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*TGFβ3 / SfaN*1 gene variant and the risk factor of nonsyndromic 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 but is still poorly understood. NS CPO is considered to be a genetically complex, multifactorial disease. Based on several studies, mutations of *TGFβ3* gene emerged as the strong candidate gene associated with NS CPO. The purpose of this study was to analyze the relationship between the *TGFβ3 / SfaN1* gene variant and the risk of NS CPO in Indonesian patients. This study was case control design using samples from 31 NS CPO subjects and 35 control subjects. DNA was extracted from venous blood and the segment of *TGFβ3* gene/ *SfaN*1 were amplified by using polymerase chain reaction (PCR) technique, then digestion products by *SfaN*1 restriction enzyme which can detect locus of gene variant / polymorphism from restriction fragment length polymorphisms (RFLP) method were evaluated. The results indicated that the gene variant as substitution of base G into A was identified in *TGFβ3* gene and the frequency of heterozygous mutant GA genotype was 63,6% in NS CPO subjects and 36,4% in control subjects. The frequency of heterozygous mutant GA genotype was associated with increased risk of NS CPO (odds ratio (OR) = 2,260, 95% CI = 0,592 - 8,625). In conclusion, *TGFβ3* gene / *SfaN*1 polymorphism can be considered as the risk factor associated with NS CPO in Indonesian patients.

Key words: Non syndromic cleft palate only; PCR-RFLP; *TGFβ3* gene; *SfaN*1; Gene variant.

Introduction

A genetic etiology for non-syndromic cleft lip with or without cleft palate (NS CL/P) and for non-syndromic cleft palate only (NS CPO) was first indicated in the population studies of Fogh-Anderson in 1942 (1, 2). Genetic and developmental studies suggest that the formation of the primary palate and secondary palate undergo different paths (2).

Cleft palate only/CPO or cleft in secondary palate is also one of the most common congenital malformations worldwide. It can appear as a part of a syndrome, with associated malformations or as isolated, non-syndromic cleft palate only (NS CPO). It has been estimated that about half of the cases are non-syndromic (3, 4). Almost 70% of human cleft palates are regarded as non-syndromic, whereas the approximately 30% remaining of cleft palate cases are syndromic (5, 6). Overall, incidence of CPO worldwide is estimated to be 1 in 1000 – 2500 with wide variability among races and regions (7). The exact prevalence of CPO in Indonesia is still unknown. CPO is usually classified into the following four categories: complete cleft palate with cleft lip; cleft of primary (anterior) palate, in which the cleft is limited to the anterior incisive fossa and may or may not involve cleft lip; cleft of the secondary (posterior) palate, in which the cleft defect is limited to the posterior incisive fossa; and submucosal cleft including a bifid uvula (6, 8). Multiple genetic and environmental factors are involved in etiology of cleft palate such as ethnic, racial, and geographic variations, socio-economic status, single-gene disorders, chromosome aberrations, and exposure to teratogens (alcohol, tobacco, anti-convulsants etc.) (6, 9-11).

In humans, some families with non-syndromic CPO show an autosomal dominant model of inheritance, yet the model is not clearly Mendelian in most cases. It has been widely accepted that the risk of recurrence is about 2 % if one child already has CPO, about 6 % if one parent has it and about 15 % if one child and one parent have it (4,12). For a monozygous twin the risk is 50-60 % (3, 4) These facts clearly show that CPO has a strong genetic component. Numerous previous studies have suggested that many extrinsic factors might influence cleft formation (4).

Table 1. PCR Components of *TGFβ3 / SfaN*1 Amplification.

Primer	Tm values (°C)	Amplicon lengths	Restricted products
forward: 5'-TGTCACTTTCCTTCCCTTCTTC-3'	47,9	326 bp	299 bp and 27 bp
reverse: 5'-TTCTTCCTGGAGATGTTTGTGA-3'	46,0		

Transforming growth factor beta 3 (*TGFβ3*) is one of the strongest candidate gene for oral cleft in humans (13-15). Animal models of clefting have focused on the formation of the secondary palate and have shown that extracellular matrix proteins and soluble factors such as epidermal growth factor, fibroblast growth factor, and transforming growth factor alpha (*TGFα*) and *TGFβ3* are important in palate formation (2). *TGFβ3* located at 14q24, has a broad spectrum of biological activities and is known to induce palatal fusion. In recent years, a large number of studies have been conducted to elucidate the relationship of *TGFβ3* and NS CL/P (15-19).

Kim et al and Ulucan et al examined the *TGFβ3* gene in Korean and Turkish NSCL/P patients and controls and significantly different in intron 5 of the gene, which was assumed as good marker for NSCL/P screening (16, 20) and these studies focused on *TGFβ3* IVS5+104AG variation as identified by *SfaN*1 restriction enzyme. Thus, this study aims to analyze the relationship between the *TGFβ3 / SfaN1* gene variant and the risk of NS CPO in Indonesian patients, considering the important role of *TGFβ3* gene in palatogenesis of the secondary palate.

Materials and Methods

Materials

The samples were collected from 31 NS CPO patients and 35 control subjects without family history of craniofacial clefts from Deuteromalay race as the majority of race among Indonesian. This study was done in Molecular Biology Laboratory, Medical Study Unit, Faculty of Medicine, Universitas Padjadjaran Bandung/ Hasan Sadikin Hospital.

Methods

DNA was isolated with informed consent 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) step. PCR was performed by using the primers (16) shown in Table 1. PCR products were digested with the specific restriction enzymes of *SfaN1* and incubated at 37^{0}C. The method adopted for PCR was RFLPs (restriction fragment length polymorphisms). The digested PCR products were separated into channels on a 1.5% agarose gel containing ethidium bromide in an electrophoretic chamber, and visualized in ultraviolet transilluminator.

Results

The initial PCR product showed DNA band of *TGFβ3 / SfaN*1 gene segment and the size of this PCR product was 326 base pairs (bp). After obtaining the initial PCR products of *TGFβ3* gene (326 bp), samples were then subjected to digestion with the specific restriction enzyme *SfaN*1. After digestion, the 326 bp products were completely digested with one restriction sites and two specific bands of 299 bp and 27 bp (could not be seen) for homozygous normal of GG genotype feature and for heterozygous mutant of GA genotype feature, it will show three specific bands of 326, 299 and also band of 27 bp. The mutant feature (homozygous mutant of AA genotype) will show the bands of 326 bp. The *SfaN*1 products were then confirmed by sequencing. PCR products of *TGFβ3* gene after restriction with *SfaN*1 and sequencing results can be seen in figure 1.

Statistical analysis through all the subjects was done to compare allelic frequency of G mutant allele and A normal allele and also to compare genotype frequency of homozygous normal of GG genotype, heterozygous mutant of GA genotype and homozygous mutant of AA genotype, in between NS CPO subjects and normal subjects by using χ^2 analysis.

Statistical analysis of allelic frequency of A mutant allele and G normal allele from 31 NS CPO subjects and 35 normal subjects shown in table 2.

Statistical analysis of genotype frequency of homozygous normal of GG genotype, heterozygous mutant of GA genotype and homozygous mutant of AA genotype from 22 NS CPO subjects and 43 normal subjects

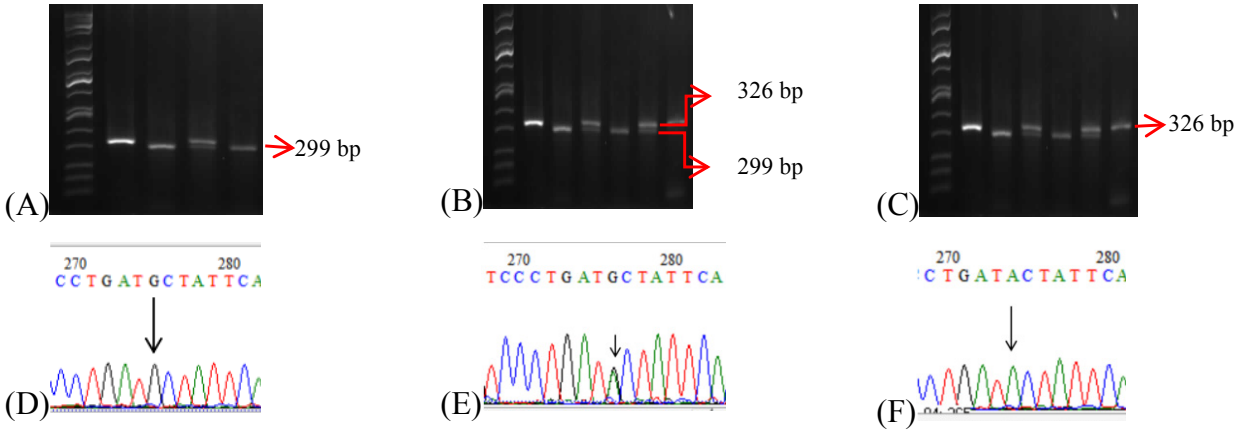

Figure 1. Digestion products from *SfaN*1 restriction enzyme to produce *TGFβ3 / SfaN*1 gene variant and sequencing results to confirm the digestion products from *SfaN*1 restriction enzyme in each genotype. (A) and (D) showed homozygous normal of GG genotype, (B) and (E) showed heterozygous mutant of GA genotype, (C) and (F) showed homozygous mutant of AA genotype.

Table 2. Allelic frequency of nucleotide G and A from *TGFβ3 / SfaN*1 gene variant (*TGFβ3* IVS5+104AG) in NS CPO subjects compared with normal subjects.

Allele	Subjects		χ^2	p	Odd Ratio	CI 95%
	NS CPO (%)	Normal (%)				
G	50,0	50,0	0,157	0,692	1,400	0,509 – 3,852
A	43,5	56,5	0,025	0,875	0,806	0,291 – 2,231

G: guanine (normal allele); A: adenine (mutant allele).

Table 3. Genotype frequency of nucleotide G and A from *TGFβ3 / SfaN*1 gene variant (*TGFβ3* IVS5+104AG) in NS CPO subjects compared with normal subjects.

Genotype	Subjects		χ^2	p	Odd Ratio	CI 95%
	NS CPO (%)	Normal (%)				
GG	25,0	75,0	0,903	0,342	0,333	0,062 - 1,790
GA	63,6	36,4	0,779	0,378	2,260	0,592 - 8,625
AA	49,2	57,1	0,037	0,847	0,784	0,276 - 2,226

GG: homozygous normal genotype; GA: heterozygous mutant genotype; AA: homozygous mutant genotype.

shown in table 3.

Discussion

Craniofacial malformations such as cleft palate are among the most common congenital birth defects in humans (22, 23). It is a failure of palatal fusion due to disturbance in palatogenesis. In humans, the palate develops from two primordiums, which are the primary and secondary palate (6). Palatogenesis of secondary palate is a complex process that involves multiple developmental events, including growth of the palatal primordia from the lateral edges of the maxillary process, reorientation of the palatal shelves from a vertical to a horizontal position, midline fusion of the palatal shelves, and disappearance of the midline epithelial seam (6,22). These processes encompass cellular activities such as cell proliferation, programmed cell death, and extracellular matrix (ECM) synthesis and remodeling, occurring in tissues that have already been patterned.

The *TGFβ* signaling pathway plays a crucial role in the development of these tissues and their interactions during palatogenesis (22). Palatogenesis involves fusion of the medial edge epithelium (MEE) of the approximating palatal shelves with each other via numerous desmosome contacts to form a midline palatal seam (15). The result of present study showed that the *TGFβ3 / SfaN1* gene variant was identified. The frequency heterozygous mutant of GA genotype was higher in NS CPO subjects compared to control subjects and the OR of heterozygous mutant of GA genotype was 2,260, 95% CI = 0,592 - 8,625, means that this GA genotype associated with increased risk of NS CPO 2,260 times. In recent years, a large number of studies have been conducted to elucidate the relationship of *TGFβ3* and cleft lip and palate (15-18). *TGFβ3* is found in the epithelial component of the shelves and in the MEE (15, 20). Later, the MEE stratify and keratinize to line the medial edge of the naturally cleft palate (24). *TGFβ3* plays a crucial role in these initial adhesive interactions (15, 25). *TGFβ3* knock out mouse exhibits cleft palate through failure of palatal shelf fusion (15, 26) Although the palatal shelves otherwise develop normally, they show a marked reduction in the filopodia and downregulation of condroitin sulphate proteoglycan on the apical surface of the MEE[15], both of which are required for efficient MEE adhesion (15, 19). Clefting anomalies also arise from dysregulation of the shelf elevation, attachment and fusion of mesenchymal and epithelial cells. During shelf elevation, *TGFβ3* regulates the proliferation of mesenchymal cells and changes in the extracellular matrix, and also regulates the shelf attachment of the epithelium by cell differentiation. During the fusion process, *TGFβ3* regulates epithelium-mesenchymal transformation (20, 27). Homozygous *TGFβ3*-null transgenic mice exhibited cleft palate (20, 28) and when mutation analyses of this gene were extended to clefting in humans, the results of these studies indicated the involvement of the *TGFβ3* gene in clefting (6, 20). Mice lacking *TGFβ3* exhibit an incomplete penetrant failure of the palatal shelves to fuse leading to cleft palate. The defect appears to result from impaired adhesion of the apposing MEE of the palatal shelves and subsequent elimination of the mid-line epithelial seam (27).

Many studies show positive correlation of *TGFβ3* gene polymorphisms in the etiopathogenesis of cleft lip and palate such as in south American population (14, 15) and Philippine population (15, 29). However, other studies did not show any significant association, such as in Japanese and Norwegian population (15, 30). Referring to studies by Kim et al and Ulucan et al, *TGFβ3 / SfaN*1 gene variant (*TGFβ3* IVS5+104AG) are a good marker for NS CL/P screening in Korean and Turkish population (16, 20). *TGFβ3 / SfaN*1 gene variant relative to the exon 5/intron 5 boundary, it may have an effect on splicing processes (20). Previous findings showed that signaling pathways including the *TGFβ3* are significant in palate development (20), so this study only focusing on NS CPO to analyze the relationship between the *TGFβ3 / SfaN1* gene variant and the risk of NS CPO in Indonesian patients.

In summary, *TGFβ3* gene / *SfaN*1 polymorphism can be considered to be the risk factor associated with NS CPO in Indonesian patients. However, the exact biochemical function of *TGFβ3 / SfaN1* gene variant in the etiology of NS CPO among Indonesian is not yet known, so further experimental studies are required.

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