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TGFA TaqI Gene Variant and the Risk Factor of Non-Syndromic Cleft Palate only among Indonesian Patients

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Abstract : Non-syndromic cleft palate only (NS CPO) is one of the most common congenital malformations that affect between 1 in 1000 - 2500 live births worldwide. The etiopathogenesis of clefts including NS CPO has been widely studied, yet it remains unclear. NS CPO is considered as a genetically complex, multifactorial disease. Transforming growth factor alpha (*TGFA*) is a candidate gene that might contribute to the development of NS CL/P. The objective of this study was to detect *TGFA TaqI* gene variant and and analyze the risk of NS CPO among Indonesian patients. This study was case control design using samples from 33 NS CPO subjects and 31 control subjects. DNA was extracted from venous blood and the *TGFA* gene was amplified using polymerase chain reaction (PCR) technique, then digestion product from *Taq*I restriction enzyme were evaluated. Results showed that the *TGFA TaqI* gene variant was identified. The frequency of C2 mutant allele (odds ratio (OR) = 2.229; 95% CI = 0,746 – 6,654) and C2C2 genotype (OR = 1.935; 95% CI = 0,167 – 2,482) were associated with increased risk of NS CPO. In conclusion, *TGFA TaqI* gene variant can be considered to be the risk factor associated with NS CPO development in Indonesian patients.
Key words: non syndromic cleft palate only, *TGFA TaqI* gene variant.

Introduction

Cleft palate only (CPO) is the most common congenital malformation in orofacial region.[1,2] Overall, incidence of CPO is estimated to be 1 in 1000 – 2500 worldwide in which its prevalence varies depending on racial and ethnic backgrounds, geographic origin, and socioeconomic status.[3] Low incidences are seen among black people while the highest incidences are seen among American Indians, Japanese and Chinese.[1] Birth prevalence of CPO and CL/P is generally higher in Asian populations compared to European populations.[4] The exact prevalence of CPO in Indonesia is still unknown.

Non-syndromic CPO (NS CPO) is the common incidence in CPO occurence which affect as much as

50% of the overall CPO phenotypes.[1] NS CPO is lower than the incidence of left lip and palate (CL/P), but the opposite result has been found in few studies.[1] Non-syndromic oral clefts can be defined as complex traits, since they do not exhibit classic Mendelian recessive or dominant inheritance attributable to any single locus, but show strong familial aggregation and have a substantical genetic component.[2] It has been widely accepted that the risk of reccurence is <2 % if one child already has CPO, <6% if one parent has it and < 15% if one child and one parent have it, and increased to 50-60%. for a monozygous twin. [1]Numerous previous studies have suggested that many extrinsic factors might influence cleft formation. Thus, NS CPO is considered to be genetically complex, multifactorial diseases.[1]

The etiology and pathogenesis of cleft formation have been extensively studied but it is still poorly understood.[5,6] On the basis of mouse studies, cleft palate seems to be either a growth or a fusion failure of the secondary palate. NS CPO might be also due to mutations in several genes. Identification of the genes involved in the development of the human craniofacial region can serve as a first step towards developing a better understanding of diagnosis, treatment and preventions of developmental anomalies of this region. Genes and genes-environmental interactions have been believed to cause CL/P or CPO.[3] Palatogenesis is a complex process that involves many genes.[4] Several loci and genes have been suggested as candidates. The current list of candidate genes for NS CPO includes transforming growth factor alpha gene (*TGFA*), transforming growth factor beta 3 gene (*TGFβ3*), orofacial cleft 1 gene (*OFC1*), Drosophila muscle segment homeobox 1 gene (*MSX1*), retinoic acid receptor alpha gene (*RARα*), orofacial cleft 2 gene (*OFC2*) and orofacial cleft gene 3 (*OFC3*). In recent years, numerous reports indicate that gene variants of *TGFα* gene might also constitute risk factors for isolated forms of orofacial clefts, supporting its critical role in craniofacial development.[7]

The process of palatal fusion is also controlled by interactive signaling from the mesenchyme to the epithelium which is mediated by growth factors and extracellular matrix (ECM) proteins. One of the key components in this process is *TGFA* gene expression which coordinates palatogenesis.[8] *TGFA* encoded by a gene mapped at 2p13, is a secretion protein that binds to the epidermal growth factor receptor (EFGR) and is situated at the palate epithelium during palate closing.[9] *TGFA* is, both structurally and functionally, similar to Epidermal Growth Factor (EGF), and induces a mitogenic response by binding to and stimulating the tyrosine kinase activity of EGF receptor. During craniofacial development, *TGFA* is expressed at the medial edge epithelium of fusing palatal shelves. In palatal cultures, *TGFA* promotes synthesis of extracellular matrix and migration of mesenchymal cells to ensure the strength of the fused palate during seam disruption.[10] Etiologically, CL/P and CPO are separate congenital defects[11] and the role of *TGFA* seems to be more important in the etiology of CPO.

The *TGFA* gene shows a restriction fragment length polymorphism when treated with *Taq*I restriction enzyme. The mutant allele shows a four-base (TAAT) deletion. *TGFA Taq*I polymorphism is located at intron 5 and has 602 bp in the 59 direction of the acceptor site of exon 6.[9] According to meta-analysis study performed by Feng C et al through different ethnic populations indicates that there is strong association between the presence of *TGFA Taq*I gene variant and the risk of CL/P.[9] Previous study among Indonesian patients with NS CL/P showed that the *TGFA Taq*I gene variant was not associated with CL/P due to genetic differences in that population.[12] To date, study of *TGFA Taq*I gene variant associated with CPO among Indonesian has not been studied yet. According to the previous study done by Shiang R et al, a significant association between alleles of *TGFA* and CPO was identified which further supports a role for this gene as one of the genetic determinants of craniofacial development.[13] For this *TGFA Taq*I gene variant, C1C1 is wild type genotype, C1C2 is heterozygote genotype, and C2C2 is homozygote mutation genotype. *TGFA Taq*I gene variant associated with NS CPO gene may vary according to race and geographical region[3], so it is possible for NS CPO among Indonesian to have distinct characteristics of the *TGFA Taq*I gene variant.

Experimental

DNA isolation

The samples were collected from 31 NS CPO patients from Deuteromalayan race as the majority of race among Indonesian and 33 normal subjects from Deuteromalayan race without family history of craniofacial clefts. This study was conducted in Molecular Biology Laboratory, Medical Study Unit Faculty of Medicine Universitas Padjadjaran /Hasan Sadikin Hospital in Bandung. DNA was isolated from venous blood of each subjects using DNA isolation kit from Phamacia, then 200 ng of DNA template was using for Polymerase Chain Reaction (PCR).

Polymerase Chain Reaction

The segment of *TGFA TaqI* gene to be ampilified was in intron V 1602 bp upstream of the acceptor site of exon VI.[14]

PCR was performed by using the primers of forward AP7: TTGTTTTGTTTTTTGAGACGG : 5'-TATGTACATTTTCTTTGATCTCCCAGG-3' and reverse AP8: GTGAGACTTTTCCAGCCCTGT.[14]

RFLPs (restriction fragment length polymorphisms).

PCR products were digested for an hour with the specific restriction enzymes *TaqI* at 65^0C. The method adopted for PCR was RFLPs (restriction fragment length polymorphisms). The digested PCR products were separated into channels on a 3% agarose gel containing ethidium bromide in an electrophoretic chamber, and visualized with an ultraviolet transilluminator.

Results

The initial PCR product showed DNA band of *TGFA TaqI* gene segment and the size of this PCR product was 662 bp (Figure 1).

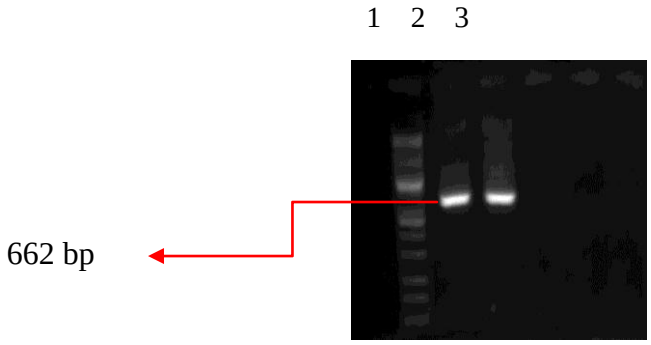

Figure 1. Initial PCR product of *TGFA TaqI* gene. Line 1. 100 bp ladder Line 2 –3. Initial PCR product

PCR products of *TGFA TaqI* gene (662 bp) were then digested with *TaqI* and generated wo restriction sites : normal sequence and polymorphism region. Two specific bands of 543 bp and 119 bp are for homozygous normal of C1C1 genotype. Deletion of 4 bases TAAT in a polimorphic sequence will create a new recognition site from *TaqI* restriction enzyme (TCTAATGA --> TCGA) and this allele is C2, as indicated by three specific bands of 349 bp, 194 bp and 119 bp. It will shows 4 fragments of 543 bp, 349 bp, 194 bp and 119 bp for heterozygous mutant of C1C2 genotype. PCR products of *TGFA TaqI* gene after restriction with *TaqI* and sequencing result can be seen in figure 2,3 and 4.

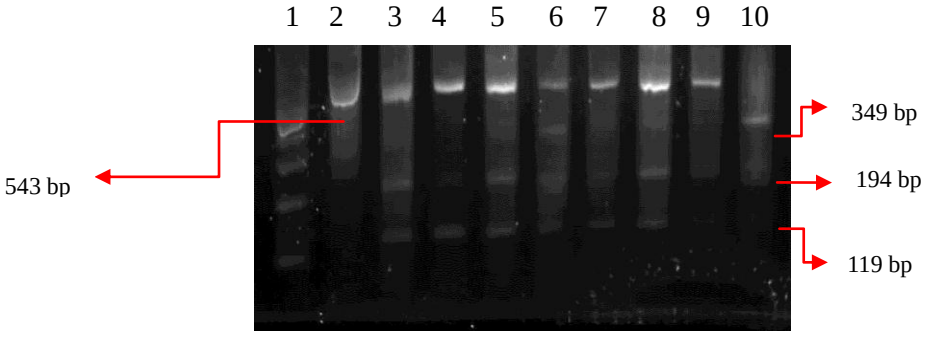

Figure 2. PCR products of *TGFA* gene after restriction with *TaqI* . Line 1. 100 bp ladder, Line 4. Homozygous normal of C1C1 genotype, Line 5,6,7 and 8. Heterozygous mutant of C1C2 genotype Line 10. Homozygous mutant of C2C2 genotype.

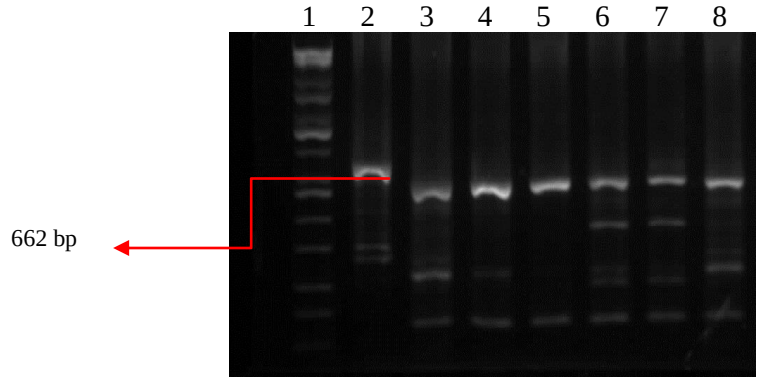

Figure 3. PCR products of *TGFA* gene after restriction with *Taq*I . Line 1. 100 bp ladder,Line 2. Uncut band for positive control. Line 3,4 and 8. Homozygous normal of C1C1 genotype. Line 6,7. Heterozygous mutant of C1C2 genotype,

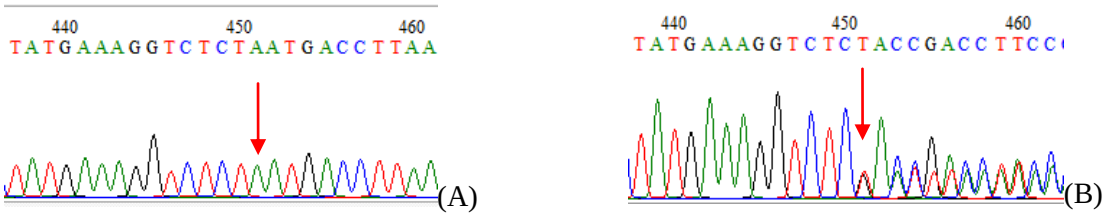

Figure 4. Sequencing result. A. The arrow shows homozygous normal of C1C1 genotype (no deletion of the base of TAAT). B. The arrow shows heterozygous mutant of C1C2 genotype.

Statistical analysis was done through all the subjects to compare allelic frequency of C2 mutant allele and C1 normal allele, as well as genotype frequency of homozygous normal of C1C1 genotype, heterozygous mutant of C1C2 genotype and homozygous mutant of C2C2 genotype, between NS CPO subjects and normal subjects using χ^2 analysis.

Statistical analysis of allelic frequency of C2 mutant allele and C1 normal allele from 33 NS CPO subjects and 31 normal subjects are shown in Table 1, and genotype frequency of homozygous normal of C1C1 genotype, heterozygous mutant of C1C2 genotype and homozygous mutant of C2C2 genotype from 33 NS CPO subjects and 31 normal subjects are shown in Table 2.

Discussion

Fewer analyses of the etiology of cleft palate only (CPO) have been performed, but a genetic component also appears important.[15] Genetic and developmental studies suggest that the formation of the primary palate (lip formation) and the secondary palate (palate formation) arise via different mechanisms.[15] Although the two processes are developmentally distinct, similar factors and mechanisms may be involved in the formation of both structures. Both processes involve the movement of mesenchymal cells and the closure of two separate regions by either apoptosis of epithelial cells or the transformation of epithelial cells to mesenchymal cells at the point of midline fusion.[13] Thus, it is possible that formation of the primary and secondary palates shares some of the same molecular developmental components.[13]

TGFA was selected as a candidate gene in the initial association studies of CL/P because of its expression in palatal tissue in culture. Based on previous study done by Ardinger et al. (1989), it was found an association between the *Taq*I and the occurrence of cleft lip and palate, suggesting that either the *TGFA* gene itself or the DNA sequences in an adjacent region contribute to the development of a fraction of cases of cleft lip and palate.[14]

Previous studies demonstrated that *TGFA* was present at high levels in epithelial tissue of the medial edge of the palatal shelves at the time of shelf fusion.[17] The role of *TGFA* in lip and palate development was then confirmed when allelic association in individuals with CL/ P was shown in Caucasian populations in widely separated geographic areas.[13] The association of CPO with the same alleles of *TGFA* that are in association with CL/P provides additional evidence that *TGFA* provides a common genetic mechanism influencing both primary and secondary palate development.

CPO is significantly associated with the *TGFA TaqI* gene variant based on the study done by Shiang R et. al through the Caucasians ethnicity in Iowa population.[13] This study also supported the theory which found that genetic and developmental differences have suggested that the mechanisms underlying CL/P and CPO are different. The sex distribution in the two is different, for example, with CL/P being more common in males, whilst CPO is commonly found in females. However, some common signals may affect formation of both the primary and secondary palate, even though they are embryologically distinct. Therefore, the same mutation in a single gene can affect primary and/or secondary palate formation. In that study, the association of CPO with the same alleles of *TGFA* that are in association with CL/P provides additional evidence that *TGFA* provides a common genetic mechanism influencing both primary and secondary palate development.[13]

Based on studies in various Asian populations, there were varying results regarding the association of the *TGFA Taq*I gene and NS CL/P subject. Studies among Filipino and Japanese populations found no significant differences between NS CL/P subject and control subject. However, a study by Zhang et al. (2004) in the Chinese population found significant differences between cleft and control subjects in frequency of the C2 allele.[18,19]

Most of previous studies have focused on CL/P and this study only focused on CPO cases. The *TGFA* gene shows a restriction fragment length polymorphism when digested with *Taq*I restriction enzyme then create a four-base (TAAT) deletion. For this *TGFA Taq*I gene variant, C1C1 is wild genotype (homozygous normal of C1C1 genotype), C1C2 is heterozygote genotype (heterozygous mutant of C1C2 genotype), C2C2 is homozygote mutation genotype (homozygous mutant of C2C2 genotype). This study results showed that the *TGFA Taq*I gene variant was identified. C2C2 is a rare allele and this study revealed that C2C2 is only found in the 3 CPO subjects, and it was different with the study done by Hwang et al. (1995) which interestingly found a rare homozygous allele (C2C2) in the patient and control samples in the same population.[18] In this study, the frequency of C2 mutant allele was 65,0% in NS CPO subjects and 35,0% in control subjects. This difference was not so significant statistically (χ^2=1,393; p > 0,05) but the frequency of C2 mutant allele (odds ratio (OR) = 2,238; 95% CI = 0,746 - 6,654) and homozygous mutant of C2C2 genotype (OR = 1,935; 95% CI = 0,167 - 22,482) were associated with increased risk of NS CPO as it means that the presence of C2 mutant alelle has a risk 2,238 times for the NS CPO and the presence of homozygous mutant of C2C2 genotype has a risk 1.935 times for the NS CPO, and this finding suggested that C2 allele might play a role in CPO among Indonesian patients.

Conclusion

In summary, *TGFA Taq*I gene variant was not associated with the etiology of NS CPO in Indonesian patients, but it can be considered as the risk factor associated with NS CPO development in Indonesian patients with NS CL/P.

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