

## ANALYSIS OF MICROSATELLITE POLYMORPHISMS IN SOUTH INDIAN PATIENTS WITH NON SYNDROMIC CLEFT LIP AND PALATE

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### ABSTRACT

Non syndromic cleft lip and/or palate (NSCLP) is a complex congenital anomaly with varying incidence among patients of different geographical origins. Multiple contributing factors are known to trigger the cleft formation. There are several genes involved in the aetiology of NSCLP and they are different in different populations. The genetic components of clefts that underlie the susceptibility to respond to the environment still remain unclear. In this study, five microsatellite polymorphisms from five candidate genes were employed to analyze the association between these genes and NSCLP in 83 patients and 90 controls. Genotyping was performed by separating and visualizing the fluorescently-labeled polymerase chain reaction (PCR) products. The association of the five microsatellite polymorphisms with NSCLP was tested by using the CLUMP v1.9 program that uses the Monte Carlo method. The genotypic distribution is in Hardy-Weinberg equilibrium in the control group for only the *MSX1* and *DLX3* genes. The *RARA* microsatellite was significantly associated with NSCLP. Our results suggest that the *RARA* gene is involved in pathogenesis of cleft lip and palate in South Indians.

**Keywords:** Non syndromic cleft lip and/or palate (NSCLP); Microsatellite; *DLX3*, *MSX1*, *RARA*, *BCL3* and *EDNI* genes; Cleft palate.

### INTRODUCTION

Cleft lip and palate are birth defects that affect children with a variable phenotype. Approximately 70.0% of cleft lip and palate cases are non syndromic, occurring as an isolated condition dissociated with any recognizable anomalies [1]. Non syndromic cleft lip, with or without cleft palate (NSCLP), is a heterogeneous, complex disease with a high incidence in the Asian populations. Non syndromic cleft lip and palate is a common craniofacial malformation with a complex genetic component. Multiple environmental factors are known to trigger the cleft formation, although both physiologic, pharmacologic and genetic studies in animal models and human populations have identified several candidate genes and pathways that regulate transcription factors, growth factors, cell signaling and de-toxication metabolisms [2,3]. The genetic components of cleft that underlie the susceptibility to respond to the environment still remain largely unclear [4]. Previous studies revealed that the disease-contributing alleles in many genes, but none of these seem to play a major role in NSCLP, and they appear to be responsible for only a fraction of NSCLP cases [5]. Linkage studies screen the whole genome and use parametric or non parametric methods such as allele sharing methods with no assumptions on the mode of inheritance, penetrance or disease allele frequency. Although single nucleotide polymorphisms (SNPs) are preferable markers for association studies, the density of SNPs required is not yet identified because of their low mutation rates.

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Microsatellites are the most preferred markers for linkage analysis and population genetic studies because of their high mutation rates. As they are highly informative, recent genome wide association studies used these markers to identify the genes involved in the complex diseases.

A large number of studies of NSCLP have been conducted mainly in Caucasian populations. However, it is desirable to perform similar studies in different ethnic groups of India to reiterate the status of the association or linkage identified in other world populations. In India, there are approximately 5,000 anthropologically well-defined populations including different caste, tribe and religious groups. Linguistically, the Indian populations are classified into four major language families, such as: Indo-European, Austro-Asiatic, Dravidian and Tibeto-Burman. Indo-Europeans are mainly found in the northern part of the country; Austro-Asiatic speakers have established themselves in central and north eastern states; Tibeto-Burmans mostly inhabit the northeastern states and the foothills of the Himalayas; and Dravidians are restricted to the southern states of India, including Tamil Nadu, Kerala Karnataka and Andhra Pradesh. In the present study, we have analyzed microsatellite markers from five different genes that play a major role in cell signalling pathways and transcriptional regulation of genes involved embryonic development.

**MATERIALS AND METHODS**

**Subjects.** In the present study, 173 South Indian subjects (Dravidian speakers) were recruited from Sree Balaji Dental College and Hospital, Chennai,

Tamil Nadu, India. Of these, 83 had NSCLP (45 males, 38 females, aged 1-2 years). Cleft status of the NSCLP group was determined by clinical examination as well as through their medical records. All the subjects in the case group are isolated NSCLPs. The cleft phenotype was divided into two sub-phenotypes such as cleft lip with or without cleft palate (74 CLP) and cleft palate only (nine CPO). The control group comprised 90 unrelated south Indian children (48 males and 42 females, aged 2-3 years) without clefts or family history of clefting or other major health problems. Children with mental retardation, serious medical problems or other congenital malformations were excluded. About 5 mL of intravenous blood was collected from all the participants. All subjects had provided informed consent prior to the sample collection. A procedure for protection of human subjects in this study is also approved by the Institutional Ethical Review Committee of Sree Balaji Dental College, Chennai, India.

**Genotyping.** Genomic DNA was extracted from blood using the standard protocol described elsewhere [6]. Five microsatellite markers within five different genes (*DLX3*, *MSX1*, *RARA*, *BCL3* and *EDN1*) were amplified using the primer sets labeled at the 5' ends (forward primer) with 6-FAM/HEX fluorescent dye (BioServe Biotechnologies India Pvt Ltd, Hyderabad, Andhra Pradesh, India; Table 1). As the forward primer is labeled with fluorescent dye, the polymerase chain reaction (PCR) product so generated will also have the fluorescence. To resolve the products, 1 µL of PCR product was mixed with 10 µL of 50.0% HiDye™ formamide and 0.1 µL LIZ (size standard; Applied Biosystems, Foster

**Table 1.** Primer sets used to amplify the different microsatellites analyzed in the present study.

Primer	Sequences (5'>3')	Chromosome Location	Forward Primer	Size Range
MSX1F MSX1R	GGG CAT GTT GAT GTC TGC TGA C TTA GAT TGT CTC AGT CCT C	4p16.1	FAM	169-175
EDN1F EDN1R	GAT GGA CAG AGA AGG CAG GTG TGA ATT TGC AAG ACG TGT GC	6p24.1	FAM	190-210
BCL3F BCL3R	TGG CAT AAA TGT TGA GTA AG TAA GGG CGA GTA TTG TTT CA	19q13	FAM	127-139
DLX3F DLX3R	CTT ATC TGG GCT GGA GCT A GCG CTG ATT GGC TGC AAG T	17q21	HEX	196-244
RARAF RARAR	GGT CTC ATC CAT CAG GTT TT ATA GAC TGT GTA CTG GGC ATT GA	17q21	HEX	164-176

F: Forward primer; R: reverse primer.

City, CA, USA). The sample plates were kept and run on the ABI PRISM™ 3730 DNA Analyzer (Applied Biosystems). In the sequencer, the fragments were separated by length from longest to shortest. The fluorescence of the PCR product when illuminated by a laser beam was read by an automatic scanner that provided the size of the allele and the data were processed by GENE MAPPER v3.0 software (Applied Biosystems).

**Statistical Analyses.** As the number of CPO samples in the group was less, we considered all clefts as a single group. Power and sample size calculation program software (version 2.1.31) (<http://biostat.mc.vanderbilt.edu/twiki/bin/view/Main/PowerSampleSize>) was used to evaluate the null hypothesis in the uncorrected  $\chi^2$  statistic model. Allele frequencies of microsatellite markers were estimated as simple proportions in patients and controls. Concordance with Hardy-Weinberg expectations was assessed through Genepop, a web based software (<http://genepop.curtin.edu.au/>). The markers were tested for association with NSCLPs by conducting a

case-control association analysis. The CLUMP v1.9 program (<http://www.smd.qmul.ac.uk/statgen/dcurtis.software.html>) was used to test each marker for association with clefts [7]. The CLUMP v1.9 program, uses the Monte Carlo method, was designed to overcome the problems of sparse contingency tables as found in a  $\chi^2$  analysis of multiallelic markers such as microsatellites. Significance was assessed for each marker by performing 10,000 simulations to generate tables with the same marginal totals as the original data. Empirical *p* values were obtained by counting the number of times the  $\chi^2$  value of the real data was achieved by the simulated tables.

## RESULTS

A total of 173 subjects were recruited in this study which includes 83 NSCLP and 90 normal controls. We examined five microsatellite markers in five genes. The allele frequencies in both patients and controls are shown in Table 2. Out of five microsatellite markers analyzed, only two (*MSX1* and *DLX3*)

**Table 2.** Allele frequencies of five short tandem repeat polymorphisms located in different chromosomal regions in non syndromic oral clefts and control samples.

<i>EDN1</i>			<i>MSX1</i>			<i>DLX3</i>			<i>BCL3</i>			<i>RARA</i>		
bp	Cleft	Control	bp	Cleft	Control	bp	Cleft	Control	bp	Cleft	Control	bp	Cleft	Control
190	8 (4.8)	5 (2.8)	169	112 (67.5)	121 (67.2)	208	0 (0.0)	2 (1.1)	127	45 (27.1)	55 (30.6)	166	18 (10.8)	30 (16.7)
192	8 (4.8)	5 (2.8)	171	21 (12.7)	14 (7.8)	210	3 (1.8)	5 (2.8)	129	63 (38.0)	55 (30.6)	168	43 (25.9)	46 (25.6)
194	51 (30.7)	57 (31.7)	173	31 (18.7)	45 (25.0)	212	3 (1.8)	3 (1.7)	131	51 (30.7)	67 (37.2)	170	32 (19.3)	18 (10.0)
196	18 (10.8)	24 (13.3)	175	2 (1.2)	0 (0.0)	214	9 (5.4)	16 (8.9)	133	2 (1.2)	2 (1.1)	172	39 (23.5)	35 (19.4)
198	23 (13.9)	17 (9.4)				216	16 (9.6)	4 (2.2)	135	5 (3.0)	1 (0.6)	174	29 (17.5)	46 (25.6)
200	21 (12.7)	16 (8.9)				218	30 (18.1)	33 (18.3)				176	5 (3.0)	5 (2.8)
202	21 (12.7)	43 (23.9)				220	26 (15.7)	33 (18.3)						
204	13 (7.8)	13 (7.2)				222	20 (12.0)	23 (12.8)						
206	1 (0.6)	0 (0.0)				224	17 (10.2)	14 (7.8)						
208	2 (1.2)	0 (0.0)				226	3 (1.8)	7 (3.9)						
						227	1 (0.6)	1 (0.6)						
						228	5 (3.0)	8 (4.4)						
						230	6 (3.6)	8 (4.4)						
						232	13 (7.8)	11 (6.1)						
						234	5 (3.0)	2 (1.1)						
						236	1 (0.6)	2 (1.1)						
						237	4 (2.4)	6 (3.3)						
						238	4 (2.4)	2 (1.1)						
<i>p</i> <sup>a</sup>	<0.001	<0.001		0.005	0.198		0.365	0.224		<0.001	<0.001		0.037	<0.001

<sup>a</sup>*p*: Hardy-Weinberg equilibrium *p* value.

**Table 3.** CLUMP tests of association for five short tandem repeats in non syndromic oral clefts and control samples.

Genes	T1: $\chi^2$ (p value)	T2: $\chi^2$ (p value)	T3: $\chi^2$ (p value)	T4: $\chi^2$ (p value)
<i>EDNI</i>	14.16 (0.117)	12.72 (0.079)	7.230 (0.053)	8.04 (0.170)
<i>MSX1</i>	5.77 (0.123)	4.56 (0.102)	2.260 (0.284)	3.34 (0.179)
<i>DLX3</i>	18.02 (0.387)	13.23 (0.287)	8.720 (0.026)	9.97 (0.442)
<i>BCL3</i>	5.821 (0.204)	2.476 (0.291)	2.102 (0.324)	3.67 (0.261)
<i>RARA</i>	10.54 (0.060)	10.02 (0.038)	6.010 (0.069)	7.29 (0.086)

T1: Pearson’s  $\chi^2$  of raw contingency table. T2: Pearson’s  $\chi^2$  of rare alleles grouped together. T3: Pearson’s  $\chi^2$  statistics of  $2 \times$  tables, each of which compares one column against the rest grouped together. T4: Pearson’s  $\chi^2$  statistics of all possible  $2 \times 2$  tables comparing any combination of columns against the rest.

were in Hardy-Weinberg equilibrium. The remaining three microsatellites showed significant deviations from Hardy-Weinberg expectations in controls (Table 2). Of these three microsatellites, one (*EDNI*) showed more homozygous genotypes and the other two (*BCL3* and *RARA*) showed more heterozygote genotypes. In the present study, the alleles did not show significant difference between the cleft cases and the controls but the *RARA* locus almost reached significance (Table 3).

**DISCUSSION**

The present study investigated possible associations between NSCLP and microsatellite markers from five different genes. We demonstrated the distribution of micro-satellite markers in the NSCLP and control groups in the South Indian samples. The differences in the frequency of different alleles were statistically significant between NSCLP and controls for only the *RARA* gene microsatellite. Retinoic acid (RA) and its signaling pathway was involved in normal development of the frontonasal and maxillary structures [8]. Retinoic acid appeared to cause G1 arrest in palatal mesenchymal cells in a similar manner as in various cancer and embryonic cells. It is likely that apoptotic cell death and cell cycle disruption are involved in cleft palate formation induced by RA [9]. The *RARA* gene that had been studied using case-control association and linkage parameters, yielded contradictory results [10,11]. Other authors, using non parametric methods, reported negative results of association [12] as well as positive results for linkage [13]. Another microsatellite marker of RA receptor- $\alpha$  (*RARA*), D17S579 was also investigated to test the relationship between the genetic susceptibility to NSCLP in Hunan Hans [14].

Endothelin-1 is synthesized by vascular endothelial cells and is found in plasma. Endothelin-1 is released from an inactive transitional form in a step catalyzed by endothelin-converting enzyme (ECE). The *EDNI* knockout mice have shown craniofacial abnormalities, including cleft palate [15]. The mouse deficient in ECE or endothelin-A receptor genes has also shown almost identical abnormalities to those of *EDNI*-deficient mice [16]. In zebra fish, *EDNI* is expressed ventrally in the primordia of the pharyngeal arches and helps in patterning of pharyngeal cartilage development. By injecting the morpholino, Kimmel *et al.* [17], demonstrated the role of *EDNI* in the sizing of pharyngeal skeletal elements of the jaw and opercular regions in zebra fish. But genes involved in the endothelin pathway (*EDNI*, *ECE1*, *EDNRA* and *EDNRB*) have not shown linkage with orofacial clefts [18]. The *MSX1* homeobox gene is expressed at diverse sites of epithelial-mesenchymal interaction during vertebrate embryogenesis, and has been implicated in signalling processes between tissue layers. Mice lacking the *MSX1* function manifest a cleft secondary palate, a deficiency of alveolar mandible and maxilla and a failure of tooth development [19,20]. Analysis of a Dutch family with CL/P, CP and selective tooth agenesis, revealed a heterozygous nonsense mutation in the *MSX1* gene. Furthermore, complete sequencing of the *MSX1* gene in 1000 unrelated CL/P individuals showed that mutations on the *MSX1* gene alone could account for 2.0% of isolated CL/P [21].

Members of the *Dlx* gene family contain a homeobox that is related to that of distal-less (*Dll*), a gene expressed in the head and limbs of the developing fruit fly. Mutations in this gene have been associated with the autosomal dominant conditions like trichodontoosseous syndrome and amelogenesis imperfecta with taurodontism [22]. In the Japanese

population, relationships between CL/P or CPO and the *DLX3* gene failed to show positive association [23]. The present study also failed to demonstrate statistically significant difference in the frequency of different alleles between NSCLP and controls for the *DLX3* microsatellite. The proto-oncogene *BCL3* has a role in the transcriptional regulation of genes involved in cell cycles [24]. Analysis of three ethnic populations using a variety of parametric and non parametric techniques showed linkage between NSCLP and the *BCL3* microsatellite [25]. Moreover, the present study also failed to show statistically significant association between CL/P and controls using the *BCL3* microsatellite.

The association study design may determine whether alleles occur together with a specific phenotype more often than in a control group. There were several limitations to this study. First, we ascertained subjects without considering the smoking status and drug usage, which prevented us analyzing gene environment interactions and second, our relatively small total samples as well as markers, would have allowed the population stratification and influenced the ability to identify association between CL/P and micro-satellite polymorphisms. Large-scale association studies may provide a powerful tool for identifying alleles associated with complex phenotypes such as orofacial clefts.

**Declaration of Interest:** The authors report no conflicts of interest. The authors alone are responsible for the content and writing of this article.

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