Exclusion of pituitary homeobox 2 gene polymorphism in vertical mandibular asymmetry patients: a preliminary study

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Abstract. Pituitary Homeobox 2 (PITX2), is an active gene as a paired-related homeobox gene that encodes multiple isoforms. Its Nodal pathway in determination of left-right patterning during embryogenesis has been reported in satellite cells and expressed in adult human skeletal muscle. PITX2A and PITX2B are produced by alternative splicing and used of different promoters. PITX2C uses an alternative promoter located upstream of exon 4. PITX2D is produced by PITX2C alternative promoter and differential splicing. The 5’-primers and 3’-antisense primer were unique for each isoforms. Variability measurement in vertical dimension showed stronger genetic component than sagittal. This study aims to obtain the genotype marker of vertical mandibular asymmetry related to PITX2A and PITX2D isoform by visualization of the amplified product on stained gel to allele specific oligonucleotide between the case and control with Restriction Fragment Length Polymorphism (RFLP). Determination of vertical mandibular asymmetry based on condylar height asymmetry index of pre-treatment panoramic radiograph using Kjellberg’s technique whilst vertical mandibular growth pattern using lateral cephalogram. The differences of condylar height asymmetry in case-control based on vertical growth pattern was compared using Pearson’s chi-squared test. DNA extraction of 129 out-coming orthodontic patients in Universitas Sumatera Utara Dental Hospital were obtained from Buccal swab. Then DNA samples were amplified by Polymerase chain reaction (PCR) and digested with NciI restriction enzyme prior to electrophoresis visualization. There was no significant statistical difference in vertical mandibular asymmetry compared to vertical mandibular growth pattern. The RFLP analysis did not show any polymorphism for PITX2A and PITX2D isoform. All of the samples showed wild type homozygote. Further analysis method, except RFLP, were required to understand the genetic factor in the variance of vertical mandibular asymmetry.

Keywords: PITX2, polymorphisms, vertical mandibular asymmetry
1. Introduction

Factors potentially contributing to timing and amount of mandibular vary considerably among individuals. One of the common craniofacial deformities is an asymmetric growth of the mandible or other diseases affecting the facial growth. It has a direct impact to aesthetic concern and functional problems of the stomatognathic system [1,2]. Factors potentially contributing to this individual masticatory action variation is related to the consistency of the diet and genetic predisposition that seems to be direct consequences of malfunction at the cellular level of condylar cartilage [3]. Some regulatory gene expressions have been reported in mandibular condyle growth and required further studies.

For the majority of malocclusion with mandibular asymmetry, we need to identify vertical, transversal, or sagittal discrepancy simultaneously. The study of malocclusion to asymmetry is quite rare nowadays. The different expression of masticatory muscle fibers that attached directly to mandibular condyle demonstrated stronger genetic component of variability measurement in the vertical dimension rather than sagittal [4-6]. Smooth and skeletal muscles in the temporomandibular joint attach directly to the mandibular condyle. The asymmetrical function and activity of jaw movement differ in both sides can contribute to the difference in condylar development. Any dental treatment during growth period might influence the downward and forward mandible growth potential. The displacement of the mandible towards the affected side such as asymmetric condylar growth could happen [2,3,7].

The emergence of Single Nucleotide Polymorphisms (SNPs) in genetic analysis can influence splicing in the intronic region and gene expression in the promoter. It can help to answer the association of DNA variants with either the presence or absence of different phenotypes among individuals from the different population, such as: multivariance of the vertical mandibular pattern [8,9]. PITX2 gene is a bicoid-like homebox transcription factor located in the 4q25 locus that is required for the early determination, development, and maintenance of extraocular muscles (EOM) compared to the limb skeletal muscle. This gene was also required for maintaining characteristic properties of the adult EOM phenotype [10]. Formerly, an inversion of this gene involved in phenotype of a child with translocation (t4;14)(q25;q13) who had mild craniofacial dysmorphism and agenesis of the corpus callosum without the limb or ocular abnormalities though not many studies have been done on PITX2 gene and craniofacial expression [11]. Human PITX2 gene mutations was genetic factor related to haploinsufficiency condition that affected the eyes, teeth and umbilicus with associated abnormalities of the craniofacial region (midfacial hypoplasia, prognathism) [12,13]. In syndromic patients related to Peters’ anomaly, Axenfeld-Rieger Syndrome patients, CHARGE syndrome, iridogoniodygenesis, and iris hypoplasia [11,13,14,15].

The PITX2 locus produces five isoforms, designated PITX2A-2E that arises through differential splicing and alternative promoter usage. An alternative promoter in exon 3 and 4 of PITX2C gene resulted in isoform of PITX2D and PITX2E gene. Both alternative splicing promoter usage which overlapped and distinct expression patterns produced different isoforms [16]. Identification of gene expression in Nodal Signaling Pathway of PITX2 expression gene was also reported to help promote development of asymmetry due to a central role in left-right asymmetry [6]. Suggestive associations (p<0.05) were also identified with PITX2 as candidate gene by genotyping 222 single-nucleotide polymorphisms in 82 genes/loci asymmetric variations of 3-dimensional dentoalveolar phenotypes in subjects with malocclusion [17].

Various radiological modalities have been evaluated for imaging of the mandibular asymmetry from 2D to 3D measurements. The panoramic radiograph is relatively accessible and provides the bilateral view of the mandible compared other radiograph. Then, the panoramic radiograph can be used to evaluate condylar mandibular asymmetry with 6% difference by Habets’ formula and 93% difference by Kjellberg’s technique [2,7,18,19]. Several authors have used this method to assess mandibular asymmetry in patients with temporomandibular disorders (TMD), unilateral and bilateral posterior crossbites, various sagittal malocclusions, and cleft lip and palate [7,20,21]. The incidence of 13% in 605 primary school students in Genoa showed malocclusions, wrong posture, and ocular
divergences disorders and required multidisciplinary medical approach [22]. Therefore the similarity in asymmetry gene expression of any organ in the vertebrae might be considered as a genomic risk factor.

One type of polymorphisms method is restriction fragment length polymorphism (RFLP) that showed the presence of different fragment lengths after DNA samples digestion with specific restriction endonucleases. This method can detect the difference in homologous DNA sequences and can be used as molecular marker specific to a single clone/restriction enzyme combination. The RFLP probes are frequently used in genome mapping and analysis, such as: genotyping, forensics, paternity tests, and genetic disease diagnosis [9].

Recent studies reported that the PITX2 gene expression in satellite cells of masseter muscle was related to asymmetry. Here, we test the hypothesis that PITX2 expression is responsible for the functional difference in the condylar asymmetry that is related to the PITX2 target gene in organogenesis, especially ramus height. The aim of this research is to identify the Single Nucleotide polymorphism (SNPs) of PITX2 gene especially related to isoform PITX2A and PITX2D in condylar asymmetry by using Restriction Fragment Length Polymorphism (RFLP) method.

2. Method

This study was approved by Research Ethics Committee of Universitas Sumatera Utara Medical Faculty and conducted in Universitas Sumatera Utara Dental Hospital. This was a case-control study by analyzing pre-treatment panoramic and lateral cephalometric radiographs. Then DNA isolation was taken by buccal swab while patients came for orthodontics treatment.

2.1. Experimental inclusion

The pre-treatment panoramic and lateral cephalometric radiographs of orthodontics patients between 11 to 25 year old were analyzed with Kjellberg’s technique. There were no previous orthodontics, prosthodontics treatment, or occlusal adjustment history. There were also no traumatic facial injury or congenital disease. Then, 79 subject with asymmetry and 50 subjects without asymmetry regardless of temporomandibular joint status were recalled for genotyping analysis.

2.2. Problem formulation

Selection of samples for vertical mandibular asymmetry used Kjellberg’s technique. The condylar height asymmetry was also estimated using 93% difference (Figure 1) [19]. The symmetry between the right and left condylar height was estimated using the following formula:

\[
Kjellberg Symmetry Index (SI) = \frac{\text{CH}_{\text{R}}}{\text{CH}_{\text{L}}} \times 100
\]

In figure 2, type of vertical mandibular growth pattern based on the SN-MP angle [23]. The validity and reliability of inter-rater and intra-rater digitized panoramic radiograph and cephalometry measurements using t-test. The differences of condylar height asymmetry in case-control based on vertical growth pattern was compared using Pearson’s chi-squared test (SPSS software, version 22.0 for windows; SPSS, Chicago IL).
Patients’ DNA was obtained using buccal cell swab procedure for genomic analysis using Presto TM (Geneaid, Taiwan). Primer pairs used for PITX2A were AAATCTCTGCTGACGTCACGT and CCAGACTCGCATTATCTCAC and PIT2XD, CAGCTCTTCCACGGCTTCT and TTCTCTCCTGGTCTACTTGG [13]. The annealing temperature for the PCR reaction was at 55°C. The target PCR results for PITX2A was 370 bp and was 374 bp for PITX2D [13]. Amplified products were then separated on agarose gel, reamplified, separated further on 2% agarose gels, purified with KAPA Taq™ EXtra HotStart ReadyMix with dye. The amplicons were subjected to restriction digestion using NCII and then analyzed on a 3% agarose gel [15].
3. Results and Discussion

Table 1. Ramus height asymmetry based on different vertical mandibular growth pattern.

| Vertical Mandibular Growth Pattern | Ramus Height Symmetry | Ramus Height Asymmetry | Total | P       |
|-----------------------------------|-----------------------|------------------------|-------|---------|
| Low Angle (<27°)                  | 10                    | 19                     | 29    |         |
| Normal (27°-37°)                  | 33%                   | 65.5%                  | 73    | 0.185   |
| High Angle (37°)                  | 25.9%                 | 74.1%                  | 27    |         |
| **Total**                         | **50**                | **79**                 | **129**|        |

*Pearson’s chi-square test, p<0.05 significant.

Figure 3. PITX2A gene of vertical mandibular patients on 3% agarose gel. 100 bp marker: wild type homozygote patterns of PITX2A gene

Figure 4. PITX2D gene of vertical mandibular patients on 3% agarose gel. 100 bp marker: wild type homozygote patterns of PITX2D gene
The mean age of 129 samples was 20.7±3.2 years old. There were no significant differences in condylar height asymmetry based on different vertical mandibular growth pattern (Table 1). We demonstrated the absence of PITX2A (Figure 3) and PITX2D (Figure 4) gene mutations in a series of 129 orthodontic patients with or without vertical mandibular asymmetry. The RFLP analysis did not show any polymorphism for PITX2. All of the samples showed wild type homozygote.

Since genetic factors were found to be responsible for only 40% of the skeletal and dental variations resulting in malocclusion, the genetic component was reported higher for the skeletal pattern than dental features. Individual differences in amount and direction of ramus growth and modelling can be explained by mandibular rotation and displacements. Multivariate assessments revealed that superior condylar growth and ramus modeling were mostly close to genetic factor.

The condylar height asymmetry using panoramic radiograph has been widely used to validate clinical tests of diagnostic categories in mandibular asymmetry. In our study there was no statistically significant differences for vertical mandibular asymmetry based on various mandibular growth pattern. The findings were different with previous CBCT imaging findings that reported a significant difference in hypodivergent patients, but not in normal and hyperdivergent patients [20]. The complexity of genetics in dentofacial variation explains why most treatments approaching malocclusion with mandibular asymmetry directed to the symptoms rather than its etiology. Despite the polygenic traits of mandible asymmetry, the study of genomic is fundamental for any clinicians in understanding the biology of underlying craniofacial growth and dental phenotype related to asymmetry [4,5,24].

The mechanism of bite force from the mandible to cranium suggest the magnitude of continuous joint loading is related to growth rotation and morphology of mandible condyle. The etiology of mandibular asymmetry is vast and often a combination of genetic and environmental factors. In this study, we analyze PITX2A and PITX2D isoform due to a difference in promoter and similar target base pair. The myogenic factor is one of the common causes in mandibular asymmetry related to Nodal signaling pathway (NSP) genes in masseter muscles. The PITX2 is a key regulator in the development process and is expressed in the brain, heart, pituitary, mandibular and maxillary region, eye, and umbilicus. Alternative splicing and the use of different promoters produced these PITX2 isoforms. PITX2A and PITX2B are generated by alternative splicing mechanisms. The PITX2C used an alternative promoter located upstream of exon 4. The PITX2D isoform acts to down-regulate the transcriptional activities of PITX2A and PITX2C. The identification of a new PITX2 isoform (PITX2D) generated by the PITX2C alternative promoter and differential splicing found in human [16,25]. This data suggests that the differences in PITX2 gene expression has emerged as a key element involved in the fine-tuning mechanism that regulates skeletal-muscle development as well as the differentiation and cell fate of satellite cells in adult muscle [6,11,25].

All isoforms can form homodimers, except for PITX2B, which can form heterodimers. The interactions between PITX2 isoforms provide a mechanism to regulate gene expression. This PITX2 isoform provided and controlled several mechanisms that interact in gene activation and repression [10]. The PITX2 isoforms were expressed and differentiate to activate genes involved in the development of the brain, craniofacial region, and pituitary. These isoforms were expressed in humans and identified from a human craniofacial library [11]. These isoforms transcriptional activity is cell-dependent with the mechanism for the regulation of PITX2 transcriptional activation through the action of a novel PITX2 isoform, like in Axenfeld-Rieger syndrome patient [14].

The screened probes for RFLPs using genomic DNA of different genotypes digested with restriction endonucleases. The previous study demonstrated the Ncil enzyme used as RFLP marker could cut both alleles in homozygous wild type sample, thus revealing a unique blotting pattern characteristic to a specific genotype at a specific locus. Separation of the amplified fragment by PCR depends on enzyme restriction site. Our study focused on PITX2A (370bp) and PITX2D (374bp) by considering the sizes in base pairs of both isoforms almost equal. PITX2D is also produced by PITX2C alternative promoter and differential splicing. Modern orthodontists need to be aware of the basis of the genetic sciences due to rapid advances in genetic techniques and application in orthodontic
practices. Genome-wide association studies, metabolic pathway analysis and candidate gene studies are necessary to further the evidence base for the practice of orthodontics to determine the diagnosis for each patient in the era of truly personalized orthodontics [5].

4. Conclusions
In the vertical mandibular asymmetry, we found the exclusion of PITX2 polymorphism in the region of PITX2A and PITX2D. Further analysis, such as sequencing or SNPshot analysis will be required for polymorphism/mutations analysis to understand the genetic factor phenotype variance in order to obtain proper diagnostic in vertical mandibular asymmetry treatment.

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