Original Article

Single nucleotide polymorphism of bone morphogenetic protein 4 gene: A risk factor of non-syndromic cleft lip with or without palate

Sathyaprasad Savitha, S. M. Sharma¹, Shetty Veena², R. Rekha³
Department of Pediatric Dentistry, KVG Dental College, ¹Department of Oral and Maxillofacial Surgery, AB Shetty Dental College, ²Department of Microbiology, KSHEMA, Mangalore, ³Department of Community Medicine, KVG Medical College, Sullia, D.K, Karnataka, India

Address for correspondence: Dr. Savitha Sathyaprasad, Department of Pediatric Dentistry, KVG Dental College and Hospital, Sullia, D.K, Karnataka, India. E-mail: drsavithaks@yahoo.com

ABSTRACT

Background: The bone morphogenetic protein (BMP) signalling pathway is crucial in a number of developmental processes and is critical in the formation of variety of craniofacial elements including cranial neural crest, facial primordium, tooth, lip and palate. It is an important mediator in regulation of lip and palate fusion, cartilage and bone formation. Aim: To study the role of mutation of BMP4 genes in the aetiology of non-syndromic cleft lip with or without palate (NSCL ±P) and identify it directly from human analyses. Materials and Methods: A case-control study was done to evaluate whether BMP4T538C polymorphism, resulting in an amino acid change of Val=Ala (V152A) in the polypeptide, is associated with NSCL ±P in an Indian paediatric population. Genotypes of 100 patients with NSCL ±P and 100 controls (in whom absence of CL ±P was confirmed in three generations) were detected using a polymerase chain reaction-restriction fragment length polymorphism strategy. Logistic regression was performed to evaluate allele and genotype association with NSCLP. Results: Results showed significant association between homozygous CC genotype with CL ±P (odds ratio [OR]-5.59 and 95% confidence interval [CI] = 2.85-10.99). The 538C allele carriers showed an increased risk of NSCL ±P as compared with 538 T allele (OR - 4.2% CI = 2.75-6.41). Conclusion: This study suggests an association between SNP of BMP4 gene among carriers of the C allele and increased risk for NSCLP in an Indian Population. Further studies on this aspect can scale large heights in preventive strategies for NSCLP that may soon become a reality.

KEY WORDS

Aetiology of non-syndromic cleft lip and palate; bone morphogenetic protein 4 gene; bone morphogenetic protein gene

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INTRODUCTION

The formation of mammalian palate is a multistep process coordinated by a network of transcription factors and signalling molecules together with proteins conferring cell polarity and cell — cell interactions that includes palatal shelf growth, elevation of shelves, fusion between paired shelves and the disappearance of midline epithelial seam, which degenerates by apoptosis; and the fusion is complete. Moreover, ossification occurs in the anterior two-third of the palate and form the hard palate. Disturbance of this tightly controlled cascade can result in a facial cleft when the facial primordia fail to meet and fuse. Recent successes in genome-wide linkage and association studies have identified novel loci significantly associated with cleft lip and palate. Several genes have been proposed based on the understanding of the molecular and cellular processes that orchestrate morphogenesis of the midface. The use of mouse models and modern molecular techniques have greatly facilitated the process of craniofacial research and extended our understanding of craniofacial development in the past decade. The bone morphogenetic protein (BMP) signalling pathway has been shown to be involved in a number of developmental processes and critical in the formation of variety of craniofacial elements including cranial neural crest, facial primordial, tooth, lip and palate. BMP-regulated genes control self-renewal, osteoblast differentiation and negative feedback regulation, suggesting that BMP signalling regulates facial skeletal morphogenesis by controlling the balance between self-renewing progenitors and differentiating lineage-restricted cells, is an important mediator in the process of orofacial union is indicated by Alk deficient mice, in which orofacial clefts is shown. BMP4 is highly expressed in the putative orofacial epithelia prior to the facial union. A recently generated mouse model with inactivation of BMP4 from facial primordial showed delay of Fgf8, a critical gene for early facial development, which fails to be unregulated at a crucial developmental stage and results in cleft lips. BMP4 expression localises at the site of fusion of the mice facial prominences. The conditional inactivation of BMP4 in a transgenic mice line results in an isolated cleft lip confirms a role in lip fusion. Several BMP genes including BMP2, BMP4 and BMP7 are expressed in developing mouse palatal shelves. The importance of BMP4 signalling in palate development was also demonstrated in Mx1 mutant mouse, which exhibits cleft palate phenotype. Msx 1 was required for the expression of BMP4 and BMP2 in the palatal mesenchyme and sonic hedge hog (SHH) in the medial edge epithelium. BMP4 appears to bypass a requirement for Msx 1 and to function upstream of SHH and BMP2 to regulate palate development. Inactivation of BMP4 — BMPr1a genetic pathway by Nestin-Cre transgenic line mutant mice demonstrated orofacial clefting, thus, revealing the role of BMP signalling in the orofacial union. Authors demonstrated that the BMP4/ BMPr1a pathway was critical for the lip development. BMPr1a gene, encoding a type I receptor for BMPs is preferentially expressed in the primary palate and anterior secondary palate during palatal outgrowth, tissue-specific inactivation of BMPr1a in the developing palatal mesenchyme in mice cause reduced cell proliferation in the primary and anterior secondary palate, resulting in partial cleft of the anterior palate at birth. The expression of Msx1, Fgf10 and SHH was down-regulated in the anterior palate mesenchyme indicating that BMP signalling regulates mesenchymal-epithelial interactions during palatal outgrowth. In addition, the formation of the palatal processes of the maxilla was blocked while the formation of the palatal processes of the palate was significantly delayed; resulting in the submucous cleft of the hard palate. BMP signalling also plays critical roles in the regulation of palatal mesenchyme condensation and osteoblast differentiation during palatal bone formation. Hence, BMP4 is considered a promising candidate gene and is intensely studied in association studies using case-control and case-parent trio designs. The present case-control study was undertaken to evaluate whether BMP4T538C polymorphism, resulting in an amino acid change of valine to Alanine is associated with non-syndromic cleft lip with or without cleft palate (NSCLP) in an Indian population so that knowledge regarding the risk factor should eventually result in improved prevention, treatment and prognosis for individuals with these conditions.

MATERIALS AND METHODS

Our sample consisted of 200 individuals; case group composed of 100 individuals with NSCLP (52 males and 48 females) belonging to age group of 6 months to 18 years old enrolled for various rehabilitation procedures in cleft lip and palate centres. Subjects were recruited between 2010 and 2012 from the centres of research involved in Craniofacial Project in Karnataka, India. The morphological nature of our study group was cleft lip with or without palate including both unilateral and
bilateral cleft, but not associated with any syndromes and no family history of clefts or neural crest cell disorders. All cases selected using the same clinical protocol, including family history. Control group were recruited from 100 phenotypically normal unrelated individuals reporting to the same hospital for treatment of trauma or other injuries they were age and gender matched (54 males and 46 females) confirming absence of family history of absence of oral cleft in three generations, also malformations of neural tube defects, monogenic twins and children with syndromes were excluded. The study was approved by the local Institutional Review Boards. All participants provided informed consent. The informed consent for patients under 18 years old was obtained from their parents.

Polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) was done after DNA extraction; genomic DNA was extracted from ethylenediaminetetraacetic acid peripheral blood leucocytes by HiMedia blood DNA extraction kit. Briefly, 200 μl blood samples were taken into the microcentrifuge tube, and 20 μl protease was added. Then, 200 μl buffer AL was added to the tube and mixed by pulse-vortexing for 15 s and then incubated at 56°C for 10-20 min. Two hundred microlitres ethanol was added to the microcentrifuge tube and mixed transferred to mini spin column placed in a 2 ml collection tube and centrifuged at 8000 rpm for 1 min. The flow through was discarded after centrifugation at 6000 ×g for 1 min, the bound DNA was washed by adding 500 μl buffer AW1 followed by centrifugation at 6000 ×g for 1 min. Then, flow through was discarded, and 500 μl buffer AW2 was added and centrifuged at 2000 ×g for 3 min. The DNA was eluted by adding 100 μl buffer AE to the mini spin column placed in a fresh microcentrifuge tube and collected by centrifuging the column at 6000 ×g for 1 min. The extracted DNA was stored at −20°C till further use. The standardisation of PCR condition for BMP4F/R primer was done in a programmable gradient thermocycler. Polymerisation chain reaction was carried out in 50 μl reaction mixture containing 5.0 μl of template DNA, ×1 assay buffer, 100 μM of each of the four dNTP’s, 10 picomoles of forwards and reverse primers and 1.25 U of Taq DNA polymerase PCR was performed using a thermocycler. The primer sequences used were BMP4F-5’CCTAACTGTGCCTAG3’ and BMP4R-5’CATAAACCTCATAATGTTTATACGG3’. The optimised PCR programme consisted of an initial denaturation step at 94°C for 12 min followed by 35 cycles of 1 min at 94°C; 1 min at 54°C; 1 min at 72°C and a final elongation at 72°C for 10 min. The PCR products were detected by agarose gel electrophoresis. About 2.5% agarose gels were prepared in ×1 TAE buffer. The molten agarose was cooled to below 65°C, ethidium bromide was added to a final concentration of 0.5 μg/ml mixed and poured to gel mould and allowed to set. Ten microlitres of the PCR products were mixed with 4 μl of ×6 loading buffer and loaded into the wells in agarose gel. Gene Ruler 50 bp DNA ladder was used as a molecular weight marker. Electrophoresis was carried out at 100-120 V resolved on 2.5% agarose gels and visualised under ultraviolet transilluminator [Figure 1]. The blood DNA samples were used to amplify desired fragments of the BMP4 gene by PCR, using BMP4F/R primers. Restriction digestion; the PCR products were digested with HphI for 3-4 h at 37°C and inactivated by increasing temperature to 80°C for 20 min. The digested PCR products after standardisation of PCR conditions, the optimum annealing temperature was selected and used for further amplification. The BMP4 primers generated PCR amplicons of 197 bp the PCR product (197 bp) was digested with HphI. Digestion produced a 197 bp fragment in the A147 allele that lacks the HphI site while the digestion detected 110 bp and 87 bp fragments in the V147 allele having the recognition site.

RESULTS

We analysed the single-nucleotide polymorphism (SNP) of rs17563 and difference in the frequency of SNP rs17563 in cases compared with controls, to estimate the relative risk of genotypes of BMP4T538C on the occurrence of NSCLP using odds ratio and 95% confidence interval (CI). The gender distribution was similar between groups (Fisher’s exact test). Significant differences in genotype distribution and allele polymorphism rs17563 mutations...
were found in BMP4 gene in a sample of patients and in a control population. Logistic regression revealed association of CC genotype and CLP susceptibility compared to TT [Table 1] (odds ratio [OR]-5.59 and 95% CI = 2.85-10.99). A significant association was also observed between C allele and CL ± P compared with T allele ($\chi^2 = 43.89, P < 0.05$) (OR - 4.2% CI = 2.75-6.41) for BMP4 T > C the frequency of 538C allele was 118%. We can conclude that when haplotypes and cleft were investigated, increased risk of NSCLP was found for homozygous CC and heterozygous TC genotype compared with genotype. These results suggested that one or more SNPs of BMP4 (BMP4T538C) are associated with increased occurrence of NSCLP and confirms the role of polymorphism of BMP4 gene in the aetiology of NSCLP. Thus, there is a significant relationship between SNP and non-syndromic cleft lip with or without palate patients and $P < 0.005$.

**DISCUSSION**

BMP4, located at 14q22-q23 in humans, is a member of the transforming growth factor-beta superfamily. Have a number of distinct roles in embryonic development, including facial development, by regulating cell proliferation, differentiation, apoptosis and chemotaxis (Hogan, 1996; Ebara and Nakayama, 2002). In particular, the overexpression of BMP4 has been found in the maxillary prominence (Gong and Guo, 2003), one of the regions of prefusion in face development of embryo, which form the lateral parts of the upper lip and the secondary palate (Tapadia et al., 2005). In addition, the presence of a cleft phenotype in BMP4 knockout mice (Cobourne, 2004) or in BMP4 null mutant mice (Liu et al., 2005) reveals the importance of BMP4 during embryonic facial fusion. Based on its role in craniofacial development, as well as evidence from animal experimental studies and human linkage studies, BMP4 has been considered a promising candidate gene for NSCL/P and intensely studied in association studies using case-control and case-parent trio designs. Expression studies of BMP4 and their antagonist Noggin. In the embryonic chicken face has suggested that BMP signals are important for the closure of the upper lip or primary palate. Gain- and loss-of-function experiments showed that BMPs regulate outgrowth and epithelial survival during avian lip fusion. Conditional inactivation of BMP4 in a transgenic mouse line has been found to result in an isolated CL. In view of its role in the regulation of skeletal development including cartilage and bone formation during craniofacial development, Fengfer used Noggin mutant mice as a model for assessing role of BMP4 gene in palatogenesis he found prominent Noggin expression in the palatal epithelium along the anterior-posterior axis during palate development and loss of Noggin leads to overactive BMP signalling that resulting in deregulation of cell proliferation, excessive cell death and change in gene expression leading to formation of palatal cleft. several genome-wide association studies have been conducted across different populations to identify genetic variants of the BMP4 gene, which could be associated with NSCLP in humans. Was first suggested in a case-control study performed with Chinese children in 2008 by Lin, authors analysed the SNP of rs17563 and found an increased risk of NSCL/P among carriers of the C allele. L. Jianyan, in his hospital-based case-control study in Chile identified the interactions between 538 (T-C) polymorphic site of BMP4 gene and the exposures in pregnancy with NSCLP showed homogyosity for polymorphism (CC) resulted in significantly raised risk compared to TT genotypes, the authors did not find an increased risk for heterozygous TC genotype compared with the TT genotypes. Lin supported the role of BMP4 in NSCLP through their study on 150 unrelated trios showed significant transmission distortion for haplotypes rs1957860-rs762642specially for C-T and T-T, which include the genomic region where the promoter and an enhancer of BMP4 are located. Bings case-control study on Chinese population evaluated BMP4T583C polymorphism resulting in amino acid change V152A out of genotypes of 184 patients with NSCLP and 205 controls detected using PCR-RFLP strategy the 583C allele carriers were associated with significantly increased risk of NSCLP as compared to no carriers. Suazo in his case-control study on Chilean population confirmed the possible mutation of BMP4 promoters BMP4.1 and BMP4.2 on three novel variants of BMP4 (C-5514G > A, C-5365G > T and 5049C > T) concluded that risk variants detected could potentially alter BMP4 promoter activity in NSCLP. A missense

| Table 1: Genotype distribution of rs17563 in NSCLP patients and controls and results of logistic regression |
|---|
| **SNP rs17563** | **Controls** | **Cases** | **$\chi^2$** | **P** | **OR** | **95% CI** |
| Genotype | 32 | 68 |
| TC | 13 | 18 | 6.527 | 0.011 | 2.94 | 1.29-6.71 |
| CC | 50 | 19 | 24.98 | 0.000 | 5.59 | 2.85-10.99 |
| Allele | T | 82 | 149 |
| C | 118 | 51 | 43.59 | 0.00 | 4.2 | 2.75-6.41 |

SNP: Single-nucleotide polymorphism; NSCLP: Non-syndromic cleft lip with or without cleft palate; OR: Odds ratio; CI: Confidence interval
and nonsense mutations were found in BMP4 gene in a sample of patients from different genetic background mutations were not found in a control population by Suazo et al. [18,29] Searched for NSCLP risk variants within the two BMP4 promoters by direct sequencing in a 167 Chilean NSCLP cases and 336 controls and found three novel variants considered as cleft risk factors, as they are not present in controls. Lately, Chen et al. [19] provided further evidence of an association between BMP4 gene and NSCLP in Asian and Maryland trios. These studies point that BMP4 gene polymorphisms clearly have different effects in different populations, Arauja did a case-control study in a Brazilian population and found a different results of the 538C allele having a protective effect on NSCLP [16] our study showed homozygosity for polymorphism (CC) resulted in significantly increased susceptibility compared to TT genotypes, also association was found for heterozygous genotype TC compared with the TT genotypes. Confirming the association between polymorphism of rs17563 of BMP4 gene with NSCLP in Indian population, this result was in accordance with findings of Lin and Suzuki and Jin and against the findings of Arauja study in a Brazilian population and Antunes, [16] this study support the hypothesis that this polymorphism might play a definite role in different ethnicity. However, NSCLP is a multifactorial disease where multiple aetiological factors, both genetic and environmental could either act solely or in combination with different levels of cell to cell interactions or during different signalling pathways, so it is very hard to declare a single variant of a particular gene to be responsible for NSCLP-based on gene association studies and several other restriction segments of different genes are studied individually or in combination with environmental factors thus there is a need for further research in this area in different population to be elucidated.

CONCLUSION

Cleft lip and palate are common congenital anomalies which pose considerable and often lifelong taboo and suffering for the patient as well as the family. Despite the complete rehabilitation of the defect that is possible now, the repeated number of hospital visits and surgeries can leave the patient and the caretaker burdened and depressed. Relentless research in evaluating the different speculated stoppable aetiological factors is growing on in this purview but still there is no definite answer to the aetiology of the defects. The identification of the modifiable risk factors for cleft lip and palate is the first step towards the primary prevention and recognition of risk factor is the wanting for further genetic counselling in the prevention of such congenital anomalies. Research was done in different ethnic groups and geographic locations can help us clearly be in a better position to find a solution to this global problem and this should eventually result in improved prevention. The results of our study suggest an association of the SNP of BMP4 gene among carriers of the C allele and increased risk for NSCLP in an Indian Population. Further studies on this aspect can scale large heights in preventive strategies for NSCLP that may soon become a reality.
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