Identification of Single Nucleotide Polymorphism on Bone Morphogenetic Protein 2 Gene in Non-Syndromic Cleft Lip/ Palate Patient

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Abstract

Cleft lip/palate (CL/P) is one of the most common birth defects in humans. Haploinsufficiency in genes Bone Morphogenetic Protein (BMP) 2 is thought to play an important role in the incidence of CL/P. This study aimed to identify changes in the nucleotide (Single Nucleotide Polymorphism/SNP) BMP 2 rs235768 A>T gene in CL/P patient in Indonesia. Seventy samples of DNA that were successfully amplified and restricted consisted of patient and control samples with the three of which were used for sequencing. Based on the analysis using restriction enzymes and Finch TV and Bioedit software programs, this study identified a change from nucleotide A to nucleotide T which is a mutation missense (Serine-Arginine/TCA-TCT). Based on the results of the Fisher's exact test, there was difference in genotype frequency between the CL/P group and the control. Meanwhile, there was no difference in allele frequencies between the two groups. The allele frequency T has a higher value than the frequency of the allele A.

Keywords: *Gene BMP2, SNP, Cleft lip/palate, Indonesia.*

Introduction

Cleft lip and/or palate (CL/P) is the most common congenital abnormality caused by a complex multifactorial interaction between gene variation and environmental exposure.[1,2] Individuals with CL/P face many challenges, such as anatomical deformities, dental problems, eating and speaking difficulty, hearing loss, and impaired maxillofacial growth. Psychologically, individuals with CL/P also experience a low quality of life index. [3] The incidence of CL/P varies between countries, socioeconomic status, race, or ethnic group. This variation causes the Asian population to have the highest prevalence (1/500 births) and the African have the lowest (1/2500 births) while Caucasians have the prevalence 1/1000 births.[4,5]

In recent years, advances in genetics and molecular biology have begun to reveal the basis for craniofacial development. A number of genes associated with the incidence of CL/P have been identified and developed

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to study the etiology of CL/P, namely genetic and environmental.[1,6] Bone Morphogenetic (BMP) is an important gene candidate in the craniofacial pattern located on chromosome 14q22-23 in humans.[7,8] More than 20 BMP genes have been identified. Currently, BMP 2 and 4 are classified as subfamily of dpp (decapentaplegic) due to similarities with the dpp gene in Drosophila. BMPs 5, 6, 7, and 8 are classified as the 60A subfamily, BMP3, and 3b (GDF10) in which are classified as separate subfamilies. BMP has receptors, namely BMP (BMPR) type I and type II (BMPRI and BMPRII).[7,9]

A small proportion of SNPs become biological markers for the determination of a disease on the human genome map because these SNPs are located on genes that are found to be associated with the disease.[10,11] The SNPs associated with a disease can be used to find and isolate the gene that causes the disease. The SNP pattern in target genes from the results of comparative studies between case and control groups in association studies can be used to design therapeutic targets and response of drugs in a population.[10–12]

In the study of Sahoo et al., haploinsufficiency in the BMP 2 gene plays an important role in cleft palate formation. Two individuals with similar cleft defects are reported to have microdeletions that only included BMP2. Microdeletions associated with CL/P have also been reported in several studies including 20p12.3 in which BMP2 was deleted.[9] In 2018, Saket et al. published the results of a study on the variation of the BMP2 gene sequence at risk for the incidence of NSCL/P in the Iranian population. It is noted that there is a significant association between the polymorphism of BMP2 rs235768 A>T and the incidence of CB/L.[13] This study aimed to identify changes in nucleotide bases (Single Nucleotide Polymorphism/SNP) of the BMP 2 rs235768 A>T gene in CL/P patients in Indonesia.

Materials and Methods

This study examined patient (CL/P) and control groups. The CL/P patient group consisted of 34 samples and 36 samples from the control group. DNA was extracted from peripheral blood using Promega A1120Wizard® Genomic DNA Purification KitDNA at the Human Genetic Laboratory, Institute of Tropical Diseases, Universitas Airlangga. This research has been approved by the ethics committee of the Faculty of Dentistry, Universitas Airlangga with a certificate number 606/HRECC.FODM/IX/2019.

Polymorphism of the BMP2 gene rs235768 A>T was analyzed with PCR-RFLP method. The primers used were produced by Integrated DNA Technologies, namely 5' GAAACGAGTGGGAAAACAACC-3' and 5'GAGACACCTTGTTTCTCCTCCA-3.[13] The reaction among PCR 25 µl 2.5 µl Promega Go Taq ™ Master Mixes, primary forward 2.5 µl (10 µmol), reverse primer 2.5 µl (10 µmol), template 7.5 µl was amplified with the BioRadCycler PCR machine. The PCR machine procedure is at 58ºC for 00:30 seconds and 34 cycles followed by elongation at 72ºC for 00:40 seconds. Electrophoresis was conducted using 2% of agarose gel (Promega) at 100 V for 30 minutes and a PCR product was observed at 353 bp length. Furthermore, the RFLP method was carried out using BsrI restriction enzyme from Thermo Scientific. Afterwards, the enzyme mixture and PCR products were incubated at 65ºC for 1 hour and inactivated at 80ºC for 20 minutes. Visualization of the RFLP band was electrophoretic 100 V for 35 minutes and the cut tape could be seen in a UV light to read the results.

There are three variations of the genotype results, namely 353 bp for wildtype homozygote (AA); 353 bp, 200 bp, and 153 bp for mutant heterozygote (TT); 200 bp and 153 bp for mutant homozygote (AT). All statistical analysis was performed using SPSS Inc., IBM Corporation, NY, and USA Statistics Version 16. The Fisher's exact test was used to analyze the distribution of genotypes 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 and Discussion

This study consists of 70 samples from the

Indonesian population with the 34 of whom were the CL/P group and 36 were the control group. Genotype distribution between CL/P groups and control for BMP2 gene polymorphisms 235768 A>T is significantly different (Table 1).

Table 1. Genotypes distribution of the BMP2 gene rs235768 A>T polymorphism in the CL/P and control group

Group	Genotype			P value
	AA	AT	TT	
CL/P	2 (5.9%)	4 (11.8%)	28 (82.4%)	**0.05**
Control	2 (5.6%)	13 (36.1%)	21 (58.3%)	
Total	4 (5.7%)	17 (24.3%)	49 (70%)	

*Significant at P < 0,05

Allele frequency distribution BMP2 gene rs235768 A>T in the CL/P and control groups is not significantly different (p>0.05, Fisher's exact test) (Table 2).

Table 2. Distribution of the alleles of BMP2 gene rs235768 A>T polymorphism in the CL/P and control group

Group	Allele		P value
	A	T	
CL/P	8 (12%)	60 (88%)	**1.000**
Control	17 (24%)	55 (76%)	
*Significant at P < 0,05			

PCR products and RFLP results with the restriction enzyme Bsr1 onBMP2 gene rs235768 A>T are presented in Figures 1 and 2.

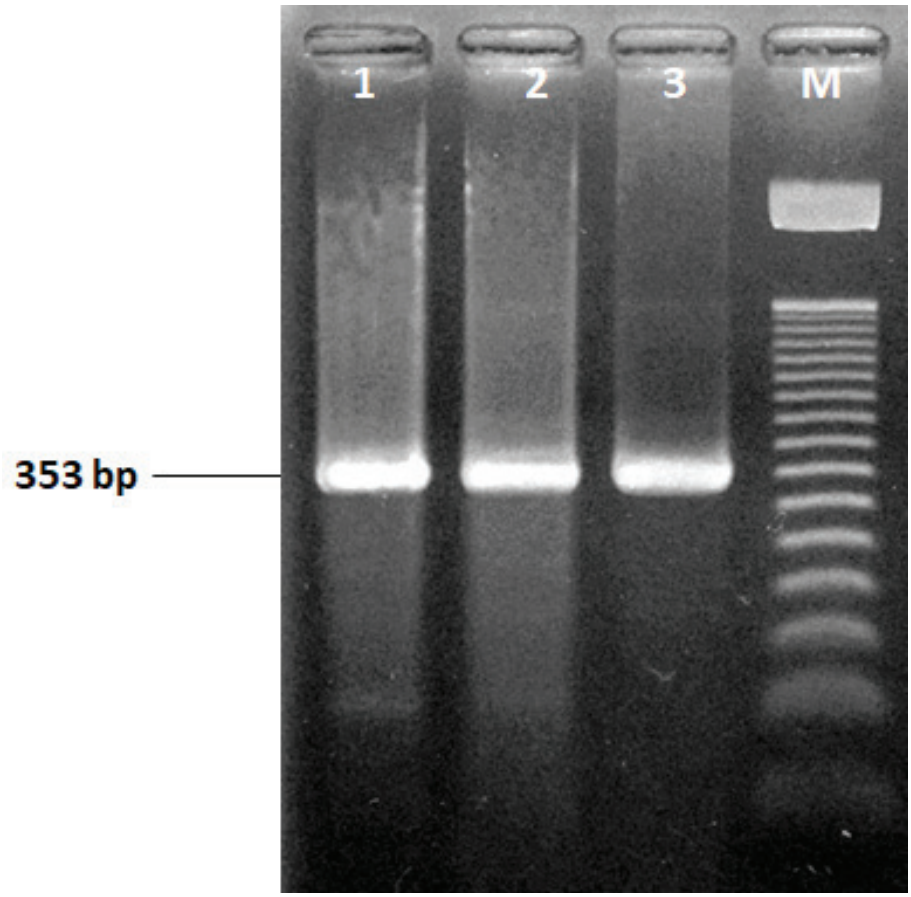

Figure 1. Visualization of the PCR BMP2 gene rs235768 A> T product.

Lanes 1, 2, 3, show bands at 353 bp, and lane 4 shows a 50-bp DNA ladder marker.

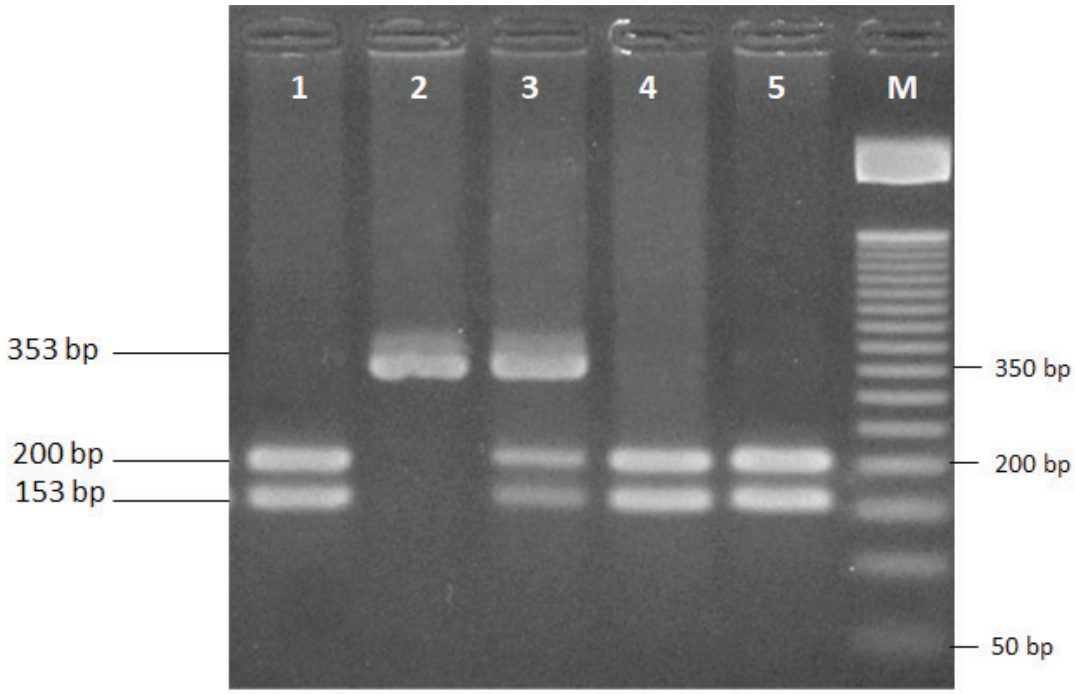

Figure 2. Results of PCR products carried out by RFLP with the enzyme Bsr1.

Lane 1, 4, and 5 indicate TT; lane 2 indicates AA; and lane 3 indicates AT genotype.

Lane M is 50 bp ladder marker.

The conformation of PCR results using sequencing read with Finch TV and Bioedit programs from three samples that were examined and obtained three SNP genotypes on the BMP2 gene rs235768 A>T, namely AA (homozygous wild type), AT (heterozygous), and TT (homozygous mutant).

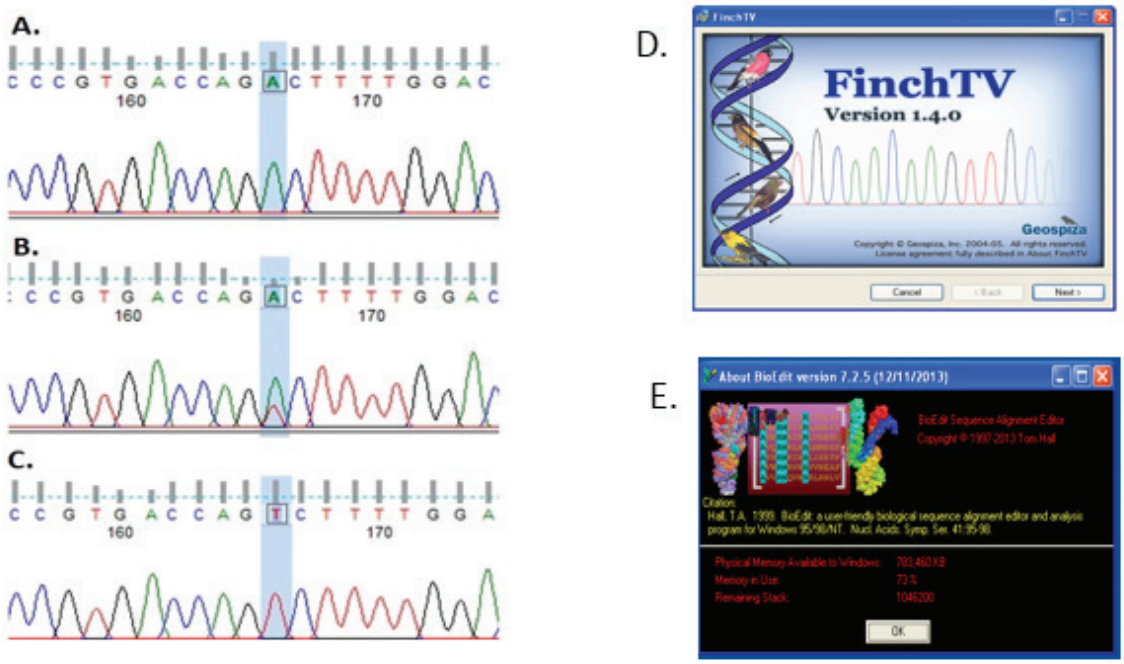

Figure 3. (A) AA genotype (wild type), (B) AT genotype (heterozygous),

(C) TT genotype (homozygous mutant), (D) Finch TV application, (E) Bioedit application

The BMP2 gene encodes a ligand that is secreted from the TGF-β superfamily. 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. The BMP2 protein plays a role in bone and cartilage development. In addition, BMP2 stimulates myoblast differentiation into osteoblasts via the EIF2AK3-EIF2A-ATF4 pathway. Activation of BMP2 EIF2AK3 stimulates phosphorylation of EIF2A which leads to increased expression of ATF4 which plays a central role in osteoblast differentiation. BMP2 also stimulates TMEM119 which can increase the regulation of ATF-4 expression.[14]

The BMP2 gene is located on chromosome 20p12.3 (short arm of chromosome 20 area 1, band 2, and sub band 3). The length of the BMP2 sequence in the NCBI annotation (GRCh38.p12) starts from 6,768,098 to 6,780,280 along 10.6 kb (Homo Sapiens Annotation Release). Consisting of three exons, the focus of this study was Exon 3 with reference to SNP (rs235768), a change in the amino acid Serine to arginine at position 190 in the BMP2 gene. Other names for the BMP2 gene are BDA2, BMP2A, and SSFSC.[14]

*Single Nucleotide Polymorphism*are is a small change to genetics or variations that can occur in a person's DNA sequence. The genetic code is determined by the four nucleotides A (adenine), C (cytosine), T

(thymine), and G (guanine). SNP variation occurs when one nucleotide, such as A replaces one of the three other nucleotides, such as C, G, or T [1115]. Based on molecular analysis using restriction enzymes and bioinformatics analysis of Finch TV and Bioedit programs, it was identified that there was a change from nucleotide A to nucleotide T which was a mutation missense (Serine - Arginine/TCA-TCT). Missense mutase is a change in the composition of nitrogen bases which causes changes in amino acids in a polypeptide chain. Changes in amino acids can produce a mutant phenotype if the changed amino acid is an essential amino acid for the protein. This type of mutation can be caused by transitional and transformation events.[15]

The genotype frequency showed that the highest percentage of 82.4% in the CL/P group was the TT genotype (homozygous mutant). The results of the Fisher's exact genotype test in the two groups were significantly different. The allele frequency in the CL/P group was the highest in the T allele which was a polymorphic allele, namely 88% and the Fisher's exact test results were not significant between the alleles and the two groups. There are differences in the research results obtained by researchers from previous research, in Iran which indicate there was an association between BMP2 and the incidence of CL/P.[13] This difference can be due to the different number of samples even though the procedural laboratories were the same from the primary arrangement, the annealing temperature of the PCR, and the type of restriction enzymes used. The difference in results was also seen in the PCR product and the resulting RFLP cut results between the Iranian and Indonesian samples. However, the differences between PCR and RFLP products have been confirmed by sequencing the PCR results and studying the nucleotide bases one by one with the Finch TV and Bioedit programs. The difference in each place is influenced by the number of samples in the study as well as differences in ethnicity and sex.[16]

From this study, the SNP genotype results can be ascertained that the changes are the same, namely AA (wild type), AT (heterozygous), TT (homozygous mutant) which differ only in their position. Thus, the BMP 2 gene still needs to be examined more deeply in this study, one of which is the addition of research samples and analysis studies at certain points in the BMP 2 gene. The high percentage yield for the mutant genotype (TT) and T allotype could potentially suggest a possible etiological link between CL/P and mutations in the BMP 2 gene.

Conclusion

There is a change from nucleotide A to nucleotide T on BMP2 gene rs23576 which is a missense mutation (Serine - Arginine/TCA-TCT). There was significant relationship between the rs235768 A>T polymorphisms of the BMP2 gene in CL/P patients. The allele frequency T (Polymorphic) has a higher value than the frequency of the A allele (non-polymorphic).

Ethical Clearance

This study was approved by the ethical committee of the Faculty of Dentistry, Universitas Airlangga number 606/HRECC.FODM/IX/2019.

Conflict of Interest

There was no conflict of interests regarding the publication of this study.

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