BMP4 SNP Rs17563 T>C Gene Polymorphism on Non-Syndromic Cleft Lip/Palate in an Indonesian Population

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Abstract

Cleft lip with or without cleft palate (CL/P) is one of the most common congenital abnormalities with a complex etiology. The prevalence of these cases varies according to ethnicity and place. One of the candidate genes associated with non-syndromic cleft lip with or without cleft palate (NSCL/P) incidence is the Bone Morphogenetic Protein 4 (BMP4) gene. BMP4 is a candidate gene that is thought to play a role in the incidence (CL/P) through signal polarization. This study aimed to analyze the genotype of BMP4 rs17563 T>C with the incidence (CL/P). The research method used was polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP). The number of DNA samples analyzed by single-nucleotide polymorphism (SNP) was 70 samples, consisting of 34 patient samples and 36 control samples. The statistical analysis used was the Fisher's exact test to analyze the significance of the difference in frequency in the two groups. The results show that there is a variation in the genotype of BMP4 polymorphism rs17563 T>C, but there is no significant difference between genotypes or alleles of NSCL/P patients and healthy individuals among the sampled Indonesian population.

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Introduction

One of the most common orofacial deformities is a cleft lip with or without a cleft palate (CL/P).[1] A cleft lip is an abnormality in the form of a gap in the upper lip. This gap can extend to the gums and palate which is formed in the first trimester of pregnancy. This abnormality occurs because the mesoderm is not formed in the area, thus the nasal and maxillary processes that have united become separate.[2] This abnormality indicates the failure of the two parts of the lips to join together.[3] A cleft palate is a cleft in the palate that can extend to the hard palate and soft palate into the nasal cavity.[4] A cleft palate occurs due to failure of the unification of the lateral palatine process, nasal septum, and median palatine process.[5]

Factors that influence the incidence of non-syndromic cleft lip with or without cleft palate (NSCL/P) are multifactorial, which means it is influenced by genetic and non-genetic factors.[6,7] In this case, it is the environment that is modified by external agents, as well as environmental conditions including the age of parents, drug consumption during pregnancy, smoking, stress, trauma, nutritional intake, and infection.[7] Differences in ethnicity and sex as well as the relatedness of genetic factors have an influence on the prevalence rate for this incidence.[1,8] It is known that the influence of geographic origin indicates a different prevalence between populations. This variation causes the Asian population to have the highest prevalence with 1/500 births and the African population to have the lowest of this incidence, with 1/2500 births, whereas the Caucasians have the prevalence of 1/1000 births.[9]

On the genetic aspect, there are many genes involved with CL/P incidence. Some genes

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involved function as growth factors like TGF-α and TGF-β as well as transcription factors, namely MSX1, IRF6, and TBX22. Xenobiotic metabolism consists of CYP1A1, GSTM1, NAT2; meanwhile, nutrient metabolism consists of MTHFR and RARA. In the immune response, there are PVRL1 and IRF6; and the ones function as signal polarization are BMP2 and BMP4.[6,10,11] Growth factors are required in the process of cell signaling, epithelial cell differentiation, and palate cleft remodeling.[4,12] Bone Morphogenetic Protein (BMP) is a TGF-β superfamily that plays important roles in a number of development processes and the formation of various craniofacial elements, including cranial neural crest, facial primordium, teeth, lips, and palate.[13,14] The disruption of the canonical BMP signal pathway results in inhibition of the signaling process and decreases in BMP levels, thus it can interfere with SMAD protein activation, namely the SMAD 1,5,8 and SMAD 4 protein complexes, which will translocate to the nucleus to be bound to transcription factors – regulating gene expression.[13]

Polymorphism of the BMP4 gene shows a changed amino acid, namely valine to alanine.[15] Hao et al. (2018) conducted a study to find a link between the BMP4 gene polymorphism and the incidence of cleft lip with or without cleft palate in the South Chinese population. The results obtained from 165 patients and 52 healthy individuals show that the BMP4 gene polymorphisms rs762642, rs17563, and rs10130587 gave different results for each NSCL/P phenotype and showed that these phenotypic differences were thought to have different etiologies.[16] Polymorphism of the BMP4 rs17563 T>C gene in Indonesia has not been studied much further in molecular biology-based research. Therefore, hopefully, the results of this study can contribute to the development of existing science.

Materials and methods

This study involved 70 samples divided into the patient and control groups. The CL/P patient group consisted of 34 samples and the control group consisted of 36 samples. DNA samples were extracted from peripheral blood, and then, using Promega A1120Wizard® Genomic DNA Purification Kit, DNA samples were extracted from blood samples and stored at -20°C at the Human Genetic Laboratory, Institute of Tropical Diseases, Universitas Airlangga. This study was approved by the ethical committee of the Faculty of Dentistry, Universitas Airlangga, number 606/HRECC.FODM/IX/ 2019.

Polymorphisms of the BMP4 SNP gene rs17563 T>C were analyzed using the PCR-RFLP method. Specific forward and reverse primers made by Integrated DNA Technologies were forward 5'-CCTAACTGTGCCTAG-3' and reverse 5'-CATAACCTCATAAATGTTTATACGG-3'. The resulting 197 bp PCR fragment was amplified using a 25 µl reaction mixture containing 12.5 µl Promega Go Taq™ Master Mixes, 2.5 µl (10 µmol) forward primer, 2.5 µl (10 µmol) reverse primer, and 7.5 µl template and amplified with the BioRadCycler PCR machine. The procedure of using the PCR machine was operating it at 54°C for 1:00 minute for 33 cycles followed by elongation at 72°C for 1:00 minute. Electrophoresis was conducted using 2% of agarose gel (Promega) at 100 V, for 30 minutes.

After successfully electrophoresed with agarose, the samples whose bands were visible were cut by the RFLP method using HphI restriction enzyme from Thermo Scientific. The mixture of RFLP formula with PCR results was incubated at 37°C for 3 hours. After that, this was followed by enzyme inactivation at 80°C for 20 minutes. To see the visualization, the results of the RFLP band were electrophoresed at 100 V for 35 minutes. The gel that had been electrophoresed was viewed in UV light and documented, and then read the results.

There are three variations of the genotype results, namely 197 bp for wildtype homozygote (TT), 197 bp, 110 bp, and 87 bp for mutant heterozygote (TC), and 110 bp and 87 bp for mutant homozygote (CC). All statistical analysis weas performed using SPSS Inc., (IBM Corporation, NY, USA) Statistics Version 16.

The Fisher's exact test was used to analyze the distribution of genotypes and allele between two groups. Genotype and allele frequencies were calculated and assessed by the Hardy Weinberg Equilibrium, where a p-value <0.05 was considered to be statistically significant in all groups.

Results

Examples of PCR products and RFLP

results in this study are depicted in Figure 1 and 2, respectively. This study consists of 70 samples taken from the Indonesian population, 34 of them were patients suffering from CL/P and 36 others were healthy individuals as control samples. The genotype distribution between the patient and control groups for BMP4 SNP gene polymorphism rs17653 does not differ significantly (p>0.05) (Table 1).

Type		Group		*P* value
		CL/P	Control	
Genotype	TT	17 (50%)	20 (55,6%)	0.935
	TC	14 (41,2%)	13 (36,1%)	
	CC	3 (8,8%)	3 (8,3%)	
	Total	34 (100	36 (100)	
Allele	T	48 (12%)	53 (75%)	0.834
	C	20 (88%)	19 (76%)	

Table 1. Distribution of the genotypes and alleles of BMP4 rs17563 T>C polymorphism in the CL/P and control groups.

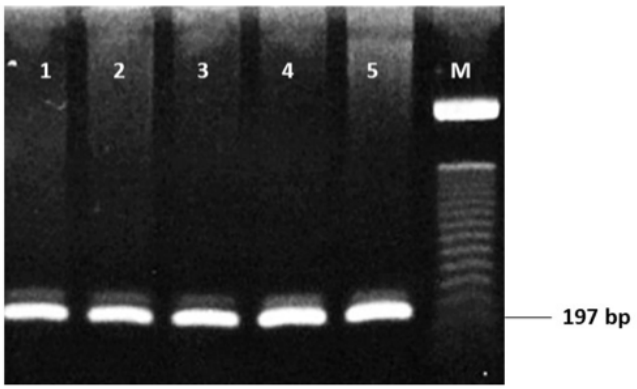

Figure 1. Visualised PCR products of BMP4 rs17563 T>C; lanes 1, 2, 3, 4, 5 show bands at 197 bp, and lane 6 is a 50-bp DNA ladder marker.

Discussion

The BMP4 gene encodes the ligands that are secreted from the TGF-β superfamily.[13] The ligands of this superfamily bind to various TGF-β receptors that play a role in the activation of transcription factors, especially the SMAD protein in regulating gene expression.[14] Mutations in this gene are associated with orofacial and microphthalmia deformities.[17] The encoded protein may also be involved in the pathology of several cardiovascular diseases and cancers in humans.[18] The BMP4 gene has a complete DNA sequence of 11.2 kb, consisting of four exons, located on chromosome 14q22.2 (on the long arm of chromosome 14 and area 2, band 2, and sub band 2).[19]

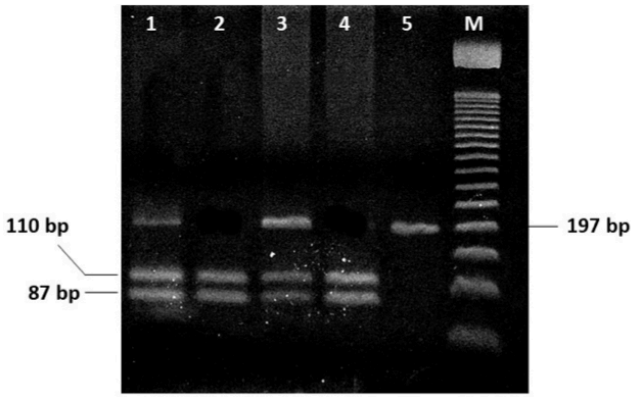

Figure 2. The results of RFLP product after electrophoresis, lane 1, 4 and 5 indicating CC, TC, and TT genotype. Lane M: 50 bp ladder marker.

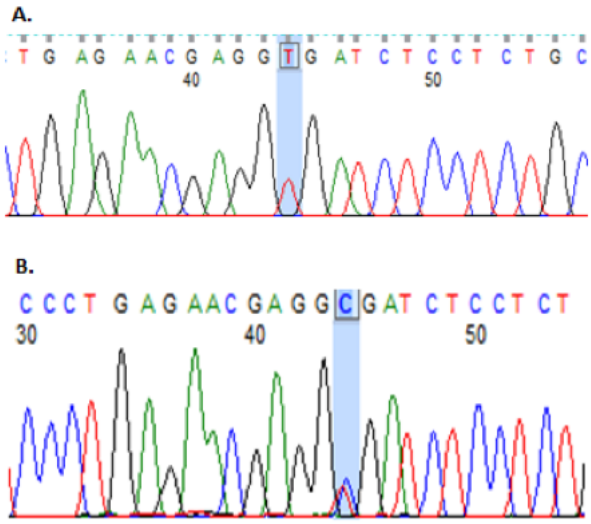

Figure 3. (A) Example of homozygous TT genotype as demonstrated by the sequencing result. (B) Example of heterozygous CT genotype as demonstrated by the sequencing result.

Chen et al. (2012) provided preliminary evidence of the relationship between BMP4 and NSCL/P in Asian population. After conducting a research on 12 SNP points around the BMP4 gene, it turns out that several SNPs show statistically significant results as risk factors of NSCL ±P.[20] A study by Suazo et al. in 2011 aimed to determine the risk associated with sequence variations in the BMP4 gene promoter in NSCL/P in a Chilean population. The results found three novel variants in BMP4, namely c-5514 G>A, c-5365 C>T, and c-5049 C>T. Bioinformatics prediction analysis shows that all risk variants detected in this study could create

new transcription factor binding motives.[21]

A Study in India conducted by Shavita et al. (2015), which aimed to evaluate the polymorphism of BMP4 and produced changes in the amino acid (Val152Ala) in the polypeptide associated with NSCL ±P in the Indian population. The results show a significant relationship between homozygous CC genotype and carrier C allele, indicating an increased risk of NSCL ±P compared to the T allele (OR 4.2% CI = 2.75-6.41).[15] Li et al. (2017) published the results of a meta-analysis of research from various publications related to the BMP4 gene polymorphism rs17563 in non-syndromic cleft lip patients with or without cleft palate. In Brazilian and Caucasian populations, the BMP4 gene shows that SNP rs17563 is a protective factor. Meanwhile, in Asian population, several studies show statistically different results and odds ratios. This shows that the existence of ethnic differences has an influence on the results of the SNP study of the BMP4 gene rs17563.[22]

Hao et al. (2018) conducted a study to find a link between the BMP4 gene polymorphism and the incidence of cleft lip with or without cleft palate in the South Chinese population. The results obtained from 165 patients and 52 healthy individuals show that the BMP4 gene polymorphisms rs762642, rs17563, and rs10130587 gave different results for each NSCL/P phenotype and showed that these phenotypic differences were thought to have different etiologies.[16] In 2018, Saket et al. published the results of a study on variations of the BMP2 and BMP4 gene sequences and their risk of the incidence of NSCL/P in the Iranian population. It was noted that there is a significant relationship between BMP2 polymorphisms rs235768 A>T and BMP4 rs17563 T>C with the incidence of CB/L. The association or allele relatedness of the two genes was considered a risk factor for CB/L in the Iranian population.[23] The analysis results on BMP4 SNP rs17563 T>C were not significant and different from studies in other countries. The difference in each place is influenced by the number of samples in the study as well as differences in ethnicity and sex. This suggests that the BMP4 gene may have an important role in the pathogenesis of CL/P.

Conclusions

There is a no significant association of BMP4 rs17563 T>C polymorphism of the BMP-4 gene on non-syndromic orofacial cleft patients in Indonesia.

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Declaration of Interest

The authors report no conflict of interest.

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