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Research article

Genetic influences on non-syndromic cleft lip palate: The impact of *BMP4*, *RUNX2*, *PAX7*, and *TGFB3* allelic variations

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Abstract: Several genes have been implicated in the etiology of cleft lip palate (CLP). Although genes such as *BMP4*, *RUNX2*, *PAX7*, and *TGFB3* have been studied in various populations, their role in familial cases within the Indian population remains unexplored. Hence, the current research was conducted to understand whether *BMP4*, *RUNX2*, *PAX7*, and *TGFB3* gene polymorphisms are involved in the etiology of Non-Syndromic Cleft Lip Palate (NSCLP) in Indian familial cases. Twenty multiplex families affected by NSCLP were selected for the research, with 50 NSCLP patients and 38 unaffected subjects from these families. Polymorphisms rs2819861 of *RUNX2*, rs17563 of *BMP4*, rs2743218 of *PAX7* and rs2268626 of *TGFB3*, which were considered high-risk in a different population, were analyzed for their role in Indian families. The DNA was extracted from each participant using the salting-out method. The isolated DNA was sent for genetic analysis by Single Nucleotide Polymorphism (SNP) genotyping using the MassArray method. The Hardy-Weinberg equilibrium (HWE) was computed using a genotype distribution, the PLINK software was utilized to

make statistical comparisons, and allelic associations were analyzed for the selected polymorphisms. All polymorphisms followed the HWE. None of the polymorphisms on these four genes showed a significant p-value in the allelic association. Therefore, no discernible variation in the allelic frequencies existed between the healthy controls and the NSCLP patients. The odds ratios were 1.28, 0.83, 0.37, and 1.01 for polymorphisms rs2819861, rs17563, rs2743218, and rs2268626, respectively. The current study indicates that the polymorphisms rs2819861 of *RUNX2*, rs17563 of *BMP4*, rs2743218 of *PAX7*, and rs2268626 of *TGFB3* were not associated with increased risk of NSCLP among the Indian population.

Keywords: cleft lip and palate; genetics; gene; polymorphism; genome-wide association studies

1. Introduction

Cleft lip palate (CLP) is a prominent congenital anomaly that affects individuals worldwide. According to a World Health Organization (WHO) study, an infant is born with this anomaly every two minutes [1]. Global studies have shown that the CLP frequency varies from nation to nation. With a prevalence ratio of 1:2500, it is lowest in Africans and East Asians, and Indigenous North Americans have the highest prevalence ratio of 1:500. With an incidence ratio of 1:800, around three babies are born every hour with clefts. The incidence of CLP in India is around 1:800 to 1:1000, and 3 infants are born with some type of cleft every hour [2]. Clefts can be either a non-syndromic (70%) or a syndromic variety (30%). The etiopathogenesis of Non-Syndromic Cleft Lip Palate (NSCLP) has to be thoroughly evaluated. The etiology of CLP is multifactorial. It can be due to consanguineous marriages, genetic variations, undernourishment, and endocrine abnormalities. A literature review showed that approximately one-fifth of the CLP cases had a history of consanguinity. There is a familial history of the clefts in 3.5% of cases, and a cleft of lip and/or palate is seen in over 600 syndromic cases [3].

Genetic research utilizes either association or linkage analyses to establish the genetic factors of oral and facial clefts. The results of the genome-wide association studies performed in multiple ethnic groups were largely indecisive or contradictory, with only a limited number of loci showing their involvement. This variation is due to genetic conglomeration. These studies revealed various candidate genes linked to NSCLP, such as *MTHFR*, *TGFB2*, *BCL3*, *BMP4*, *P63*, *PAX3*, *PAX7*, *PVRL1*, *IRF6*, *MSX1*, *CRISPLD*, *ABC4*, *RARA*, *TGFβ3*, *MYH9*, *BCL3*, *SATB2*, *FOXE1*, and *RUNX2* [4–9]. Some of the crucial genes responsible for protein coding, neural crest development, embryogenesis, cell differentiation, and osteogenesis are Bone Morphogenic Protein-4 (BMP4), Runt-related Transcription Factor-2 (*RUNX2*), Paired Box Protein-7 (*PAX7*), and Transforming Growth Factor Beta-3 (*TGFB3*).

The high-risk Single Nucleotide Polymorphisms (SNPs) rs2819861 of *RUNX2*, rs17563 of *BMP4*, rs2743218 of *PAX7*, and rs2268626 of *TGFB3* were reported to be involved in different populations [10–13]. Most genetic studies were performed on isolated cleft cases. Thus, the present research objective was to assess the role of these markers in the etiology of familial cases of NSCLP.

2. Materials and methods

2.1. Ethics approval of research and study design

The Institutional Review Board (IRB) of the GSR Institute of Craniofacial Surgery, Hyderabad, India, accepted the study. Multigenerational families with NSCLP were selected. Patients with abnormalities of chromosomes, growth retardation, and the mentally challenged were excluded from the study. Unaffected individuals from these families were utilized as controls. Twenty multigenerational families were chosen based on the population genetics power calculation for family association studies. These include one family with five probands, two families with four probands, five families with three probands, and 12 families with two probands. Four multigenerational families reported consanguinity. Written consent was obtained from all the participants, including the subjects and/or their parents or guardians from these multiplex families who participated in the study.

2.2. Blood sample collection and DNA retrieval

Four to five millilitres of venous blood were collected, and their Deoxyribonucleic Acid (DNA) was isolated using the salting-out technique [14]. Then, the concentration and purity of the isolated DNA were then assessed using an ultraviolet (UV) spectrometer. Subsequently, polymorphism genotyping was performed. The characteristics of the analyzed polymorphisms are presented in Table 1.

Table 1. Features of the SNPs analyzed.

Gene	SNP	Alteration form	Alleles	Ancestral trait	Global MAF
BMP4	rs17563	Mis-sense variant	A/G	A	0.37
RUNX2	rs2819861	Intron variant	A/G/T	G	0.20
PAX7	rs2743218	Intron variant	G/T	G	0.40
TGFB3	rs2268626	Intron variant	C/T	T	0.24

Note: Abbreviations: SNP: Single Nucleotide Polymorphism; A: adenine; G: guanine; C: cytosine; T: thymine; MAF: Minor allele frequency

2.3. Genotyping and statistical analysis

MassARRAY (Agena Bioscience, Inc., San Diego, CA, USA) was employed to genotype the SNPs selected. The polymorphism allele information of the affected patients and the healthy controls were subjected to statistical analyses. This study utilized PLINK (Version 1.09), which is an open-source genetic toolset [15]. The Hardy–Weinberg equilibrium (HWE) was calculated using the genotype distribution. Statistical analyses were conducted between the cleft patients and healthy individuals' controls. The Odds Ratio (OR) and the 95% confidence intervals (CI) were given. The Chi-square test was utilized to analyze the allelic association.

3. Results

All the selected SNPs followed the HWE. No high-risk marker showed any association with NSCLP in the allele association (Table 2). The p-value < 0.05 was not seen for any SNP analyzed on the *BMP4*, *RUNX2*, *PAX7*, and *TGFB3* genes. Moreover, the ORs were less than 2 for all the SNPs analyzed.

Table 2. Correlation between SNPs and NSCLP.

SNP	BP	Major Allele	Minor Allele Frequency (Affected)	Minor allele Frequency (Unaffected)	Minor Allele	Chi-squared test	P-value	Odds Ratio
rs17563	8	G	0.17	0.19	A	0.21	0.64	0.83
rs2819861	17	A	0.27	0.22	G	0.49	0.48	1.28
rs2743218	28	G	0.01	0.02	A	0.68	0.40	0.37
rs2268626	16	C	0.12	0.11	T	0.00	0.97	1.01

Note: Abbreviations: BP: Base pair, SNP: Single Nucleotide Polymorphism, A: adenine; G: guanine, C: cytosine, T: thymine.

4. Discussion

The etiology of the CLP is multifactorial. They involve the influence of genetic, environmental, a combination of genetic and environmental causes. Studies in the form of genetic analyses on the etiology of CLP are increasing. With improvements in molecular genetics, our scope of research has increased. Genetic polymorphism identification in our population would be vital in identifying the mechanisms involved in causing the defect. Information from animal models, where clefts arise either spontaneously or as a result of mutagenesis, combined with an analysis of how expression patterns correlate with gene function and examining the effects of gene-environment interactions have proven themselves as powerful tools to identify genes for intricate traits, such as clefts. Multiple investigations indicated that syndromic forms with Mendelian inheritance patterns may offer an understanding of the genetic etiology of non-syndromic clefts.

Phenomenal advances in gene identification studies on CLP identified numerous new genes involved in the genetic etiology of NSCLP. In different populations, associations have been identified between polymorphic markers for *RUNX2*, *BMP4*, *TGFB3*, *PAX7*, *NTN1*, *IRF6*, *PTHFR*, *GHR*, and the risk of clefts. Genetic research was conducted on varied populations in both non-syndromic and syndromic cases. Only variants in the *IRF6* gene consistently showed a connection to the etiology of CLP across different populations. There is little research on familial non-syndromic cases and the trio of cases-parents in India. The percentage of familial cases comes to a meager 3.5 % of the total cleft cases. The GSR Institute of Craniofacial surgery is a high-volume cleft center located in India. The subjects were identified from this center as patients from various states of the country who were offered treatment with the generous help of various national and international agencies. Familial and non-syndromic clefts were recognized after a full medical history and clinical examination.

This study was designed to investigate the genetic factors within specific families, which could reveal heritable patterns and potential genetic risks that were specific to those families. Using cases

and controls from the same family, we aimed to control for shared environmental and genetic backgrounds, which might otherwise confound population-based studies. By selecting controls from the same family, we controlled for shared environmental factors and background genetic variations that could confound the association between the SNPs and cleft lip/palate. This approach reduced the noise that could obscure potential genetic associations in a more diverse population.

The genes selected (*RUNX2*, *BMP4*, *PAX7*, and *TGFB3*) for this study play an essential role in protein-coding, neural crest development, embryogenesis, cell differentiation, and osteogenesis. Four polymorphisms on these genes, which were reported as significant genetic markers with CLP in various populations, were selected for the study.

The *BMP4* gene, which is located at 14q22-q23 in humans, is a transforming growth factor-beta superfamily member. It is useful in embryonic development, including facial development, by regulating cell proliferation, differentiation, apoptosis, and chemotaxis. In one of the earlier studies conducted by Lin JY et al. in the Chinese population, *BMP4* genetic polymorphisms were described to be important in the development of NSCLP [16]. The present study suggested that the rs17563 polymorphism of *BMP4* was not a risk marker in the etiology of NSCLP. A study on *BMP4* rs17563 on NSCLP found a significantly increased risk for the Chinese population, but it had a protective effect on the Brazilian population [17]. In a study of the population in southern China, *BMP4* rs17563 was reported to be a risk factor solely for cleft lip [18]. In the southeastern Iranian population, the *BMP4* rs17563 variant exhibited a protective effect against the occurrence of NSCLP [19]. A single non-family-based study reported that this marker had increased the risk of NSCLP in Indians [20].

This study showed that the rs2819861 *RUNX2* polymorphism did not show an increased risk of NSCLP. However, in a case-parent trio study on four populations (Taiwan, Singapore, Korea, and Maryland), the *RUNX2* rs2819861 polymorphism influenced the risk of NSCLP [8]. The *PAX* genes play a critical role during the development of the face by regulating the differentiation programs and organogenesis [21,22]. In a replication study of Poland's population, the authors confirmed that the *PAX7* gene was strongly associated with NSCL/P, as they revealed the high-risk nature of rs2743218 of *PAX7* [13]. However, in the present study, there was no significant risk of this marker in the Indian Population.

TGFB3 belongs to a large family of cytokines called the Transforming Growth Factor-Beta superfamily. This is vital in cell differentiation, embryogenesis, and development and is expressed in epithelial cells on the medial edge of the palatal shelves [23]. For the first time within the Japanese population, Ichikawa E et al., reported that there was a positive correlation between CLP and *TGFB3*-SNP based on population and family analyses [24]. Later, studies by Reutter H et al., also revealed a significant role of *TGFB3* polymorphisms, including rs2268626, in the CLP families of central European descent [11]. Additionally, a case-parent trio study of the Chilean population reported the association of the rs2268626 SNP with NSCLP [12]. However, the present study's results suggested no significant risk in the etiology of NSCLP in familial cases.

The variability or discrepancies in results observed across different populations regarding the etiology of CLP could stem from a multitude of factors, including multifactorial origins, ethnic diversity, epigenetic influences, and interactions between genes. Moreover, the selection of familial cases of cleft may be a reason for the difference in the insignificant nature of the polymorphisms.

5. Conclusions

The current study indicated that the polymorphisms rs2819861 of *RUNX2*, rs*17563* of *BMP4*, rs2743218 of *PAX7*, and rs2268626 of *TGFB3* were not associated with an increased risk of NSCLP among the Indian population. This highlights the complexity of genetic markers, since a marker that is identified as a risk factor in one ethnicity, population, or family may not hold the same significance in another. Factors such as multifactorial etiology, epigenetics, and gene interactions may contribute to the variability or inconsistency in results. Nevertheless, it remains imperative to persist in studying the etiology of CLP.

The current study was constrained by a relatively small sample size and the examination of only four SNPs. Future investigations should incorporate a larger sample size and scrutinize additional SNPs within the selected genes (*RUNX2*, *BMP4*, *PAX7*, and *TGFB3*) in Indian multiplex families to enhance the comprehension of their involvement in NSCLP etiology. Furthermore, forthcoming research should prioritize functional analyses of these polymorphisms, thereby exploring other genetic models to validate our findings further.

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Conflict of interest

All authors declare no conflicts of interest in this paper.

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