

# In Search of Mutations of MSX1 Gene in Indian Nonsyndromic Cleft Palate Patients

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## ABSTRACT

**Objective:** To understand the role of MSX1 gene in Indian nonsyndromic cleft palate patients.

**Materials and methods:** A cross-sectional study was devised consisting of nonsyndromic cleft lip and palate and cleft palate only patients. The study was conducted in an Orthodontic Department of a Tertiary Teaching Hospital. Fifty-two patients were included in the study, inclusion criteria were: (A) nonsyndromic oral clefting, CI/P: (B) absence of a tooth or teeth (tooth agenesis).

**Interventions:** Case history, clinical examination and radiographic evidence of missing teeth were recorded. Blood samples, obtained after a written consent, were collected in 1.5 ml polypropylene microfuge tubes and transferred to the laboratory in dry ice. Genomic DNA was isolated and exon 1 site was subjected to polymerase chain reaction (PCR) amplification. The PCR product was further subjected to restriction enzyme analysis with 0.5 units of MbO<sub>2</sub>.

**Main outcome measures:** Mutations in exon 1 region of MSX1 gene were reported by Van den Boogard et al (2000), in a Dutch family with nonsyndromic oral clefting, this study was done to ascertain if mutation of exon 1 region could be involved in Indian nonsyndromic oral clefting.

**Results:** There was perfect digestion of MbO<sub>2</sub> enzyme, thus confirming the absence of mutation in the patient samples.

**Conclusion:** The absence of mutation in the selected patient samples correlates with those reported for a Caucasian population (Lidral et al, 1998), but not with those reported from a Dutch family (Van den Boogaard et al, 2000). The findings of this study are significant as they establish the genetic diversity involved in nonsyndromic clefting. This study can be further expanded to include the noncoding regions of MSX1 gene.

**Keywords:** Nonsyndromic clefting, MSX1 Gene, Polymerase chain reaction (PCR), Mutation.

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## INTRODUCTION

Cleft lip and/or cleft palate are among the most common birth defects. They seem to occur even before the time of Christ. The children afflicted with this anomaly are handicapped right from birth for breastfeeding, deglutition, recurrent nasobronchial infection, defective speech, improper growth and development of maxillary arch and overall facial development, affecting the total personality of the individual.

## NONSYNDROMIC CLEFT LIP AND PALATE

The most often proposed and accepted genetic model for non-syndromic CLP is that of multifactorial threshold (MF/T)

inheritance.<sup>1</sup> According to this model, the occurrence of the anomaly depends upon the additive effects of several minor abnormal genes (polygenes) and environmental factors. An accumulation of these factors is tolerated by the developing fetus to a threshold point beyond which there is a risk for malformation (MF/T) model.

## HOMEBOX GENES

Recent research has brought to light the existence of a large group of genes, which are highly conserved, and are responsible for coding for transcription factors involved in the regulation of expression of downstream target genes, known as the homeobox genes.<sup>2-4</sup>

The homeobox was originally discovered in the homeotic selector genes of the fruitfly *Drosophila melanogaster*, where they are responsible for specifying segment identity in the developing fly. Homeotic genes exhibit a feature known as co-linearity, their spatial arrangement along the chromosome is in the same order as their patterns of expressions along the anteroposterior axis of the fly embryo. Mutations in these genes can lead to bizarre homeotic transformations where one segment of the fly can be transformed into another segment.<sup>4</sup>

## MSX GENES

The MSX genes of vertebrates comprise a small family of chromosomally unlinked homeobox containing genes related

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to the *Drosophila* gene muscle segment homeobox (MSH). Despite their ancient pedigree, the MSX genes are expressed in a range of vertebrate specific tissues, including neural crest, cranial sensory placodes, bone and teeth. There are two main classes of MSX genes, the MSX1 and MSX2 genes. A third class of MSX gene (MSX3) found in the mouse, has been partly characterized; its homeobox places it in the MSX1 subclass.

The most direct evidence concerning the function of MSX genes at the tissue comes from mutations. MSX1 deleted mice fail to form teeth and have craniofacial abnormalities, which include absence of alveolar bones in the jaw and abnormalities in parietal, frontal, nasal membrane bones, malleus of the middle ear and cleft palate.<sup>5-8</sup>

As most of current research is centered in western countries, a strong need was felt to study the Indian nonsyndromic cleft lip and palate, so as to understand the genetic cause for clefting in India. A thorough understanding of genetics of clefting will help to understand the development of cleft palate, and also in the field of genetic counseling and most importantly the possibility of development of a specific cleft palate marker.

## AIMS AND OBJECTIVES

The aim of the study was to understand the role of MSX1 in the development of Indian nonsyndromic cleft lip and palate.

*Objectives of the study include:*

1. To amplify the chosen region (Exon1) of MSX1 gene from a South Indian cleft palate patient sample
2. To perform a mutation search, using a restriction enzyme analysis
3. To discuss the results and significance of this study.

## MATERIALS AND METHODS

### Case Sample

Fifty-two cleft palate patients were selected for this study, the patient's consent was obtained, and appropriate institutional review board approval was obtained. The study conformed to the protocols given in the Helsinki declaration for human studies.

The patients were selected on the basis of the following criteria:

1. Nonsyndromic cleft lip and/or palate
2. Clinical evidence of selective tooth agenesis
3. Both bilateral and unilateral cleft lip and palate were included in the study.

A clinical proforma was prepared, case history and diagnosis were recorded. A written consent was taken from all the patients.

### Chemicals

Trizma base, sodium dodecyl sulfate (SDS), ethylene diacetate (EDTA), boric acid, glycerol, formamide, mixed bed resin and dimethyl sulfoxide (DMSO) were obtained from Sigma Chemical Company, USA. The dideoxynucleotide triphosphates (dNTPs) were procured from Pharmacia Biotech,

Sweden. Agarose was purchased from FML bioproducts, USA. Molecular weight (100 base pair, Gene Ruler) was purchased from Genei Private Ltd, Bengaluru, India.

### TAQ Polymerase

TAQ Polymerase was purchased from Perkin Elmer, Sweden and Pharmacia Biotech, Sweden.

### Primers

Primers for Exon 1 of MSX1 gene FOR-5'CGG CTG CTG ACA TGA CTT C-3', MSX1 REV 5'-GCC TGG GTT CTG GCT ACT AC-3'.

### Restriction Enzyme

#### MbO<sub>2</sub>

*Source:* An *E. coli* strain that carries the cloned MbO<sub>2</sub> gene from *Moraxella bovis*.

### Reaction Buffer

Ne buffer 2, 50 mM, 10 nM MGCL 2, 1 nM dithiothreitol (pH 7.9 at 25°), incubated at 37°.

### Storage Conditions

50 mM KC, 10 nM Tris-HCl (pH 7.4), 0.1 mM, EDTA, 1 nM dithiothreitol, 200 µg/mlBSA and 50% glycerol store at -20°C.

- 5'....GAAGA(N)8Δ.....3'
- 3'....CTTCT(N)7Δ.....5'

## METHODS

The blood samples from nonsyndromic cleft lip and palate patients were collected by venipuncture using a sterile 2 ml disposable syringe.

1.5 ml of venous blood from each patient was collected and transferred to 2 ml polypropylene microfuge tubes, containing trisodium citrate, mixed thoroughly by inversion, and transported on dry ice.

Further, the blood samples were stored in -20°C deep freezer until the DNA isolation procedure.

## EXTRACTION OF GENOMIC DNA

Genomic DNA can be extracted by different methods. The method used, in the present study, was PCR. It is a simple procedure but great care should be taken to avoid any contamination of DNA from any other sources like contaminated glasswares or plastic tubes and buffers used for DNA extraction. PCR (polymerase chain reaction) being a powerful method of DNA amplification, even a small amount of contaminated DNA may be amplified, thus interfering in the results.

An easy source of nucleated cells for DNA extraction are the white blood cells in the buffy coat of peripheral blood. These can be obtained by venipuncture and the DNA can be extracted.

**RESULTS**

The search for the genetic cause of nonsyndromic cleft lip and palate has brought to focus the role of a major homeobox gene, MSX1 in the development of nonsyndromic clefting. As the linkage of a genetic disease is attributed to a defective gene (s), the presence of a mutation in the MSX1 gene would conclusively prove its role in nonsyndromic clefting of the Indian patients.

Genomic DNA were isolated from the peripheral lymphocytes of 52 blood samples obtained from individuals with cleft lip and palate.

The DNA was isolated by proteinase K/SDS digestion, followed by phenol/chloroform/isoamyl alcohol extractions and 0.1 M/95% ethanol precipitation (Fig. 1).

The precipitated DNA were suspended in MQ and was electrophoresed in a 0.7 agarose gel, containing 0.5 µg/ml of ethidium bromide (Fig. 2).

Placental DNA was used as normal control and was amplified with specific primer pairs in a 20µl reaction, and an aliquot of 5 µl was electrophoresed in 2% agarose gel containing 0.5 µg/ml of ethidium bromide.

*Lane 1:* Molecular weight marker (MW) (100 base pair ruler)

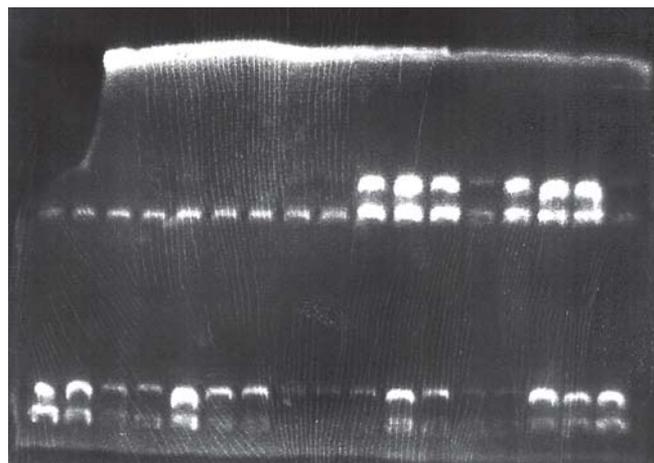
*Lane 2:* A 482 base pair product of MSX1 Exon1 amplified from placental DNA.

A 15 µl aliquot of PCR product of MSX1 Exon1 of placental DNA was digested with 2 units of MbO<sub>2</sub> in a 50 µl reaction, at 37°C overnight. The following day, the DNA in the reaction was precipitated by addition of 2.5 volumes of 95% ethanol and 0.1M sodium acetate. The precipitated DNA was suspended in 10 µl MQ and resolved in a 2% agarose gel. The restriction digestion results in 2 products of sizes 322 base pairs and 160 base pairs respectively (Fig. 3).

A band corresponding to a region between 300 base pair and 400 base pair marker and a band corresponding to a region between 100 base pair and 200 base pair marker confirmed a perfect digestion, and hence the required product.

Lymphocytes DNA were amplified with specific primer pairs in a 20 µl reaction and an aliquot of 5 µl was electrophoresed in 2% agarose gel containing 0.5 µg/ml of ethidium bromide.

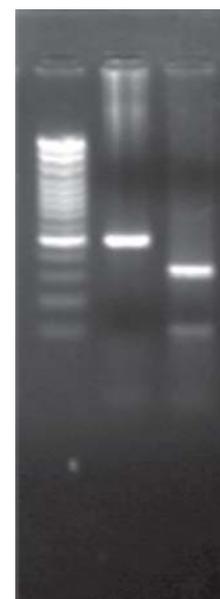
*Lane 1:* Molecular weight marker (MW) (100 base pair ruler)



**Fig. 1:** Genomic DNA isolates from patients' blood samples



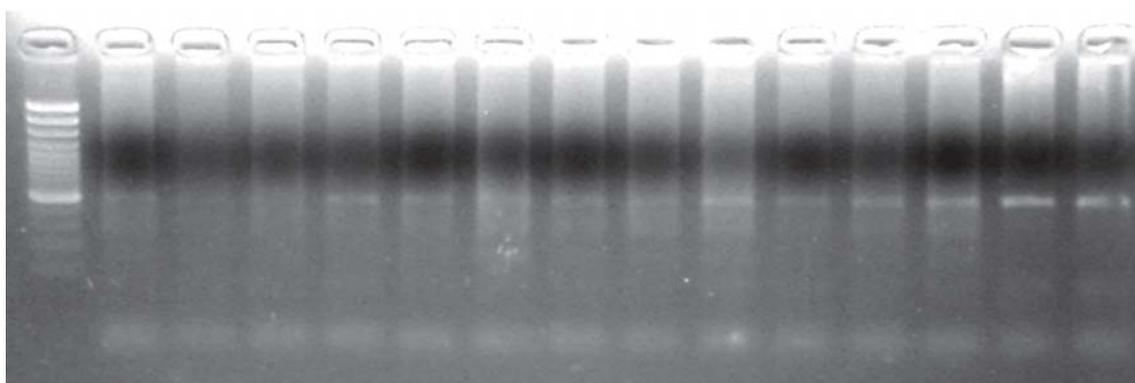
**Fig. 2:** PCR amplification of MSX1 Exon1 of the placental DNA samples



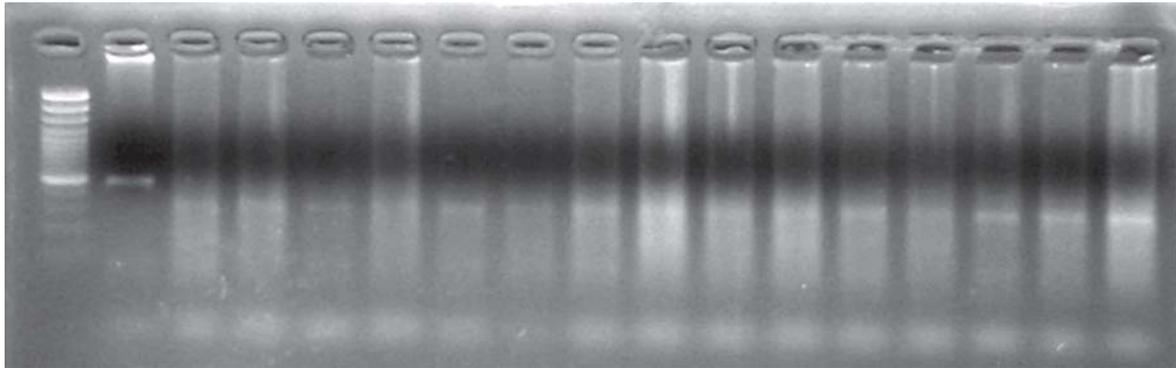
**Fig. 3:** Restriction digestion analysis of MSX1 Exon1 PCR product of placental DNA samples

*Lane 2-16:* A 482 base pair product of MSX1 Exon1 amplified from peripheral lymphocyte DNA (Fig. 4).

A 15 µl aliquot of PCR product of MSX1 Exon1 of DNA isolated from CL/P was digested with 2 units of MbO<sub>2</sub> in a



**Fig. 4:** PCR amplification of MSX1 Exon 1 of the CLP



**Fig. 5:** Restriction digestion analysis of MSX1 Exon1 PCR product of DNA sample isolated from CLP

50  $\mu$ l reaction, at 37°C overnight. The following day the DNA in the reaction was precipitated by addition of 2.5 volumes of 95% ethanol and 0.1M sodium acetate. The precipitated DNA was suspended in 10  $\mu$ l MQ and resolved in a 2% agarose gel.

A band corresponding to a region between 300 base pairs and 400 base pair marker confirmed perfect digestion, and hence the required product. However, the band corresponding to a region between 100 base pair and 200 base pair marker is not visible in this picture, due to a higher DNA template concentration.

*Lane 1:* Molecular weight marker (MW) (100 base pair ruler)

*Lane 2:* Undigested placental DNA

*Lane 3-17:* Digested CL/P samples (Fig. 5).

## DISCUSSION

Nonsyndromic cleft lip palate is a common congenital anomaly with significant medical, psychological, social and economic ramifications.

Traditionally, nonsyndromic cleft lip/palate is considered to have a multifactorial etiology,<sup>9,10</sup> Same authors have reported a significant increase in dental and dermatoglyphic asymmetry within individuals with cleft lip and palate. They postulated that polygenic systems normally buffer developmental processes against environmental effects. However, substitution of deleterious genes caused the level of buffering to be lowered beyond the point where environmental disturbances may be compensated and a developmental defect results.

Recent studies have shown evidence of the role of a homeobox gene, MSX1 (previously Hox1), in the normal formation of craniofacial structures and tooth.<sup>3</sup>

The homeobox genes are regarded as the master genes of the head and face controlling patterning, induction, programmed cell death and epithelial-mesenchymal interaction during development of the craniofacial complex.

Those of interest on craniofacial development include MSX1, MSX2 (Muscle segment), Dlx (Distalless), OTX (Orthodontical), GSC (Goosecoid) and SHH (Sonic Hedgehog).<sup>3</sup>

The proteins encoded by these homeobox genes are transcription factors which control the transcription of RNA from native DNA template. These transcription factors can switch genes on and off by activating or repressing gene

expression and therefore control other genes producing a coordinate cascade of molecular events which in turn control patterning and morphogenesis.<sup>11</sup> At a cellular level, this control is expressed through two main groups of regulatory proteins, the growth factor family and the steroid/thyroid/retinoid acid superfamily.<sup>12</sup> These regulatory molecules in the mesenchyme, such as fibroblast growth factor (FGF), epidermal growth factor (EGF), transforming growth factor alpha (TGF $\alpha$ ), transforming growth factor beta (TGF $\beta$ ) and bone morphogenetic proteins, are vehicles through which homeobox gene information is expressed in the coordination of cell migration and subsequent cell interactions that regulate growth.<sup>13</sup> By this means different parts of the DNA are activated in different cells regulating the different proteins, enzymes, etc. produced by different tissues and organs. These mechanisms will hold the key to understanding disease and dysmorphology and are the current subjects of intensive research in craniofacial biology.

In the search of genetic cause of nonsyndromic cleft lip/palate, screening of several candidate genes including TGFA, BCL3, DLX2, MSX1 and TGFB3 for linkage disequilibrium, on a predominantly Caucasian population, was done. Following this, significant LD was found between CL/P and both MSX1 and TGFB3 and CPO (cleft palate only) and MSX1, suggesting that these genes were involved in the pathogenesis of nonsyndromic clefting.<sup>14</sup>

A study on a Dutch family with tooth agenesis and various combinations of cleft palate only and cleft lip and palate showed a nonsense mutation (SER 104 stop) in the Exon 1 of MSX1 gene.<sup>15</sup> The mutant phenotype of the family was similar to that of the MSX1 mutant mouse.

Studies have shown that transforming growth factor Beta 3 (TGFB3) is essential to the development of normal palate and mice lacking as its orthologues have isolated cleft palate. Clearly there are diagnostic implications for determining susceptibility to human cleft palate, which is one of the most common birth defects.<sup>16,17</sup>

In the case of TGFB3, there are already therapeutic possibilities. Exogenous TGFB3 can correct palatal fusion defect in TGFB3 null embryos, raising the possibility of fetal therapy.<sup>17</sup> Fortunately, maternally administered recombinant TGFB3 crosses the mouse placental barrier. Exogenous TGFB3 is also known to reduce the severity of scarring following wound

healing in rats.<sup>18</sup> Raising the possibility that some individuals with cleft palate caused by genetic abnormalities in the TGFB3 pathway might be prone to excessive scarring following surgical correction thereby compounding an otherwise distressing abnormality.

Exogenous application of TGFB3 as an antiscarring therapy at the time of surgical correction of cleft may be particularly beneficial to such individuals.<sup>6</sup> The study on the Dutch family, reported in nature<sup>15</sup> with the mutation in 12 affected members, prompted an interest to conduct a similar study in the context of South Indian nonsyndromic patients, aimed to understand the genetic abnormality in such cases.

Blood samples from 52 CLP patients were obtained with consent, Exon 1 of MSX1 was amplified with PCR, restriction enzyme MbO<sub>2</sub> was used for mutational search. The study showed a perfect digestion of the MbO<sub>2</sub> enzyme, thus conforming absence of mutation in the patient samples. The results of the study correlate with the results of Lidral AC et al (1998),<sup>14</sup> who reported absence of mutation in the coding region of MSX1 gene in 69 nonsyndromic cleft lip and palate patients.

Our results are significant as they indicate to diversity in the genetic etiology of nonsyndromic clefting.

Future perspective of the present study should include (1) a familial study of CLP families, (2) extension of mutational search in the Exon 2 and non-coding region intron of the MSX1 gene.

A positive finding in the form of a mutation would help to understand a genetic abnormality in the Indian CLP patients. Such a finding is very important to understand the development of clefting, and for therapeutic possibilities in these unfortunate patients.

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