**TGFβ3 / SfaN1 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 / SfaN1 polymorphism was identified using polymerase chain reaction (PCR) technique, then digestion products by SfaN1 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 / SfaN1 polymorphism can be considered as the risk factor associated with NS CPO in Indonesian patients.

**Key words:** Non syndrome cleft palate only; PCR-RFLP; TGFβ3 gene; SfaN1; 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).
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 SfaN1 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 Pharmacia, 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°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 / SfaN1 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 SfaN1. 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 SfaN1 products were then confirmed by sequencing. PCR products of TGFβ3 gene after restriction with SfaN1 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 χ² 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

### Table 1. PCR Components of TGFβ3 / SfaN1 Amplification.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Tm values (°C)</th>
<th>Amplicon lengths</th>
<th>Restricted products</th>
</tr>
</thead>
<tbody>
<tr>
<td>forward: 5’-TGTCACCTTTCTCCCTTCTTC-3’</td>
<td>47,9</td>
<td>326 bp</td>
<td>299 bp and 27 bp</td>
</tr>
<tr>
<td>reverse: 5’-TTCTCTCCTGGAGATGTTTGTGA-3’</td>
<td>46,0</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Figure 1. Digestion products from SfaN1 restriction enzyme to produce TGFβ3 / SfaN1 gene variant and sequencing results to confirm the digestion products from SfaN1 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.
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 primordia, 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 processes, reorganization 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 he laboratory found a significant association between the TGFβ3 / SfaN1 gene variant and the risk of cleft palate in Indonesian patients.

<table>
<thead>
<tr>
<th>Allele</th>
<th>Subjects</th>
<th>NS CPO (%)</th>
<th>Normal (%)</th>
<th>χ²</th>
<th>p</th>
<th>Odd Ratio</th>
<th>CI 95%</th>
</tr>
</thead>
<tbody>
<tr>
<td>G</td>
<td>50,0</td>
<td>50,0</td>
<td>0,157</td>
<td>0,692</td>
<td>1,400</td>
<td>0,509 – 3,852</td>
<td></td>
</tr>
<tr>
<td>A</td>
<td>43,5</td>
<td>56,5</td>
<td>0,025</td>
<td>0,875</td>
<td>0,806</td>
<td>0,291 – 2,231</td>
<td></td>
</tr>
</tbody>
</table>

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

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Subjects</th>
<th>NS CPO (%)</th>
<th>Normal (%)</th>
<th>χ²</th>
<th>p</th>
<th>Odd Ratio</th>
<th>CI 95%</th>
</tr>
</thead>
<tbody>
<tr>
<td>GG</td>
<td>25,0</td>
<td>75,0</td>
<td>0,903</td>
<td>0,342</td>
<td>0,333</td>
<td>0,062 - 1,790</td>
<td></td>
</tr>
<tr>
<td>GA</td>
<td>63,6</td>
<td>36,4</td>
<td>0,779</td>
<td>0,378</td>
<td>2,260</td>
<td>0,592 - 8,625</td>
<td></td>
</tr>
<tr>
<td>AA</td>
<td>49,2</td>
<td>57,1</td>
<td>0,037</td>
<td>0,847</td>
<td>0,784</td>
<td>0,276 - 2,226</td>
<td></td>
</tr>
</tbody>
</table>

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


