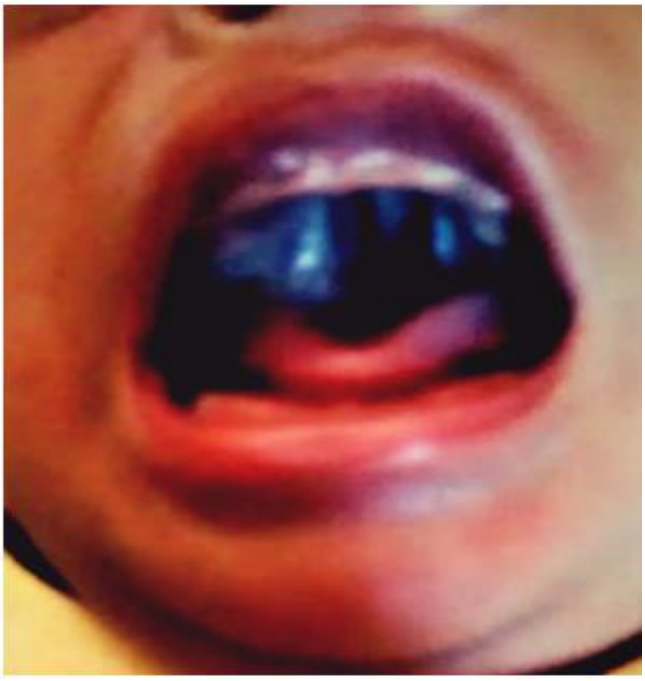

Figure 2: Offspring suffered Cleft Palate who caused by homozygote mutation on A1298C. The C677T phenotype Offspring is mutated so that offspring suffered Cleft palate, but the genotype of C677T Offspring is normal. (table 4)

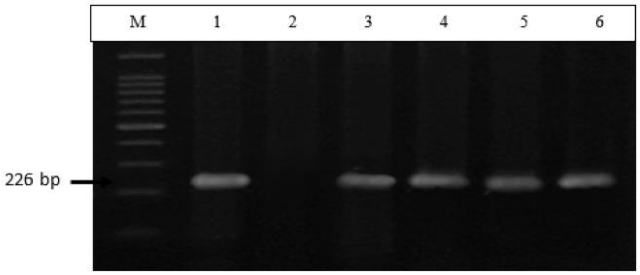

Figure 3: The electrophoresis image of affected subject against A677C (Mutant = 226 bp; Normal = 226 bp). The symbols in Figure 3 (numbers 1 and 2 relate to offspring, 3 and 4 to the mother of the offspring and 5 and 6 to the father of the offspring) (as shown in Table 4).

In contrast, disease is considered an autosomal recessive inheritance when both parents possess normal phenotypes but may carry disease allele leading to less frequent recurrence in every generation of the affected family. The analysis suggests that an individual could not be identified as a carrier or non-carrier based on their normal phenotype. However, unlike other hereditary diseases, NSCLP may not follow typical Mendelian inheritance patterns, despite slight familial tendency.[19] Numerous studies attempted to analyze the genetic factor of NSCLP in studies of twins and families, but only a few of them present Mendelian inheritance patterns. The majority of cases seem to demonstrate irregular patterns, supporting the idea that NSCLP is conjointly regulated by genetic and environmental risk factors.[20]

Normal phenotypes in non-cleft individuals are potentially deceptive as they could possess mutated alleles, resulting in sub-phenotypes with minor clinical features that are prone to under-diagnosis.[21] The reality is that genetic variants of susceptible genes are not identified in affected children but, predominantly, in their mothers. This provides researchers with opportunities for further exploration into possible methods of preventing NSCLP. The presence of environmental teratogenic agents can be confirmed through genetic analysis of pregnant woman, supporting the woman to minimize exposure to that agent. The crucial initial step towards primary prevention of NSCLP is that of identifying modifiable risk factors.[22]

Maternal nutritional status, particularly folic acid deficiency, is considered to contribute to the pathogenesis of NSCLP due to its ability to prevent neural tube defect (NTD). However, high levels of folic acid may not be able to reduce the occurrence of NSCLP because the availability of folic acids are firstly needed to be biologically activated. MTHFR is an enzyme responsible for catalyzing the conversion of 5,10-methylenetetrahydorfolate to 5-methyltetrahydrofolate. Thus, the MTHFR gene is one of the most widely studied genes related to NSCLP. Its susceptibility due to the role in folic acid metabolism and defect of MTHFR gene could alter the biological response of affected individual.[23]

The data obtained, despite the fact that NSCLP is inherited in various cases, it may occur without following typical Mendelian inheritance patterns. The irregular inheritance pattern is probably due to the interaction between genetic susceptibility and environmental stimulus.[20] This study indicated that an NSCLP patient with MTHR polymoprhism has parents who do not present MTHFR polymorphism. The relationship between MTHFR C677T and A1298C polymophism with the risk of NSCLP within the Indonesian population may support future diagnosis and early treatment. However, identification of parental MTHFR polymorphism will not significantly affect the prevention of recurrent neural tube defects in their offspring. Measuring the level of maternal serum folate and encouraging maternal supplement consumption could substitute genetic analysis due to folate supplementation potentially reducing neural tube defect-related disease by approximately 70%.[24]

Conclusions

The mutation of the C677T and 1298C genes can cause the failure of fusion within oral and maxillofacial development. Nevertheless, both genes are related to folic acid metabolism because of its role in pregnancy which has a great impact pathogenesis of CLFP because of the prevention of its ability to prevent neural tube defect (NTD).

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Declaration of Interest

The authors report no conflict of interest.

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