Abstract: Background: Growth hormone plays a significant role in determining craniofacial morphology. Mutations of its receptor gene might be associated with mandibular prognathism (MP). Purpose: The aim of the current study was to evaluate growth hormone receptor (GHR) gene polymorphisms in relation to facial dimensions. Material and Method: The study enrolled 65 participants with class III profile in MP group and 60 orthognathic control participants. Genomic DNA was extracted from a blood sample from the patients and the P561T and C422F polymorphisms of GHR gene were screened by PCR-RFLP method followed by Sanger sequencing of randomly selected samples to validate the genotyping results. Chi square was used to compare distribution of polymorphism in MP and control groups ($p<0.05$). Results: Heterozygous P561T mutation was found in 10.77% and 8.33% of MP and control groups, respectively ($p=0.644$) while none of the subjects had the C422F mutation. Sanger sequencing confirmed the genotyping results from the PCR-RFLP method. P561T polymorphism was significantly associated with ramus and lower facial height in MP patients and with ramus height in orthognathic patients ($p<0.05$). Conclusion: The results indicate that the P561T polymorphism of the GHR gene is associated with the vertical dimension of the mandible in an Iranian population.

Keywords: Growth hormone; polymorphism, genetic; mandible; malocclusion; Malocclusion, Angle Class III; Iran.
INTRODUCTION.

The clinical presentation of Class III malocclusion is highly heterogeneous and as such, the term "Class III" is used to describe pure mandibular prognathism (MP), maxillary retrognathism, or a combination of both.\(^1\)\(^-\)\(^3\) When this type of craniofacial skeletal deformity is severe enough, it can lead to both social and functional disability.\(^4\)\(^-\)\(^6\) The prevalence of class III malocclusion is reported to be high in Asians and relatively lower in Caucasians.\(^7\)\(^,\)\(^8\) Based on a recent meta-analysis, the prevalence of class III malocclusion in Iranian children is 6.01%.\(^9\)

It seems that this malocclusion is a poly-genetic anomaly caused by interaction of both environmental and genetic factors; however MP seems to be more related to genetic factors.\(^10\) Although familial studies revealed an autosomal dominant inheritance of MP,\(^11\) still it is difficult for clinicians to predict mandibular overgrowth.\(^2\)

Growth hormone (GH) is a peptide secreted by the anterior pituitary gland and plays a considerable role during childhood and adolescence and also in craniofacial complex growth and development.\(^12\) GH binds to its specific receptor in order to initiate internal cell signaling.\(^13\)

The growth hormone receptor (GHR) is found in areas with endochondral growth such as mandibular condyle.\(^14\) The GHR gene (NM_000163.4) is located on chromosome 5 and has 10 exons of which nine exons encode the receptor (NP_000154.1). Until now, 88 different mutations of GHR gene have been recorded.\(^15\) The effect of some of these mutations on mandibular growth has been investigated. Yamaguchi et al.\(^16\) reported the association between mandibular height growth and the P561T variant in a Japanese population. In addition, the C422F and P561T polymorphisms of the GHR gene were reported to be related to effective mandibular length and lower face height in a ‘Turkish population’.\(^17\) Tomoyasu et al.\(^18\) evaluated the C422F, S473S, P477T, I526L and P561T polymorphisms in a Japanese population and reported that only the C422F and P561T mutations were associated with ramus height. The same results were reported in a Korean population.\(^19\)

However, in a Japanese population study using cone beam computed tomography (CBCT), the P561T mutation was not associated with cephalometric measurements of the mandible.\(^20\) Mandibular growth was negatively influenced by the P561T polymorphism during early childhood in a study by Sasaki et al.\(^21\) These differences among studies are partly due to the fact that the P561T variation occurs at different frequencies in different ethnic backgrounds (i.e. less than 1% prevalence in Caucasian populations and 5.2-15% in Japanese populations).\(^16\)\(^-\)\(^21\)

Due to this inconsistency of the results of previous studies and the necessity of assessment of genetic factors in different populations, the current study was performed to investigate the prevalence of the P561T and C422F polymorphisms and their association with mandibular dimensions in an Iranian population.

MATERIALS AND METHODS.

Study designs and population.

This observational case control study was performed at the Genomic Research Center of Shahid Beheshti University of Medical Sciences, Tehran, Iran during 2015-16. Samples were collected from orthodontic patients at four private clinics in Tehran and patients who had orthognathic surgery in Taleghani and Shariati hospitals, both in Tehran. The study protocol was approved by the ethical committee of the university and an informed consent in accordance with Helsinki Declaration was taken from all participants.

This study included two groups of subjects: Patients with a class III appearance who were candidate for orthognathic surgery were included in the mandibular prognathism (MP) group, and patients with class I appearance served as control group. Only patients older than 16 years were included. In the MP group, patients with skeletal class III appearance (ANB and Wits less than zero) and mandibular prognathism (SNB>82°) were included while patients with class III appearance due to maxillary retrognathism (SN<80°) were excluded. In the control group, patients with orthognathic appearance (2º ≤ ANB ≤ 4º and 0 mm ≤ Wits ≤ 2mm) were included. In both groups, patients with syndromic, endocrine or systemic diseases that affect craniofacial morphology were excluded.

A sample size of 57 subjects in each group was calculated considering proportion of polymorphism 0.2 and 0.02 in case and control groups, respectively, with confidence level of 0.95 and 80% power.

Sample collection, DNA extraction and genotyping

Five milliliters of fresh venous blood were obtained...
from the participants and stored in EDTA/K tubes, kept at -20°C until DNA extraction. Genomic DNA was prepared using salting out method. The DNA concentration was estimated at 260 nm using a spectrophotometer.

Genotyping of the partial sequence of exon 10 of the GHR gene was performed using the polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) method as previously described, with optimized PCR condition. PCR amplifications were performed in a total volume of 25 µl, containing 100ng template DNA, 12.5 µl Taq DNA Pol. 2_Master mix Red (Ampliqon, Denmark), 0.5 µl of each primer pair (10pmol/µl) and distilled deionized water up to 25 µl. PCR thermal parameters consisted of initial denaturation at 95°C for 3 min, and 35 cycles of denaturation at 95°C for 30s, annealing of primers 57°C for 30s and extension at 72°C for 40s followed by a final extension step at 72°C for 5 min. The following primers were used to assess polymorphism on rs6182 and rs6184: 5’-GGGAAGCAGATCTCTTATGC-3’ and 5’-TATAGTCTGGGACAGGCATCT-3’.

Following digestion of the amplicon DNA with Eco147I and Cac8I, which digests the GHR gene at codon 561 and 422, respectively, the products were separated by electrophoresis on a 2.5% agarose gel and finally allele genotypes at 561 and 422 codons were recorded. Then, the PCR products of randomly selected samples were sequenced to corroborate the genotyping data by RFLP method.

**Cephalometric analysis**

Lateral cephalograms of patients were traced using Dolphin software version 10 (Patterson Inc., Chatsworth, CA) by two trained examiners. The average values of the measurements by the two examiners were calculated. Intraclass correlation coefficient (ICC) was used to assess inter-examiner reliability. All the cephalograms had proper quality and a ruler for linear measurement calibration. Linear measurements included Wits appraisal, ramus height (articulare-gonion; Ar-Go), mandibular length (gonion-menton; Go-Me), effective length of mandible (condylion-gnathion; Co-Gn), anterior cranial base length (sella-nasion; S-N), lower face height (anterior nasal spine-menton; ANS-Me) as well as Wits appraisal and angular measurements comprising SNA, SNB and ANB. In order to assess intra-examiner reliability, measurements were performed by one of the examiners after two weeks and ICC was calculated.

**Statistical analysis**

Cephalometric measurements were calculated as mean and standard deviation (SD) while polymorphism occurrence was reported as percentage. In order to compare polymorphism between groups a chi square analysis was used and to compare cephalometric measurements between groups’ independent samples the Student’s t-test and Mann–Whitney U test were applied. Statistical analysis was performed using SPSS version 21 (SPSS, Chicago, IL) with a significant level of 0.05.

**RESULTS.**

A total of 65 patients (23 males and 42 females) with a mean age of 24.64±5.86 years were included in the MP group, and 60 patients (22 males and 38 females) with a mean age of 22.41±7.49 years were included in the control group. No significant difference was found between the two groups regarding age and gender (p>0.05). Mean cephalometric measurements of both groups are demonstrated and compared between MP and control groups in Table 1. All measurement except Go-Me and S-N were significantly different between the two groups. ICC was 0.879 and 0.812 for intra-examiner and inter-examiner reliability, respectively, which are considered an excellent correlation.

No subject with a C422F polymorphism was found. Regarding P561T, no homozygous polymorphisms were detected while 7 (10.77%) and 5 (8.33%) cases of heterozygous polymorphism was found in MP and control groups, respectively. The difference was not statistically significant (p=0.644). In order to combine results of polymorphism with cephalometric analysis, average measurements of cephalometric indices were compared based on presence of polymorphisms.

The results showed that the P561T polymorphism was significantly associated with larger Ar-Go and ANS-Me in MP group and larger Ar-Go in control group (p<0.05). Further, the patients were divided based on P561T variation and the results showed the polymorphism is related with larger Ar-Go and ANS-Me in the whole study population, regardless of group. (Table 2)
Table 1. Comparison of cephalometric measurements between MP and control groups.

| Index   | Group            | Mean   | Std. Deviation | p-value |
|---------|------------------|--------|----------------|---------|
| Witts   | Mandibular prognathism | -6.7538 | 4.32468        | <0.001  |
| Control |                  | .8400  | .62415         |         |
| ANB     | Mandibular prognathism | -3.2292 | 2.28129        | <0.001  |
| Control |                  | 1.0200 | .62675         |         |
| SNA     | Mandibular prognathism | 83.4677 | 4.37822        | <0.001  |
| Control |                  | 80.9583| .81184         |         |
| SNB     | Mandibular prognathism | 86.6031 | 3.82949        | <0.001  |
| Control |                  | 80.0133| .60013         |         |
| Ar-Go   | Mandibular prognathism | 46.6077 | 2.38578        | <0.001  |
| Control |                  | 44.7383| 2.05782        |         |
| Go-Me   | Mandibular prognathism | 67.3985 | 7.37990        | .339    |
| Control |                  | 66.4467| 2.90980        |         |
| ANS-Me  | Mandibular prognathism | 66.2615 | 3.94128        | .026    |
| Control |                  | 64.8800| 2.77390        |         |
| Co-Gn   | Mandibular prognathism | 134.5485 | 4.33949       | <0.001  |
| Control |                  | 111.4300| 5.09151        |         |
| S-N     | Mandibular prognathism | 64.9600 | 3.49409        | .198    |

Table 2. Cephalometric measurements based on P561T polymorphism.

| Index  | 561 region | Mandibular prognathism | Mean | SD     | p-value |
|--------|------------|------------------------|------|--------|---------|
| Ar-Go  | C/A        | 50.2286                | 2.74695 | .001   |
|        | C/C        | 46.1707                | 2.53585 |        |
| Go-Me  | C/A        | 63.9000                | 4.6761 | .172   |
|        | C/C        | 67.8207                | 7.56067 |        |
| ANS-Me | C/A        | 70.9429                | 2.35291 | <0.001 |
|        | C/C        | 65.6966                | 3.72054 |        |
| Co-Gn  | C/A        | 132.8714               | 4.15039 | .204   |
|        | C/C        | 134.7509               | 4.35241 |        |
| S-N    | C/A        | 64.4286                | 3.73529 | .688   |
|        | C/C        | 65.0241                | 3.49292 |        |

DISCUSSION.

The present study investigated a correlation of mandibular morphology with GHR gene polymorphisms in an Iranian population. The reason for choosing C422F and P561T polymorphisms of the GHR gene was that a recent study in Turkey, which has a relatively similar population to Iran, showed an association between these polymorphisms and facial dimensions. The GHR gene was selected due to its influence on craniofacial growth. Laron syndrome (GH insensitivity syndrome), which is associated with underdeveloped facial bones, is caused by mutation in the GHR gene. In addition, GHR deficiency would result in relatively poor vertical growth of the face.

The results showed that none of the subjects had genotype GT of variants rs6182 (C422F) while genotype CA of rs6184 (P561T) was found in 10.77% and 8.33% patients in the MP and control groups, respectively. Although the P561T variation was more common in the MP group, the difference was not significant. This finding is in harmony with previous studies.
was reported in approximately 5.2%-15% of a Japanese population, 18,20,21,25 1% of a Turkish population 17 and more than 10% of a Chinese population 26 with normal occlusion while it was found in 15% of Japanese 21 and 5% of a Turkish population 17 with class III malocclusion. In contrast to the results of the current study, the C422F polymorphism was found in 5.9% of Japanese 18 and 1-2% of Turkish 17 people.

A noteworthy finding was relatively larger ramus and lower facial height in association with P561T polymorphism. This polymorphism is defined by transversion of cytosine to adenine (C to A) which changes the codon 561 from proline to threonine (P561T) and alters the intracellular domain of the GHR. Similar to these results, a study in a Turkish population of 200 normal and class III Angle patients showed a relationship between the P561T variant of the GHR gene and effective mandibular length and lower face height. 17

In contrast, Sasaki et al., 21 assessed the P561T polymorphism in 60 Japanese children (age range 3 to 13 years old) and revealed that this GHR gene mutation is related with decreased mandibular dimensions. However, in their study patients’ growth was not evenly distributed. In another study, the P561T polymorphism was associated with smaller mandibular ramus height in 167 Japanese individuals with normal occlusion. 18

A study of Kang et al., 19 in a group of 159 Koreans with normal occlusion also revealed an association between P561T polymorphism and smaller ramus height. This inconsistency might be due to ethnic differences and higher prevalence of class III Angle in Asian populations compared to Middle Easters.

It has been demonstrated that Iranians have a relatively steeper mandibular plane resulting in higher lower facial height. 27,28 On the other hand, Nakawaki et al., 20 used CBCT images to measure mandible dimensions and reported that there was no association between the P561T polymorphism and mandible size.

One of the limitations of this study was small sample size relative to the prevalence of the polymorphism in the target population. This study could not find a significant correlation between MP and P561T polymorphism, which might be due to the study power rather than a real lack of correlation.

Clinical significance of the results includes pediatric patients with a class III tendency, in whom the orthodontist should estimate mandibular growth potential and decide whether to start orthodontic treatment and class III camouflage or defer treatment until adolescence for orthognathic surgery. In future genetic tests in conjugation with other indices could be used to estimate mandibular growth potential. 29,30 For this purpose, longitudinal studies evaluating the presence polymorphisms in related genes and mandibular growth pattern are required.

**CONCLUSION.**

Within the limitations of this study, is could be concluded that there was no difference on the incidence of heterozygous mutation of GHR gene at codon 561 between MP and control groups.

However, the P561T polymorphism was significantly associated with ramus and lower facial height in MP patients and with ramus height in orthognathic patients. No mutation at codon 422 of the GHR gene was found.

**Conflict of interests:** The authors declare no conflict of interest of any kind.

**Ethics approval:** Study protocol was approved by ethical committee of Shahid Beheshti University of Medical Sciences and an informed consent in accordance with Helsinki Declaration was taken from all participants.

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REFERENCES.

1. Ngan P, Moon W. Evolution of Class III treatment in orthodontics. Am J Orthod Dentofacial Orthop. 2015;148(1):22-36.
2. Zere E, Chaudhari PK, Sharan J, Dhingra K, Tiwari N. Developing Class III malocclusions: challenges and solutions. Clin Cosmet Investig Dent. 2018;10:99-116.
3. Rongo R, D'Anto V, Bucci R. Skeletal and dental effects of Class III orthopaedic treatment: a systematic review and meta-analysis. J Oral Rehabil. 2017;44(7):545-62.
4. Zhou Y, Li Z, Wang X, Zou B, Zhou Y. Progressive changes in patients with skeletal Class III malocclusion treated by 2-jaw surgery with minimal and conventional presurgical orthodontics: A comparative study. Am J Orthod Dentofacial Orthop. 2016;149(2):244-52.
5. Palomares NB, Celeste RK, Miguel JA. Impact of orthosurgical treatment phases on oral health-related quality of life. Am J Orthod Dentofacial Orthop. 2016;149(2):171-81.
6. Grewal H, Sapawat P, Modi P, Aggarwal S. Psychological impact of orthodontic treatment on quality of life - A longitudinal study. Int Orthod. 2019;17(2):269-76.
7. de Frutos-Valle L, Martin C, Alarcon JA, Palma-Fernandez JC, Iglesias-Linares A. Subclustering in Skeletal Class III Phenotypes of Different Ethnic Origins: A Systematic Review. J Evid Based Dent Pract. 2019;19(1):34-52.
8. Alhammadi MS, Halboub E. Global distribution of malocclusion traits: A systematic review. Dental Press J Orthod. 2018;23(6):40.e1-e10.
9. Akbari M, Lankarani KB, Honarvar B, Tabrizi R, Mirhadi H, Moosazadeh M. Prevalence of malocclusion among Iranian children: A systematic review and meta-analysis. Dent Res J. 2016;13(5):387-95.
10. Duraczynska-Kowalik A, Nelke KH, Pawlak W, Sasiadek MM, Gerber H. Genetic Factors Involved in Mandibular Prognathism. J Craniofac Surg. 2017;28(5):e422-e31.
11. Liu H, Wu C, Lin J, Shao J, Chen Q, Luo E. Genetic Etiology in Nonsyndromic Mandibular Prognathism. J Craniofac Surg. 2017;28(1):161-9.
12. Ramirez-Yanez GO, Smid JR, Young WG, Waters MJ. Influence of growth hormone on the craniofacial complex of transgenic mice. Eur J Orthod. 2005;27(5):494-500.
13. Pilecka I, Whatmore A, Hooft van Huijsduijnen R, Destenaves B, Clayton P. Growth hormone signalling: sprouting links between pathways, human genetics and therapeutic options. Trends Endocrinol Metab. 2007;18(1):12-8.
14. Simmons KE. Growth hormone and craniofacial changes: preliminary data from studies in Turner's syndrome. Pediatrics. 1999;104(4 Pt 2):1021-4.
15. Stenson PD, Mort M, Ball EV, Shaw K, Phillips AD, Cooper DN. The Human Gene Mutation Database: building a comprehensive mutation repository for clinical and molecular genetics, diagnostic testing and personalized genomic medicine. Hum Genet. 2014;133(1):1-9.
16. Yamaguchi T, Maki K, Shibasaki Y. Growth hormone receptor gene variant and mandibular height in the normal Japanese population. Am J Orthod Dentofacial Orthop. 2001;119(6):650-3.
17. Bayram S, Basciftci FA, Kurar E. Relationship between P561T and C422F polymorphisms in growth hormone receptor gene and mandibular prognathism. Angle Orthod. 2014;84(5):803-9.
18. Tomoyasu Y, Yamaguchi T, Tajima A, Nakajima T, Inoue I, Maki K. Further evidence for an association between mandibular height and the growth hormone receptor gene in a Japanese population. Am J Orthod Dentofacial Orthop. 2009;136(4):536-41.
19. Kang EH, Yamaguchi T, Tajima A, Nakajima T, Tomoyasu Y, Watanabe M, Yamaguchi M, Park SB, Maki K, Inoue I. Association of the growth hormone receptor gene polymorphisms with mandibular height in a Korean population. Arch Oral Biol. 2009;54(6):556-62.
20. Nakawaki T, Yamaguchi T, Isu M, Kawagushi A, Tomita D, Hikita Y, Suzuki-Tomoyasu Y, Adel M, Ishida H, Maki K, Kimura R. Growth hormone receptor gene variant and three-dimensional mandibular morphology. Angle Orthod. 2016;87(1):68-73.
21. Sasaki Y, Satoh K, Hayasaki H, Fukimoto S, Fujiwara T, Nonaka K. The P561T polymorphism of the growth hormone receptor gene has an inhibitory effect on mandibular growth in young children. Eur J Orthod. 2009;31(5):536-41.
22. Miller SA, Dykes DD, Polesky HF. A simple salting out procedure for extracting DNA from human nucleated cells. Nucleic Acids Res. 1988;16(3):1215.
23. Laron Z. Laron syndrome (primary growth hormone resistance or insensitivity): the personal experience 1958-2003. J Clin Endocrinol Metab. 2004;89(3):1031-44.
24. Schaefer GB, Rosenbloom AL, Guevara-Aguirre J, Campbell EA, Ullrich F, Patil K, Frias JL. Facial morphometry of Ecuadorian patients with growth hormone receptor deficiency/Laron syndrome. J Med Genet. 1994;31(8):635-9.
25. Chujio S, Kaji H, Takahashi Y, Okimura Y, Abe H, Chihara K. No correlation of growth hormone receptor gene mutation P561T with body height. J Endocrinol. 1996;134(5):560-2.
26. Zhou J, Lu Y, Gao XH, Chen YC, Lu JJ, Bai YX, Shen Y, Wang BK. The growth hormone receptor gene is associated with mandibular height in a Chinese population. J Dent Res 2005;84(11):1052-6.
27. Rachkhan V, Ghorbanyjavapour F. Anteroposterior and vertical soft tissue cephalometric norms of Iranians, interethnic comparisons, sex dimorphism, and the effect of age on cephalometric variables. Oral Maxillofac Surg 2019;23(2):167-78.
28. Azarbeyjani S, Omrani A, Kalaantar-Motamedi A, Abdellahi M, Taaleb V, Teimoori F. Cephalometric norms for 6-17 year-old Iranians with normal occlusion and well-balanced faces. Dent Res J. 2014;11(3):327-35.
29. Tehranchi A, Motamedian SR, Rohani K. Radiographic Evaluation of Frontal Sinus Dimensions and Anatomic Variations. Br J Med Res. 2015;8(5):454-62.
30. Tehranchi A, Motamedian SR, Saiidi S, Kabiri S, Shidfar S. Correlation between frontal sinus dimensions and cephalometric indices: A cross-sectional study. Eur J Dent. 2017;11(1):64-70.