Analysis of *FOXE1* rs4460498 and *GSTP-1* I105V associated with non syndromic cleft lip and palate among Deutero Malay Subrace in Indonesia

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ABSTRACT

Introduction: *FOXE1* rs4460498 and *GSTP-1* I105V gene polymorphisms are suspected of having a role in some of the non-syndromic cleft lip and palate (NS CLP) populations worldwide. This study aims to analyze *FOXE1* rs4460498 and *GSTP-1* I105V polymorphisms associated with NS CLP as the risk factor among Deutero Malay Subrace in Indonesia. **Methods:** This study was a case-control design, using samples from the venous blood of 102 NS CLP subjects and 102 healthy control subjects. After DNA was extracted, the PCR-RFLPs method was performed using TasI restriction enzyme on 100 blood samples of *FOXE1* rs4460498 group and Alw26I restriction enzyme on 105 blood samples of the *GSTP-1* I105V group. The Chi-Square test was used with the Kolmogorov Smirnov and Exact Fisher alternatives. **Results:** T mutant allele (OR= 0.926, p>0.05) and CT genotype (OR= 0.0, p>0.05) of *FOXE1* rs4460498 and the G mutant allele (OR= 0.988,p>0.05) and AG genotype (OR= 0.675,p>0.05) of the *GSTP-1* I105V are not the risks of NS CLP. **Conclusion:** *FOXE1* rs4460498 and *GSTP-1* I105V gene polymorphisms are not associated with non-syndromic cleft lip and palate among Deutero Malay Subrace in the Indonesian population.

Key words: deutero malay; *FOXE1* rs4460498; *GSTP-1* I105V; NS CLP

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INTRODUCTION

An orofacial cleft is a common congenital malformation in the craniofacial area which can include cleft lip (CL), cleft palate (CP) or cleft lip and palate (CLP).[1] CLP disorders are characterized by incomplete formation of the upper lip, palate caused by failure of normal fusion of the lip and palate at the midline during embryonic period.[2] The prevalence of CLP is estimated to be 1.5 per 1000 live births worldwide[3] and it can be different in each region depending on geographical

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conditions, racial groups and socioeconomic factors.[4] The continent of Asia has the highest prevalence of CLP among other continents.[5] The prevalence of CLP may vary among different countries or populations due to racial, climatic, cultural diversity, and differences in pregnant mother's treatment, and also the influence of different environment situations may create various risk factors.[6] The case of CLP can be in the form of syndromic (S) or non-syndromic (NS) based on the presence or absence of other organ malformations.[7,8]

The incidence of NS CLP cases is around 65%-70%, while the prevalence of syndromic cases is 30%. NS CLP disorder occurs more in the Asian population than in the African population.[7,8] Syndromic CLP (S CLP) is usually associated with the presence of other malformations or syndromes such as Stickler's syndrome, Van der Woude's syndrome and DiGeorge syndrome while NS CLP is not associated with other disorders,[9,10,11] and the cases are due to monogenic or Mendelian disorder.[12] The prevalence rate of NS CLP is estimated at 76.8% of a total of 5,918 cases of CLP, and 7.3% of cases were S CLP, this results may vary based on geographic area, ethnicity, and socioeconomic status.[13]

Impairment of highly complex process in craniofacial morphogenesis resulting in CLP, that is characterized by a failure of fusion of the frontonasal and maxillary processes and also palatal shelves of the maxillary processes during embryonic period.[14,15] The etiology of NS CLP is multifactorial with complex interactions between genetic and environmental factors.[16,17] Genetic factors are believed to be the main factor causing CLP.[10,18] There are candidate genes that are involved in NS CLP disorders, some of them are *FOXE1* rs4460498 and *GSTP-1* I105V gene polymorphisms.[7,19]

Forkhead Box E1(FOXE1) is located on chromosome 9q22.q33 which consists of 1 exon and is expressed transiently in the developing thyroid and the anterior pituitary gland. *FOXE1* rs4460498 polymorphism located in the downstream region and cause a substitution of base C into T (C>T),[20] which is a point mutation that form a change in a single base pair.[21] *The FOXE1 gene belongs to a family of transcription factors that contains a DNA-binding forkhead domain that can bind and open chromatin structures and can also aid the binding of transcription factors to DNA.*[3] *FOXE1* is known to play an important role in the formation of lip and palate during embryonic period, and overexpression of FOXE1 contribute to the formation of cleft palate (CP). This is based on experiments in mouse models that have been genetically modified by activating various components of the *FOXE1* gene resulting in abnormal development of the lips and palate, indicating an essential function of *FOXE1*.[22,23]

Glutathione S-Transferase P1 (GSTP-1) gene is located on chromosome 11, 11q13 which consists of 5 exons. *GSTP-1* I105V gene polymorphisms cause a substitution of base A to G at 313 base pair (bp) which will eventually result in substitution of isoleucine (ATT, ATC, ATA) to valine (GTT, GTC, GTA).[19,24] *GSTP-1* gene is the most important isoform at the embryonic development stage. Polymorphism in *GSTP-1* will cause a decrease in protein enzymatic activity and reduce the catalytic activity involved. will affect the enzymatic activity.[25]

FOXE1 rs4460498 and *GSTP-1* I105V polymorphisms have been studied among different populations with various results but have not been examined in Deutero Malay subrace among Indonesian population, so we are interested to study *FOXE1* rs4460498 and *GSTP-1* I105V polymorphisms associated with the risk of NS CL/P disorders in Deutero Malay Subrace among Indonesian population, which is the largest population in Indonesia. This study aims to analyze *FOXE1* rs4460498 and *GSTP-1* I105V polymorphisms associated with NS CLP as the risk factor among Deutero Malay Subrace in Indonesia.

METHODS

Subjects of study

Sampling was done by consecutive sampling method by using 102 patients with NS CL/P and 102 healthy controls without a family history of NS CL/P. among them, the PCR-RFLPs method was performed on 100 samples *FOXE1* rs4460498 gene group (50 NS CLP subjects and 50 control subjects) and 105 samples from *GSTP-1* I105V gene group (52 NS CLP subjects and 53 control subjects). All the sample was from venous blood, then DNA isolation was done by using the manual method from Home Brew.

***FOXE1* rs4460498 and *GSTP-1* I105V genotyping**

The PCR mixture with a total volume of 25 µl consisted of 1 µl of DNA, 1 µl of forward primer, 1 µl of reverse primer, 9,5 µl Nuclease Free Water and 12,5 µl PCR Mix was prepared.

Then, the tube containing the PCR mixture was put into the Thermalcycler machine with PCR conditions for both polymorphisms consist of: the denaturation temperature was 95°C for 1 minute, the annealing temperature was 51,5°C for 1 minute and the extension temperature was 72°C for 1 minute. The first cycle of denaturation was added up to 5 minutes, while the final extension was added up to 3 minutes, and the total cycle was 35 cycles. The primer for *FOXE1* rs4460498 was 5'ATTCCGCTGTATGTCTTGG3'(forward) and 5'TTTGTTGCTGGTTCCCTA3' (reverse)[22] and for *GSTP-1* I105V was 5'GTAGTTTGCCCAAGGTCAAG3' (forward) and 5'AGCCACCTGAGGGGTAAG3' (reverse).[25]

The optimal PCR results were evaluated using by 2% agarose gel electrophoresis. The 100 bp DNA ladder marker from the universal ladder was used as a marker of DNA size. The amplified DNA fragments that had been stained with Nucleic Acid Dye were then visualized by using UV transluminator. After optimal PCR results were obtained, PCR-RFLPs was perfomed by using the TasI restriction enzyme to evaluate the *FOXE1* rs4460498 polymorphism, and Alw26I to evaluate *GSTP-1* I105V polymorphism.

The PCR-RFLP mixtures of *FOXE1* rs4460498 were incubated at 65°C for 15 minutes and *GSTP-1* I105V were incubated at 37°C for 15 minutes. The results of PCR-RFLPs were re-evaluated by using 3% agarose gel electrophoresis. The results of PCR-RFLPs would be evaluated by the Sanger sequencing method.

Statistical analysis

The data was from examination results and processed descriptively, numerical scale data were presented with the mean, standard deviation, median and range. The categorical scale data will be analyzed by unpaired T-test if normally distributed and the Mann Whitney test if it is not normally distributed to test the significance of the comparison between two groups characteristics. To analyze allele and genotype frequencies of *FOXE1* rs4060498 and *GSTP-1* I105V polymorphisms between patient and control subjects, the chi-square test was used. Fisher's Exact test and the Kolmogorov Smirnov would be used for other alternatives.

The odds ratio (OR) would be determined from the contingency table to evaluate the risk factor of *FOXE1* rs4060498 and *GSTP-1* I105V gene in NSCL/P. Then, if $p \leq 0.05$, it means that it is statistically significant or significant, and if $p > 0.05$, it means that it is not statistically significant. This study was approved by Research Ethics Commission of Universitas Padjadjaran with the number of 988/UN6.KEP/EC/2021 and 987/UN6.KEP/EC/2021. This study was done at the Molecular Genetics Laboratory, Faculty of Dentistry, University Padjadjaran Bandung, from September until December 2021. This study is a molecular epidemiological study with case control design.

RESULTS

The results of thePCR products, RFLPs and DNA sequencing of *FOXE1* rs4460498 gene polymorphisms showed in Figure 1,2, and 3, also Figure 4,5 and 6 for *GSTP-1* I105V gene polymorphisms. The optimal PCR products and PCR-RFLPs results are described in Figure 1 for *FOXE1* rs446098 and Figure 4 for *GSTP-1* I105V. For *FOXE1* rs446098, the optimal PCR product was a single band of 315 base pairs (bp) and for *GSTP-1* I105V was a single band of 433bp. In Figure 2, PCR-RFLPs for *FOXE1* rs446098 resulted in the feature of CC genotype (wildtype) (236 and 79 bp),CT genotype (mutant heterozygous) (236, 196, 79 and 40 bp) and TT genotype (mutant homozygous) (196, 77, and 40 bp).

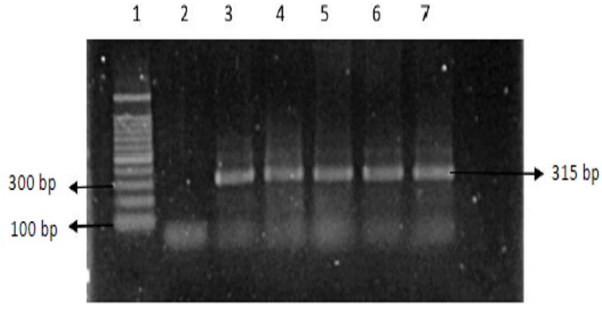

Figure 1. Optimal PCR product of *FOXE1* rs446098. Line 1 shows DNA Ladder of 100 bp. Line 2-7 DNA bands of optimal PCR products (315 bp)

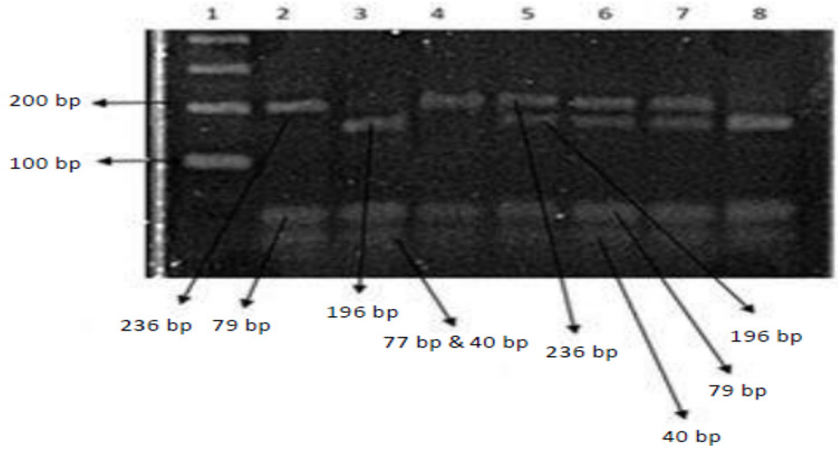

Figure 2 . Results of PCR-RFLPs of *FOXE1* rs4460498 by using TasI restriction enzyme. Line 1 shows DNA ladder of 100bp. Line 2 shows CC genotype (wildtype) (236 and 79 bp). Line 3 shows TT genotype (mutant homozygous) (196, 77, and 40 bp). Line 5 shows CT genotype (mutant heterozygous) (236, 196, 79 and 40 bp).

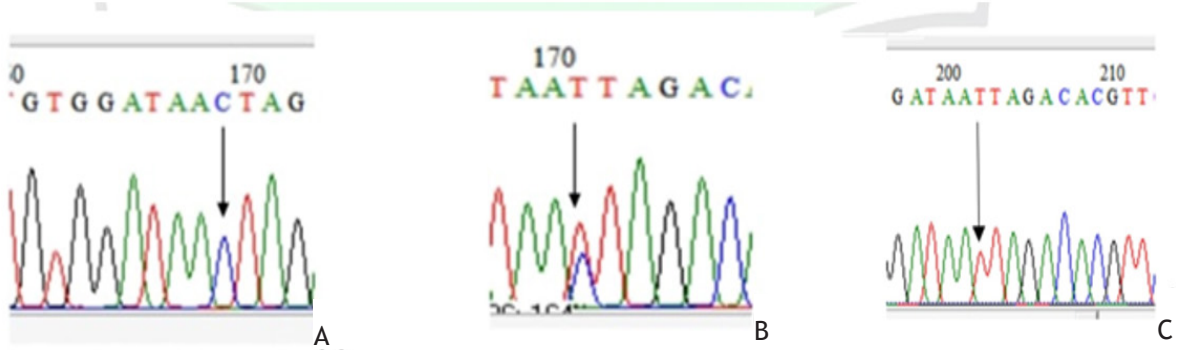

Figure 3. Sequencing results of *FOXE1* rs4460498: A. CC genotype (wild type); B. CT genotype (mutant heterozygous) C. TT genotype (mutant homozygous)

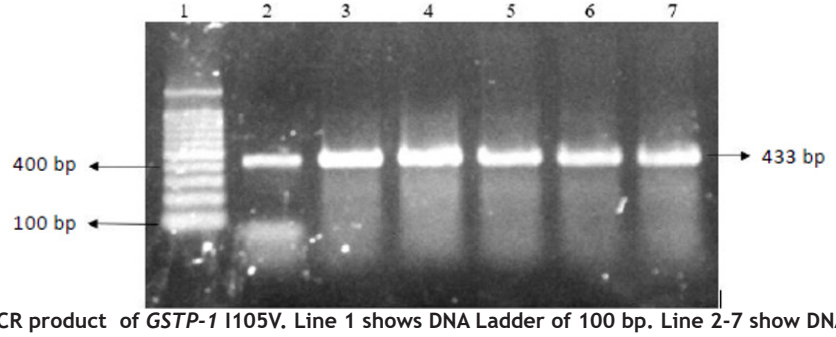

Figure 4. Optimal PCR product of *GSTP-1* I105V. Line 1 shows DNA Ladder of 100 bp. Line 2-7 show DNA bands of optimal PCR products (433bp).

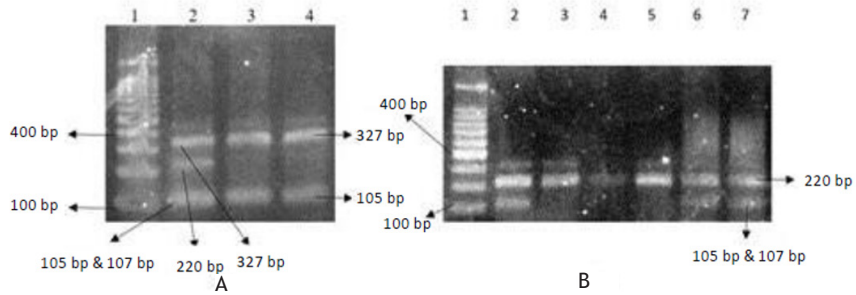

Figure 5. Results of PCR-RFLPs products by using Alw26I restriction enzyme. Line 1 shows DNA ladder of 100 bp. A. Line 4 shows AA genotype (wildtype) (327 bp, and 105 bp). Line 2 shows AG genotype (mutant heterozygous) (105 bp, 107 bp, 220 bp and 327 bp). B. Line 7 shows GG genotype (mutant homozygous) (105 bp, 107 bp, and 220 bp).

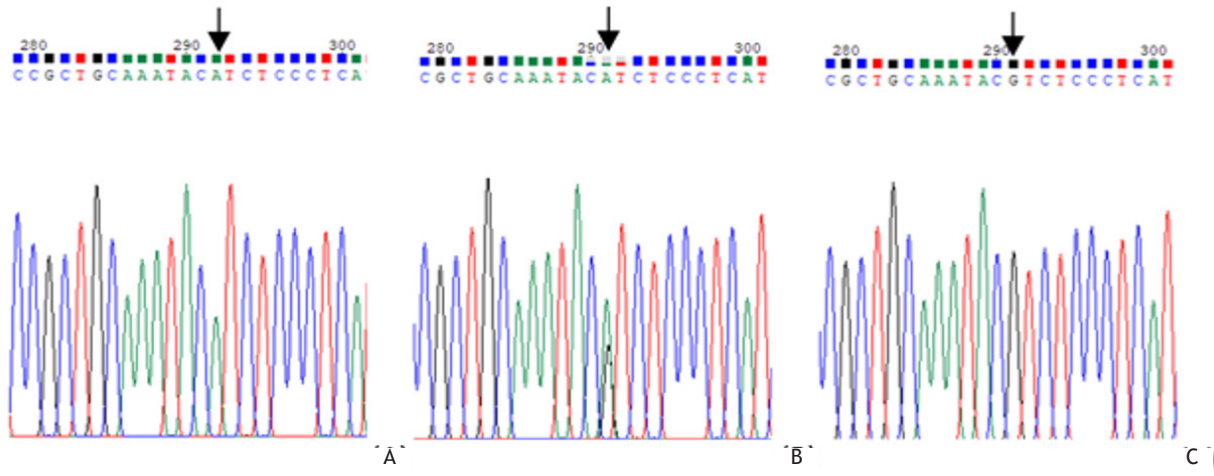

Figure 6. Sequencing results of *GSTP-1* I105V: A. AA genotype (wildtype); B. AG genotype (heterozygous mutant); C. GG genotype (homozygous mutant)

Table 1. Genotypes and alleles distribution for *FOXE1* rs4460498 and *GSTP-1* I105V in case-control analysis

		NS CLP (n=50) n(%)	Control (n=50) n(%)	OR CI (95%)	p value
***FOXE1* rs4460498**	**Genotypes**				
	CC	36(72.0%)	35(70.0%)	0.778 (0.319-1.895)	1.000*
	CT	12(24.0%)	15(30.0%)	0.0 (0.0-0.0)	
	TT	2(4.0%)	0(0.0%)	0.0 (0.0-0.0)	
	Alleles				
	C	84(84.0%)	85(85.0%)	0.926 (0.431-1.993)	0.845*
	T	16(16.0%)	15(15.0%)		
***GSTP-1* I105V**	**Genotypes**	**NS CLP (n=52) n(%)**	**Control (n=53) n(%)**		
	AA	30(57.7%)	27(51.9%)	1.263 (0.582 - 2.737)	1.000*
	AG	20(38.5%)	25(48.1%)	0.675 (0.309 - 1.472)	
	GG	2(3.8%)	0(0.0%)	0.00 (0.0-0.0)	
	Alleles				
	A	80(76,9%)	79(76,7%)	0.988 (0.518-1.883)	0.970*
	G	24(23,1%)	24(23,3%)		

p-value is not significant statistically (p>0.05) C/Cytosine (wildtype allele), T/Thymine (mutant allele), CC (wildtype genotype), CT (heterozygous mutant), TT (homozygous mutant); A/Alanine (wildtype allele), G/Guanine (mutant allele), AA (wildtype genotype), AG (heterozygous mutant), GG (homozygous mutant)

Table 2. Comparison of CC and CT genotypes of *FOXE1* rs4460498

Variable	Group		OR CI (95%)	P
	NS CLP	Control		
CC	36(75.0%)	33570.0%)	1.286 (0.528-3.132)	0.580*
CT	12(25.0%)	15(30.0%)		

***p-value statistically is not significant (p>0.05)**

Table 3. Comparison of CT and CC genotypes of *FOXE1* rs4460498

Variable	Group		OR CI (95%)	P
	NS CLP	Control		
CT	12(25.0%)	15(30.0%)	0.778	0.580*
CC	36(75.0%)	35(70.0%)	(0.319-1.895)	

*p-value statistically is not significant (p>0.05)

Table 4. Comparison of CT and TT genotypes of *FOXE1* rs4460498

Variable	Group		OR CI (95%)	P
	NS CLP	Control		
CT	12(85.7%)	15(100.0%)	0.0	0.224*
TT	2(14,3%)	0(0.0%)	(0.319-1.895)	

*p-value statistically is not significant (p>0.05)

Table 5. Comparison of CC and TT genotypes of *FOXE1* rs4460498

Variable	Group		OR CI (95%)	P
	NS CLP	Control		
CC	36(94.7%)	35(100.0%)	0.0	0.494*
TT	2(5.3%)	0(0.0%)	(0.0-0.0)	

*p-value statistically is not significant (p>0.05)

Table 6. Comparison of AA and AG genotypes of *GSTP-1* I105V

Variable	Group		OR CI (95%)	P
	NS CLP	Control		
AA	30(60.0%)	27(51.9%)	1.389	0,411*
AG	20(40.0%)	25(48.1%)	(0.634-3.045)	

*p-value statistically is not significant (p>0.05)

Table 7. Comparison of AG and AA genotypes of *GSTP-1* I105V

Variable	Group		OR CI (95%)	P
	NS CLP	Control		
AG	20(40.0%)	25(48.1%)	0.720	0,411*
AA	30(60.0%)	27(51.9%)	(0.328-1.578)	

*p-value statistically is not significant (p>0.05)

Table 8. Comparison of AG and GG genotypes of *GSTP-1* I105V

Variable	Group		OR CI (95%)	P
	NS CLP	Control		
AG	20(90.9%)	25(100.0%)	0	0,214*
GG	2(9.1%)	0(0.0%)	(0.0-0.0)	

*p-value statistically is not significant (p>0.05)

Table 9 Comparison of AA and GG genotypes of *GSTP-1* I105V

Variable	Group		OR CI (95%)	P
	NS CLP	Control		
AA	30(93.8%)	27(100.0%)	0	0.495*
GG	2(6.3%)	0(0.0%)	(0.0-0.0)	

*p-value statistically is not significant (p>0.05)

In Figure 5, PCR-RFLPs for MTHFR A1298C, resulted in the feature of AA genotype (wildtype) (327 bp, and 105 bp), AG genotype (mutant heterozygous) (105 bp, 107 bp, 220 bp and 327 bp) and GG genotype (mutant homozygous) (105 bp, 107 bp, and 220 bp). To examine PCR-RFLPs results from both polymorphisms, we performed Sanger sequencing method over some samples (Figure 3 and 6).

The distribution of alleles and genotypes of *FOXE1* rs4460498 and *GSTP-1* I105V in NS CLP and control groups are presented on Table 1 and 2. There was no significant association of both polymorphisms in NS CLP risk. In order to reveal the role of each genotypes, we did a comparison of each CC, CT and TT genotypes of *FOXE1* rs4460498 that can be seen in tables 2,3,4. and 5. Case-control analysis revealed no significant differences in genotype comparisons of CC and CT, CT and CC, CT and TT, CC and TT. Comparison of each genotypes AA, AG and GG of *GSTP-1* I105V can be seen in tables 6,7,8,and 9. Case-control analysis revealed not significant in comparison AA and AG, AG and AA, AG and GG, AA and GG ($p>0.05$)

DISCUSSION

According to our result, the T mutant allele (Table 1) and the CT heterozygous mutan and TT homozygous mutant genotypes (Table 4) were not significantly associated with NS CLP (p value > 0,05) . This indicates that the *FOXE1* rs4460498 gene polymorphism is not a risk factor for the NS CLP in Deutero Malay sub race in Indonesian population. This result was in contrary with a study conducted by Ludwig et al in 2014 in Central Europe and Mesoamerica Maya which was found that there were significant results between the *FOXE1* rs4460498 polymorphism and NS CLP abnormalities ($p=6.5 \times 10^{-5}$ and $p=0.015$).[26]

Study conducted by Liu et al in 2015 in Northeast China found that there were also significant results between the *FOXE1* rs4460498 polymorphism and NS CLP abnormalities ($p=0.006$).[22] A study conducted by Lammer et al in 2016 showed that there was a contribution of the *FOXE1* gene polymorphism to the incidence of NS CLP and NS CP in Hispanic and non-Hispanic populations in California.[27] These different study results revealed that the prevalence of *FOXE1* rs4460498 polymorphism may varies across geographic areas and ethnic groups means that there is also different role of this polymorphism associated with NS CLP among different population.

The *FOXE1* gene is very important in embryonic development and it is part of a family of transcription factors that contains a forkhead winged helix DNA binding domain and it is an intron-less single exon gene that encodes transcription factor FOXE1 (or Thyroid Transcription Factor-2 (TTF-2)).[7] *FOXE1* regulates transcription of the Thyroglobulin (TG) and Thyroid Peroxidase (TPO) genes by binding to specific regulatory DNA sequences in the promoter region via its forkhead DNA binding domain.[28] Genome-wide association study (GWAS) have related *FOXE1* with NS CLP in different populations. This *FOXE1* rs4460498 gene polymorphism is associated with a disturbance in *FOXE1* activity which can decrease DNA binding and transcriptional activity which in turn will disrupt embryonic development and prevent fusion of palate processes.

The *FOXE1* rs4460498 gene polymorphism can affect the specific expression pattern of *FOXE1* at the time of fusion between the maxillary and nasal processes which plays an important role in palatogenesis. This expression pattern is found in the oropharyngeal epithelium and the thymus.[7] The *FOXE1* gene also regulates 2 candidate NS CLP genes, namely the *MSX1* and *TGF3* genes.[22] Study by Venza et al demonstrated that *MSX1* and *TGFB3* genes can be upregulated in response to *FOXE1* at the transcriptional and translational levels as well as recruitment of *FOXE1* to specific binding motifs.[28] However, the role of FOXE1 rs4460498 in NS CLP among Deutero Malay subrace in Indonesian population can not be explained yet based on our study result.

In this study, G mutant allele (Table 1) and the AG and GG genotypes were not significantly risk factors for the incidence of CLP NS (p value > 0.05) (Table 8). This indicates that the *GSTP-1* I105V gene polymorphism is not a risk factor for the CLP NS in Deutero Malay subrace among the Indonesian population. In table 8 it was found that the 2 homozygous GG genotype was only found in the group of NS CLP patients but the results were not significant. This finding can also mean that the GG genotype homozygous mutant can indicate

that the GG genotype was still likely to have an influence on the incidence of CLP NS if the sample is larger. In a study conducted by Krapels I et al in the Netherlands, it was found that there were significant results between the *GSTP-1* I105V gene polymorphism with or without maternal smoking on NS CLP disorders.[25] In contrast to the study conducted by Lie RT et al in Norway, it was found that there was no significant change between the *GSTP-1* I105V gene polymorphisms and NS CLP disorders.[29]

The *GSTP-1* gene is the most important isoform at the embryonic development stage and *GSTP-1* I105V gene polymorphism cause the substitution of amino acids A to G, this substitution change will cause a decrease in protein enzymatic activity and reduce the catalytic activity involved. will affect the enzymatic activity. The *GSTP-1* I105V gene polymorphism as a risk factor of NS CLP is closely associated with smoking during pregnancy, smoking can affect the expression of genes involved in palatogenesis, such as matrix metalloproteinases (MMPs) or modify the concentrations of important transcription factors including folic acid. Teratogens in cigarette smoke include nicotine, polycyclic aromatic hydrocarbons (PAHs), arylamines, N-nitrosamines and carbon monoxide. These compounds absorb into the mother's blood and reach the fetus, however the mechanism by which cigarette smoke causes abnormal development is still poorly understood. The presence of developmental abnormalities in infants whose mothers smoked during pregnancy may be related to the level of exposure to teratogens in the fetus. Exposure may be related to number of cigarettes smoked, rate of placental and fetal transfer, and maternal and fetal metabolic biotransformation.[23]

Detailed information regarding the location and process of mutation in a gene is not yet fully known. Several mechanisms in DNA synthesis can be one of the suspects for the emergence of several abnormalities in humans. In this study the *FOXE1* rs4460498 and *GSTP-1* I105V gene polymorphism did not affect the risk during the development of NS CLP, therefore there was no influence of environmental and ethnic factors associated with the *FOXE1* rs4460498 and *GSTP-1* I105V gene. In this study, there was no association between the *FOXE1* rs4460498 and *GSTP-1* I105V gene and NS CLP abnormalities in the Indonesian Malay Deutero population.

CONCLUSION

FOXE1 rs4460498 and *GSTP-1* I105V gene polymorphisms are not associated as a risk factor of NS CL/P among Deutero Malay Subrace in the Indonesian population.

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