



Original Research Article

Runx2 gene single nucleotide polymorphism in Class II and Class III malocclusions

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ABSTRACT

Aim and Objective: To assess the prevalence of RUNX2 gene polymorphism in skeletal class II and class III malocclusions.

Materials and Methods: Unstimulated salivary samples of 36 patients (18- 30 years of age group), comprising 18 with skeletal class II and 18 with skeletal class III were collected from a tertiary care hospital in Mangalore. Salivary DNA samples were collected and analyzed using Sanger sequencing. Digital tracing was performed on lateral cephalometric radiographs by using AutoCAD software for digitization to assess the antero-posterior and vertical relationship of the maxillary and mandibular arch.

Results: When polymorphism of 36 samples comprised of 18 Class II and 18 Class III were assessed, 83.3% Class III malocclusion and 5.6% Class II showed RUNX2 gene polymorphism in the population. Chi Square test which indicated that the results are statistically significant in Class III malocclusion for RUNX2 single nucleotide polymorphism ($p < 0.001$).

Conclusions: RUNX2 gene polymorphism was statistically significant in skeletal Class III malocclusion when compared to skeletal Class II malocclusion. This is a preliminary study done on a smaller sample, we need a larger sample size to confirm our findings.

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1. Introduction

Genetics is a branch of biology deals with the mechanisms of inheritance and the causes of diversity in living beings.¹ The size, shape of the maxilla and mandible, morphology of teeth present, and soft tissue morphology are under genetic influences.^{2,3}

Growth and development is mainly the result of interaction between different genetic and environmental factors overtime.^{4,5} Higher the genetic component, the lower the rate of a successful orthodontic treatment outcome.^{6,7} The identification of the most vital genes and the biochemical action of these genes to a specific jaw discrepancy is the first approach essential for the search of

a solution.^{8,9}

Malocclusions usually believed to have a negative interaction of hereditary and environmental factors.^{10,11} From the beginning, the role of inheritance and environment as causes of malocclusion and dentofacial deformities have been the subject of great controversy.¹²⁻¹⁴ During foetal development, the mandibular and maxillary growth is affected, resulting in skeletal malocclusion.¹⁵⁻¹⁷ The significant prognathic mandible in the Hapsburg Royal family's ancestry strongly suggests a genetic component in the inheritance of this craniofacial feature.¹⁸⁻²⁰ One parent with a comparable phenotype was found in 1/3 of the affected Hapsburg family members with severe class III malocclusion.^{21,22} Orthodontists must consider the genetic basis of a skeletal anomaly during the diagnosis and

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treatment planning.^{23–25} Genetic basis of skeletal anomaly can be identified using different methods like single gene sequencing, site specific mutation testing, gene panels, molecular testing, immunohistochemistry, microsatellite instability.^{26,27}

Runt – related transcription factor 2 (RUNX2) plays an important role in osteoblast differentiation, tooth development and chondrocyte maturation; hence its involvement in the growth of the craniofacial area is crucial.^{28,29} Genetic studies therefore are important to know if this polymorphism affects different classes of malocclusion.³⁰

2. Aim and Objective

1. To assess the prevalence of RUNX2 gene polymorphism in different classes of malocclusions mainly class II and class III malocclusion.

2.1. Source of data

Unstimulated salivary samples of 36 patients (18- 30 years of age group), comprising 18 with skeletal class II and 18 with skeletal class III were collected from a tertiary care hospital in Mangalore. Salivary DNA samples were collected and analyzed using Sanger sequencing. Digital tracing was performed on lateral cephalometric radiographs by using AutoCAD software for digitization to assess the antero-posterior and vertical relationship of the maxillary and mandibular arch.

2.2. Ethics statement

This prospective study was approved by the Institutional Research Ethics committee of, AJIMS (AJIEC/REV/271/2019)

2.3. Inclusion criteria

1. Patients with skeletal class II and class III malocclusions.
2. Patients between 18- 30 years of age group.

2.4. Exclusion criteria

1. Presence of systemic diseases.
2. Presence of congenital deformities.

3. Materials and Methods

Cephalometric measurements: Skeletal class II and class III malocclusion cases were assessed using following parameters.

- Steiner's SNA angle: Class II > 82°, Class III < 82°
- Steiner's SNB angle: Class II > 80°, Class III < 80°
- ANB angle: Class II > 6°, Class III < 2°
- Down's A-B Plane angle: Class II < -4.6°, Class III > -4.6°

Wits appraisal (AoBo): Class II: BO is behind AO, Class III :BO is ahead of AO > 2mm

3.1. Dna extraction

DNA isolation is done with Favorgene kit which involves following steps:

1. Transfer up to 200µl saliva to a micro centrifuge tube
2. If RNA-free genomic DNA is required, add 10ul of 20 mg / ml RNase A. Mix thoroughly by vortexing and incubate at room temperature for 2 min
3. Add 20 ul Proteinase K to the sample, and then add 200 ul FATG2 Buffer to the sample. Mix thoroughly by pulse-Vortexing. Incubate at 60°C for 30 min. Vortex occasionally during incubation.
4. Incubate at 70° C for 10 min
5. Add 200 ul ethanol (96-100% to the sample mixture Mix thoroughly by pulse- vortexing
6. Briefly spin the tube to remove drops from the inside of the lid
7. Place a FATG Mini Column in a Collection Tube. Transfer the mixture (including any precipitate) carefully to the FATG Mini Column. Centrifuge at full speed(18,000 xg) for 1 min then place the FATG Mini Column to a new Collection Tube.
8. Add 400 ul WI Buffer to the FATG Mini Column. Centrifuge at full speed for 1 min then discard flow-through. Make sure that ethanol has been added into WI Buffer when first open.
9. Add 750 ul Wash Buffer to the FATG Mini Column. Centrifuge at full speed for 1 min then discard flow-through. Make sure that ethanol has been added into Wash Buffer when first open.
10. Centrifuge at full speed for an additional 3 min to dry the column. This step will remove the residual liquid.
11. Add 100 ul of preheated Elution Buffer to the membrane of the FATG Mini Column. Stand the FATG Mini Column for 3 min. For effective elution, make sure that the elution solution is dispensed onto the membrane center and is absorbed completely. If less sample to be used, reduce the elution volume to 50 ul to increase DNA concentration and do not elute the DNA using less than suggested volume (50 ul). It will lower the final yield.
12. Centrifuge at full speed for 2 min to elute DNA.

Additional requirement: RNase A, 96- 100% ethanol, PBS
Hint: Set dry or water baths: 60° C for step 3 and 70 °C for step 4.

3.2. Polymerase chain reaction

Amplification of RUNX2 gene was performed from the extracted DNA (n=27) using 2X PCR Master Mix (SMOBio, Hsinchu City, Taiwan). The primer sets (Eurofins

Genomics India Pvt. Ltd., Bangalore) used for amplification were:

rs6930053 RUNX2 Forward: 5'-GTATGTCATTTCTGTACTTTTCG-3'

rs6930053 RUNX2 Reverse : 5'-GTGCTATTTTCCTGTCCTTATC-3'

The reaction mixture contained 0.2 μ M of each primer per sample and the rest of reaction mixture was prepared as per the kit protocol. Typically one reaction contained the following ingredients:

0.5 μ M of each primer, 2mM MgCl₂, 0.2 mM of dNTP, 200 mg of gDNA, 1.75U of DNA polymerase.

Standard amplification conditions are: 95 degree for 4min followed by 35 cycles and final extension for 10 min at 72 degree Celsius.

The PCR products (639bp) were visualised in 1% Agarose gel and subsequently sent for sequencing.

3.3. Gene sequencing

The RUNX2 PCR amplified gene was sent to Eurofins Genomics Pvt. Ltd., Bangalore for Sanger sequencing with forward primers.

3.4. Sample size estimation

To detect a difference of 50% of RUNX2 gene in class II and class III malocclusions using 95% confidence interval and 90% power. The sample size estimated for the study is 18 saliva samples of each class II and class III.

$$n = \left[z_{1-\alpha} + z_{1-\beta} \right]^2 \frac{p_1 q_1 + p_2 q_2}{(\text{the difference in proportions})^2}$$

3.5. Sampling technique

Convenience sampling will be adopted.

4. Results

When polymorphism of 36 samples comprised of 18 Class II and 18 Class III were assessed, the following results were obtained.

SNP's are found in 44.4% of the total sample

83.3% Class III malocclusion and 5.6% Class II showed RUNX2 gene polymorphism in the population. Chi Square test which indicated that the results are statistically significant in Class III malocclusion for RUNX2 single nucleotide polymorphism (p<0.001)

There is alteration in the nucleotidic sequence where in T is replaced by C in the mutant sample.

For rs6930053, the ancestral allele is T, allele C is mutant.(Figure 1)

Electrophoresis gel shows a band of amplified dna at 639 bp which would indicate the dna extraction is successful.(Figure 2)

Polymorphism in Malocclusion

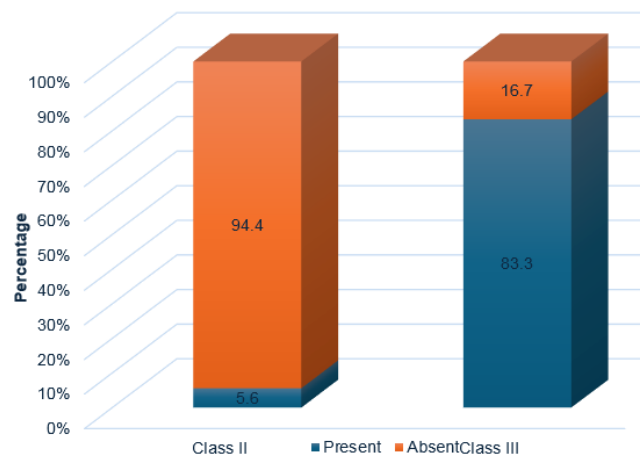


Chart 1: Comparison and association of polymorphism in different classes of malocclusions.

4.1. Results of this study can be summarized as follows

1. RUNX2 gene polymorphism (rs6930053) in skeletal Class II malocclusion was 5.6% and in skeletal Class III malocclusion was 83.3% from the samples obtained from a tertiary care hospital in Mangaluru.
2. RUNX2 gene polymorphism was statistically significant in skeletal Class III malocclusion when compared to skeletal Class II malocclusion.

5. Discussion

Skeletal malocclusion is generally predisposed by genetic and environmental factors as it's etiology. In this study RUNX2 gene mutation has been assessed to identify it's relationship with the malocclusion.

The RUNX2 genes are found on chromosomes 6p21 and 17 in humans and mice, respectively.^{31,32}

This gene encodes a nuclear protein with a Runt DNA-binding domain that belongs to the RUNX transcription factor family.^{33,34} This protein is required for osteoblast development and skeletal morphogenesis, and it serves as a scaffold for nucleic acids and regulatory factors involved in the expression of skeletal genes.³⁵

Bones are formed through one of two ossification processes: (i) intramembranous or (ii) endochondral ossification.³⁶

Both endochondral and intramembranous mechanisms are used in the ossification of the skull³⁷ both procedures entail the conversion of pre-existing mesenchymal tissue into bone tissue. Intramembranous ossification is the direct conversion of mesenchymal tissue to bone and endochondral ossification is a process by which a cartilage intermediate is formed initially which will be replaced by

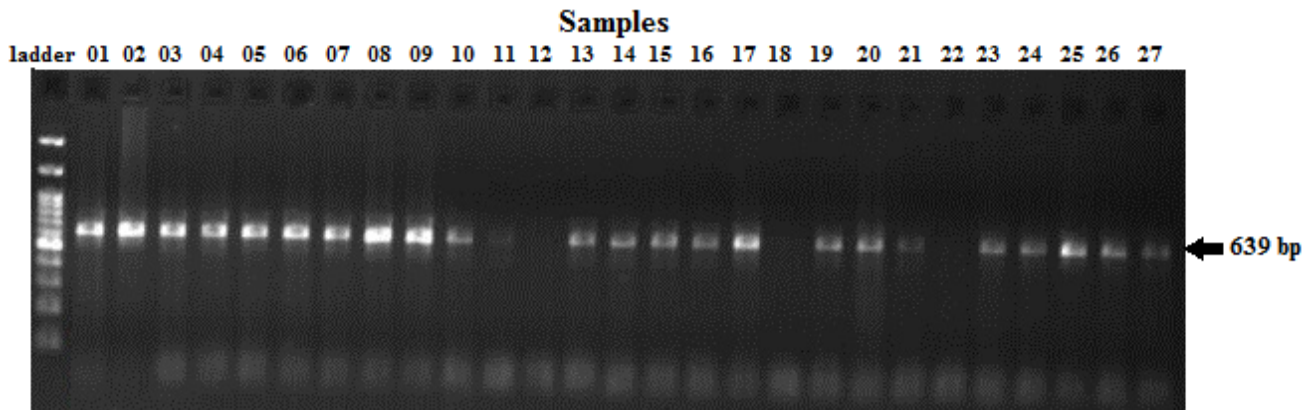


Figure 1: Genotypic analysis shows the following results:

Table 1: Prevalence of RUNX2 polymorphism in different classes of malocclusions using Chi square test.

		Polymorphism in malocclusion		Total
		Malocclusion		
Absent	Count	Class II	Class III	20
	%		17 94.4%	
Present	Count	1	15	16
	%	5.6%	83.3%	44.4%
Total	Count	18	18	36
	%	100.0%	100.0%	100.0%

. $\chi^2=22.05$ $p<0.001$ vhs

**Figure 2:** Agarose gel picture

bone cells.^{38,39}

RUNX2 is involved in the differentiation of chondrocytes into hypertrophic chondrocytes and further differentiation processes, such as ossification.⁴⁰

The TMJ's general structure is similar to that of most synovial joints, but the cartilage that caps the mandibular condyle (mandibular condylar cartilage or MCC) is a secondary cartilage, that indicates the morphogenesis of the TMJ and its components begins after the mandible has formed and analogous joints in the limbs have formed.⁴¹ Primary cartilages of the limbs are formed by the interplay of the mesenchyme and epithelium, whereas secondary cartilages form in response to local biomechanical stimuli. RUNX2 is responsible for osteogenic response, as evidenced by the presence of mRNA for osteogenic lineage markers (e.g., collagen, RUNX2, and Osterix) in mandibular condylar cartilage.^{42,43}

The skeletal form had a strong correlation to the presence of type II collagen fibres in the masseter muscle. In both the sagittal and vertical dimensions, these correlations have substantial implications for facial structure. Mechanism through which RUNX2 may function in the sagittal dimension is through its effect on condylar development,⁴⁴ and periosteal activation of osteoblast gene expression.

Cleidocranial dysplasia (CCD), an autosomal-dominant heritable skeletal disorder characterised by open or delayed

closure of calvarial sutures, hypoplastic or aplastic clavicles, and supernumerary teeth, is caused by RUNX2 mutations in humans.⁴⁵

An allele is one of two or more versions of a gene. A gene's allele is one of two or more variants. For each gene, an individual inherits two alleles, one from each parent. The individual is homozygous for that gene if the two alleles are same. The individual is heterozygous if the alleles are different. Though the term allele was originally intended to describe gene variation, it is now also used to denote variation in non-coding DNA sequences.⁴⁵

The two members of a pair of alleles separate during gamete production, according to Mendelian law of segregation, which is one of the Mendelian Laws of Inheritance. As a result, each gamete only has one member of each gene pair.

Mendelian law of dominance when applied says that "It is stated that one factor in pair of traits dominates while the other remains suppressed in inheritance unless the two factors in the pair are recessive. In the next generation of parents who are pure for contrasting traits, there will be only one type of trait."^{42,43}

While most genes exist in two allele forms, some have multiple alleles for a trait.⁴⁴ Likewise, there is polygenic inheritance where in the traits are determined by more than one gene.⁴⁵

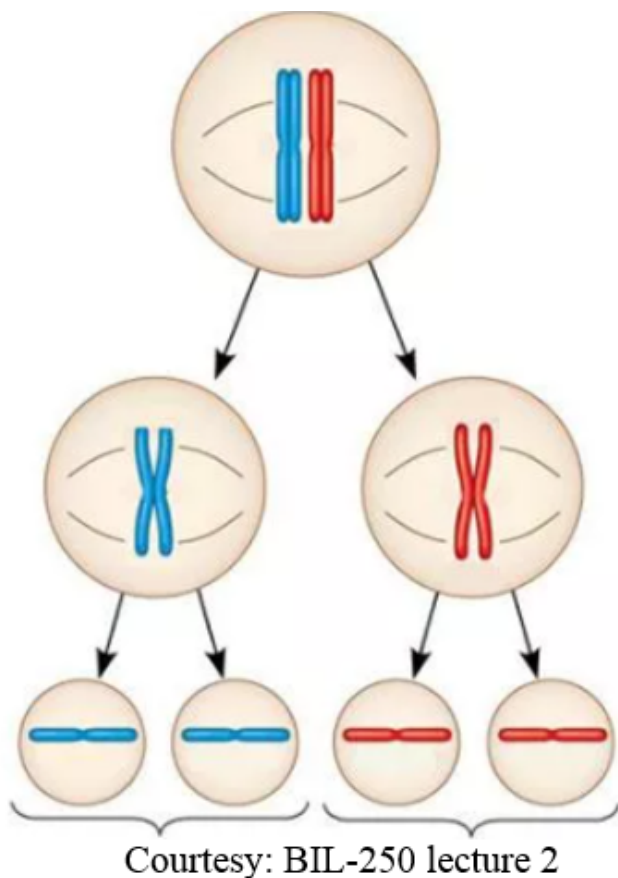


Figure 3: Segregation of parent chromosomes

Alteration in even one non coding sequence would result in a definitive phenotypic variation. Identification of the mutant allele in one generation can significantly predict the probability of transfer of this allele to the future generations based on the laws of genetics.

This study was conducted to determine whether there was an association of class II and class III skeletal malocclusion and the gene RUNX2 in Mangalore population. The genomic DNA was extracted from unstimulated saliva of 18 class II and 18 class III patients based on the skeletal parameters.

In the present study, it is observed on forward gene sequencing that the nucleotide T has been replaced by C in the non coding sequence which has resulted in the phenotypic Class III malocclusion in the patients.

SNP's are found in 44.44 % of the total sample.

In previous studies conducted by Mokhtar KI et al significant association of class II malocclusion and the gene RUNX2 in Malaysian population was found where as 83.3% Class III malocclusion and 5.6% Class II showed RUNX2 gene mutation in population.

Sample 13 was taken from a cleft patient with Class III malocclusion, Sample 14 and sample 23 patient

had hypodontia. This indicates that RUNX 2 plays a vital role in the development of craniofacial skeleton and dentition, thus opening up new horizons for future studies in this direction.

Sample 25 whose sample had a mutation when her family tree was traced, it was found that patient's other sibling and mother had similar phenotype. However the patient's prognathism was more severe in comparison to her other family members. This would suggest the severity of the skeletal malocclusion can increase in the subsequent generation constantly. This tells us how important it is to identify a genetic malocclusion and plan treatment accordingly with all the measures available. Sample 11 and sample 18 were dead during the transportation for gene sequencing.

It is difficult to control skeletal malocclusion due to genetic causes by the conventional orthodontic treatment. Orthodontic treatment coupled with orthognathic surgery was the only resort to overcome this problem. That's why it is important to diagnose early these skeletal malocclusions to carry out definitive treatment.

The results of this study enable us to identify a genetic malocclusion which would aid in accurate diagnosis and treatment planning, thus providing appropriate treatment. So it is always better to determine the cause first than the effects. Genetic analysis aids in planning definitive treatment and reduces the treatment duration for the patient. Knowing the exact treatment that has to be carried out right after the patient enters the clinic in an early age would resolve the problem early without worsening the malocclusion, as this would be cost effective and treatment effective.

Orthopedic and functional appliances can be used to treat malocclusions of genetic origin(skeletal discrepancies) when detected in growing period except in extreme cases where surgical intervention is needed after the growth completion. Genetic testing would give information regarding the need of treatment for a child and age at which treatment can be begun to control a skeletal malocclusion from becoming a more severe one.

This is a preliminary study done on a smaller sample, we need a larger sample size to confirm our findings. Also forward and reverse sequencing give confirmatory results for the sample.

6. Conclusions

The study concluded that:

1. RUNX2 gene polymorphism (rs6930053) in skeletal Class II malocclusion was 5.6% and in skeletal Class III malocclusion was 83.3% from the samples obtained from a tertiary care hospital in Mangalore.
2. RUNX2 gene polymorphism was statistically significant in skeletal Class III malocclusion when

compared to skeletal Class II malocclusion.

- This is a preliminary study done on a smaller sample, we need a larger sample size to confirm our findings.

7. Source of Funding

None.

8. Conflict of Interest

None.

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