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Association of *MTHFR, BMP4, TGFA* and *IRF6* Polymorphisms with Non-Syndromic Cleft lip and Palate in North Indian Patients

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Abstract

Background: Non-Syndromic Cleft Lip and Palate (NSCL/P) is a multifactorial birth defect. The world-wide prevalence of NSCL/P is 1 in 1000 live births; it differs with race, ethnicity and gender. The aim of the present study was to find out the status of candidate gene polymorphisms in NSCL/P cases and its association in phenotype of the patients.

Methods: We have screened five polymorphisms in four candidate genes *MTHFR* (rs1801133, rs1801131) *BMP4* (rs17563), *TGFA* (rs1146297) and *IRF6* (rs2235371) by restriction fragment length polymorphism and results were validated by Sanger sequencing. Our dataset consists of 200 NSCL/P cases and 200 healthy controls from the Indian population. Statistical data analysis was performed by SPSS software.

Results: *MTHFR* (rs1801133), *BMP4* (rs175563) and *TGFA* (rs11466297) gene polymorphisms showed significant association with NSCL/P and act as a risk factor in the Indian population (p=<0.05). However, *MTHFR* (rs1801131), and *IRF6* (rs2235371) gene polymorphisms did not show significant association with NSCL/P in the Indian population.

Conclusion: The result of the study suggests an association between *MTHFR* (rs1801133), *BMP4* (rs175563) and *TGFA* (rs11466297) polymorphisms with NSCL/P in Indian population.

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Keywords: BMP4, Indian population, IRF6, MTHFR, NSCL/P, TGFA

Introduction

Non-Syndromic Cleft Lip and Palate (NSCL/P) is the world's second most prevalent congenital birth defect, with incidence of 1 in 700 live births and varies by ethnicity or geographical region. Balaji *et al*, reported a prevalence of NSCL/P 1.3 in 1000 live births in India [1]. Since patients of NSCL/P suffer with problems of feeding, speech difficulties, malnutrition, hearing injuries, infections and mental disorders from birth to adulthood, they need multidisciplinary care like surgical or dental treatment, speech therapy and psychosocial interventions throughout life [2]. A sequence of closely orchestrated events are needed during development of lip and palate formation, including cell proliferation, growth, differentiation and apoptosis. The disruptions in any of these events affects unacceptable facial structure morphology resulting in manifestation of disease [3,4]. Thus it is considered as a complicated hereditary disorder but polygenic in nature. Shi *et al*, reported involvement of environmental risk factors like tobacco, smoke and alcohol intake during early pregnancy [5].

Epidemiological surveys and animal model studies have also shown that antiepileptic medications or hormonal treatment, are the risk factor for NSCL/P [6]. In past linkage analysis, association studies, direct sequencing, and more recently genome-wide association studies have been done in relation to NSCL/P and found suitable for genetic predisposition studies [2]. Approximately 20 gene loci are identified in NSCL/P etiology, among those genes; we have selected five polymorphisms from four genes for present study, which are *MTHFR* (rs1801135, rs1801131), *BMP4* (rs17563), *TGFA* (rs1146297) and *IRF6* (rs2235371).

Methylenetetrahydrofolate reductase (MTHFR) is

one of the most important enzymes which plays a crucial role in the folate metabolism regulation. The gene coding for MTHFR is on the long arm of chromosome 1 (1p36.3), which contains 11 exons [7]. MTHFR (C677-Tand A1298C) has two common single nucleotide polymorphisms responsible for a moderately variable enzymatic action. *MTHFR* gene polymorphism studies have been reported from several regions suggesting a strong association with NSCL/P. Bone Morphogenetic Proteins (BMPs) plays an important role in the fusion of the upper lip, main palate, and craniofacial growth, primarily expressed in palatal shelf epithelial and mesenchymal cells [8-10]. Saket *et al*, have suggested that the polymorphism of *BMP4* (rs17563) variant plays a significant role in the frequency of CL/P in the Iranian population [11]. The importance of BMP4 (rs17563) variations in the development of CL/P was addressed in previous investigations [9,10]. Throughout craniofacial growth, TGFα is expressed in the inner epithelium boundary of fusing palatal shelves and activates the extracellular matrix synthesis [12,13]. Ardinger *et al*, and Ebadifar *et al*, assessed the role of *TGFA* gene polymorphism in the event of CL/P and results show a strong association [14,15]. Interferon Regulatory Factor 6 (IRF6) is a transcription factor and is located on chromosome 1q32.3-q41 [16]. *IRF6* gene assembly consists of a highly conserved helix-turn-helix DNA-binding domain and a less conserved protein binding domain. The *IRF6* gene is the gene between the candidates involved in both syndromic and non-syndromic form CL/P, with variations in this gene associated with van der Woude syndrome [17]. The *IRF6* gene polymorphism rs2235371 is well established in many studies of NSCL/P [18].

As the genetic diversity of NSCL/P is present in different ethnic groups, the role of these gene remains speculative in Indian populations; therefore, in this study, we have aim to investigate the association of genetic polymorphism of *MTHFR, BMP4, TGFA,* and *IRF6* in NSCL/P in Indian population.

Materials and Methods

Study design and ethical approval

This study was carried out from September 2017 to December 2020 in the Department of Medical Genetics, Sanjay Gandhi Postgraduate Institute of Medical Sciences, Lucknow. The research was carried out according to the World Medical Association (Declaration of Helsinki) for Experiments in humans. The study was approved by the Institutional Ethical Committee (IEC No: 2018-107-EMP-EXP) and informed consent was obtained from cases and controls.

Sample collection

NSCL/P patient's sample (n=200, age=7±5 years) and age and sex matched healthy controls (n=200, age=7±5 years) were recruited for the study, after signed informed consent was obtained from patients and parents. The exclusion criteria for NSCL/P cases were patients with any other history of developmental disorder, Syndromic form of CL/P (*e.g.*, eye, brain, limb anomalies and cardiac defects). The healthy controls were Indian healthy children without cleft lip and palate and other known genetic diseases.

Genotyping analysis

DNA isolation: Genomic DNA was extracted from 3 *ml* peripheral venous blood samples of patients and controls. DNA was extracted by using standard Phenol-chloroform method, quantification of the DNA was measured by spectrophotometer at wavelength of 260 *nm* and the quality was checked on 0.8% agarose gel.

Restriction fragment length polymorphism (RFLP)

Primers were designed to amplify candidate gene variants *MTHFR* (C677T, A1298C), *BMP4* (T<C), and *TGFA* (A<C). Polymerase Chain Reaction (PCR) performed details of primers, annealing temperature and restriction sites are mentioned in table 1. The PCR amplified products were digested using restrictions enzymes (Hinfl for C677T, MboII for A1298C, Hphl for T<C and BamHI for A<C) and were kept at 37°C overnight; products were visualized by standard Ethidium Bromide-Agarose Gel Electrophoresis method (Figures 1-4).

Sanger sequencing

Sanger sequencing was done for the *IRF6* (G<A) gene polymorphism followed by amplification and PCR product purification. Sequencing of PCR products were performed using the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, California, United States), and products were resolved on the ABI 3130XL Genetic Analyser (Applied Biosystems). Sequence electro-pherograms were analysed using Finch TV (Figure 1-5).

Statistical analysis

Genotype and allele frequency distributions of *MTHFR BMP4, TGFA* and *IRF6* polymorphisms were calculated by counting the genotypes and compared with the predicted values using the chi-square test, based on the assumption of Hardy-Weinberg equilibrium. The Odds Ratios (OR) were calculated with the 95% confidence intervals (95% CI) and p-values <0.05 were considered to be significant. All analyses were performed using SPSS for Windows, version 18.0 (SPSS Inc., Chicago, USA).

Results

This study consisted of 200 NSCL/P patients (102 males, 98 females) and 200 healthy individuals (105 males, 95 females). The observed genotype frequencies of cases and controls in all polymorphic sites were in Hardy-Weinberg equilibrium. The genotyping results, OR (95% CI) and p-value calculations for five Single Nucleotide Polymorphisms (SNPs) of the *MTHFR* (rs1801133, rs1801131), *TGFA* (rs1146297), *BMP4* (rs17563) and *IRF6* (rs2235371) are reported in table 2 (Figures 1-5).

Table 1. Candidate gene Variants Details

Genes	Variant/ Alleles	Ch. Loc	Role	Primers for PCR amplification (5'–3')	Annealing Temp (°C)	PCR product length (*bp*)	Restriction enzymes	Genotypes
MTHFR								
	rs1801133	1p36.3	Nutritional	F 5'-TGAAGGAGAAGGTGTCTGCGGGA-3' R 5'-AGGACGGTCCGGTGAGAGTG-3'	62	198	Hinfl	CC-198 CT-198, 175 TT-175
	rs1801131		metabolism	F 5′-CTTTGGGGAGCTGAAGGACTACTAC-3′ R 5′CACTTTGTGACCATTCCGGTTTG-3′	62	163	MboII	AA-163 AC-84, 51, 28 CC-51, 28
BMP4	rs17563	14q22-q2	Transcrip-tion factors	F 5'-CCTAACTGTGCCTAG-3' R 5'-CATAACCTCATAAATGTTTATACGG-3'	56	197	HPhl	TT-197 TC-197,110,87 CC-110,87
TGFA	rs11466297	2p13.3	Growth factors	F 5'-GCCTGGCTTATTTGGGGATT-3' R 5'-AAGGGCAAGGAAACACAGG-3'	58	174	BamHl	AA-174 AC-174,120,54 CC-120,54
IRF6	rs2235371	1q32.2	Immune system	F 5'-GAGTCACAGGGATGAACAGG-3' R 5'-GCTTCTGCTTCTCATTGGTA-3'	55	263	Sanger Sequencing	GG GA AA

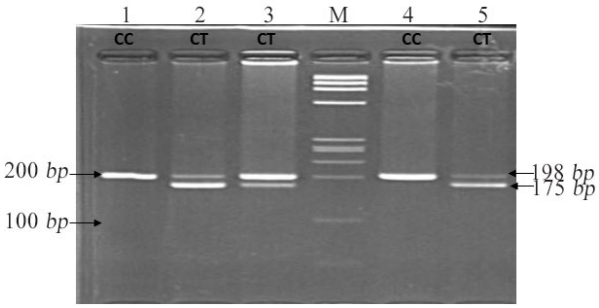

Figure 1. Restriction fragment length polymorphism result of MTHFR rs1801133 (C>T).

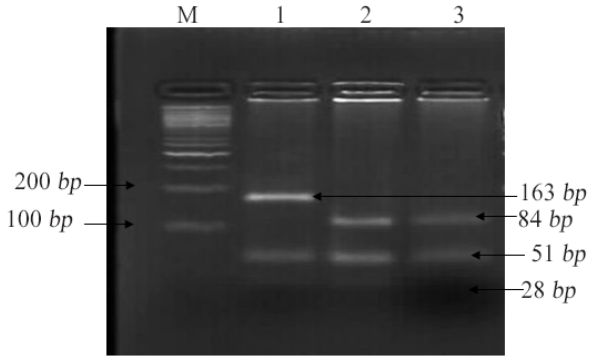

Figure 2. Restriction fragment length polymorphism result of MTHFR rs1801131 (A>C).

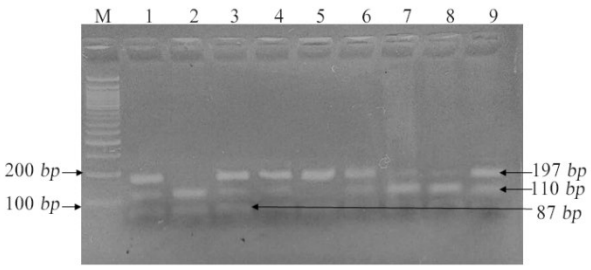

Figure 3. Restriction fragment length polymorphism result of BMP4 rs17563 (T>C).

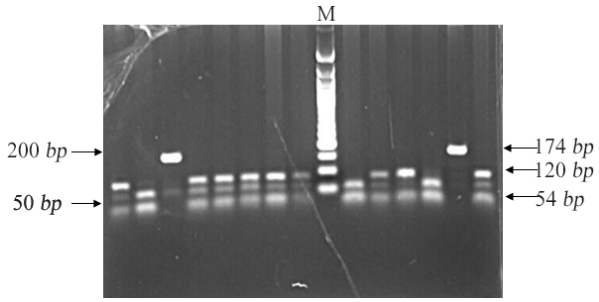

Figure 4. Restriction fragment length polymorphism result of TGFA rs11466297 (A>C).

Findings revealed that *MTHFR* rs1801133 (OR= 1.56, 95% CI: 1.02-2.39, p=0.041), *BMP4* rs17563 (OR=1.85, 95% CI: 1.19-2.89, p=0.005) and *TGFA* rs1146297 (OR=1.69, 95% CI: 1.01-2.82, p=0.045) polymorphisms are significantly associated with NSCL/P. Our findings did not support an association between *MTHFR* rs7224837 and *IRF6* rs861019 polymorphisms and risk/protection of NSCL/P (Table 2).

Discussion

The aetiology of orofacial clefts remains largely unknown, although genetic factors are thought to play the most important roles. There is compelling evidence

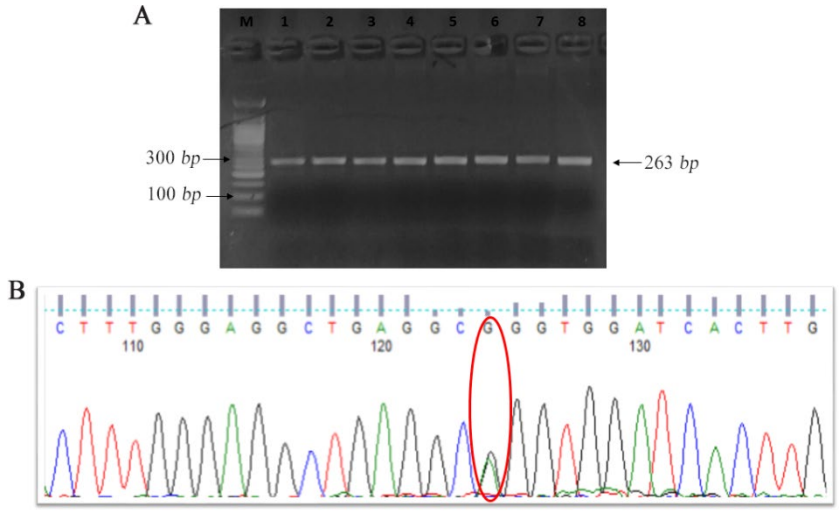

Figure 5. Sanger Sequencing result of IRF6 rs2235371 (G>A).

Table 2. The genotype and allele frequency of the MTHFR, BMP4, TGFA and IRF6 polymorphisms in the case and control groups

Gene/SNPs	Genotype/Allele	Controls n=200 (%)	Cases n=200 (%)	OR (95% CI)	p-value
MTHFR (rs1801133)					
	CC	135 (67.5)	109 (54.5)	Referent Genotype	
	CT	58 (29)	73 (36.5)	1.56 (1.02-2.39)	0.041*
	TT	7 (3.5)	18 (9)	3.18 (1.28-7.90)	0.012
	C	328	291	Reference Allele	
	T	72	109	1.70 (1.21-2.39)	0.001
MTHFR (rs1801131)					
	AA	159 (79.5)	150 (75)	Referent Genotype	
	AC	38 (19)	46 (23)	1.28 (0.79-2.08)	0.312
	CC	3 (1.5)	4 (2)	1.41 (0.31-6.42)	0.654
	A	356	346	Reference Allele	
	C	44	54	1.26(0.82-1.93)	0.281
BMP4 (rs17563)					
	TT	139 (69.5)	110 (55)	Referent Genotype	
	TC	49 (24.5)	72 (36)	1.85 (1.19-2.89)	0.005*
	CC	12 (6)	18 (9)	1.90 (0.88-4.10)	0.104
	T	327	292	Reference Allele	
	C	73	108	1.66 (1.18-2.31)	0.003
TGFA (rs11466297)					
	AA	161 (80.5)	143 (71.5)	Referent Genotype	
	AC	30 (15)	45 (22.5)	1.69 (1.01-2.82)	0.045*
	CC	9 (4.5)	12 (6)	1.50 (0.61-3.67)	0.372
	A	352	331	Reference Allele	
	C	48	69	1.53 (1.02-2.28)	0.036
IRF6 (rs2235371)					
	GG	167 (83.5)	152 (76)	Referent Genotype	
	GA	28 (14)	40 (20)	1.59 (0.93-2.69)	0.087
	AA	5 (2.5)	8 (4)	1.77 (0.57-5.55)	0.321
	G	364	344	Reference Allele	
	A	36	56	1.64 (1.05-2.56)	0.027

suggesting that common genetic variations contribute to NSCL/P susceptibility [2]. In this study, we systematically evaluated the associations between a comprehendsive panel of five polymorphisms of the four genes *MTHFR, BMP4, TGFA* and *IRF6* involved in NSCL/P. *MTHFR* C677T allele will increase the incidence of NSCL/P in Asian and Chinese populations [19,20]. Furthermore, *MTHFR* 677TT homozygotes are associated with NSCL/P and 6777CT heterozygotes is the minor risk factor. However, a study from southern Han Chinese population reported involvement of *MTHFR* 677CT in cleft lip only [21]. Studies from southern and northern part of India also reported association between *MTHFR* C677T and NSCL/P [22,23]. Studies reported from China and Thailand showed no association between *MTHFR* C1298A allele risk factor for NSCL/P, which supports our results [24-27]. Also *MTHFR* A1298C poses no risk of any combination with NSCL/P in the eastern Uttar Pradesh [23].

Several studies reported earlier have shown that *BMP4* may be involved in CL/P formation. Hu, *et al* found in meta-analysis, depending on the ethnicity range, the *BMP* rs17563 variant plays a different role in NSCL/P. This variant significantly increased the risk of NSCL/P in the Chinese population, while the Brazilian population showed a protective effect [28,29]. By contrast, Chen *et al*, reported that this variant was not associated with NSCL/P in the Asian population [30].

First report in NSCL/P with *TGFA* gene polymorphisms was reported by Ardinger *et al*, and shows significant association with *TGFA* rs11466297 [14]. Several studies have been published on TGFA. Studies of British, Japanese and French populations indicate involvement of the TGFA rs11466297 polymorphism in the occurrence of NSCL/P (31-33). Lidra *et al*, from Philippines published a contradictory study; it may be due to genetic differences in different populations [34]. Past research findings are contradictory and may be due to variations in sample size, demographic history and environmental conditions. Ebadifar *et al*, found that there is a link between BamHI variant polymorphism and prevalence of CL/P in the Iranian community, so that the incidence of AC genotype and C allele in the patient sample was substantially higher [15].

Zucchero *et al* investigated 36 SNPs in *IRF6* gene in 10 populations including Asian, European and American; among these *IRF6* gene polymorphism rs2235371 was reported as a risk factor for NSCL/P in Filipinos of Asia [16]. Association studies between *IRF6* gene rs2235371 polymorphism and NSCL/P are well documented in Norway and western Chinese population [35,36]. Rahimnov *et al*, reported a lack of involvement of polymorphism rs2235371 and NSCL/P in the Brazilian population [18]. A study in south Indian population indicated *IRF6* (rs2235375) gene polymorphism is significantly associated with increased risk of NSCL/P [37]. Study from eastern part of Uttar Pradesh reported minor risk of *IRF6* 820GG with NSCL/P [23].

Conclusion

The present study assessed the interaction effects of *MTHFR* (rs1801133, rs1801131), *BMP4* (rs17563), *TGFA* (rs11466297), and *IRF6* (rs2235371) polymorphisms on the occurrence of NSCL/P in Indian population. The results showed that the *MTHFR* (1801133), *BMP4* (rs17563), and *TGFA* (rs11466297) polymorphisms have a significant effect on the occurrence of NSCL/P.

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