Identification of Single Nucleotide Polymorphism Gen MTHFR C677T in Non-Syndromic Cleft Lip and/or Palate

Agung Sosiawan[1]*, Mala Kurniati[2], Indah Nuraini[3], Qurrota A'yun[3], Raden Mas Coen Pramono Danudiningrat[4], Indra Mulyawan[4], Regina Purnama Dewi Iskandar[5], Rozita Hassan[6]

1. Dept. of Dental Public Health, Faculty of Dental Medicine, Universitas Airlangga, Surabaya, Indonesia.
2. Dept. of Biology, Faculty of Medicine, Universitas Malahayati, Lampung, Indonesia.
3. Human Genetic and Forensic Laboratory, Institute of Tropical Disease, Airlangga University, Surabaya, Indonesia.
4. Dept. of Oral & Maxillofacial Surgery, Faculty of Dental Medicine, Universitas Airlangga, Surabaya, Indonesia.
5. Dept. of Orthodontics, Faculty of Dental Medicine, Universitas Airlangga, Surabaya, Indonesia.
6. Orthodontic Unit, School of Dental Sciences, Universiti Sains Malaysia, Kelantan, Malaysia.

Abstract

Cleft lip/palate (CL/P) is considered to be the most common congenital abnormality caused by genetic and environmental factors. Certain genes related to CL/Ps have been identified and explored to determine their etiology. One of the most influential genes in the pathogenesis of CL/P is methylenetetrahydrofolate reductase (MTHFR) gene. Polymorphism of MTHFR gene impair craniofacial development during first trimester of pregnancy.

This study aims to identify the single nucleotide polymorphism (SNP) C6777T of the MTHFR gene in non-syndromic CL/P patients.

DNA samples were obtained from 30 non-syndromic CL/P patients. The Deoxyribonucleic acid (DNA) was amplified for Polymerase Chain Reaction (PCR) examination. Electrophoresis was performed on agarose gel to visualize PCR product, and sequencing was conducted on three specimens. The polymorphism of MTHFR C677T was examined using Restriction Fragment Length Polymorphisms (RFLP) technique.

Based on analysis using restriction enzyme and bioinformatic software, cytosine was substituted into thymine in nucleotide 222 on exon 4. Valin was synthesized instead of alanine. Genotype CC was found in 22 patients (73%) and genotype CT in eight patients (73%), while the mutant homozygote TT was not found in this study.

The SNP C6777T of the MTHFR gene is associated with non-syndromic CL/P.

Clinical article(J Int Dent Med Res 2022; 15(4): 1620-1624)

Keywords: Cleft Lip and/or Palate, methylenetetrahydrofolate reductase, Single Nucleotide Polymorphism, Medicine.

Received date: 14 July 2022 **Accept date:** 21 September 2022

Introduction

The pathogenesis of CL/P has been widely studied to reveal the basis of craniofacial development[1]. Non-syndromic CL/P is the most common congenital defect worldwide. The prevalence may vary between regions, but it reaches 1:730 in low-income countries[2]. Genetic variation is defined as the subtle genomic differences among individuals that makes each or a group of organisms different from others. Genetic variation interferes with gene function by modifying the splicing process between individuals, leading to diseases. Genetic variants are accurately predicted during splicing and that occurred in the intron are important to observe the mechanism of certain diseases. In order to accurately identify gene structure, the impact of genetic variants that produce inactive protein could be analyzed through bioinformatic methods[3].

Previous studies have demonstrated significant links between CL/P and the polymorphism of folic acid-related genes, particularly 5,10-MTHFR[4]. The MTHFR gene is located in chromosome 1p36.3. The gene is translated into the MTHFR enzyme to catalyze the conversion of 5,10-metilenatetrahidrofolat

Corresponding author:
Agung Sosiawan,
DDS., M.Sc., LLM., Ph.D. (Dean and Associate Professor)
Department of Dental Public Health, Faculty of Dental Medicine, Universitas Airlangga, Indonesia.
E-mail: agung-s@fkg.unair.ac.id

into 5-methyltetrahydrofolate. It is responsible for the methylation of homocysteine and the production of methionine[5]. The MTHFR enzyme plays an important role in folic acid metabolism and DNA synthesis. The SNP of MTHFR rs1801133 C677T is known to reduce the activity of enzymes. Thus, the activity of MTHFR must be properly regulated to maintain the cellular homeostasis of methionine and S-adenosylmethionine[6].

A healthy diet combined with sufficient folic acid reduces the susceptibility of NSCL/P[6]. Despite an adequate supply of maternal folic acid being important during pregnancy, a developmental abnormality may result from the changes in the activity of the MTHFR enzyme related to folic acid metabolism[7]. In a previous case-control study related to MTHFR C677T, it was found that the mutant TT homozygote genotype is considered a risk factor of NSCL/P in the Asian population (OR=1.96; P<0.001). This contradicts another study stating that maternal TT genotype increases the morbidity of NSCL/P in the Caucasian population. The meta-analysis of 22 case-control studies asserts that the polymorphism of MTHFR C677T is strongly associated with NSCL/P. Sosiawan et al. (2020) discovered the association between the polymorphism of MTHFR C677T and A1298C with non-syndromic CL/P. Parents with the heterozygote C677T mutant allele have offspring with C677T normal allele. Cleft palates were discovered in the offspring despite of their "normal" parents. It shows that non-syndromic CL/P patients may have different genetic variants from their parents[8].

The identification of SNP in polygenic disease must be continuously explored in order to discover the ideal method for early detection of CL/P. The aim of this study is to detect SNP in the MTHFR C677T gene in CL/P patients to support diagnosis accuracy and initiate treatment as early as possible.

Materials and methods

This study received ethical clearance by the ethical commission at The Faculty of Dental Medicine, Airlangga University (156 /HRECC.FODM /VI/2020). The 30 subjects are CL/P patients who underwent reconstructive surgery provided by the Department of Oral and Maxillofacial Surgery, Faculty of Dental Medicine, Airlangga University. Children with non-syndromic cleft lip and/or palate appearance were included in this study. All the objectives, risk, and details of this study were fully explained to children and their parents of legal guardians, and written informed consent was obtained from all participants.

DNA was extracted from the peripheral blood of CL/P patients using the A1120 Wizard® Genomic DNA Purification Kit. The laboratory process was performed at the Human Genetic Laboratory, the Institute of Tropical Diseases, Airlangga University. The Polymerase Chain Reaction-Restriction Fragment Length Polymorphisms (PCR RFLP) method was used in this study to analyze the polymorphism of MTHFR C677T (NCBI Reference Sequence: NC_000001.11; GenBank Graphics >NC_000001.11:c11889723-11706103 *Homo sapiens* chromosome 1, GRCh38.p13). The DNA purity and concentration were examined using nanodrop with 260 and 280 nm wavelength.

Integrated DNA Technologies developed the primer with the sequence of 5'- TGA AGG AGA AGG TGT CTG CGG GA-3' dan 5' AGG ACG GTG CGG TGA GAG TG -3'[9]. The PCR mix consists of 25 µl, 2.5 µl Promega Go Taq™ Master Mixes, 2.5 µl (10 µmol) of forward primer, 2.5 µl (10 µmol) of reverse primer, and 7.5 µl of a template. The mixture was amplified using BioRadCycler PCR. The temperature was set to 55°C for 45 seconds and 72°C for 60 seconds with 30 cycles for the PCR work.

The electrophoresis method was conducted using 2% agarose gel (Promega) at 100V for 30 minutes. The PCR product was observed in 198 bp. It was followed by RFLP using the Hinf1 restriction enzyme (Thermo Scientific). The mixture of the enzyme and PCR products was incubated overnight at 37°C. The RFLP band was visualized through electrophoresis (100V; 35 minutes).

The restricted band was observed through UV light. There were three variations of genotype: 198 bp for wildtype homozygote (CC), 198 bp + 175 bp for mutant heterozygote (TT), and 175 bp + 23bp for mutant homozygote (TT). The statistical analysis was performed and tabulated using SPSS Statistical Software version 20 (IBM Corporation, NY, USA).

Results

The mean of the DNA concentration in all subjects is 98.30 ng/µl. The purity value of 1.8 between 260 nm and 280 nm wavelength examination indicates that the DNA is eligible for further analysis. The temperature for PCR amplification in this study was presented in figure 1.

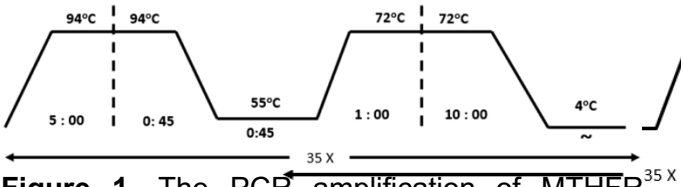

Figure 1. The PCR amplification of MTHFR C677T gene.

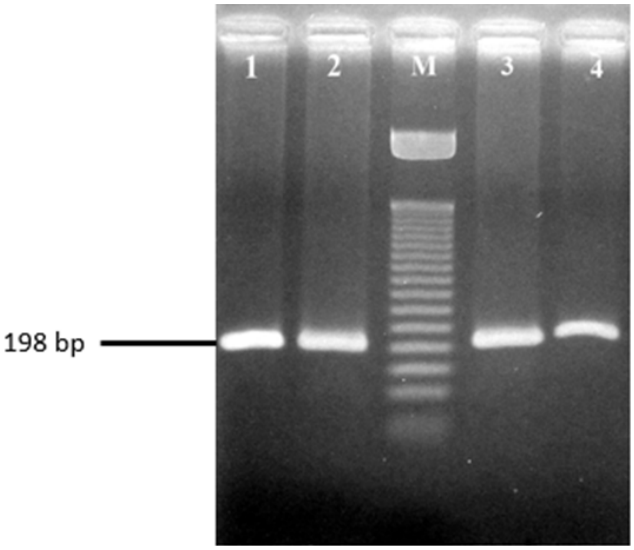

Figure 2. The visualization of the PCR of MTHFR C677T shows thick single band of 198 bp. (M = 50 kb is DNA marker; Line 1,2,3, and 4 are DNA samples).

Figure 2 shows a single band of 198 bp was observed in this study, indicating that the primer amplified the target area of MTHFR C677T gene. The band was generated by annealing the temperature at 55°C and 35 cycles. The ratio of genetic and non-genetic factors that lead to a certain phenotype in diseases may vary due to genetic variants known as heritability, which is measured by concordance rate (CR)[10]. Monozygotic twins have 40%–60% higher CR of CL/P than dizygotic twins[11,12]. Figure 3 shows the RFLP result of MTHFR gene using Bsr1 enzyme. Lines 1, 2, and 3 indicate the CC genotype. Lines 4 and 5 indicate the CT genotype. Lane M (50bp) is ladder marker.

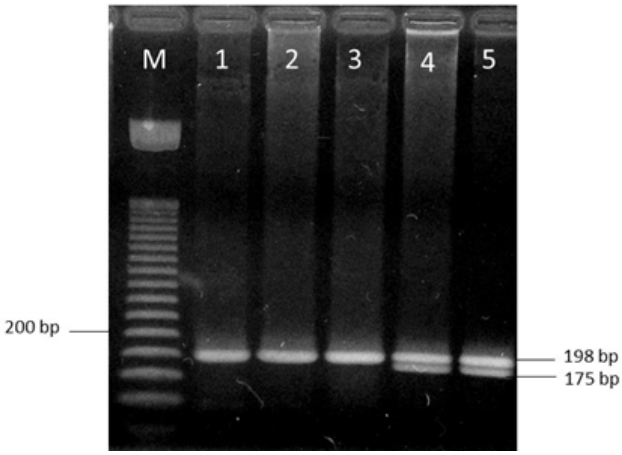

Figure 3. The RFLP result performed with the Bsr1 enzyme.

The CC genotype was found in 22 out of the 30 patients (73%) and the CT genotype in eight patients (27%), but no TT genotype was found in these patients. Figure 4 describes sequence analysis of MTHFR C677T gene.

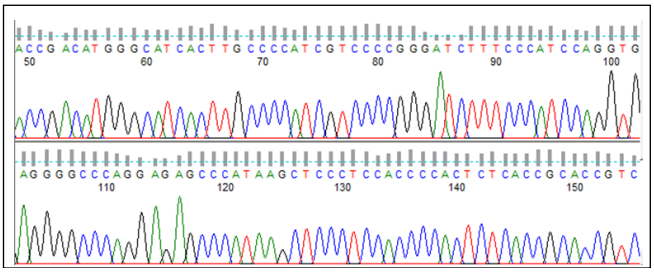

Figure 4. Sequence analysis using the primer of the MTHFR C677T gene.

Discussion

Gene mutation is the change of nitrogen in the DNA that leads affected gene to synthesize irregular proteins and followed by different phenotype. The etiologies of mutation are: (1) impaired DNA replication mediated by mutagen; (2) the breakdown of oxygen-phosphate bond; (3) the base-pair substitution; and (4) the changing amount of the nitrogen base. Missense mutation causes the related gene to produce different types of amino acids because of the changed nitrogen base sequence[13]. The SNP C677T (rs1801133) of the MTHFR gene is the most common mutation of CL/P that has been widely studied. C677T is located in exon 4 of the

MTHFR gene, where cytosine is replaced by thymine in nucleotide 677. It produces valine instead of alanine in position 222 (A222V). The mutation C677T causes the enzyme to become more unstable[14].

The MTHFR enzyme functions as dimer or tetramer, while flavin adenine dinucleotide (FAD) acts as a cofactor. The mutation C677T alters the secondary structure of a peptide and the interactions between monomers. The substitution of amino acid from alanine into valine increases the dissociation rate of FAD. The loss of FAD is associated with the impaired quaternary structure of protein and reduced enzyme function. The docking study has established that the mutant enzyme 222V has a lower affinity against its FAD cofactor compared to the normal enzyme 222A[15]. The mutant protein is more unstable and losses FAD faster than the normal type. However, that effect could be minimized by folic acid administration, which increases the affinity of FAD[16].

The result of studies from three different populations: Asia (China, Philippines, Laos, and Cambodia), the Middle East, and Northern Africa show that the severity of CL/P vary between geographical regions. Southeast Asia has the highest severity rate compared to other regions. The variation that both genetic and environmental factors contribute to the pathogenesis of CL/P[17]. Various factors may contribute to the difference of clinical appearance between patients, such as gender, history of similar cases in the family, environmental issue, nutritional status, maternal age, and teratogenic effect[18,19]. It is reported that the polymorphism of the MTHFR gene for C677T in the Brazilian population is not associated with CL/P[20], while the polymorphism A1298C of the MTHFR gene is associated with CL/P in Turkish populations[21]. The study of the Iranian population shows no significant result for C677T, but A1298C is significant for CL/P cases[22], which is identical to a similar study conducted in the Sasak population in Indonesia[9] and the Uyghur population in China[23]. There are currently two methods to detect genetic variations in CL/P: a focused study of the potential gene target related to folic acids, such as MTHFR, and a wide genome analysis in certain populations or races to detect genes related to CL/P to support the polygenic concept[24,25].

Conclusions

The pathogenesis of CL/P remains unclear despite of numerous studies that had been conducted relating to CL/P. Variations C6777T in exon 4 of the MTHFR gene identified in this study indicates that it contributes to the pathogenesis of CL/P. It is a potential candidate for early detection of CL/P in high-risk community.

Acknowledgements

This study was funded by Directorate of Research and Community Services, Deputy of Research and Development Reinforcement, Ministry of Research and Technology/ National Agency for Research and Innovation, Republic of Indonesia (No. 302/UN3.15/PT/2021). The authors were grateful of the support of Bima Hospital in West Nusa Tenggara, Indonesia and Nahdlatul Ulama Hospital in Tuban, Indonesia. All authors have made substantive contribution to this study and/or manuscript, and all have reviewed the final paper prior to its submission.

Declaration of Interest

The authors report no conflict of interest.

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