ORIGINAL ARTICLE

# EFFECT OF THE PAIRED RELATED HOMEOBOX GENE ON MANDIBULAR SIZE: A CASE-CONTROL STUDY

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## ABSTRACT

***Objective:*** *To evaluate the relationship between mandibular size and polymorphisms of the paired related homeobox-1 (PRX-1) gene in selected human subjects.* ***Materials and Methods:*** *The study was conducted on 20 individuals subdivided into two groups of 10 individuals each; Group A- Orthognathic individuals and Group B- Patients with mandibular retrognathia. Venous blood samples were collected and after DNA extraction, amplification of the extract was done using PCR followed by sequencing and analysis for polymorphisms at the selected site rs387906667 as referenced from the National Centre for Biotechnology Information (NCBI) database.* ***Results:*** *Analysis of the PRX-1 gene revealed no change at SNP rs387906667 between Groups A and B. Thymine remained unchanged in the homozygous and heterozygous forms for both the groups. Chromatograms of each of the samples displayed no change in the base pair at the concerned site corresponding to the SNP listed in the NCBI database.* ***Conclusion:*** *For the selected site (SNP rs387906667), no demonstrable correlation was found between mandibular size at nucleotide polymorphism within the Paired Related Homeobox-1 gene.*

***Keywords:*** *Mandibular micrognathia, PRRX1, Genetics, Single Nucleotide Polymorphism, Homeobox Genes.*

## INTRODUCTION

Ever since the pioneering and extensive work by Mendel in the 19th century in the field of genetics, the field has made significant advances in research, analysis and understanding the impact of genetics in the health sciences. Today we understand that genetics plays a vital role in the underlying etiology of various pathologies in general and, multiple craniofacial and dentofacial anomalies in specific.

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As understanding the cause of a given dentofacial anomaly plays a crucial role in the treatment planning phase for any orthodontist, it becomes clearer that fully rationalizing the genetic component for the same becomes an indispensable tool in the arsenal of the well informed orthodontic clinician.[1]

Essentially, malocclusion can be described as the physical manifestation of genetic and environmental factors or a combination of the two and their interactions on the development of the orofacial region. Mandibular growth in particular occurs under ardent genetic control. However, there isn't a single gene that by itself regulates the entire developmental process. Instead the system is managed by a variety of genes through a complex set or interactions with each other and their systems; a fault in any single of which could result in a pathologic phenotype.[2]

Mandibular development is a tortuous process beginning with mandibular mesenchymal formation from the first pharyngeal arch. Here, the mandibular processes consists of mesenchymal tissue enclosed in an epithelial capsule. The skeletal elements in the mandibular arch are derivatives of the cranial neural crest cells (NCC), whose patterning information is species exclusive.[3]

Mandibular micrognathia features inadequacy in mandibular bone growth. This prevents the formation of proper occlusal contacts during mastication and has also been found to hinder proper phonation andhas been indicated as a risk factor in the development of Sleep Apnea. It has been proven that the complex process of mandibular development is under the control of a number of genes including but not limited to Msx1, Shh, Bmp, Wnt, Dlx and Fgf. Studies regarding the process of its development have concentrated on the effects of various gene interactions that regulate the expression of the mandibular mesenchyme and also the various growth factors and transcription factors involved in the process.[4]

The role played by the paired related homebox (PRRX1) gene in the development of the mandible is also of crucial importance, given its requirement for proper formation of the proximal derivatives of the first arch. Additionally studies on mice with mutations introduced specifically to cancel the expression of the PRRX1 gene have resulted in deformed craniofacial

phenotypes including marked mandibular recessive traits in the mutant specimens.[5]

With regards to human studies, the role the PRX1 gene plays though not studied in great extent has shown a definite correlation with development abnormalities of the craniofacial region. Numerous studies have shown repeatedly that still born babies suffering from agnathia otocephaly. The condition is a fatal developmental malformation which is a result of mutations at either one of two specific sites within the PRX1 gene. Of import is the fact that the fetuses show completely missing mandibles and severely deformed ears amongst other symptoms.[6,7]

It seems prudent thus, from the perspective of an orthodontist, to evaluate the possible relation between this gene and mandibular micrognathia to better understand the condition in our patients so as to allow for possible early detection thereby altering existing treatment protocols for the earliest possible intervention.

## MATERIALS AND METHODS

This Case-control study was conducted between 2015 and 2016 on patients visiting the Department of Orthodontics and Dentofacial Orthopedics at A. B. Shetty Memorial Institute of Dental Sciences, Mangalore. Ethical clearance necessitated by the study requiring collection of human blood samples; was obtained from the Ethical Committee of Nitte University, where the study was conducted.

10 patients (5 males/5 females) with mandibular micrognathia and 10 patients (5 males/5 females) with orthognathic mandible were selected based on cephalometric and clinical measurements.

### Inclusion criteria

- Patients with Mandibular retrognathia / micrognathia
- Individual consent obtained to participate in the study
- Non syndromic individuals

### Exclusion Criteria

- Previous history of Orthodontic treatment, Maxillofacial or Plastic surgery
- Gross facial asymmetry determined clinically and radiographically
- Individuals with other skeletal or obliterating medical condition

## CLINICAL ANALYSIS

A case report with adequate patient details was obtained. Clinical profile examination was recorded by a single operator for all the samples who consented to take part in the research. Patients exhibiting a deficient mandible clinically were then assessed radiographically to confirm true mandibular skeletal jaw base deficiency. Profile radiographs were acquired, on which linear measurements were taken and recorded (Fig.1).

The subjects were clinically diagnosed for mandibular retrognathism. After obtaining the informed consent, lateral cephalograms were made for all individuals and following measurements were made:

a. Condylion (Co) to Gnathion (Gn)

b. Articular Point (Ar) to Gonial Point (Go)

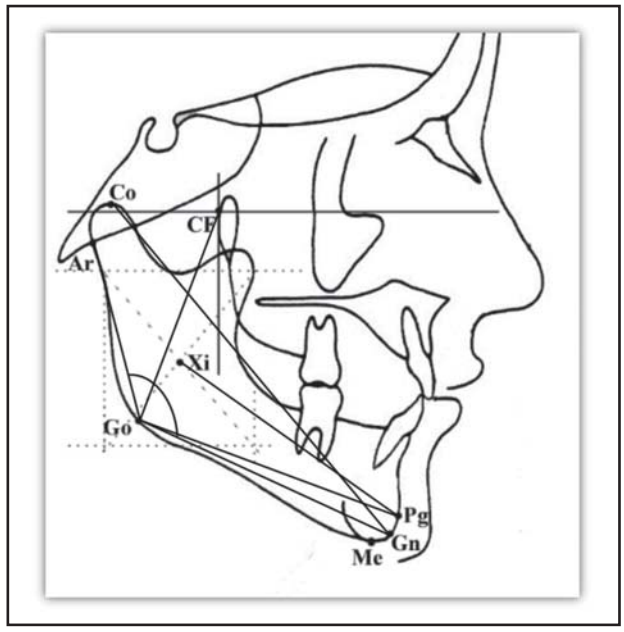


**Fig. 1: Measurements recorded for the purpose of Cephalometric Analysis**

Individuals were grouped into 2 Groups:

1. Control group (Orthognathic mandible) and a
2. Test group (Retrognathic mandible)

## DNA EXTRACTION & AMPLIFICATION BY PCR

DNA isolation: Fresh blood sample (6ml) was obtained from the above mentioned sample and selected DNA extraction/isolation protocol was followed.

PCR Amplification: The fragments corresponding to polymorphisms SNP at sites 57095 (T-C) and were subjected to amplification by running PCR (Polymerase

Chain Reaction), using the primers devised from the PRIMER 3 program (http://primer3.ut.ee/cgibin/primer3/rimer3web_results.cgi), under the conditions listed in Fig. 2.

```
OLIGO          start  len      tm     gc%   any    3' seq
LEFT PRIMER       55   20   59.98   50.00  3.00  0.00 agcagcgaaggaataggaca
RIGHT PRIMER     217   22   59.82   50.00  8.00  2.00 cagcctctcacagcttgagtta
SEQUENCE SIZE: 220
INCLUDED REGION SIZE: 220

 start                  len    tm     gc%   any    3' seq

 1 LEFT PRIMER         47   20   59.84   50.00  3.00  0.00 gaagagaaagcagcgaagga
   RIGHT PRIMER       217   22   59.82   50.00  8.00  2.00 cagcctctcacagcttgagtta
   PRODUCT SIZE: 171, PAIR ANY COMPL: 6.00, PAIR 3' COMPL: 1.00
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**Fig. 2 Selection of the Forward and Reverse Primer sequence SNP rs387906667**

For a total volume of 50µl master Mix, 0.4 µl of taq polymerase, 2 µM of each primer, 1.2 µM of dNTPs, 30µl of MH ∈O, 5µl of buffer and 1O µl of DNA (50 ng/µl). The fragment was viewed in 1.5% agarose gel using ethidium bromide (Table 1).

**Table 1: Primer used for assessment of Prrx1 gene SNPs.**

| SNPs | Primer Forward | Primer Reverse | Fragment size |
|---|---|---|---|
| rs387906667 | GGGACTCCTA CAGTGAATTTGG | ATGTGCCTCT GAGGAGGGTA | 284bp |

Detection of Polymorphism: The single nucleotide polymorphism (SNP) of the PRRX1 gene reported in the NCBI SNP Database, (www.ncbi.nlm.nih.gov) was recorded. The PCR samples were then sent for Sequencing. Table 1 highlights the primer used for the assessment of SNPs.

## RESULTS

## CEPHALOMETRIC MEASUREMENTS:

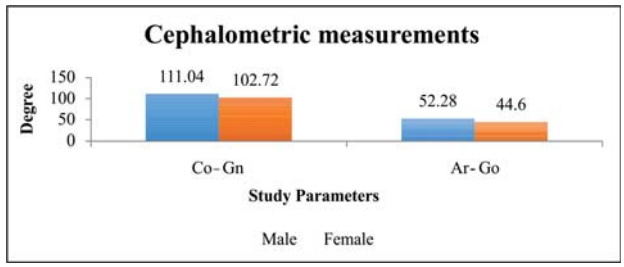
The cephalometric measurements for determining mandibular micrognathia were recorded. The mean values for males and females in the test group are listed in Table 2. It was noted that the cephalometric measurements for Ar-Go and Co-Gn were statistically significant. This was in accordance to the criteria considered for including the samples as exhibiting mandibular micrognathia. (Graph 1)

**Table 2: Cephalometric variables measured in the study**

| | Gender | N | Mean | SD | Mean difference | 95% Confidence Interval of the Difference | | p-value |
|---|---|---|---|---|---|---|---|---|
| | | | | | | Lower | Upper | |
| Ar-Go | Male | 5 | 52.28 | 0.94 | 7.68 | 5.05 | 10.30 | <0.001* |
| | Female | 5 | 44.60 | 2.36 | | | | |
| Co-Gn | Male | 5 | 111.04 | 3.56 | 8.32 | 3.43 | 13.20 | 0.004* |
| | Female | 5 | 102.72 | 3.11 | | | | |

Independent Sample t test, P<0.05 statistically significant

**Graph. 1: Cephalometric measurements for Test samples**




## CLINICAL ANALYSIS

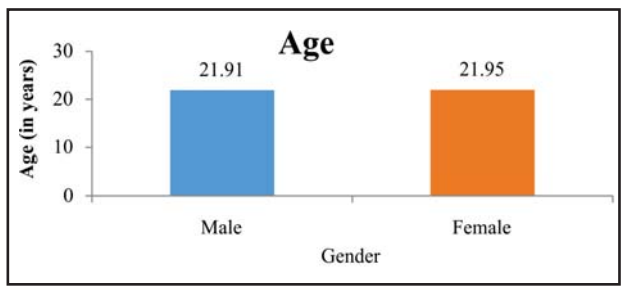
Twenty patients visiting the orthodontic clinic were identified according to the abovementioned criteria. 10 patients (5 Males / 5 Females) comprised the test group and 10 patients (5 Males / 5 Females) comprised the control group. The test and the control groups were matched for age and sex (Table 3, Graph 2)

**Table 3: Sample distribution for Age and Gender**

| Gender | N | Mean | SD | Mean difference | 95% Confidence Interval of the Difference | | t | p value |
|---|---|---|---|---|---|---|---|---|
| | | | | | Lower | Upper | | |
| Male | 10 | 21.91 | 1.63 | -0.04 | -1.59 | 1.51 | -0.05 | 0.95 (NS) |
| Female | 10 | 21.95 | 1.66 | | | | | |

Independent Sample t test, p < 0.05 statistically significant, p > 0.05 Non significant, NS

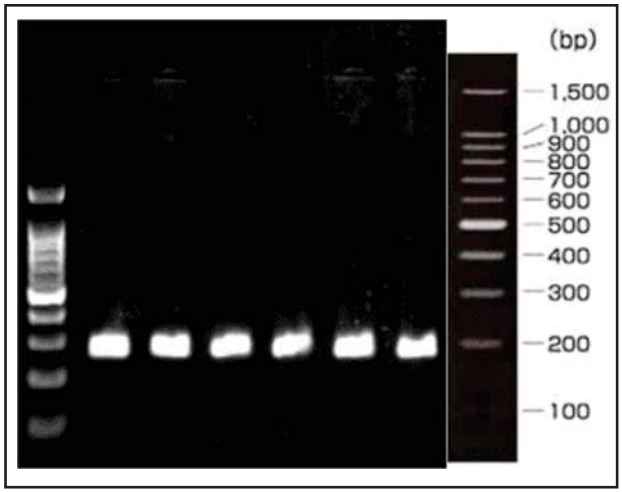
**Graph. 2: Sex distribution of the samples included in the study**



## PCR GEL ELECTROPHORESIS

The samples (10 test /10 control) were amplified by PCR analysis. They were then subjected to gel electrophoresis to confirm the presence of the desired bands of the amplified products at 284bp (284 Base Pair) region. Distinct bands were seen on running the gel electrophoresis confirming a positive amplification (Fig.3)

**Fig. 3: Agarose gel picture depicting the PCR products at 284bp**




## DETECTION OF POLYMORPHISM

Direct Sequencing was carried out using ABI 3130 (48 capillary) and / or 3730Xl (96 capillary) electrophoresis. The analysis of PRX1 gene polymorphism through sequencing of the SNP rs387906667, revealed no change in the base pair of either the test or the control samples present in homozygote form. Thymine remained unchanged in the homozygous and heterozygous forms for both the test and control samples. These results were confirmed from individual chromatograms of each sample where in the Thymine base pair remained unchanged consistently in all the samples studied.

The control samples included in the study were also analyzed to check for any irregularities or mutations in their genetic constitution with the database control taken as standard. The results showed that there was no polymorphism exhibited in all the control samples that were tested. Thymine remained unchanged as was seen in the test samples (Fig. 4-5).

## DISCUSSION

There are various components participating in the development of the mandible. The diversity of events unfolding from the beginning of the primordial germ formations to the establishment of the epithelial signals

**Fig. 4: Chromatogram for the Test samples showing no change in Thymine base pair**

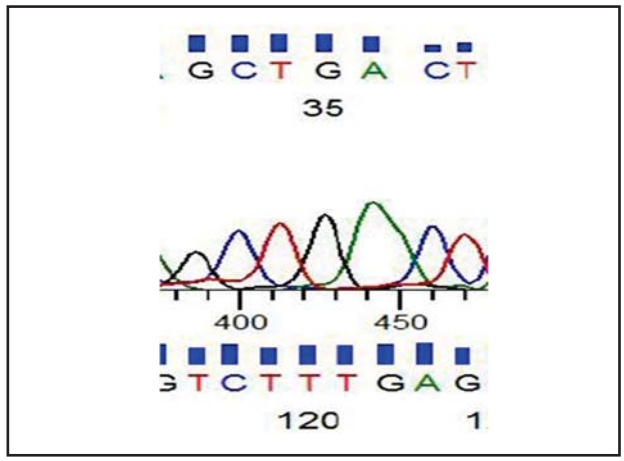
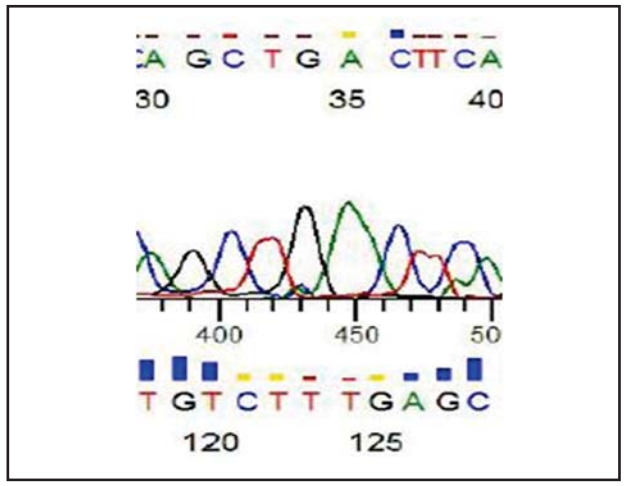



**Fig. 5: Chromatogram for the Control samples showing no change in Thymine base pair**




regulating mandibular mesenchymal development, are under the scrutiny of a wide variety of genes and their corresponding signaling pathways in a dose dependent and time specific manner. Regulation of specific genetically dependent pathways is important for the mandibular mesenchyme to achieve its full potential in transforming into a mandible of normal shape and size.[8]

The visible or measurable characteristics of an individual is their phenotype. A phenotype can be explained based on the expression and combination of the following three factors: (1) the genetic information passed on through generation (the individual's genotype); (2) the environment regulating the expression of these regulatory proteins; and (3) any genotype-environment interactions that could have an effect on the expression.[9]

Although there have been studies enumerating the genetic components affecting growth of the mandible, there are few reports verifying the association between

the phenotypic expression and the associated genotypic constitution. This may be attributed to the wide variety of genes involved in facial development, including mandibular growth. The intricacy and complexity of various processes involved in mandibular development make it difficult to pin point a specific cause for derangement in normal physiological growth.[4]

Ten Berge et al deduced based on their observation of Pax-9 markers in Prx1 and Prx2 double mutant mice that the resultant phenotypic jaw defects were the result of an upstream regulatory defect. Additionally they postulated further that the appearance of a single lower incisor rather than two, was the result of a single pax-9 domain being present upon fusion of the medial process of the mandible rather than two domains. This further bolstered their assertion that Prx1 and Prx2 had definitive roles to play in conjunction with other homeobox genes in the development of the medial region of the mandible. In addition the double mutant mice also showed a decrease rate of proliferation in the distal mesenchyme of the mandibular process which resulted in a overall reduction in the mandibular size of the affected mice.[10,11]

In the present study, Prx1 was singled out for additional evaluation of polymorphism because Berge et al in their study on mice noted that $Prx1^{-/-}$ mutant mice tend to show severe development anomlies albeit less aggravated than those seen in $Prx1^{-/-}$ and $Prx2^{-/-}$ double mutant mice. However single mutant $Prx2^{-/-}$ mice showed no significant phenotypic defects.[12] Additionally, the role of Prx1 has been implicated in the regulation of the Shh pathway which as we have seen earlier plays a concomitant role with BMPs and FGFs in the formation of the mandible. However, an additional implication of the role of Shh is in the development of the lower anterior teeth, wherein its expression or antagonism has been shown to facilitate or retard tooth development respectively.[13]

Human studies as mentioned earlier have been limited to stillborn foetuses suffering from agnathia otocephaly complex. In nearly all the studies conducted, the role of Prx1 has been clearly outlined. Additionally, two polymorphism sites have been implicated with regard to the mistranslation of protein coding sequences in Exon 2 and Exon 4 of Prx1. In this study the SNP in Exon 2 at site rs387906667 was singled out for study. Polymorphism at this site is the consequence of a Thymine to Cytosine amino acid conversion which results in a protein translation of Phenylalanine to Serine (F113S).[14]

In this study, sequencing was carried out for the known SNP s387906667 as identified through the NCBI database.[15] All samples collected for the purpose of the study were subjected to direct Sequencing. The results showed that the selected site for SNP rs387906667 for the Prx1 gene did not show any polymorphisms in all the individuals sampled for the study. The Thymine base pair remained unaltered in both Control and Test samples.

The NHLBI Exome Sequencing Project (ESP) database shows over 1481 variations seen in the PRX1 gene. It might be possible that polymorphisms in any one of the other SNPs noted might be responsible for mandibular micrognathia and hence further studies should be carried out in this context to establish the role of Prx1 gene polymorphism.

In this study, familial history wasn't obtained. As it has been shown in previous studies, environment and heredity have a major role to play in the phenotypic expression of an individual's genotype. Thus, lack of records of the patient's family history could be considered as a limiting factor of the study.

## CONCLUSION

The selected site of the Single Nucleotide Polymorphism (SNP rs387906667) of the PRX1 gene did not show any alteration in the base pairs in all the samples studied and thus we can conclude that for the selected Single Nucleotide Polymorphism site, it can be said that there is no correlation between mandibular size and genetic polymorphisms.

## CONFLICTS OF INTEREST DECLARATION

The authors do hereby declare that no conflicts of interest exist in terms of participants of the research study, the suppliers for equipment used and the laboratories where DNA sequencing was carried out.

## SOURCE OF FUNDING

The study in its entirety was solely self-funded by the lead author, with no external funding from any academic or commercial institution.

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