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The P561T polymorphism of the growth hormone receptor gene has an inhibitory effect on mandibular growth in young children

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SUMMARY P561T heterozygous missense mutation in the growth hormone receptor (GHR) is a candidate genetic polymorphism (single-nucleotide polymorphism) for human mandibular growth. The purpose of this study was to assess whether this mutation affects mandibular growth during early childhood. The difference in mandibular growth between P561T heterozygous and wild-type individuals was analysed by cephalometric measurements during childhood. The subjects included 33 children with mandibular protrusion (aged 3–12 years, 16 males and 17 females) and 27 normal children (aged 3–13 years, 14 males and 13 females). Genomic DNA extracted from buccal epithelial cells was genotyped for the P561T heterozygous mutation with a molecular analysis (polymerase chain reaction—restriction fragment length polymorphism method). Two of the patients with normal occlusion and five with mandibular protrusion were heterozygous for the mutation.

Chi-square analysis showed that the frequency of this mutation did not differ statistically between the normal and mandibular protrusion subjects. Multilevel model analysis of the 101 cephalograms showed that the mutation reduced the linear measurements of the mandible. These findings suggest that P561T heterozygous mutation affects mandibular growth during early childhood, and this mutation in the GHR gene is hypothesized to function as an inhibitory factor in the process of mandibular growth.

Introduction

Many factors regulating mandibular growth have been identified in the formation of the mandibular arch (Pirinen, 1995; Mina, 2001). Determining the factors affecting mandibular growth contributes to early diagnosis and treatment of mandibular protrusion. Longitudinal cephalometric observation of the mandibles in offspring produced by a complete Dilallel cross among five strains of rats showed that mandibular size was primarily affected by genetics (Nonaka *et al.*, 1991). Quantitative trait locus (QTL) analysis in an inbred strain of mice was used to determine the chromosomal regions responsible for mandibular length between menton and gonion (Dohmoto *et al.*, 2002). Two significant QTLs were detected within the regions 13cM and 16cM in chromosome 11, including orthodenticle homeobox, suggesting the possibility that some major genes are responsible for mandibular length.

Growth hormone (GH) plays a major role in regulating growth during childhood and adolescence and also regulates metabolism through its binding to the growth hormone transmembrane receptor (GHR; Piwien-Pilipuk *et al.*, 2002). Laron syndrome is caused by the dominant-negative defects of the intracellular domain of GHR, and affected individual have underdevelopment of the facial bones (Laron, 2004). In the Human Gene Mutation Database, 56 different *GHR* gene mutations, including 32 missense and nonsense mutations, have been registered (Stenson *et al.*, 2003). In a few reports concerning the effect of GHR gene mutations on craniofacial growth, Chinese Han individuals with a genomic polymorphism at codon 526 of the GHR gene have a greater mandibular ramus length (Co–Go/Ar–Go) (Zhou *et al.*, 2005). At position 1777 in GHR, a transversion of amino acid from cytosine to adenine changed codon 561 from proline to threonine (P561T), affecting the cytoplasmic domain of the GHR. In the Japanese population, no significant correlation has been observed between body height and P561T variants at the GHR gene locus (Chujo *et al.*, 1996; Yamaguchi *et al.*, 2001), but a relationship has been reported between mandibular ramus length and the heterozygous missense mutation P561T (Yamaguchi *et al.*, 2001). Japanese carrying the P561T variant had a significantly smaller mandibular ramus length (condylion–gonion) than those who were wild type at this locus. These findings suggest that the change in the cytoplasmic domain of GHR produced by P561T does not play a significant role in determining final body height and may affect mandibular growth independently of systemic growth. However, it is currently unclear whether P561T affects craniofacial growth in children.

To determine whether the P561T heterozygous missense mutation affects mandibular growth during the early

stages of growth and development in humans, differences in mandibular growth between wild-type and P561T individuals genotyped with restriction fragment length polymorphism were analysed using cephalometric measurements.

Subjects and methods

All subjects were either patients at the Department of Pediatric Dentistry, Faculty of Dentistry, Kyushu University, or volunteers of their respective families. This study was approved by the ethics committee of Kyushu University Faculty of Dental Science and Nagasaki University, and informed consent was granted by the parents/guardians of all children.

Genetic analysis

Thirty-three Japanese children with mandibular protrusion and 27 'normal' children with a Class I occlusion and without other types of malocclusion, who had no general physical diseases or congenital disorders, participated in this study. As the focus of the research was the effect of a GHR gene polymorphism on mandibular growth, subjects with a Class II occlusion with maxillary protrusion were initially excluded to avoid confusion. Genomic DNA was extracted from buccal epithelial cells and employed as the primary source for polymerase chain reaction (PCR) amplification (Liu *et al.*, 1995; Sasaki *et al.*, 2007). Briefly, buccal epithelia cells were collected by twirling a sterile cytology brush on the inner cheek for 30 seconds. DNA was extracted using a BuccalAmp™ DNA extraction kit (Epicentre, Madison, Wisconsin, USA) according to the manufacturer's protocol. The partial sequence of exon 10 of the GHR gene, which encodes the cytoplasmic domain of GHR, was amplified by PCR using primer pairs deduced from the published DNA sequence (Chujo *et al.*, 1996) and then, the PCR-amplified DNA was digested with *Stu* I, which digests a wild-type GHR gene at codon 561, but not a GHR gene with mutation P561T (Figure 1a,b). DNA samples were analysed by electrophoresis using 1.8 per cent (w/v) agarose gel (Figure 1c). For wild-type subjects, digestion resulted in two restriction fragments of 808 and 229 base pairs (subjects 1, 2, 3, and 7). In contrast, P561T heterozygous subjects showed three bands of 1037, 808, and 229 base pairs (subjects 4, 5, and 6).

Cephalometric analysis

A total of 101 lateral cephalograms were obtained for 24 of the mandibular protrusion subjects and 17 of the normal subjects during the treatment or management period according to the informed consent. Nine of the patients with mandibular protrusion and 10 of the normal subjects were excluded because of the absence of lateral cephalograms.

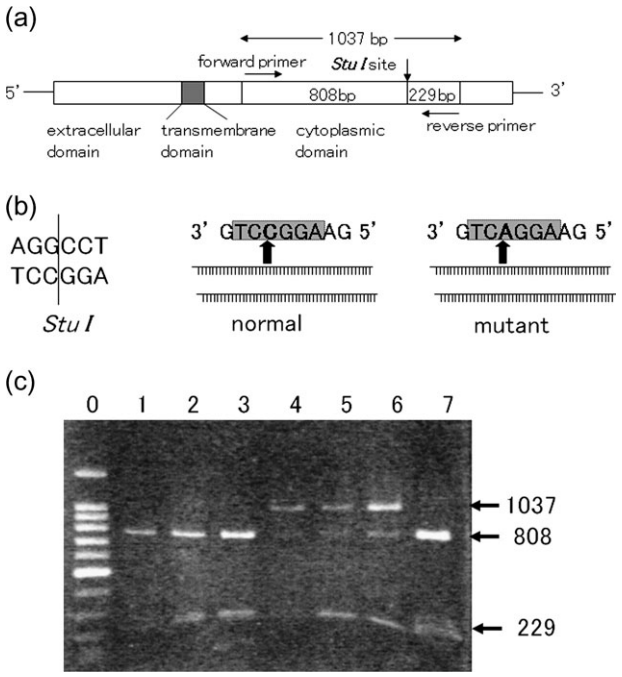

Figure 1 Analysis of the polymerase chain reaction (PCR)–restriction fragment length polymorphism method of the growth hormone receptor (GHR) gene mutation P561T in seven of the 60 subjects was examined. (a) Human GHR cDNA was represented. GHR gene including *Stu* I site (or codon 561) is amplified by PCR using forward and reverse primers. (b) The PCR-amplified DNA was digested by Stu I, which is able to digest the normal GHR gene, but not that with mutation P561T. (c) DNA samples were analysed by electrophoresis using 1.8 per cent (w/v) agarose gel. Lane 0: 100 bp DNA marker.

Of the 101 cephalograms, 43 were from 19 males (mean age: 7 3 ± 2 2 years) and 58 from 22 females (mean age: 7 1 ± 20 1 years). The age range of subjects was from 3 years 3 months to 13 years 3 months. The lateral cephalometric radiographs were analysed according to the landmarks shown in Figure 2. The radiographic enlargement was 100 per cent. Linear measurements associated with mandibular size (Cd–Go, ramus length; Pog'–Go, mandibular body length; and Gn–Cd, mandibular length) and maxillary length (A'–Ptm') and angular measurement associated with Gn–Cd (Ar–Go–Mn) were compared between patients who were either wild type or carried the heterozygous mutation. All the measurements were made with an electric digitizer (model KD 4320, Graphtec Ltd, Yokohama, Japan) online with a computer. The resolution and angular variables were 0.1 mm and 0.2 degrees, respectively.

Statistical analysis

Chi-square analysis was performed to test the difference in mutantion frequencies between the normal and mandibular protrusion subjects. Multilevel model analysis has been previously applied to the analysis of jaw movements (Strenio *et al.*, 1983; Goldstein and Rasbash, 1996) and was used in the present study to evaluate the growth curves. The

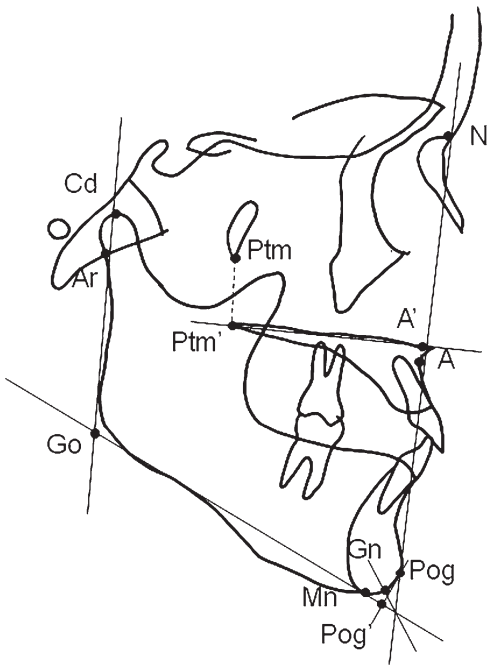

Figure 2 Landmarks and reference lines used for linear and angular measurements on the lateral cephalograms. N, nasion; A, A point; A', crossover point of a vertical line from point A to the nasal plane; Ptm, pterygomaxillary fissure; Ptm', crossover point of a vertical line from Ptm to the nasal plane; Gn, gnathion; Mn, menton; Pog, pogonion; Pog', crossover point of a vertical line from Pog to the mandibular plane; Go, gonion; Ar, articulare; and Cd, condylion.

variances were based on the results of an iterative generalized least squares method. Significance for all analyses was determined at $P < 0.05$. In order to compensate for the small sample size, these cephalometric measurements were also used to calculate the standard deviation score (SDS) to investigate the trend of mandibular size in both mutant and wild-type subjects. SDS was calculated in P561T heterozygotes and wild-type homozygotes, respectively, using the formula: (individual value minus the mean value of the data of normal Japanese children)/standard deviation. The values for normal Japanese children were derived from a previous study (Izuka, 1958). Furthermore, the percentage distribution was calculated and compared between mutant and wild-type subjects.

Results

Genetic analysis

There were no subjects with P561T homozygous mutation. Two of the patients with normal occlusion and five with mandibular protrusion were heterozygous for the P561T missense mutation. The frequency of this mutation did not statistically differ between normal and mandibular protrusion patients. Therefore, the data was combined in order to determine the effect of P561T mutation on mandibular longitudinal growth.

Cephalometric analysis

After calculating the SDS, the percentage contribution of SDS between P561T heterozygotes ($n = 13$) and wild-type homozygotes ($n = 88$) for four different cephalometric linear measurements and Ar–Go–Mn (Figure 3) were compared. The median values for wild type and mutant were −0.15 and −0.69 for A'–Ptm', 0.62 and −0.42 for Gn–Cd, 0.87 and −0.14 for Pog'–Go, −0.19 and −0.65 for Cd–Go, and −0.82 and −0.90 for Ar–Go–Mn. The median and mode in P561T were relatively small compared with those in the wild type samples for all measurements except for mode in A'–Ptm' length and Ar–Go–Mn, which showed the same values of mode between heterozygous mutation and wild-type samples.

Multilevel model analysis was used to examine longitudinal differences during the observation period. A significant difference was found in the growth curves of heterozygote subjects and wild type for Ar–Go–Mn and all linear parameters except A'–Ptm' (Figure 4). Generally, the heterozygote subjects tended to show smaller linear measurements than the wild type; however, heterozygote and wild type growth curves for Cd–Go and Ar–Go–Mn crossed over and back again during the middle stage of the analysed period.

Discussion

The GHR gene mutation P561T is considered to be a single-nucleotide polymorphism because heterozygosity existed in approximately 15 per cent of 96 randomly selected Japanese subjects in one investigation (Chujo *et al.*, 1996) and 14 per cent of 100 Japanese in another study (Yamaguchi *et al.*, 2001). The heterozygote frequency with the P561T variant in the present investigation was approximately 12 per cent of 60 subjects, similar to that previously observed.

No statistically significant difference in the frequency of the P561T variant between normal and mandibular protrusion subjects was found in the present study. The mechanism of mandibular protrusion is complex and is caused by an imbalance in linear growth of the alveolar or basal bone between the mandible and maxilla, with or without imbalance of incisal inclination, and functional effects of mandibular location. More samples and additional data related to relevant functional and morphological factors would help to clarify the effects of the P561T heterozygous mutation on occlusion type.

Cephalometric measurements were compared with Japanese norms from the 1950s (Izuka, 1958) because of the lack of recent data of cephalometric measurements of Japanese children. When the variations of linear and angular measurements from a young adult population were compared, the mandible was found to be significantly larger (Yamauchi *et al.*, 1995). Regardless of the relatively small normal standards used in the study, analysis of the percentage contribution of SDS showed that all mandibular linear

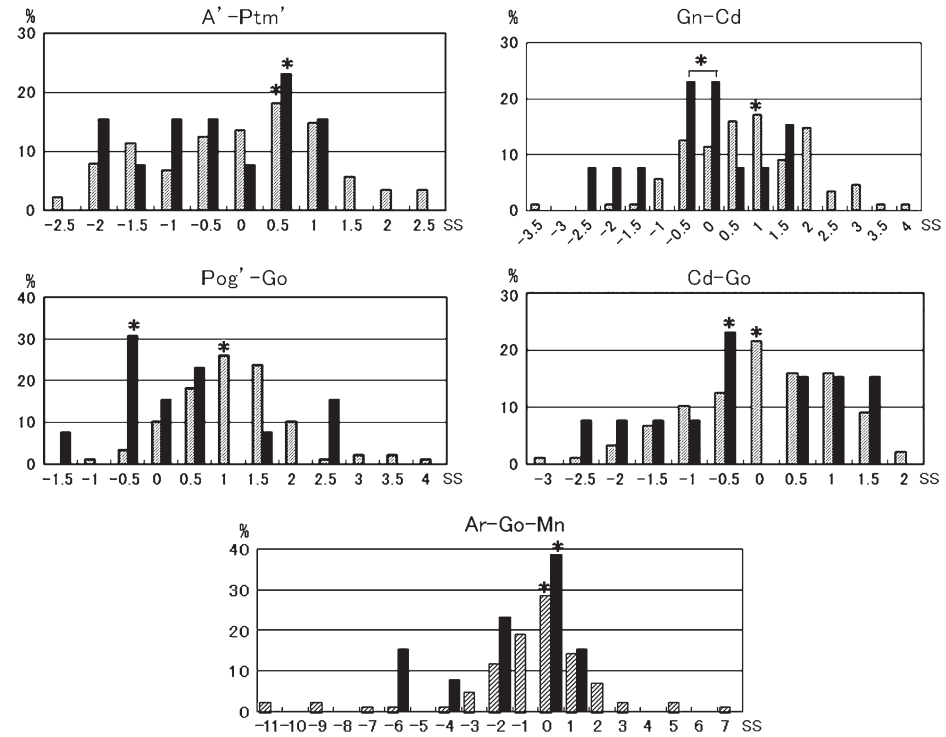

Figure 3 Percentage distribution in wild and heterozygous mutation by standard scores. Shaded boxes show the wild type and black boxes the heterozygous mutation ($n = 101$). Asterisks indicate the mode of wild type and heterozygous mutation.

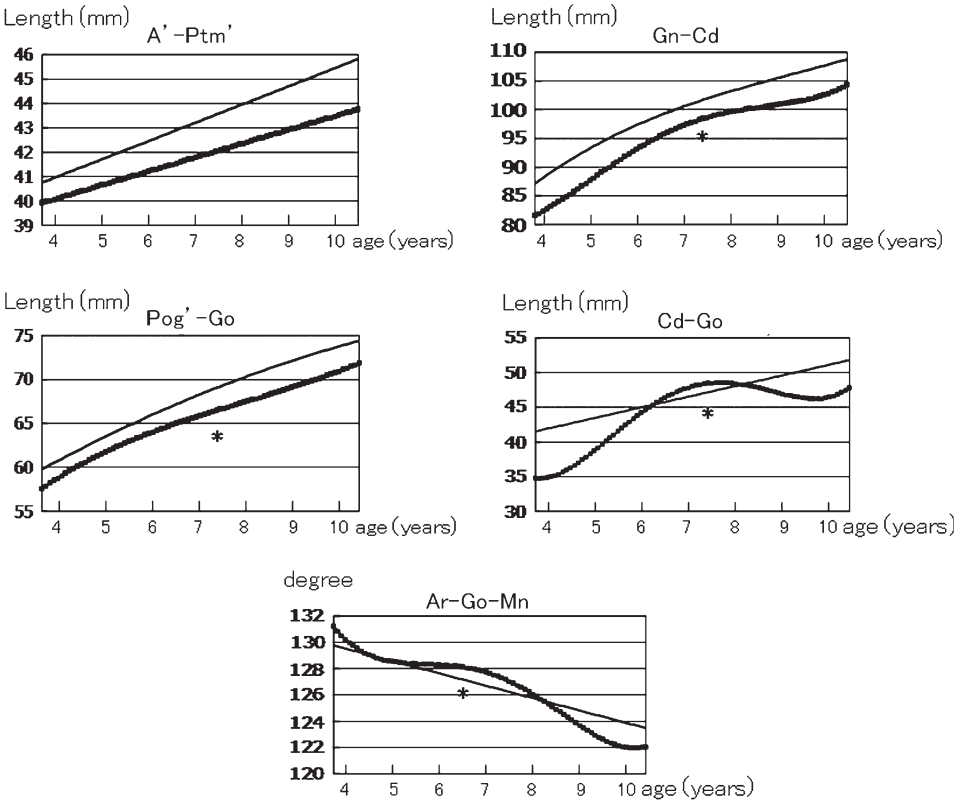

Figure 4 Cephalometric measurement growth curves by multilevel model analysis. Thin lines show the wild type and bold lines the heterozygous mutation ($n = 101$). *Statistically significant difference between the growth curves ($P < 0.05$).

parameters tended to be smaller in subjects carrying the heterozygous mutation than in normal and wild types. Furthermore, the growth curves of all mandibular parameters were significantly different between the P561T variant and wild type during the observation period, based on multilevel model analysis. These findings indicate that mandibular growth is negatively affected by the P561T mutation during early childhood.

Interestingly, the temporal and spatial effects of the P561T variant are characteristics depending on the measured parameters; the growth curves of P561T and wild type crossed over during the middle stage of the analysed period for both Cd–Go and Ar–Go–Mn, while Gn–Cd and Pog'–Go parameters consistently increased at different levels throughout the study. As mandibular length (Gn–Cd) can be determined in part by mandibular body length (Pog'–Go), ramus height (Cd–Go), and mandibular angulation (Ar–Go–Mn), differences in growth pattern among these measurements suggest first that mandibular length and mandibular body length are affected by the P561T mutation regardless of the contribution of ramus height and Ar–Go–Mn. The mandible, which is a single bone, developmentally and functionally consists of several units, including the mandibular body, condyle, chin, angular, coronoid, and alveolar processes. The length of the mandible was found to increase significantly after human GH therapy, but when it ceased, the rate of increase gradually diminished (Hwang and Cha, 2004). GHR genotype had a significant effect on the response to GH during 2 years of administration (Dos Santos *et al.*, 2004). Taken together, this evidence supports the hypothesis that the mutation in GHR, including the P561T variant, might affect mandibular growth in a region-specific manner, resulting in a morphological difference between heterozygote and wild type. In addition, a different temporal effect of P561T mutation on mandibular growth has been supported by the results of another study. Mandibular condyles from young (3 month) and old (20 month) female ICR mice were cultured up to 72 hours in the presence of recombinant rat GH. Cartilage from the young animals responded *in vitro* only slightly to GH, whereas a significant response was observed in cartilage from the old animals (Livne *et al.*, 1997).

The functional mechanism explaining how the mutant allele in P561T may be responsible for growth inhibition during mandibular growth and development in children is still unclear. Although a number of findings have been reported on the GH receptor, little is known about the function of the cytoplasmic domain of GHR (Billestrup *et al.*, 1995; Tiulpakov *et al.*, 2005), and the effect of P561T on the structure of GHR likewise remains unclear. The functional significance of homozygous defects on the intracellular domain of the GHR was examined in growth hormone insensitivity (GHI), also known as Laron syndrome, a congenital disorder in which defective GH receptor function results in severe post-natal growth failure (Woods *et al.*, 1996). In a classic GHI patient, the GHR-1776del mutation, which is predicted to result in GHR truncation to 581 amino acids and a nonsense sequence of residues in the 560–581 region, showed lower STAT5-mediated transcriptional activation as well as STAT5 Tyr694 phosphorylation compared with wild-type GHR (Tiulpakov *et al.*, 2005). In one patient with partial GHI, a heterozygous missense mutation (P561T) was identified in the cytoplasmic domain of GHR (Kaji *et al.*, 1994). Therefore, it was speculated that, in normal patients, the P561T-containing allele affects the downstream signalling of GHR (including JAK and STAT families), but the other wild-type allele might at least partially counteract its influence. The features of Laron syndrome patients is characterized as short stature and craniofacial dimorphism, including midfacial retrusion (Woods *et al.*, 1996). In the present study, body height was not recorded and it is still unclear whether the midfacial length, A'–Ptm', was affected by the P561T mutation.

Conclusion

P561T heterozygous mutation did not account for the difference between mandibular protrusion and normal occlusion, but may have an effect on mandibular growth itself during early childhood, indicating that this mutation in the GHR gene functions as an inhibitory factor in the process of mandibular growth.

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