IS MAXILLARY GROWTH AFFECTED BY THE COMMONLY OCCURRING MUTATIONS IN FGFR3 GENE?

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ABSTRACT:

Background: Mutations in several Fibroblast Growth Factors and Fibroblast Growth Factor Receptors (FGFR) were found to cause a variety of bone/osteo-genic diseases. More than 97% of achondroplasia cases were reported to be caused by one of two mutations G1138A and G1138C of the gene FGFR3. These commonly occurring SNP sites were targeted to evaluate whether they are responsible for altered maxillary growth in the selected subjects.

Material and Methods: Genomic DNA of 30 subjects having Prognathic Maxilla, 30 individuals having Retrognathic Maxilla were compared with that of 30 individuals having normal maxilla. Genomic DNA was extracted from serum using HiPura™ Forensic Sample Genomic DNA Purification Kit as per the manufacturer’s instructions. A programmable gradient Thermo cycler (Corbett) was used for standardization of PCR conditions for FGFR3 Forward and Reverse primers. Agarose Gel Electrophoresis was used to detect the PCR products and digested with MspI and Sfcl (Thermo Scientific) in separate tubes and visualized under UV Trans-illuminator.

Results & Conclusion: None of the analyzed DNA samples of the selected subjects showed any presence of the targeted mutation sites in selected subjects analyzed in the present study.

Keywords: FGFR3 Gene, PCR analysis, Prognathic Maxilla, Retrognathic Maxilla, SNP

INTRODUCTION:

Craniofacial morphology is a complex but interesting trait that reveals individual phenotypic differences within a species as well as morphological divergence among species. Multiple genetic factors and environmental variables account for the large degree of variability in human craniofacial morphology. A genetic component has been reported for 60–90% of craniofacial traits, including facial height, position of the lower jaw, and cranial base dimensions as suggested by Savoye et al. Johannsdottir et al [1]
Most current research activities in relation to genetic control of facial growth and development are concentrating on the role of Hox genes and various Growth factors and signalling molecules in influencing the facial growth [2]. In recent years growth factors have shown to influence the proliferation and differentiation of wide variety of cell types. It has been postulated that they are well suited to control the complex morphogenic events taking place in various organs during embryonic development or during the adult phase [1]. It is shown that Growth Factor such as Fibroblast Growth Factor (FGF) can regulate proliferation and migration of variety of cell types including osteoblastic cells. The pio trophic fibroblast growth factor (FGF) and fibroblast growth factor receptors (FGFR) play a critical role in morpho-genesis by regulating of cell proliferation, differentiation and cell migration during embryonic development. In adult organism FGF play an important role in the control of the nervous system, in tissue repair and wound healing [3-8].

Researchers believe that this receptor regulates bone growth by limiting the formation of bone from cartilage (a process called ossification), particularly in the long bones.

Understanding the underlying molecular mechanism for the role of FGF / FGFR in the regulation of bone development and disease will ultimately lead to better prevention and treatment of FGF signalling related bone deformities and disorders [3].

Fibroblast Growth Factor Receptor 3 (FGFR3) Gene:

The human Fibroblast growth factor receptor-3 was identified by Keegan et al who demonstrated also that it binds aFGF and bFGF. Human and mouse FGFR3 share 92 % homology at the amino acid sequence level as indicated by Perez-Castro et al in 1997. The receptor is known also as Cek2. In the nomenclature of CD antigens the receptor has been given the designation CD333.[9]

The FGFR3 gene is located in chromosome region 4p16.3 9Thompson et al. 1991) and encodes a 4.4-kb mRNA (Keegan et al. 1991). (4p16.3: short (p) arm of chromosome 4 at position 16.3.)

Molecular Location on chromosome 4: base pairs 1,795,038 to 1,810,597.

This gene encodes a member of the fibroblast growth factor receptor (FGFR) family, with its amino acid sequence being highly conserved between members and among divergent species. FGFR family members differ from one another in their ligand affinities and tissue distribution. A full-length representative protein would consist of an extracellular region, composed of three immunoglobulin-like domains, a single hydrophobic membrane-spanning segment and a cytoplasmic tyrosine kinase domain. [10].The extracellular portion of the protein interacts with fibroblast growth factors, setting in motion a cascade of downstream signals, ultimately influencing mitogenesis and differentiation. This particular family
member binds acidic and basic fibroblast growth hormone and plays a role in bone development and maintenance. Mutations in this gene lead to craniosynostosis and multiple types of skeletal dysplasia.\textsuperscript{[11]}

**Normal functions of the FGFR3 Gene:**

The FGFR3 gene provides instructions for making a protein called fibroblast growth factor receptor 3. This protein is part of a family of fibroblast growth factor receptors that share similar structures and functions. These proteins play a role in several important cellular processes, including regulation of cell growth and division, determination of cell type, formation of blood vessels, wound healing, and embryo development.

FGFR3 signals influence a variety of cellular events and processes largely through inducing or repressing expression of target genes in a cell-specific context. The tyrosine kinase-mediated transmembrane receptor FGFR3 is an important negative regulator of linear bone growth acting mainly through signaling pathways to inhibit chondrocyte proliferation and terminal differentiation in the growth plate. The ultimate effect of FGFR 3 signaling is inhibitory \textsuperscript{[12,13]} Similar opinion was also expressed by Chuxia Deng et al in their experiment on mouse and they concluded that certain human disorders such as achondroplasia can be interpreted as gain-of-function mutations that activate the fundamentally negative growth control exerted by FGFR3 kinase \textsuperscript{[14]. Several gain –of-functions FGFR mutations induce pre mature ossification of the cranial sutures and most of the mutations in FGFR gene family are gain –of-function.\textsuperscript{[4]}

Nakajima et al (2003) have suggested a role of FGFR3 in fracture repair. FGFR3 mRNA is expressed strongly in resting and proliferating chondrocytes, weakly in hypertrophic chondrocytes, and not in osteoblasts. During fracture repair, it is expressed strongly in pre-hypertrophic chondrocytes.\textsuperscript{[8]}

**Role of FGFR3 in Craniofacial disorders:**\textsuperscript{[5]}

Mutations in the FGFR3 gene have been shown to cause a variety of skeletal dysplasias (Spranger, 1988), ranging from mild (hypochondroplasia) and more severe forms (achondroplasia) to lethal neonatal dwarfism (thanatophoric dysplasia) (Passos-Bueno MR et al, 1999; Bellus et al, 1995, 1996; Muenke et al, 1997; Rousseau et al, 1996; Tavormina et al, 1995; Webster and Donoghue, 1996). These mutations can involve activating mutations and loss-of-function mutations. Cho et al (2004) have reported that activated FGFR3 found in patients with achondroplasia and related chondrodysplasias lead to recycling of activated receptors and amplification of FGFR3 signals.

Mutations in the fibroblast growth factor receptor (FGFR) genes 1, 2, and 3 are causal in a number of craniofacial dysostosis syndromes featuring craniosynostosis with basi-cranial and mid-facial deformity. Great clinical variability is displayed in the pathologic phenotypes encountered \textsuperscript{[16,17].}
Mutations in FGFR receptor genes are known to affect suture development in mice and humans, and such mutations have been found to occur in Apert, Crouzon and Pfeiffer syndromes (Wilkie, 1997). Mutations in the FGFR3 gene have been demonstrated as the underlying cause of several chondro-dysplasias, a heterogeneous group of disorders affecting the growing skeleton.\(^\text{[6,18]}\)

Specific mutations were identified in achondroplasia (ACH), hypochondroplasia (HCH) and in thanatophoric dysplasia (TD) (Rousseau et al. 1994, 1995; Shiang et al. 1994; Bellus et al. 1995; Tavormina et al. 1995). Recently, Meyers et al. (1995) made the unexpected observation that FGFR3 mutations may cause Crouzon syndrome with acanthosis nigricans. Meanwhile FGFR3 mutations have been found in other cases of Crouzon syndrome with acanthosis nigricans (Wilkes et al. 1996), and in another cranio-synostosis syndrome (Bellus et al. 1996). Because of the recently shown pleiotropic effect of FGFR3 mutations, it seems likely that further mutations might be responsible for a variety of related subtypes of chondrodysplasia or cranio-synostosis syndromes.\(^\text{[19]}\)

New mutations in FGFR3 have been identified in patients with craniosynostosis syndromes (Meyers et al. 1995; Bellus et al. 1996; Wilkes et al. 1996), a group of disorders that are usually caused by FGFR1 and FGFR2 gene mutations (for review see Muenke and Schell 1995). These cases and the observation of additional FGFR3 phenotypes presenting with affected bone and brain (Franccomano et al. 1996) indicate that mutations in the FGFR3 gene other than those already known may result in different, unexpected clinical entities. This underlines the need for screening the complete FGFR3 gene in disorders with symptoms of the known FGFR3 disease spectrum and especially in cases of ACH, HCH and TD, where none of the known mutations can be found.\(^\text{[19]}\)

More than 97% of achondroplasia cases have been reported to have caused by one of the two commonly occurring mutations in FGFR3 gene. These two mutations, G1138A and G1138C, in exon 10 of the FGFR3 gene result in specific amino acid substitution (G380R). It has been shown that the FGFR3 nucleotide 1138 is among the most highly mutable single nucleotides known in human genome.\(^\text{[20]}\)

With this review of literature background, we have planned and designed this study with an objective of exploring the possibility of implicating the most commonly occurring mutation sites in FGFR3 gene to the maxillary morphology in individuals with either maxillary prognathism or maxillary retrognathism.

**MATERIAL AND METHODS:**

Individuals visiting the Institution were selected for the study as per the inclusion criteria and grouped in to three groups based on their clinical maxillary morphology in anterior-posterior dimension as decided by latewral cephalometric analysis.
Sample size: 30 subjects in each group consisting of similar age group and similar ethnic/geographic background.

Inclusion Criteria:

- Non syndrome individuals
- No history of previous Orthodontic and or Maxillo-facial surgical Treatments
- No oblivious facial asymmetry and gross deformity
- Individuals consenting to participate in the study

Group I: Individuals with normal maxillary morphology. (Control Group)

Group II: Individuals with Retrognathic Maxilla

Group III: Individuals with Prognathic Maxilla

After obtaining the informed consent from the subjects and the Institutional Ethics Committee approved the research proposal, Cephalometric radiographs were made for all the selected subjects and analysis of dento-facial characteristics were carried out to confirm the clinical diagnosis (Table1) (Fig 1).

Genetic Protocol:

1. DNA Isolation:
Fresh blood samples were obtained from the above mentioned subjects. Genomic DNA was extracted using HiPura™ Forensic Sample Genomic DNA Purification Kit. 200 µl of blood was mixed with 300µl of Lysis solution AL, 20 µl proteinase K (20mg/ml) and 20 µl of IMDTT in a micro-centrifuge tube. The mix is thoroughly mixed by pulse-vortexing for 10-15 seconds and incubated at 55°C for one hour. 300µl of Lysis solution C1 was added and again mixed thoroughly for 10-15 seconds and incubated at 70°C for 10 minutes. Then the mix was centrifuged at 12000xg for 1 minute at room temperature. The supernatant was transferred in to another collection tube and mixed with 300 µl of ethanol (96-100%) and transferred to HiElute mini prep spin column placed in a 2ml collection tube and centrifuged at 600 x g for one minute. The floe through was discarded and 700 µl wash solution (WS) was added and centrifuged at 1200 x g for one minute. The DNA was eluted by adding 100 µl of elution buffer (ET) to the mini spin column placed in a fresh micro centrifuge tube and collected by centrifuging the column at 6500 x g for 1 minute. Thus extracted DNA was stored at -20°C for further use.

2. Identification of specific mutation sites / SNP:
Based on the back reference research works and registered genetic research canters, the two commonly occurring mutation sites were identified as a) G1138A and b) G1138 C and were targeted to evaluate whether these mutation sites are also responsible for the maxillary morphology in the selected subjects for this research study.

3. PCR Amplification:
A programmable gradient Thermo cycler (Corbett) was used for standardization of PCR conditions for FGFR3 Forward and Reverse primers. The sequences of primers used are as follows: FGFR3 F-5’-AGGAGCTGGTGAGGCTGA-3’ and FGFR3 R-5’ GAGATCCTGTCAGGCTGG-3’.

PCR was carried out in 5.0µl of template DNA.IX assay buffer (10mM Tris-HCl, Ph

9.0, 1.5 mM MgCl₂, 50 mM KCl, 0.01% Gelatin), 100 µl of each of the four dNTP’s, 10 picomoles of forward and reverse primers and 1.25 U of Taq DNA polymerase (Hi media).

Using the gradient function of the PCR machine, a gradient of 60°C to 68°C was set for determining the Optimal annealing temperature of FGFR3 F/R primers. The annealing temperature of 68°C was considered for further evaluation as a solid single band was observed at that annealing temperature.

The optimised PCR protocol consisted of an initial denaturation step at 94°C for 12 minutes followed by 40 cycles of 30 seconds at 94°C; 30 seconds at 68°C, 30 seconds at 72°C and a final elongation at 72°C for 10 minutes.

**Agarose Gel Electrophoresis** was used to detect the PCR products. 2.5% (w/v) agarose gel was prepared in IX TAE buffer. The molten gel was cooled to below 65°C and mixed with Ethidium Bromide to a final concentration of 0.5 µg / ml. It was then poured to gel mould and allowed to cool. 10 µl of PCR products were mixed with 4 µl of 6X loading buffer and then loaded in to the wells of agarose gel. Gene Ruler 50bp DNA ladder (Thermo Scientific) was used as a molecular weight marker. Electrophoresis was carried out at 100-120 V and the bands were visualized under UV transilluminator.

**4. Restriction Digestion:**

The PCR products were digested with MspI and Sfcl (Thermo Scientific) in separate tubes for 3-4 hours at 37°C and inactivated by increasing temperature to 80°C for 20 minutes. The digested PCR products were resolved on 2.5 % agarose gels and visualised under UV transilluminator.

**RESULTS & DISCUSSION:**

The two most commonly occurring SNP sites in FGFR3 gene causing achondroplasia, G1138A and G1138C can be detected by PCR amplification of a 164–nt portion of FGFR3 Gene and digestion with enzymes, Sfcl and MspI. Since more than 97% of achondroplasia are caused by these two mutation sites, we have selected the same SNP sites to target in our study to evaluate whether same SNPs are also responsible for maxillary morphology in the selected subjects. We have analyzed the genomic DNA of 30 subjects having Prognathic Maxilla, 30 individuals having Retrognathic Maxilla and compared them with that of 30 individuals having normal maxillary morphology (control Group). None of the analysed DNA samples of the selected subjects in our study showed any presence of the targeted mutation sites (Figure 2). This indicates that the two commonly occurring SNPs causing achondroplasia have no role to play in the morphology of maxilla in the selected individuals of our study.

Sequencing of 164 bp corresponding to the targeted mutation sites also demonstrated normal sequence of amino acids and did not suggest any role for the Gene FGFR3 in the maxillary morphology of the selected individuals (Figure 3).

In view of the findings of the study, further studies are required to sequence the complete gene in order to evaluate
the presence of any novel mutation sites in the FGFR3 gene that can be attributed to the morphology and growth of maxilla. It may also be possible that some other genes are involved and are responsible for the control of growth of the maxilla. These aspects of the genetic analysis need to be explored in different phenotype expressions in different ethnic groups.

CONCLUSION:

Based on the results of the present study, it may be concluded that the targeted mutation sites in FGFR3 gene are not responsible for either the excess or the deficient growth of maxilla in sagittal plane in the selected subjects.

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Acknowledgements: Authors would like to thank

1. Prof. Dr. U.S.Krishna Nayak, HOD, Department of Orthodontics and Dean, A.B. Shetty Memorial Institute of Dental Sciences, Nitte University, Mangalore.

2. Dr.Pravin Kumar Rai and Staff Members of Mangalore BioTech Laboratory, Mangalore for helping us with the Genetic analysis
### TABLE:

**Table 1: Selection of subjects based on Cephalometric analysis**

<table>
<thead>
<tr>
<th>Group</th>
<th>Sample size</th>
<th>Mean SNA angle (Degrees)</th>
<th>Mean SNB angle (Degrees)</th>
<th>Mean N-A mm</th>
<th>Mean N-B mm</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Group I Control</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control 15 M</td>
<td>83.5</td>
<td>80.5</td>
<td>1.5</td>
<td>0.5</td>
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<tr>
<td>Control 15 F</td>
<td>82</td>
<td>79.5</td>
<td>1.0</td>
<td>1.0</td>
<td></td>
</tr>
<tr>
<td><strong>Group II Retrognathic Maxilla</strong></td>
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<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>17M</td>
<td>75</td>
<td>80.5</td>
<td>-5.5</td>
<td>1.5</td>
<td></td>
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<tr>
<td>13F</td>
<td>75.5</td>
<td>79.5</td>
<td>-6.5</td>
<td>0.5</td>
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<tr>
<td><strong>Group III Prognathic Maxilla</strong></td>
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<td></td>
<td></td>
<td></td>
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<tr>
<td>16M</td>
<td>89.5</td>
<td>80.5</td>
<td>8.0</td>
<td>1.5</td>
<td></td>
</tr>
<tr>
<td>14F</td>
<td>88.5</td>
<td>79.5</td>
<td>7.5</td>
<td>1.0</td>
<td></td>
</tr>
</tbody>
</table>

### FIGURES:

1. Angle SNA
2. Angle SNB
3. A to N perpendicular
4. B to N perpendicular

**Fig 1: Cephalometric analysis**
Fig 2: Sample figure of PCR analysis of FGFR 3 Gene

Lane M: 50 bp molecular weight marker
Lane 1: Sample A digested with MspI
Lane 2: Sample A digested with SfcI
Lane 3: Sample P digested with MspI
Lane 4: Sample P digested with SfcI
Lane 5: Sample Po digested with MspI
Lane 6: Sample Po digested with SfcI
Lane 7: Sample J digested with MspI
Lane 8: Sample J digested with SfcI
Lane 9: Sample R digested with MspI
Lane 10: Sample R digested with SfcI
Lane 11: Sample A undigested (Control)
Lane 12: Sample P undigested (Control)
Fig 3: Sample Sequencing of 164 bp