

Optimizing examination of *methylenetetrahydrofolate reductase* gene promoter methylation in cleft lip with or without cleft palate non-syndromic patients using the pyrosequencing method

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ABSTRACT

Background: Cleft lip with or without cleft palate (CL/P) is the most common congenital anomaly found in Indonesia. CL/P is caused by hereditary (genetic) and environmental factors. Environmental factors can result in methylation in the promoter of the methylenetetrahydrofolate reductase (MTHFR) gene, affecting its expression. Methylation takes place at the CpG site found at chromosome 1, coordinates 11,805,406–11,806,509. Pyrosequencing technology can detect the percentage methylation of a gene quickly, simply, and accurately. **Purpose:** The aim of the study is to optimize detection of methylation of the MTHFR gene using the pyrosequencing method. **Methods:** Methods used in this study were DNA extraction from blood, DNA bisulfite conversion, polymerase chain reaction (PCR), and methylation detection using CpG pyrosequencing assay. Samples were taken from 20 CL/P patients (C) and 44 normal patients (N). **Results:** The pyrosequencing method was successful in detecting methylation at three MTHFR gene sites at coordinates 11,805,507–11,805,529. The methylation level at the third site was higher in group C than in group N, while at the first and second positions, group C had a lower methylation level than group N. In general, the percentage of methylation for both groups was low or hypomethylated (less than 5%). **Conclusion:** The pyrosequencing method can be used to determine methylation levels in the MTHFR gene with the results presented as percentages (quantitative data). Hypomethylation occurs in groups C and N at the coordinates 11,805,507–11,805,529 of the MTHFR gene promoter.

Keywords: cleft lip; cleft palate; methylation; MTHFR; pyrosequencing

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INTRODUCTION

Cleft lip with or without cleft palate (CL/P) is a birth defect that affects the upper lip and palate. Oral-facial cleft, especially CL/P, is the most common craniofacial deformity, with worldwide prevalence reaching 1 in 700 to 1000 live births.¹ CL/P is divided into two categories, namely syndromic and non-syndromic (NS). In Indonesia, the birth of babies with CL/P NS abnormalities is quite high, namely around 1.7/1000 live births, and in Hasan Sadikin Hospital in Bandung the incidence is around 1.47/1000 live births.² In Asia, the prevalence of babies born with CL/P NS conditions reaches 1 per 500 births,

the prevalence is highest in Asia, followed by Europe and Africa.³ In Indonesia, it is estimated that 7,500 children suffer from cleft lip or cleft palate.⁴ In the West Java area, there were 1,087 CL/P NS patients from January 2016 to December 2019.^{5,6}

CL/P is a hereditary disease caused by a gene mutation. It can also be caused by environmental factors such as viral and microorganism infection in early pregnancy or exposure to hazardous chemicals (mutagenic causes). The influence of environmental factors and their interaction with various genes involved in embryogenesis also play an important role in the development of CL/P.⁷ Previous studies show that mutations in the methylenetetrahydrofolate reductase

(*MTHFR*) allele 677T are the most common polymorphisms found in CL/P patients. The *MTHFR* gene is a genetic component involved in the folate cycle. This gene is responsible for the process of converting folate from the food or supplements we consume daily into the active form of folate used by the body as an essential nutrient.⁸

DNA methylation is the most common epigenetic process and is one of the mechanisms of epigenetic control over hereditary (inherited) gene expression, but it does not change the base arrangement of DNA. Epigenetic changes can affect gene expression through the addition of a methyl group at the fifth carbon position of cytosine on CpG dinucleotides. These CpG locations are always clustered to form a kind of island, known as a CpG island, that contains between 200 bases and several kilobases. CpG islands are predominantly found in the promoter region of a gene.⁹ The methylation of nitrogen bases is higher in DNA segments that are rich in cytosine and guanine bases, which bind via phosphodiester bonds (CpG islands). Environmental factors can affect the expression of a gene by causing the addition of methyl groups to the promoter region of a gene. Excessive methylation levels will cause gene expression levels to decrease, and conversely, low methylation levels will cause gene expression levels to increase.¹⁰

Cleft lip is a phenomenon whereby the lip area fails to close during the process of facial formation when the embryo is between four and seven weeks of gestation. Cleft palate is a phenomenon in which the palate in the mouth fails to close during the joining process of the upper and lower parts of the mouth, occurring when the embryo is six to nine weeks of gestation. CL/P is caused by multiple factors, including genetic factors, the environment during pregnancy, carcinogens, and exposure to chemicals.¹¹

There are several methods for detecting methylation in a gene. It is often done with a polymerase chain reaction (PCR) method using primers that are designed to recognize methylated and unmethylated areas. The weakness of this method is that we can only observe it qualitatively by looking at the presence or absence of DNA bands in electrophoresis.¹² Low levels of methylation cannot be detected with this method. The latest technology today, namely pyrosequencing, can determine the percentage methylation of a gene (quantitative data) in a way that is simpler, more accurate and faster than the PCR method, so it is now possible to detect a low percentage of methylation.¹²

This study aims to optimize detection of *MTHFR* gene methylation using the pyrosequencing method.

MATERIALS AND METHODS

The research subjects were CL/P sufferers and their families who were registered with the Foster Foundation for Cleft Lip and Palate Patients. The study protocol was approved by the Research Ethics Committee, Universitas Padjadjaran, Bandung (No. 773/UN6.KEP/EC/2019). This study used blood samples from 20 patients with CL/P and 44 normal patients and was conducted at the Molecular Genetics Laboratory, Faculty of Medicine, Universitas Padjadjaran in 2022. This research is exploratory, and several stages of examination are needed to detect the level of methylation in the *MTHFR* gene.

As much as 3 ml of venous blood was collected in EDTA tubes. DNA was isolated from the blood of CL/P patients and normal controls using the Quick-DNA™ Miniprep Plus Kit from Zymo Research (catalog No. D4069). DNA was dissolved in 100 µl of buffer fluid, and the bisulfite conversion step followed.

DNA bisulfite conversion is a technique used to differentiate between methylated and unmethylated cytosine bases in DNA methylation studies. The bisulfite modification technique uses sodium bisulfite, which changes the unmethylated base of cytosine to uracil, while 5-methylcytosine will not change. Extracted DNA was first treated with bisulfite using the reagent EZ DNA Methylation Kit from Zymo Research (Catalog No. D5001).

Assays for examining methylation using the pyrosequencing method were carried out in silico using the help of several genome browsers (Ensembl and UCSC) as well as Pyromark Assay Design software by Qiagen. The location of the *MTHFR* gene is known due to the help of the browser genome Ensembl; it is on the chromosome 1 reverse strand with coordinates 11,785,723–11,806,455 (Figure 1).

The nucleotide target for methylation is located on the CpG island on the promoter of the *MTHFR* gene. The UCSC Genome browser can make it easier to detect the location of the CpG Island, which is at position chr1:11,805,406–11,806,509. The CpG Island sequence of the *MTHFR* gene was entered into the Pyromark Assay Design software and the area where the methylation level will be examined was

Gene: MTHFR

Gene: MTHFR ENSG00000177000

Description methylenetetrahydrofolate reductase [Source:HGNC Symbol;Acc:HGNC:7436]

Location [Chromosome 1: 11,785,723-11,806,455](#) reverse strand. GRCh38:CM000663.2

About this gene This gene has 19 transcripts ([splice variants](#)), [205 orthologues](#), [4 paralogues](#) and is associated with [9 phenotypes](#).

Transcripts [Show transcript table](#)

Figure 1. Search results for the location of the gene with Ensembl genome browser.

determined. In this study, we aimed to detect the percentage of methylation at three methylation points located at 11,805,507–11,805,529. Considerations for choosing a methylation target were that it is easily amplified by forward and reverse primers and that the maximum length of the PCR product is not more than 200 bp.

From this software, we obtained the information needed to carry out methylation tests using the pyrosequencing method. The primary sequence obtained was as follows:

Forward primer 5'-AGG AGG GGT TAT GAG AAA AG-3', reverse primer 5'-biotinACC TAA AAA CCC CAA CCA AAA CTC T-3', sequencing primer 5'-GGT TAT GAG AAA AGA TTT TAG ATT-3', and sequence to analyze TAG GTA YGT GAA GTA GGG TAG AYG TTT YGA GAG TTT TGG TTG. In pyrosequencing, the reverse primer should be labeled by biotin.

DNA bisulfite conversion results in PCR using forward and reverse primers that have been designed to produce

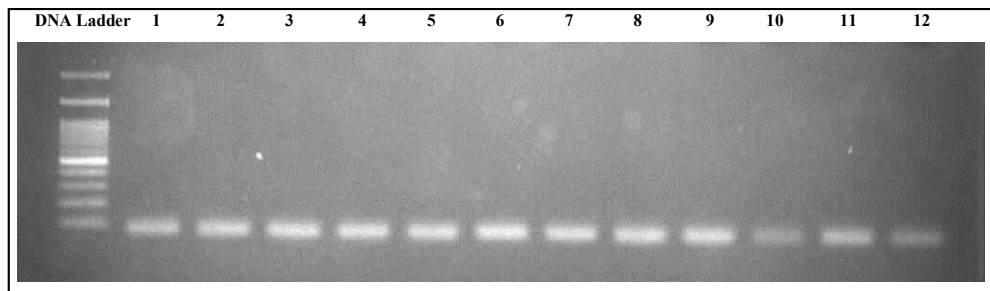


Figure 2. Electrophoresis results of the *MTHFR* gene with a product length of 84 bp. Lane 0: Ladder 100 bp, Line 1–6: DNA C1–C6, Line 7–12: DNA N1–N6.

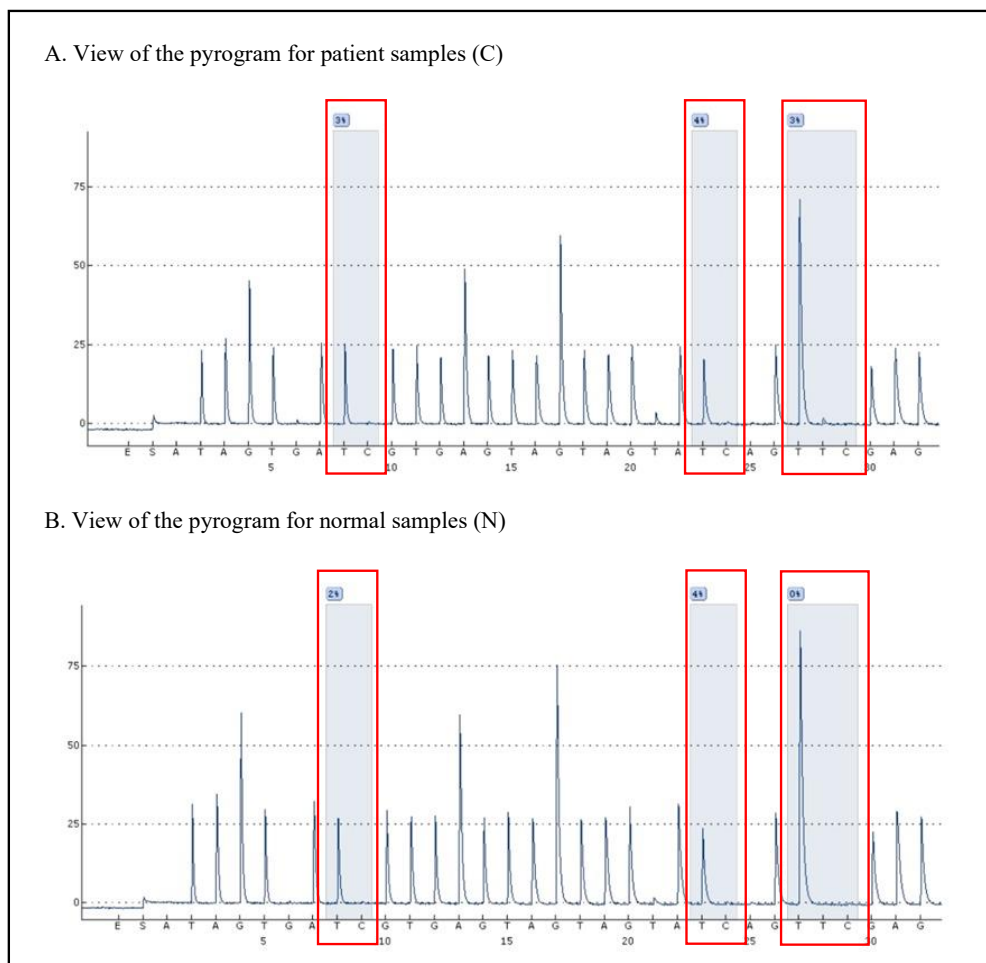


Figure 3. Results of *MTHFR* gene pyrosequencing. The red boxes indicate the location of methylation points. (A) Pyrogram view of sample C4 (patient sample); Point 1: 3%, Point 2: 4%, Point 3: 3%, (B) Pyrogram view of sample N5 (normal control sample); Point 1: 2%, Point 2: 4%, Point 3: 0%.

DNA strands with a length of 84 bp when viewed on gel electrophoresis. In the bisulfite process, the C base (cytosine) is converted to the U base (uracil), while the mC base (methylated cytosine) remains unchanged, so that in the PCR process the U base pairs with the T base (thymine) and the mC base pairs with the C base. The PCR conditions used were initial denaturation at 94 °C, 3 minutes, thereafter, cycling 40 times: 94 °C, 30 seconds; 60 °C, 30 seconds; 72 °C, 30 seconds. The final extension was 72 °C, 3 minutes.

Pyro Q-CpG is useful for DNA methylation studies, as it can quantitatively measure the methylation level of the CpG island region. Cytosine methylation in CpG is an important regulator in the process of gene expression in the human genome. Pyrosequencing is a useful method for CpG methylation analysis because it can measure methylation quickly and easily. In the pyrogram produced during the pyrosequencing process, mC and C are represented as C and T peaks respectively. The proportion of the peak heights is proportional to the number of alleles methylated at each CpG site; the number of data calculations is displayed as a percentage. In the process of detecting *MTHFR* gene methylation, the sequencing primers diluted with annealing buffer and the sequence to analyze the sequence must be entered into Qiagen's PyroMark Q96 software.

RESULTS

The concentration and purity of DNA samples from CL/P sufferers and normal controls were calculated using the Nanodrop tool. DNA extraction was carried out on all sample groups C and N; after being measured by nanodrop, it was found that all samples had a high DNA concentration of more than 50 ng/μl. DNA purity was also very good; all samples showed a ratio of 260/280, 1.8 (good DNA purity: 1.7–1.8). This shows that all samples met the quality criteria, so they were eligible to enter the next stage of examination, namely bisulfite conversion and PCR. Before entering the pyrosequencing stage, all samples to be tested (both CL/P patient samples and normal samples) were amplified with *MTHFR* gene primers designed according to the methylation target of interest. The PCR product obtained was 84 bp; an overview of the gel electrophoresis results can be seen in Figure 2.

Good quality PCR products entered the next stage of examination, namely methylation detection by pyrosequencing. The result of pyrosequencing can be

seen in the form of a pyrogram, and the percentage of methylation in the target area is shown. In Figure 3 the shape of the pyrogram shows that there are three methylation points in the sample CL/P (C) and the normal sample (N) that can be analyzed. The pyrosequencing process was successfully carried out on 20 samples from group C and 44 samples from group N.

Pyrosequencing can detect a low percentage of methylation. Overall, the methylation level at these three points was low (hypomethylation). Almost all were below 5%, both in the patient sample group (C) and the normal control sample (N). The average results at each sample point in groups C and N can be seen in Table 1.

DISCUSSION

The methylation point at location three corresponds to the data seen in Table 1, i.e., the group C sample has a higher methylation level than the group N sample. However, at point 1 the results look different to point three, as group C samples have lower levels of methylation than group N. These data do not show a significant difference between the values for groups C and N. In general, the percentage of methylation in the two groups was low or hypomethylated (average less than 5%). The results of this study show that all samples examined experienced hypomethylation at the target point of the *MTHFR* promoter region, so in this case methylation in the *MTHFR* gene was not a factor causing CL/P.

The results are inconsistent with the hypothesis that the methylation level of the CL/P sample will be higher than that of the normal sample, causing suppression of *MTHFR* gene expression and resulting in a decrease in the activity of the 5,10-methylenetetrahydrofolate reductase (*MTHFR*) enzyme which becomes homocysteine compound remethylation agents.¹³

Cleft lip and palate is a phenomenon whereby the tissues fail to cover facial areas, and it is thought to have a relationship with vitamin levels and genetic factors.¹⁴ A study of *MTHFR* gene polymorphism showed that patients with the C/T genotype with low levels of folic acid had higher rates of chromosomal damage and heterochromatin dimethylation-related gene variation.¹⁵ Another study showed that people with CL/P had lower levels of folic acid and vitamin B12, as well as higher levels of homocysteine, compared to normal controls.^{16,17} Homocysteine accumulation can cause a decrease in transcription levels in endothelial cells. The mechanism for decreasing transcription occurs in the promoter region by causing the demethylation of proteins associated with the CpG region, resulting in the acetylation of histone proteins which play a role in the process of chromatin transcription.¹⁸ As a result, the accumulation of homocysteine can lead to the formation of orofacial clefts during development. The combination of accumulation of homocysteine and deficiency in folate metabolism can lead to various

Table 1. Average percentage value of *MTHFR* gene methylation

Group of Sample	Methylation percentage (%)		
	Point 1	Point 2	Point 3
Patient sample (C)	1.95	3.29	2.15
Normal sample (N)	2.15	3.29	1.90

disorders such as neurodegenerative diseases, osteoporosis, cancer, heart disease, and cleft lips.¹⁹

The results of this study show that CL/P was not caused by methylation at the three points of the promoter region of the *MTHFR* gene, coordinates 11,805,507–11,805,529, because all samples showed hypomethylation at the points examined. Cases of CL/P can be caused by other factors (multifactorial), so research on the causes of CL/P needs to be continued. Much evidence suggests that *MTHFR* hypermethylation is a risk factor for various disease disorders in humans.^{10,20}

In this study, only methylation levels were detected at three methylation points in the promoter region of the *MTHFR* gene (coordinates 11,805,507–11,805,529), so further research is needed to determine the factors causing the decrease in *MTHFR* gene expression. Further research could be carried out to detect methylation at different locations on the *MTHFR* gene promoter or to look for mutations or methylation in other genes involved in the phosphate cycle, for example, the *DNMT* gene.²¹ This study used a limited number of patient samples. Due to this limitation, more cases are needed. In addition, the pyrosequencing method can only read fewer than 300 bp; therefore, examining methylation at different locations of the *MTHFR* gene promoter is required.

From the results, it can be concluded that detecting and examining *MTHFR* gene methylation in CL/P samples can be successfully carried out using the pyrosequencing method. Pyrosequencing can detect low levels of methylation, demonstrating that this method is very sensitive and accurate. Quantitative data in the form of percentages can be obtained with this method, in contrast to methods that only use PCR and electrophoresis. The conclusion that can be drawn is that in all samples examined, no methylation occurs in the promoter of the *MTHFR* gene with coordinates 11,805,507–11,805,529. Therefore, in this case, methylation at the three methylation points of the *MTHFR* gene promoter is not a factor causing CL/P. Other assays can be designed for the *MTHFR* gene to examine different methylation point locations. In addition, methylation point locations can be searched for in other genes such as the *DNMT* gene because this gene functions as a DNA methylation catalyzer.

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