EXPRESSION OF TRANSFORMING GROWTH FACTOR ALPHA BamHI AND RsAI GENE VARIANTS ASSOCIATED WITH NON-SYNDROMIC CL/CPO OF INDONESIAN SUBJECTS USING POLYMERASE CHAIN REACTION METHOD

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ABSTRACT

Objective: This study aimed to detect and analyze transforming growth factor alpha (TGFA) BamH1 and Rsal gene variants which associated with the risk factor of non-syndromic cleft palate only (NS CPO) of Indonesian subject.

Methods: This was case-control study using samples from 32 NS CPO subjects and 28 control subjects. DNA was extracted from venous blood, and the TGFA gene was amplified using polymerase chain reaction technique, then digestion product from TaqI and Rsal restriction enzyme was evaluated. Statistical analysis to determine significant differences of gene variant frequency among NS CPO subject and control was χ². The odds ratio (OR) was used to determine a risk factor of NS CPO.

Results: The study results showed that the TGFA BamH1 gene variant was not identified in NS CPO among Indonesian but TGFA Rsal gene variant was identified. The frequency of TT/T1B1 homozygous mutant genotype was 80.0% in NS CPO subjects and 20.0% in control subjects (OR=3.857; 95% confidence interval=0.405–36.749).

Conclusion: TGFA Rsal gene can be considered a risk factor of NS CPO compared TGFA BamH1 gene of Indonesian subjects.

Keywords: Cleft palate, Non-syndromic, Gene variant, Transforming growth factor alpha BamH1, Transforming growth factor alpha Rsal.

INTRODUCTION

Orofacial clefts are defects of birth that caused by the disorder of anatomical, genetic, and embryological. They are subdivided into those affecting the lip and/or palate/primary palate (CL/P) and those involving the palate only/secondary palate (cleft palate only [CPO]) which has the distinct embryologic and pathophysiologic mechanisms of both types [1]. According to the physical and developmental anomalies, clefts categorized into syndromic and non-syndromic (NS) unto 70% CL/P and 50% CPO [2]. NS CPO reported one of the most common congenital malformations that affect between 1 in 1000 and 2500 live births worldwide [3].

The formation of the secondary palate (palatogenesis) in mammals involves the orchestration of several processes to produce the correct separation of the oral and nasal cavities. Failure of palatogenesis results in a cleft palate, one of the most common birth defects in humans [4]. Genetic and developmental differences have suggested that the mechanisms underlying CL/P and CPO are different from the formation of the primary palate and secondary palate [1,5]. The sex distribution in the two is different, for example, with CL/P being more common in males and CPO more commonly found in females. It is likely, however, that some common signals may affect the formation of both the primary and secondary palate, even though they are embryo-logically distinct [6]. The secondary palate in human begins to develop early in the 6th week from the two palatal shelves, which extend from internal aspects of the maxillary prominences [7].

The most factors of cleft palate have consumed the drug during pregnancy [8]. Dexamethasone is one of the drugs reported as the risk factor of cleft lip with or without cleft palate as well as other subsequent side effects on the child [9]. The other factors are gene mutation, including transcription factors, growth factor receptors, extracellular matrix components, and cell surface adhesion molecules also decreased malonylcarnitine [10]. Palatal fusion itself appears to be driven by several cell adhesion molecules, including nectin-1, desmosomes and Type IX collagen, and growth factors, such as transforming growth factor β3 (TGFB3) and TGF alpha (TGFA) were important to gene mutation in cleft palate cases [11]. TGFA was mapped the chromosome 2p13, comprises 80 kilobases of genomic DNA, and consists of six exons; it was selected as the candidate gene of CL/P, because its expression in palatal tissue in culture and its presence at high levels in epithelial tissue of medial edge of the palatal shelves at the time of shelf fusion [12]. TGFA alleles and NS CL/P were specifically in a Caucasian, US population, and Chilean Caucasian-Mongoloid, Japanese, and Vietnamese populations [13].

In general, TGFA has three genes of polymorphisms (Rsal, and TaqI in intron 5, and BamH1 in an exon. These genes investigated a susceptibility of CL/P [14], nevertheless it is debatable and contradictory [12]. Qian (1993) was reported who the evidence for TGFA TaqI gene variant association with NS CPO was shown in a Caucasian population and a West Bengali Indian population [13,14]. Our previous study (manuscript in preparation) showed that TGFA/TaqI gene variant can be considered to be the risk factor associated with NS CPO development.
in Indonesian patients that was associated with increased risk of NS CPO. Therefore, this research aimed to assess the TGFA BamHI and TGFA Rsal gene variants and the risk of NS CPO of Indonesian.

MATERIALS AND METHODS

Material

This research has approved with the ethical clearance No.395/UN6. C.13.2/KEPK/PN/2016 issued by Faculty of Medicine, University of Padjadjaran, Bandung, Indonesia. This study was used to 32 NS CPO subjects and 28 controls without family history of craniofacial clefts, both samples were from deutoro-malay race as the majority of race among Indonesian, and based on the molecular epidemiology of case-control study which is done in Molecular Genetic Laboratory, Faculty of Medicine Universitas Padjadjaran in Bandung, West Java, Indonesia. Venous blood samples were collected along with informed consent.

DNA extraction and polymerase chain reaction (PCR)

DNA extraction and running PCR were taken kits of Kapa Biosystems, Wilmington, MA, USA. DNA from venous blood was then extracted using DNA isolation standard method by Vemuri et al. (2016) and Bachtiar and Bachtiar (2017) [15,16]. Furthermore, TGFA BamHI and TGFA Rsal fragments were amplified with PCR that adapted by Omran et al. (2016) [17].

PCR for TGFA BamHI gene variant was performed using the primers of Forward/AP3: 5’-ACAGATGGGCAAGGAAAGGT-3’ and reverse/AP4: 5’-CTAAAAGCCCCAGACAGACAG-3’. PCR for TGFA Rsal gene variant was performed using the primers of forward/AP5: 5’TGGCTCACAGACAGACACA-3’ and reverse/AP6: 5’TGAATAACCCCAAGCAGACGG-3’. The PCR analyses have four cycles with temperatures 93°C, 1 min, annealing 57°C for AP4/AP5 primer; 58°C for AP5/AP6 primer; 1 min of each, extension 72°C, 1 min and PCR were running at 30 cycles [7,18]. Analysis for gene variant of TGFA BamHI and TGFA Rsal has been run by PCR - restriction fragment length polymorphism (RFLP) using the BamHI and Rsal restriction enzymes, respectively RFLP for TGFA BamHI and TGFA Rsal gene variant [16,19]. PCR products from TGFA gene were digested with BamHI and Rsal restriction enzymes at 37°C. PCR products were separated on a 2% agarose gel containing ethidium bromide and then visualized with an ultraviolet trans-illuminator [15-17].

Statistical analysis

A statistical analysis which was used to determine significantly (p<0.05) of differences from sequence variants frequency among NS CPO subject and control subject was $\chi^2$. The odds ratio (OR) was used to determine a risk factor of NS CPO.

RESULTS AND DISCUSSION

Statistical analysis

Statistical analysis of TGFA BamHI and TGFA Rsal gene variants was shown in Table 1. Whereas statistical analysis of genotype frequency of normal homozygous of C allele, the heterozygous mutant of CT genotype and a homozygous mutant of TT genotype shown in Table 2. In TGFA Rsal gene variant from this study, the frequency of homozygous mutant of TT genotype (OR=3.857; 95% confidence interval [CI]=0.405–36.749) will increase the risk of NS CPO for 3857 times compared with control subjects.

Cleft of secondary palate in the absence of a cleft lip (CPO) is etiologically distinct from the cleft lip with or without cleft palate (CL/P) on the basis of epidemiologic data, separate patterns of the risk of recurrence, and embryonic palate and lip formation [7]. The development of the secondary palate, or roof of the mouth, involves proliferation and differentiation of palatal epithelial cells as well as programmed cell death. The occurrence of cell death within the medial epithelial lamina between the fusing palatal processes assists in the removal of the medial palatal epithelium [18]. During the initial stage of the fusion process, the epithelial covering the tip of each palatal shelf (MEE) that surrounded by mesenchyme then undergoes dissolution and leaving confluent mesenchyme in the forming palate adhere to form a midline epithelial seam (MES) [4]. The MES disappear through the combination of programmed cell death, epithelial-mesenchymal transformation, and migration to the oral and nasal palatal epithelial failure of any of these processes can result in the isolated cleft palate [20].

Expression of TGFA BamHI and TGFA Rsal genes

The initial PCR product of TGFA BamHI gene variant showed DNA band segment with the size of 434 base pairs (bp) and TGFA Rsal gene variant size of 657 bp. (Fig. 1). TGFA BamHI gene variant means that there is substituting nucleotide of A (A2) into C (A1). After obtaining the initial PCR products of the segment of TGFA BamHI gene variant, samples were then digested with the specific restriction enzyme BamHI [21]. After digestion, the 434 bp products were completely digested with one restriction sites to create two specific bands of 313 and 121 bp for homozygous normal of AA (A2A2) genotype feature as can be seen in this present study (Fig. 2), for heterozygous mutant of AC (A1A2) genotype feature it will show three specific bands of 434, 313, and 121 bp and for the homozygous mutant of CC (A1A1) genotype feature.

In this study, we only found the feature of AA (A2A2) genotype from all subjects.

TGFA is a secretion protein that binds to the epidermal growth factor (EGF) receptor and is situated in the palate epithelium during secondary palate closing. GFA may function as a normal embryonic version of EGF-related growth factor [4]. EGF/TGFA and glucocorticoids are believed to regulate the proliferation and differentiation of palatal epithelial cells both in vitro and in vivo. Moreover, the continued presence of EGF inhibits the fusion process; TGFA is likely to have similar effects. These biological studies suggest that mutations in the TGFA gene might contribute to the development of CL/P, especially for those mutations that affect the timing of the tissue-specific expression of this gene [22].

TGFA was chosen as a candidate gene in the preliminary association studies of CL/P because it is expressed in palatal tissue in culture. In palatal cultures, TGFA promotes synthesis of extracellular matrix and mesenchyme cell migration, thereby ensuring the strength of the fused palate [21]. It subsequently revealed that TGFA was present at high levels in epithelial tissue of the medial edge of the palatal

### Table 1: Allelic frequency of C and T nucleotide of TGFA Rsal gene in NS CPO

<table>
<thead>
<tr>
<th>Allele</th>
<th>Subjects</th>
<th>$\chi^2$</th>
<th>p</th>
<th>OR</th>
<th>CI 95%</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>NS CPO</td>
<td>Control</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C</td>
<td>29</td>
<td>27</td>
<td>0.099</td>
<td>p&gt;0.05</td>
<td>1.164</td>
</tr>
<tr>
<td>%</td>
<td>70.7</td>
<td>67.5</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>T</td>
<td>12</td>
<td>13</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>%</td>
<td>29.3</td>
<td>32.5</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

C allele: Wild type allele, T allele: Mutant allele, P value based on hardy Weinberg proportion. NS CPO: Non-syndromic cleft palate only, OR: Odds ratio, CI: confidence interval, TGFA: Transforming growth factor alpha

### Table 2: Genotype frequency of C and T nucleotide of TGFA Rsal gene of NS CPO

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Subjects</th>
<th>$\chi^2$</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>NS CPO (%)</td>
<td>Control (%)</td>
<td></td>
</tr>
<tr>
<td>CC</td>
<td>12</td>
<td>13</td>
<td>1.068</td>
</tr>
<tr>
<td>%</td>
<td>37.5%</td>
<td>46.4%</td>
<td></td>
</tr>
<tr>
<td>CT</td>
<td>17</td>
<td>14</td>
<td></td>
</tr>
<tr>
<td>%</td>
<td>53.1%</td>
<td>50.0%</td>
<td></td>
</tr>
</tbody>
</table>

CC: Homozygous normal genotype, CT: Heterozygous mutant genotype, NS CPO: Non-syndromic cleft palate only, TGFA: Transforming growth factor alpha
shelves at the time of shelf fusion in the secondary palate which means the defect on the secondary palate will bring it into CPO [23]. Previous studies on TGFA have focused on CL/P, while the present study focused on CPO cases alone, based on the role of TGFA in palatal shelves fusion.

TGFA BamHI and TaqI polymorphisms are involved in oral cleft. Their results suggest that TGFA gene or adjacent DNA sequences may contribute to the development of a portion of cases with CL/P [22,24]. Ebaidar reported that the three variations of TGFA (BamHI, TaqI, and RsaI) in a British population with CL/P, and they found a significant association between the TaqI polymorphism and occurrence of cleft [12] Stoll (1993) detected a significant association with CL/P with TaqI in a French population of Alsatian ancestry with CL/P. They concluded that TGFA may be a modifier gene, not a major gene that may play a role in the development of bilateral cleft in some individuals [25]. Chenevix-Trench (1991) studied the two polymorphisms of TGFA in unrelated Australians with CL/P, and a significant association between the TGFA TaqI and BamHI polymorphism and CL/P was confirmed [26].

Shiang et al. (1993) reported that found TGFA possibly playing a role in a significant number of cases of NS CPO [27].

The TGFA Rsal gene variant, after digestion of Rsal restriction enzyme, the 657 bp products were completely digested with one restriction sites to create two specific bands of 373 and 284 bp for homoygous normal of GC (B2B2) genotype feature (Fig. 3), for heterogeneous mutant of CT (B1B2) genotype feature it will show three specific bands of 657, 373, and 284 bp (Fig. 3) and for homoygous mutant of TT (B1B1) genotype feature, the initial PCR products of 657 bp will not be restricted by Rsal.

The role of TGFA in lip and palate development has been evaluated in different populations with different results means that different phenotypes of CL/P may associate with different gene variants of TGFA which include three common polymorphisms or gene variants of the TGFA gene (TGFA RsaI, TGFA TaqI in intron 5, and TGFA BamHI in exon 5) [27]. In this study, we perform to be more specific in other CL/P phenotype that is focusing on CPO phenotype to be associated with TGFA BamHI and TGFA Rsal gene variants [12].

The TGFA gene shows RFLPs when treated with BamHI and Rsal restriction enzymes and creates TGFA BamHI in exon 6 and TGFA Rsal in intron 5 gene variants [28]. In this study, we performed to examine whether the TGFA BamHI (rs1466297 A/C) and Rsal (rs3732248 C/T) gene variants are associated with the increased risk of NS CPO among Indonesian patients including 32 NS CPO patients and 28 controls. Our results showed that TGFA BamHI polymorphism was not associated with the NS CPO in Indonesian population between the case and control groups, as all subjects showed no C (A1) mutant allele. In contrast, it was a difference in the allele and genotype frequencies of the TGFA Rsal which was found between the case and control groups. In TGFA Rsal, the frequency of the T (B1) mutant allele in the patients (57.1%) was higher than that of the control group (42.9%), the frequency of the Rsal homoygous mutant of TT (B1B1) genotype in the patients (80.0%) was significantly higher than that of the control group (20.0%), and the OR was 3.857; 95% CI = 1.450 – 9.074, and this result suggests that the TGFA Rsal may be a risk factor for NS CPO among Indonesian patients.

CONCLUSION

Finally, it can be concluded that TGFA Rsal can be considered a risk factor of NS CPO among Indonesian subjects compared TGFA BamHI gene. However, further research maybe needful the strategic to explore the agent of the cause of NS CPO.

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CONFLICTS OF INTEREST

The authors declare that there is no conflict of interest.
AUTHORS CONTRIBUTIONS

AMM carried out the conception, DNA extraction and PCR analyses also drafted the manuscript. Solid lipid nanoparticles, proprioceptive neuromuscular facilitation, and ESSS have been given the research ideas and design of research. BAG has arranged the manuscript, statistical analysis, and corresponding author. All of the authors were read and approved the final manuscript.

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