MTHFR C677T rs1801133 GENE POLYMORPHISM AS A RISK FACTOR FOR NONSYNDROMIC CLEFT PALATE ONLY AMONG DEUTERO MALAY SUB RACE IN INDONESIA

(POLIMORFISME GEN MTHFR C677T rs1801133 SEBAGAI FAKTOR RISIKO CELAH LANGIT-LANGIT NON SINDROMIK SUBRAS DEUTERO MELAYU INDONESIA)

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ABSTRACT

Nonsyndromic cleft palate only (NS CPO) is a congenital malformation that arises from palatal fusion failure process at the seventh week of palatogenesis with a prevalence of 1:1000 live birth worldwide. The *MTHFR* C677T rs1801133 gene polymorphism is one candidate gene that can be associated with NS CPO. This study aims to determine the risk factor for NS CPO through MTHFR C677T rs1801133 analysis among the Deutero Malay subrace of the Indonesian population. This study was a case-control design, using samples from venous blood of 50 NS CPO subjects and 51 healthy control subjects. After DNA was extracted, followed by PCR, the PCR-RFLPs method was performed using the *Hinf*I restriction enzyme. The Chi-Square test was used with the Kolmogorov Smirnov and Exact Fisher alternatives. The results

showed that CT genotype (OR=2,960, p<0.05) of *MTHFR* C677T rs1801133 gene polymorphism can increase the risk of NS CPO. *MTHFR* C677T rs1801133 gene polymorphism is a risk factor associated with NS CPO among the Deutero Malay subrace in the Indonesian population.

Keywords: MTHFR C677T rs1801133; polymorphism

ABSTRAK

*Celah langit-langit non sindromik (CL NS) merupakan malformasi kongenital. yang terjadi karena kegagalan fusi prosesus palatal pada minggu ketujuh palatogenesis, dengan prevalensi 1:1000 kelahiran hidup. Polimorfisme gen MTHFR C677T rs1801133 merupakan salah satu kandidat gen yang dapat dihubungkan dengan kejadian CL NS. Tujuan dari penelitian ini adalah untuk menentukan faktor risiko CL NS melalui analisis polimorfisme gen MTHFR C677T rs1801133 pada subras Deutero Melayu populasi Indonesia. Desain penelitian ini adalah kasus kontrol, menggunakan sampel darah vena 50 subjek CL NS dan 51 subjek kontrol sehat. Setelah tahap ekstraksi DNA dan PCR, metode PCR-RFLPs dilakukan dengan menggunakan enzim restriksi Hinf*I*. Uji Chi-Square digunakan dengan alternatif uji Kolmogorov Smirnov dan Exact Fisher. Hasil penelitian menunjukkan bahwa genotip CT (OR=2,960, p<0.05) polimorfisme gen MTHFR C677T rs1801133 dapat meningkatkan risiko kejadian CL NS. Polimorfisme gen MTHFR C677T rs1801133 merupakan faktor risiko CL NS pada subras Deutero Melayu populasi Indonesia.*

Kata kunci: *MTHFR C677T rs1801133; polimorfisme*

INTRODUCTION

Craniofacial anomalies, including cleft palate only (CPO), are abnormalities that can arise from failure of the palatal fusion process beginning in the seventh week during palatogenesis.[1] This disorder occurs due to a partial or complete lack of palatal plate fusion, failure of the palate

plates to reach a horizontal position, lack of contact between the palate plates, and the occurrence of rupture after fusion of the palate plates.[2] CPO can occur syndromic as well as nonsyndromic. Syndromic CP (S CPO), representing 27,2% of the total CPO cases, is a disorder in the palate accompanied by abnormalities in other body parts. Nonsyndromic CPO (NS CPO) (54,8% of the total CPO cases) is a disorder that occurs in the palate without abnormalities in other body parts.[3,4] The prevalence of CPO is more common in Asians than in Africans. The majority of CPO varies in different parts of the world and can occur according to race and genetics.[5] In general, NS CPO occurs with a prevalence of about 1:1000 livebirths. It has a different frequency, depending on geographical area and socioeconomic level.[6,7] for example, Europeans have a majority of about 1:1000 livebirths[8,] and in Iran, there are 0.97:1000 live births.[9] In Indonesia, the prevalence of NS CPO is still unknown. The Deutero Malay subrace is the majority of subrace in Indonesia. The Deutero Malay population is spread in almost all parts of Indonesia except Gayo, Alas and Batak in Sumatra, Toraja in Sulawesi and Papua, which are included in the Proto-Malay subrace.[10]

The exact etiology of NS CPO is still unknown.[11] However, several studies suggest that NS CPO can be caused by a multifactorial factor, a combination of environmental and genetic factors.[12] One of the genes that have been believed to play a role in the occurrence of NS cleft lip and palate (CL/P) in general is the *Methylenetetrahyrodfolate reductase* (*MTHFR*) gene, especially in the C677T rs1801133 gene polymorphism. Polymorphism of the gene can be a risk factor for NS CPO, and gene mutation found in DNA can cause variations in more than 1 % of the population.[13] Polymorphism can determine susceptibility to disease and is not clinically manifest.[14] MTHFR gene, located on the short arm of chromosome 1 (1p36,3)[15], plays a role in folic acid metabolism. When there is a polymorphism, it may cause a defect in palatal development fusion.[16] *MTHFR* C677T rs1801133 polymorphism is the most investigated gene in exon four as a point mutation.[3,12,17] Some studies have been done to associate this polymorphism with NS CL/P across populations. A study by Xinjuan et al. found that the *MTHFR* C677T rs1801133 polymorphism was a risk factor for NS CLP.[5] The study conducted by Jyotsna et al. found that the study did not prove that the MTHFR C677T rs1801133 gene polymorphism was a risk factor for NS CL/P in South India.[18] Study by Xianrong Xu et al. found that the *MTHFR* C677T

rs1801133 gene polymorphism was not associated with the incidence of NS CL/P NS in the Chinese Uyghur population.[19] In Turkey, Ashlar et al. showed a significant association between NS CL/P and *MTHFR* C677T rs1801133 gene polymorphism (p=0.0004).[7] However, studies on the *MTHFR* gene as a risk factor for NS CPO with statistical significance have not been conducted that much. In a meta-analysis based on five studies, with 576 NS CPO cases and 2587 controls, Luo et al. found no statistical significance between *MTHFR* C677T rs1801133 gene polymorphism and NS CPO for heterozygous, neither for homozygous.[3,20]

CPO is one of the phenotypes of CL/P. It has been believed that there are different genetic roles and etiology between CL/P phenotypes, which include CPO, cleft lip (CL), unilateral CL/P and bilateral CL/P, so we are interested to analyze *MTHFR* C677T rs1801133 gene polymorphism in NS CPO among Deutero Malay subrace as largest subrace in Indonesian population to reveal the role of *MTHFR* gene as genetic etiology in NS CPO and considering that allele and genotype susceptibility in *MTHFR* C677T rs1801133 gene polymorphism may be different or have a similarity among different races or NS CL/P phenotypes, to obtain a better understanding of NS CPO based on molecular bases.

METHOD

This study was conducted with the approval of the Research Ethics Commission (KEP) from Universitas Padjadjaran with 1130/UN6.KEP/EC/2020. This study was done at the Laboratory of Molecular Genetics, Faculty of Medicine, Universitas Padjadjaran, Bandung Indonesia, from August until December 2020. The study design was a case-control and a molecular epidemiological study.

Subject of study

Sampling was done by consecutive sampling method by using 50 patients with NS CPO subjects and 51 healthy controls subjects who did not have a family history of NS CPO disorders. The sample was from venous blood and all subjects have been confirmed as Deutero Malay subrace. DNA was then extracted by using the manual method from Home Brew.

MTHFR C677T rs1801133 genotyping

The PCR mixture with a total volume of 25 µl consisted of 0.5 µl of DNA, one µl of forwarding primer, one µl of reverse primer, ten µl Nuclease Free Water and 12.5 µl PCR Mix from myTaq (Bioline). Then, the tube containing the PCR mixture was put into the Thermalcyler machine with PCR conditions. The

temperature of the denaturation stage was 93° for 1 minute, the annealing stage temperature was 59°, the extension stage temperature was 72° for 1 minute, the first cycle in the denaturation stage added time to 5 minutes, while the extension stage was added with a time of up to 3 minutes, and the total cycle was 35 cycles. The primers for *MTHFR* C677T rs1801133 gene polymorphism are forward (F): 5'-TGAAGGAGAAGGTGTCTGCGGGA-3' and reverse (R): 3'-AGGACGGTGCGGTGAGAGTG-5'.[17,21]

The PCR results were evaluated using 2% agarose gel electrophoresis. The 100 bp DNA ladder marker from the universal ladder used a DNA size marker. The amplified DNA fragments stained with ethidium bromide were then visualized using a UV transilluminator.

The optimal product was then digested by *Hinf*I restriction enzyme for PCR-RFLPs method. The PCR-RFLPs mixtures were incubated at 37^0C for 3-4 hours, then electrophoresed in a 3% agarose gel and visualized by etidium bromide staining. The CC genotype (normal homozygous) will show a single band of 201 bp, CT genotype (mutant heterozygous) will show 3 DNA bands of 201, 176 and 25 bp, and TT genotype (mutant homozygous) resulting in 2 DNA bands of 176 and 25 bp. The results of PCR-RFLPs will be evaluated by the Sanger sequencing method.

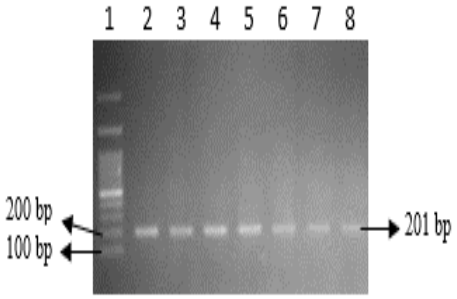
Statistical analysis

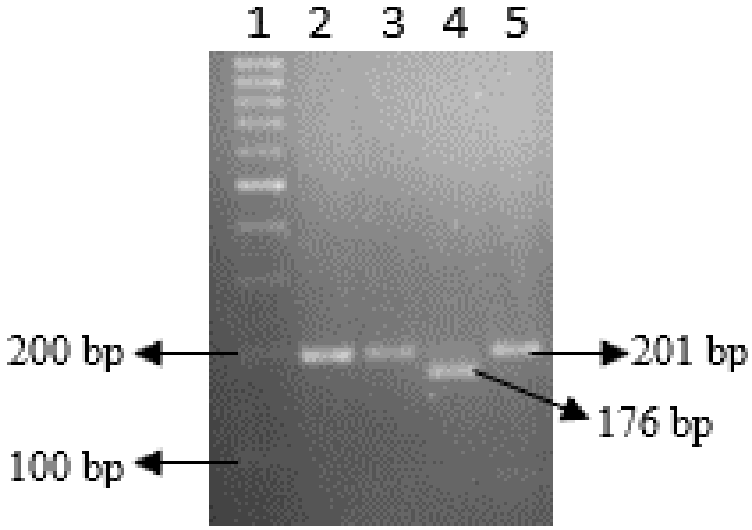
The data was from the examination results then processed descriptively. Numerical scale data are presented with the mean, standard deviation, median and range. Meanwhile, the categorical-scale data will be analyzed by unpaired T-test if the data is normally distributed and the Mann-Whitney test if the data is not normally distributed to test the significance of the comparison between the two groups' characteristics. The chi-square test was used to analyze the different alleles and genotypes frequencies in MTHFR C677T rs1801133 gene polymorphism in patients and controls. Meanwhile, to test the hypothesis of *MTHFR* C677T rs1801133 gene polymorphism as a risk factor of NS CPO, the odds ratio (OR) will be determined from the contingency table.

RESULT

The results of PCR products, PCR-RFLPs and DNA sequencing showed in Figures 1,2 and 3.

Figure 1. Optimal PCR product. Line 1. DNA marker (DNA Ladder) of 100 bp. Line 2-8 DNA bands from optimal PCR products (201 bp).





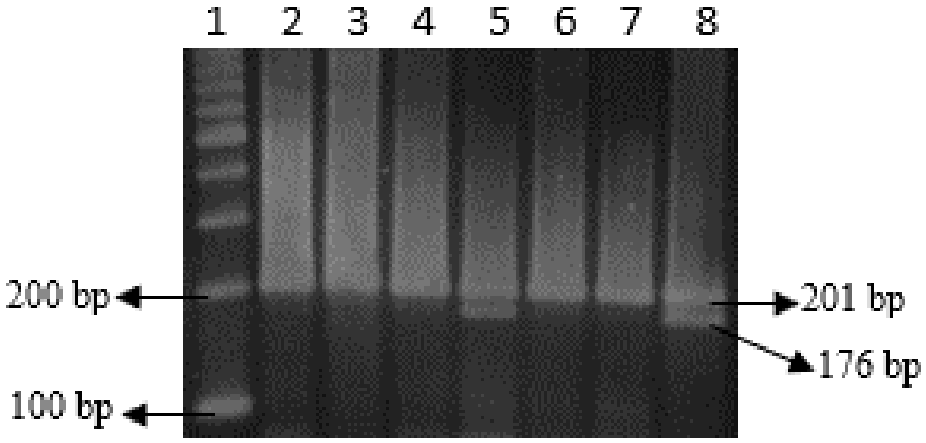


Figure 2. PCR-RFLP products by *Hinf*I restriction enzyme. A. Line 1 DNA marker (DNA Ladder) of 100 bp. Line 2,3,5 (201 bp). CC genotype. Line 4. TT genotype (176 bp and 25 bp or unseen). B. Line 5 and 8. CT genotype (201 bp, 176 bp, and 25 bp unseen).

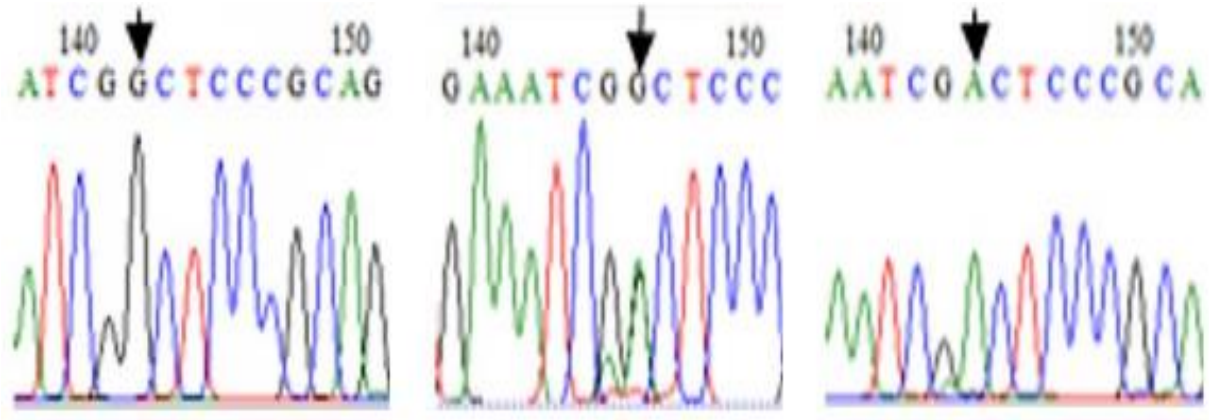


Figure 3. Sanger sequencing result. A. CC genotype. B. CT genotype. C. TT genotype.

The frequency of C and T alleles and CC, CT, and TT genotypes can be seen in Table 1. Comparison of each genotype can be seen in Table 2 (CC, CT and TT genotypes), Table 3 (CC and CT genotypes), Table 4 (CC and TT genotypes) and Table 5 (CT and TT genotypes).

Table 1. Allele and genotype frequencies

Variable	Group		OR	p	x^2
	NS CPO	Control			
C	63(63,0%)	75(73,5%)	0,613 (0,337-1,115)	0,108	2.586
T	37(37,0%)	27(26,5%)	0,613 (0,337-1,115)	0,108	2.586
CC	13(26,0%)	25(49,0%)	0,365 (0,158-0,844) ‡	0,017*‡	5,701
CT	37(74,0%)	25(49,0%)	2,960 (1,282-6,837)	0,010*	6,647
TT	0(0,0%)	1(2,0%)	0 (0,00-0,00)	1,000	0,980

C/Cytosine: wild type allele, T/thymine: mutant allele; CC: wild type genotype, CT: mutant heterozygous genotype TT: mutant homozygous genotype
* Statistically significant (p<0,05)
‡ Protective factor

Table 2. Comparison of CC, CT and TT genotypes between NS CPO and controls

Variable	Group		p
	NS CPO N=50	Control N=51	
CC	13(26,0%)	25(49,0%)	
CT	37(74,0%)	25(49,0%)	0,138
TT	0(0,0%)	1(2,0%)	

CC: wild-type genotype, CT: mutant heterozygous genotype, TT: mutant homozygous genotype

Table 3. Comparison of CC and CT genotypes between NS CPO and controls

Variable	Group		OR	p
	NS CPO N=50	Control N=50		
CT	37(74,0%)	25(50,0%)	2,846 (0,228-6,597)	0,013*
CC	13(26,0%)	25(50,0%)		

CT: mutant heterozygous genotype, CC: wild type genotype
* Statistically significant (p<0,05)

Table 4. Comparison of CC and TT genotypes between NS CPO and controls

Variable	Group		OR	p
	NS CPO N=13	Control N=26		
CC	13(100,0%)	25(96,2%)	0 (0,00-0,00)	1,000
TT	0(0,0%)	1(3,8%)		

CC: wild-type genotype, TT: mutant homozygous genotype

Table 5. Comparison of TT and CT genotypes between NS CPO and controls

Variable	Group		OR	p
	NS CPO N=37	Control N=26		
TT	0(0,0%)	1(3,8%)	0 (0,00-0,00)	0,413
CT	37(100,0%)	25(96,2%)		

CT: mutant heterozygous genotype, TT: mutant homozygous genotype

DISCUSSION

NS CPO is a complex and heterogenous congenital malformation with multifactorial etiology[22,23]. This malformation refers to any palate cleft that is posterior to the palatine foramen, which does not involve the alveolar process or lip.[3,4] In this study, the CT genotype was 2.960 times (p=0.017) at risk of causing NS CPO compared to controls (Table 1). It indicates that the *MTHFR* C677T rs1801133 gene polymorphism is

associated with NS CPO among the Deutero Malay subrace in the Indonesian population. However, the role of this polymorphism in NS CPO still cannot be explained yet. However, not much data has been found to reveal an association between *MTHFR* C677T rs1801133 gene polymorphism and an increased risk of NS CPO in other populations, especially in studies in which the subjects were from NS CP and control cases only. So far, much data was from maternal and triads compared with control subjects across different populations in Norway[24], France[25], United Kingdom[26] and Ireland[27] with no statistical significance. As a part of NS CL/P phenotypes, NS CPO has been believed to have a different genetic etiology from other NS CL/P phenotypes, so it is important to study NS CL/P genetic etiology based on each genotype. Different opinion has been concluded by Martinelly et al., as NS CPO are considered as different congenital malformations, having distinct embryologic origins and recurrence risk.[3]

The *MTHFR* C677T rs1801133 gene polymorphism causes a base substitution from C into T in the position of 677 base pairs that will eventually bring alanine substitution (GCU, GCC, GCA, GCG) into valine (GUU, GUC, GUA. GUG).[19] The *MTHF*R gene is involved in Folate and homocysteine (Hcy) metabolism3 and needs folic acid both for DNA synthesis and processing of B12 vitamin to convert HCy into methionine. Hcy is formed during the metabolism of methionine in cells. The folate-methionine reaction pathway plays a role in DNA synthesis, DNA methylation, and cellular oxidation-reduction balance. Methionine is used for the formation of protein derived from food. Hcy will indirectly cause oxidative stress through transcription and translation decrease and catalytic activity of glutathione peroxide (GPx) enzymes and superoxide dismutase (SOD). Hcy also has the potential to break the protein disulfide bonds that will change protein structure and or function.[29, 30, 31]

MTHFR is an important enzyme in folate metabolism which catalyze the conversion of 5, 10-methylenetetrahydrofolate to 5-methyltetrahydrofolate, the predominant circulatory form of Folate and the methyl donor for the remethylation of Hcy into methionine.[32] The *MTHFR* C677T rs1801133 gene polymorphism is a functional polymorphism[33,34] and was demonstrated to decrease enzyme activity[35] and folate distribution caused by impaired folate status. When this polymorphism interacts with low folate intake, disturbances in folate metabolism happen to cause an increase in Hcy levels, which is

dangerous for pregnant women with impacts such as abortion, placental infarction and fetal growth disorders.[36] Even though there is no strong association between folate supplementation during the periconceptional period and a decreased risk of NS CPO,[37] the folate pathway, with its enzyme and substrates, was suspected of having a role in NS CPO etiology and several studies evaluated associations between *MTHFR* C677T and NS CPO risk.[3] The base substitution in the *MTHFR* C677T rs1801133 gene polymorphism can cause an increase in Hcy so that the enzyme becomes damaged.[16,34] Based on the result of this study, CT genotype mutant heterozygous is supposed to be associated with decreased MTHFR activity, increased plasma Hcy levels, and lower plasma folic acid, which might contribute to NS CP. To analyze the role of each genotype, we tried to compare each genotype (Table 2-5) and especially to reveal the role of the GT genotype, which has a statistically significant result. The result showed that only when compared with the CC genotype, the CT genotype has a statistically significant result of 2,846 times at risk of causing CL NS (p= 0,013) (Table 3).

Based on previous studies, TT homozygous genotype is considered dangerous, causing high concentrations of total plasma Hcy that will increase the risk of congenital abnormalities such as NS CL/P, colorectal neoplasia and predisposition to adverse effects of drugs with antifolate effects.[33,34,38]

Decreased activity of the *MTHFR* gene will also reduce folate levels and produce hyperhomocysteinemia, increasing Hcy levels in the blood, resulting in blood blockage or atherosclerosis.[28] There is inhibition of endothelial nitric oxide synthesis by endogenous inhibitors, namely asymmetric dimethylarginine (ADMA), which causes endothelial dysfunction, which in turn causes disruption of fetal development and can be associated with NS CL/P. This condition may also affect the *MTHFR* gene to regulate the methionine synthesis process, which will affect the DNA methylation process, gene expression and the development process such as orofacial formation.[36] But it has a contrary with the result of this study which showed that TT genotype only found in one control subjects (Table 1 – 5), reveal that there was no exact role of TT genotype to increase total plasma Hcy in NS CP among Deutero Malay subrace in Indonesian population.

CONCLUSION

Based on the results of our study, *MTHFR* C677T rs1801133 gene polymorphism is a risk factor associated with NS CPO among the Deutero Malay

subrace in the Indonesian population.

CONFLICT OF INTEREST

The authors reported no potential conflict of interest.

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