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Research Article

EXPRESSION OF TRANSFORMING GROWTH FACTOR ALPHA *BAMHI* AND *RSAI* GENE VARIANTS ASSOCIATED WITH NON-SYNDROMIC CLEFT PALATE 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*) *BamH*I and *Rsa*I 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 *Taq*I and *Rsa*I restriction enzyme was evaluated. Statistical analysis to determine significant differences of gene variant frequency among NS CPO subject and control was χ^2. The odds ratio (OR) was used to determine a risk factor of NS CPO.

Results: The study results showed that the *TGFA BamH*I gene variant was not identified in NS CPO among Indonesian but *TGFA Rsa*I gene variant was identified. The frequency of TT/B1B1 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 Rsa*I gene can be considered a risk factor of NS CPO compared *TGFA BamH*1 gene of Indonesian subjects.

Keywords: Cleft palate, Non-syndromic, Gene variant, Transforming growth factor alpha *BamH*I, Transforming growth factor alpha *Rsa*I.

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INTRODUCTION

Orofacial clefts are defects of birth that caused by the disorder of anatomical, genetical, 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 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 (TGFβ3) 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 (*Rsa*I, and *TaqI* in intron 5, and *BamH*I 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 studied (manuscript in preparation) showed that *TGFa/Taq1* 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 BamH*I and *TGFA Rsa*I 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. C1.3.2/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 deutero-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 BamH*I and *TGFA Rsa*I fragments were amplified with PCR that adapted by Omran *et al.* (2016) [17].

PCR for *TGFA BamH*I gene variant was performed using the primers of Forward/AP3: 5'-ACAGATGGCGGAACGAGAGGT-3' and *reverse*/AP4: 5'-CTAAAGGGCAAGGAAACACAG-3'. PCR for *TGFA Rsa*I gene variant was performed using the primers of forward/AP5: 5'-TGCCTCACCACGACAGACACA-3' and reverse/AP6: 5'-TGAATAACCCCAAGCAGACGG-3'. The PCR analyses have four cycles with temperatures 93°C, 1 min, annealing 57°C for AP3/AP4 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 BamH*I and *TGFA Rsa*1 has been run by PCR - restriction fragment length polymorphism (RFLP) using the *BamH*I and *Rsa*I restriction enzymes, respectively RFLP for *TGFA BamHI* and *TGFA RsaI* gene variant [16,19]. PCR products from *TGFA* gene were digested with *BamH*I and *Rsa*I 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 χ^2. The odds ratio (OR) was used to determine a risk factor of NS CPO.

RESULTS AND DISCUSSION

Statistical analysis

Statistical analysis of *TGFA BamH*I and *TGFA Rsa*I gene variants was shown in Table 1. Whereas statistical analysis of genotype frequency of normal homozygous of CC genotype, the heterozygous mutant of CT genotype and a homozygous mutant of TT genotype shown in Table 2. In *TGFA Rsa*I 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 risks 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 BamH*1 and *TGFA Rsa*I genes

The initial PCR product of *TGFA BamH*1 gene variant showed DNA band segment with the size of 434 base pairs (bp) and *TGFA Rsa*I gene variant size of 657 bp. (Fig. 1). *TGFA BamH*1 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 BamH*1 gene variant, samples were then digested with the specific restriction enzyme *BamH*1 [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 Rsa*I gene in NS CPO

Allele	Subjects		χ^2	p	OR	CI 95%
	NS CPO	Control				
C n	29	27	0.099	p>0,05	1.164	0.453–2.989
%	70.7	67.5				
T n	12	13				
%	9.3	32.5				

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 Rsa*I gene of NS CPO

Genotype	Subjects		χ^2	p
	NS CPO (%)	Control (%)		
CC n	12	13	1.068	p>0.05
%	37.5%	46.4%		
CT n	17	14		
%	53.1%	50.0%		

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* (*BamH*I, *Taq*I, and *Rsa*I) 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 *BamH*I and not with *Taq*I 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 Taq*I and *BamH*I polymorphism and CL/P was confirmed [26].

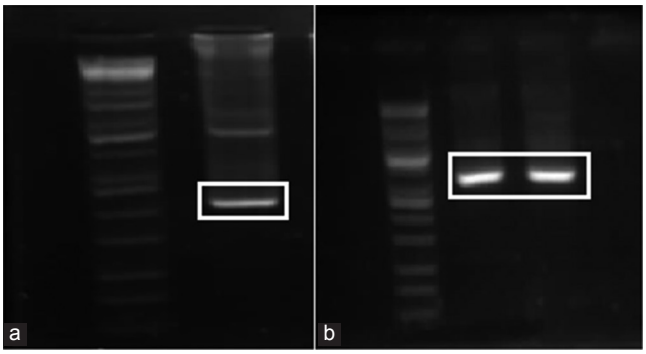

Fig. 1: Initial polymerase chain reaction product (a) Transforming growth factor alpha (*TGFA*) *BamH*1 gene variant of 434 bp and (b) *TGFA Rsa*I gene variants of 657 bp

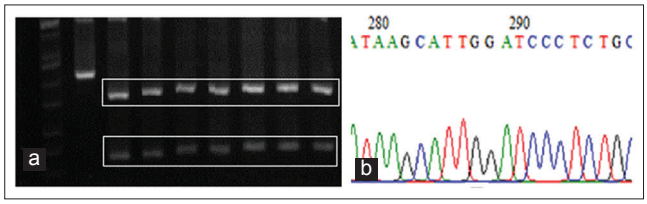

Fig. 2: (a) Polymerase chain reaction products of *TGFA BamH*1 gene after restriction with *BamH*I. Line 1. 100 bp ladder Line 3–9. The bands in the electrophoresis appearance show digested product of *BamH*I restriction enzyme (2 bands at 313 and 121 bp), which indicates normal homozygous l genotype AA (A2A2). (b) Sequencing result from *TGFA BamH*1 gene variant to confirm restriction site of *BamH*I. The arrow show A2A2 (base of A) homozygous normal genotype

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

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

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 Rsa*I, *TGFA* TaqI in intron 5, and *TGFA BamH*I in exon) [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 BamH*I and *TGFA Rsa*I gene variants [12].

The *TGFA* gene shows RFLPs when treated with *BamH*I and *Rsa*I restriction enzymes and creates *TGFA BamH*I in exon 6 and *TGFA Rsa*I in intron 5 gene variants [28]. In this study, we performed to examine whether the *TGFA BamH*I (rs11466297 A/C) and *Rsa*I (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 BamH*I 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 Rsa*I which was found between the case and control groups. In *TGFA Rsa*I, 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 *Rsa*I homozygous 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=0.405–36.749, and this result suggests that the *TGFA Rsa*I may be a risk factor for NS CPO among Indonesian patients.

CONCLUSION

Finally, it can be concluded that *TGFA Rsa*I can be considered a risk factor of NS CPO among Indonesian subjects compared *TGFA BamH*1 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.

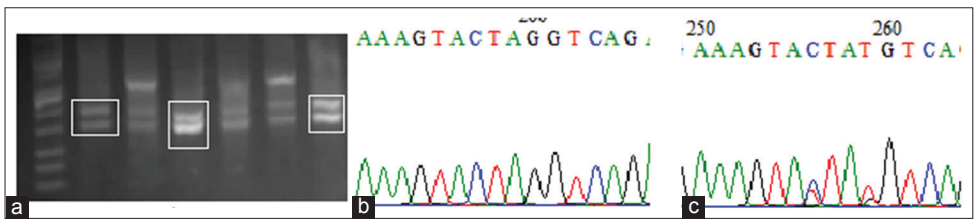

Fig. 3: (A) Polymerase chain reaction products of *TGFA Rsa*I gene after restriction with *Rsa*I. Line 1 100 bp ladder Line 2, 4, 7. The bands in the electrophoresis appearance show digested product of *Rsa*I restriction enzyme (2 bands at 373 and 284 bp), mean as B2B2 homozygous normal genotype. Line 3,5,6. B1B2 heterozygous mutant genotype (3 bands of 657, 373, and 284 bp. (b) Sequencing result from *TGF/Rsa*I gene variant. The arrow show B2B2 (base of C) homozygous normal genotype. (c) Sequencing result from *TGFA Rsa*I gene variant. The arrow show B1B2 (base of C and T) heterozygous mutant genotype

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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