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Whole-exome sequencing in a Japanese pedigree implicates a rare non-synonymous single-nucleotide variant in *BEST3* as a candidate for mandibular prognathism

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ABSTRACT

Mandibular prognathism is a phenotype of facial deformity seen in populations around the world, but with higher incidence among East Asian populations. Five genome-wide nonparametric linkage analyses and a genome-wide association study to identify susceptibility loci of the phenotype have shown inconsistent results. To explore variants related to mandibular prognathism, we undertook whole-exome sequencing in a Japanese pedigree. The pedigree was ascertained as mandibular prognathism. The pedigree comprised 15 individuals from 4 generations. Four affected individuals across 2 generations and 5 unaffected individuals were chosen for whole-exome sequencing. Five non-synonymous single-nucleotide variants (SNVs) of *UBASH3B*, *OR6M1*, *OR8D4*, *OR8B4*, and *BEST3* genes were detected in all 4 affected individuals, but in none of the 5 unaffected individuals. A non-synonymous SNV of the *BEST3* gene, Chr12(GRCh37):g.70048878G > T, NM_032735.2:c.1816C > A, p.(L606I), was identified as rare missense variant. *BEST3* is located on chromosome 12q15 and encodes bestrophin 3 from the bestrophin family of anion channels. The 4 other non-synonymous SNVs of *UBASH3B*, *OR6M1*, *OR8D4*, and *OR8B4* were not considered plausible candidates for mandibular prognathism. Our whole-exome sequencing implicates a rare non-synonymous SNV of *BEST3* as a candidate for mandibular prognathism in the Japanese pedigree.

1. Introduction

Mandibular prognathism (Online Mendelian Inheritance in Man [OMIM] #176700), a skeletal Class III malocclusion in orthodontics, is a morphological disorder of the face (phenotype) that occurs in populations throughout the world but with higher incidence among East Asian populations [1]. The prevalence is less than 1% in Caucasians [2] while approximately 10% in Japanese [3]. The phenotype can induce deficiencies in speech articulation and low efficiency of mastication. Not only orthodontic treatment but also orthognathic surgery is frequently necessary to treat adult mandibular prognathism. The majority of cases of mandibular prognathism evidently represent a multifactorial phenotype attributed to the interactions of susceptibility genes with environmental factors [4–6].

The results of a genome-wide linkage analysis of mandibular prognathism identified chromosomes 1p36, 6q25, and 19p13.2 as showing suggestive linkage to mandibular prognathism in Japanese and Korean families [7]. Supplementary association studies on 1p36 of the susceptibility locus suggested *MATN1* (matrilin 1, cartilage matrix protein) (164 Korean patients and 132 controls) [8] and *EPB41* (erythrocyte membrane protein band 4.1) (211 Han Chinese patients and 224 controls) [9] as candidate genes for the phenotype. However, another linkage analysis of Brazilian families [10] did not reveal any suggestive linkages between the phenotype and 1p36, 6q25, or 19p13.2. On the other hand, a genome-wide linkage analysis of Colombian Hispanic families [11] showed 5 susceptibility loci: 1p22.1; 3q26.2; 11q22; 12q13.13; and 12q23 with suggestive linkage to mandibular prognathism. A supplementary association study [12] of multiracial 44

Abbreviations: GWAS, genome-wide association studies; ANB, point A-the Nasion-point B; SNP, single-nucleotide polymorphism; SNV, single-nucleotide variant; EGFR, epidermal growth factor receptor; PDGF, platelet-derived growth factor

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patients and 35 controls suggested *MYO1H* (myosin 1H) located on 12q24.11, close to 12q23, as a candidate gene for the phenotype. Three more linkage analyses [13–15] on susceptibility loci of the phenotype have been reported in Han Chinese families. However, the loci shown by those linkage analyses for mandibular prognathism were inconsistent.

To reach a better understanding of the genetic basis for mandibular prognathism, we undertook the first genome-wide association studies (GWAS) [16,17], recruiting 240 patients and 360 controls and suggesting six loci (1p22.3, 1q32.2, 3q23, 6q23.2, 7q11.22 and 15q22.22) as susceptibility regions for mandibular prognathism. *SSX2IP* (synovial sarcoma, X breakpoint 2 interacting protein), *PLXNA2* (plexin A2), *RASA2* (Ras p21 protein activator 2), *TCF21* (transcription factor 21), *CALN1* (calneuron 1), and *RORA* (RAR [retinoic acid receptor]-related orphan receptor α) genes were suggested as the respective candidate genes [17]. The 1p22.3 locus was supported by a previous linkage analysis [11], while the remaining 5 loci (1q32.2, 3q23, 6q23.2, 7q11.22 and 15q22.22) were novel.

A few exome sequencing [18] studies in Estonian [19], Chinese [20], and Italian [21] families have reported on candidate genes for mandibular prognathism using families of subjects. However, inconsistent genes have also been shown within exome sequencing for the phenotype. These inconsistencies seem to be due to variable subphenotypes (maxillary hypoplasia [11,19], mandibular hyperplasia, or a combination of both) for mandibular prognathism.

To explore variants related to mandibular prognathism without maxillary hypoplasia, we carried out whole-exome sequencing in a Japanese family.

2. Materials and methods

2.1. Pedigree recruitment

The study protocol was approved by the ethics committees of the institutions with which the authors were affiliated. Each individual from the family provided written informed consent to participate in the study. A Japanese family was ascertained as showing mandibular prognathism. The pedigree was comprised of 15 individuals from 4 generations residing in Tomamae-town in the Province of Hokkaido, Japan (Fig. 1A). Participant III:3 was a male orthodontic patient who had undergone orthognathic surgery (bilateral sagittal split ramus osteotomy to set back the distal segment of the mandible). On initial examination, he had been 18 years of age and had displayed anterior crossbite and lateral openbite (Fig. 1B). For sagittal jaw relationship, the angle formed by point A-the Nasion-point B (ANB) [22] was −4.1° (norm for Japanese adult male: +4.0°), maxillary antero-posterior length was 50.6 mm (distance between the pterygomaxillary fissure and point A; norm for Japanese adult male: 50.4 mm), and mandibular length was 127.9 mm (distance between points articulare and menton; norm for Japanese adult male: 115.6 mm), as determined by cephalometric analysis (Fig. 1C).

The first-generation individual affected by mandibular prognathism was deceased. Clinical examination, facial and oral photographs, and model casts were collected for all available participants. Diagnostic criteria for mandibular prognathism included at least 2 of the following: concave facial profile with prognathic mandible; negative overjet of anterior teeth; and Angle Class III molar (or cuspid) relationship [16,21]. To increase phenotype homogeneity, participants showing a retrognathic maxilla on facial profile were excluded from consideration as affected individuals [16,21]. None of the participants had any congenital disorders such as cleft palate or general physical disease. None of the participants other than participant III:3 had undergone orthognathic surgery.

2.2. Whole-exome sequencing

From this pedigree of mandibular prognathism, we could obtain saliva samples from 4 affected individuals across 2 generations (II:3, II:6, III:1, III:3) and 5 unaffected individuals (I:2, II:5, II:7, III:2, III:4), so that the 9 individuals were used for whole-exome sequencing and Sanger sequencing. An ORAgene•DNA kit (DNA Genotek Inc., Ottawa, ON, Canada) was used to extract DNA from saliva samples obtained from each individual. Exon capture was performed using the Agilent SureSelect v6 + UTRs kit (Agilent Technologies Inc., Santa Clara, CA, USA), followed by massive parallel sequencing on Illumina HiSeq2500 platform (Illumina, San Diego, CA, USA). Paired-end sequencing reads with a read length of 100 bp were mapped to the human reference genome (hg19, GRCh37) using Burrows-Wheeler Aligner (BWA, version

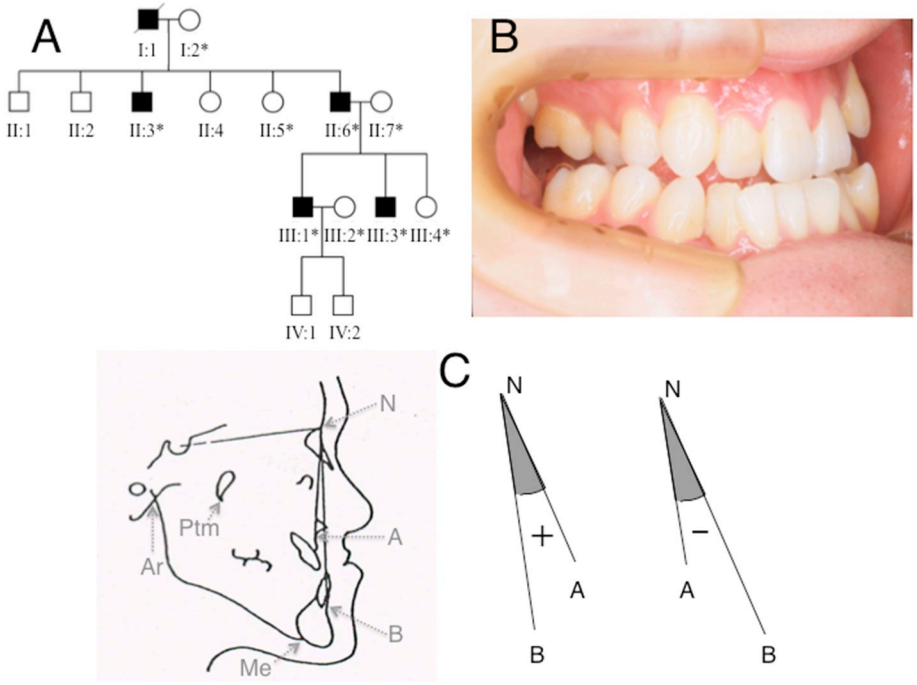

Fig. 1. Pedigree recruitment. (A) Pedigree of a Japanese family with mandibular prognathism without maxillary hypoplasia. Nine individuals (asterisks *) were used for whole-exome sequencing. (B) View of anterior crossbite and lateral openbite of participant III:3. Anterior crossbite is usually found in mandibular prognathism (skeletal Class III malocclusion). Lateral openbite is usually found with long face due to vertically excessive growth of the mandible. (C) Cephalometric landmarks and explanation of ANB angle. N: nasion, A: point A, B: point B, Me: menton, Ar: articulare, Ptm: pterygomaxillary fissure. ANB angle shows the anteroposterior relationship of the maxillary and mandibular bones. A negative value for ANB indicates an anterior mandibular position.

0.7.8). Single-nucleotide substitutions and small indelible variants were called using SAMtools (version 0.1.19), Picard tools (version 1.105), and a Genome Analysis Toolkit (GATK, version 3.5).

Variants were annotated for functional consequences at gene and protein sequence levels using ANNOVAR [23]. To assess allele frequencies of the variants identified in affected individuals in control populations, we used public databases of single-nucleotide polymorphisms (dbSNP), including dbSNP144 and gnomAD. ClustalW was used to compare multiple alignments of annotated protein sequences across species.

2.3. Confirmation of the variant in BEST3 by Sanger sequencing

Confirmation of the variant in *BEST3* was sequenced using PCR-based capillary Sanger sequencing. Oligonucleotides were purchased from Greiner Bio-One (forward primer: TGGCACTCATTTGGGTGAT TCC, reverse primer: TGATCACAGCAGCCGAAGCA). PCR was performed in a reaction volume of 10 µl containing 5 ng of genomic DNA, 0.2 U of KOD FX Neo (TOYOBO Life Science, Osaka, Japan), 5 µl of 2 × PCR Buffer, 2 µl of dNTP (2 mM each), and 0.2 µM (final concentration) of each of the primers. The thermal cycling profile was as follows: initial denaturation at 94 °C for 2 min and 35 rounds of amplification at 98 °C for 10 s, then 66 °C for 30 s and 68 °C for 1 min. PCR products were purified using an AMPure XP (Beckman Coulter, Fullerton, CA, USA), according to the manufacturer's protocol. Purification and sequencing of the PCR products were carried out using a BigDye Terminator v3.1 Cycle Sequencing kit (Thermo Fisher Scientific, Waltham, MA, USA) and BigDye XTerminator Purification kit (Thermo Fisher Scientific), following the manufacturer's instructions. Automated electrophoresis was performed with an ABI PRISM 3730 Genetic Analyzer (Thermo Fisher Scientific). Sequencing data were analyzed using Sequencher (v. 5.1, Gene Codes Corporation, Ann Arbor, MI, USA).

3. Results

Mean coverage of depth of the targeted exomes was 78.2 reads, covering a mean of 98.0% of the exomes in at least 10 reads.

In total, 836,372 variants were observed as a result of sequencing (Fig. 2). Among these variants, 47,181 variants were located in exons. To reduce the number of pseudo-positive results, 5952 small insertion-deletion and 6388 variants in segmental genomic duplications were excluded from this analysis. Synonymous single-nucleotide variants (SNVs) were also excluded from the results of sequencing. Homozygous missense mutations were likewise excluded from the results, because there are far fewer genes in each exome that are homozygous or compound heterozygous for rare non-synonymous SNVs. Human autosomal dominant phenotypes refer to genetic conditions that occur when a variant is present with only one copy of a given gene (i.e., the individual is heterozygous). We did not filtered based on the deleterious prediction by SIFT and PolyPhen. Finally, 5 non-synonymous SNVs were identified as shared variants in all 4 affected individuals and none of the 5 unaffected individuals (Fig. 2, Table 1).

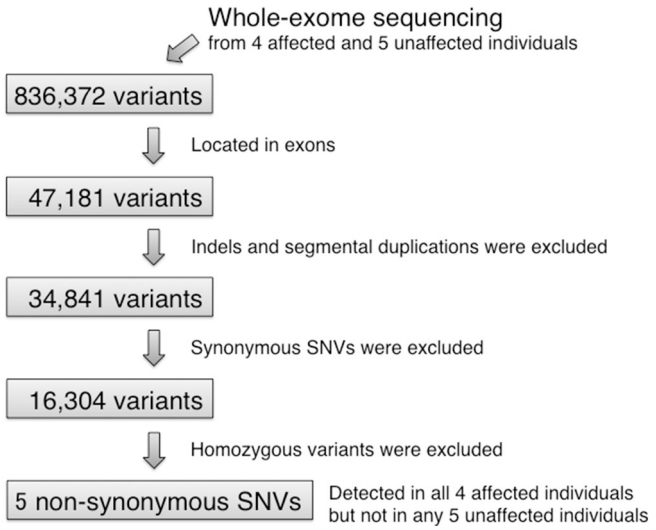

Fig. 2. Schematic outline of the experimental procedure. Indels: insertion-deletion variants, SNVs: single-nucleotide variants.

UBASH3B gene encodes a protein that contains a ubiquitin associated domain at the N-terminus, an SH3 domain, and a C-terminal domain with similarities to the catalytic motif of phosphoglycerate mutase. The gene is located on chromosome 11q24.1. A non-synonymous SNV of the gene, Chr11(GRCh37):g.122646967G > A, NM_032873:c.202G > A, p.(A68T), was rs12790613 in the gnomAD (Table 1). Allele frequencies in the Japanese [24] (https://ijgvd.megabank.ac.jp/search/) and Caucasian [25] (http://evs.gs.washington.edu/EVS/) populations were shown to be 0.1745 and 0.2409, respectively (Table 1).

The *OR6M1*, *OR8D4*, and *OR8B4* genes encode olfactory receptors, which share a 7-transmembrane domain structure with many neurotransmitter and hormone receptors. *OR6M1* and *OR8D4* are located on chromosome 11q24.1, and *OR8B4* is located on chromosome 11q24.2. Two non-synonymous SNVs (Chr11(GRCh37):g.123676231G > T, NM_001005325:c.827C > A, p.(T276K) of *OR6M1*, and Chr11(GRCh37):g.123777736T > C, NM_001005197:c.598T > C, p.(F200L) of *OR8D4*) were rs4936845 and rs10790610, respectively (Table 1). Allele frequencies in the Japanese population [24] were shown to be 0.2824 and 0.3123 (Table 1). A non-synonymous SNV of *OR8B4*, Chr11(GRCh37):g.124294125T > C, NM_001005196:c.643A > G, p.(I215V), was detected (Table 1). Allele frequency in the Japanese population [24] was shown to be 0.0008 (Table 1).

BEST3 gene encodes bestrophin 3, a transmembrane protein that shares a homology region containing a high content of aromatic residues, including an invariant arginine (R)-phenylalanine (F)-proline (P) motif. The gene is located on chromosome 12q15. A rare non-synonymous SNV of the gene, Chr12(GRCh37):g.70048878G > T, NM_032735.2:c.1816C > A, p.(L606I), was detected (Table 1). The variant has not been shown except Japanese population (Table 1). Allele frequency in the Japanese population [24] was shown to be 0.0001 (Table 1). Sanger sequencing confirmed the G > T transition in all 4 affected individuals and none of the 5 unaffected individuals (Fig. 3). L606 was conserved across six species (human, macaque, mouse, phascolarctos, elephant, and medaka) (Fig. 4).

4. Discussion

OR6M1, *OR8D4*, and *OR8B4* genes encode olfactory receptor proteins, which are members of a large family of G-protein-coupled receptors arising from single coding-exon genes [26]. Two non-synonymous SNVs rs4936845 (of *OR6M1*) and rs10790610 (of *OR8D4*) represented common missense variants (Table 1), and a non-synonymous SNV of *OR8B4* was detected as a rare missense variant (Table 1). However, more than 50% of the loci are annotated as nonfunctional due to frame-disrupting mutations, and the *OR8B4* gene is a segregating pseudogene, whereby some individuals have an allele that encodes a functional olfactory receptor, while others possess an allele encoding a protein predicted to be non-functional [27]. These variants were thus not considered plausible candidates for mandibular prognathism.

Excessive mandibular growth causes mandibular prognathism. Mandibular growth includes cartilaginous growth (endochondral ossification) of the condyle and periosteal growth (intramembranous ossification) of the cortical bone. Active antero-posterior growth of the mandible occurs in endochondral growth of the condyle of the temporomandibular joint [28]. Endogenous cytokines are reportedly

Table 1
Non-synonymous single-nucleotide variants (SNVs) in all 4 affected individuals with mandibular prognathism.

Chr	Position	dbSNP ID	Alleles (Ref)	Alleles (Alt)	Func	Gene	CDS_pos	Substitution	Cytobands	Frequency in gnomAD (worldwide)	Frequency in Japanese	Frequency in Caucasians
11	122646967	rs12790613	G	A	Exonic	*UBASH3B*	c.202G > A	p.A68T	11q24.1	0.2663	0.1745	0.2409
11	123676231	rs4936845	G	T	Exonic	*OR6M1*	c.827C > A	p.T276K	11q24.1	0.2815	0.2824	0.2390
11	123777736	rs10790610	T	C	Exonic	*OR8D4*	c.598T > C	p.F200L	11q24.1	0.3137	0.3123	0.2625
11	124294125	N/A	T	C	Exonic	*OR8B4*	c.643A > G	p.I215V	11q24.2	0.000007	0.0008	N/A
12	70048878	N/A	G	T	Exonic	*BEST3*	c.1816C > A	p.L606I	12q15	N/A	0.0001	N/A

Notes: Chr: chromosome, dbSNP: database of single-nucleotide polymorphism, Ref: reference, Alt: alternative, Func: function, CDS: coding sequence, pos: position, rs: reference SNP. Allele frequencies of the variants identified in affected individuals were assessed in the Japanese (Integrative Japanese Genome Variation Database, https://ijgvd.megabank.ac.jp/search/) and Caucasian (Exome Variant Server, http://evs.gs.washington.edu/EVS/) populations.

produced by non-inflammatory cells and interact with their receptors in the temporomandibular joint [29].

UBASH3B, also called *Suppressor of T-cell Receptor Signaling 1* (*STS-1*), encodes ubiquitin-associated and SH3 domain-containing protein B [30]. A non-synonymous SNV rs12790613 represented a common missense variant (Table 1). The encoded protein was found to inhibit endocytosis of epidermal growth factor receptor (EGFR) and platelet-derived growth factor (PDGF) receptor [30]. In endocytosis, ubiquitin ligase Cbl mediates ubiquitination of EGFR and PDGF receptor for degradation in lysosomes [30]. A culture study of rabbit costal chondrocytes suggested that the number of EGFRs offers a negative marker of chondrocyte differentiation [31]. That report and the present study suggest that endocytosis of EGFR attributed to a missense mutation of *UBASH3B* gene may decrease the number of EGFRs and subsequently promote chondrocyte differentiation. However, allele frequency of the missense variant in the Japanese population was lower than that in the Caucasian population (Table 1), whereas the prevalence of mandibular prognathism is approximately 10% in Japanese [3] and less than 1% in Caucasians [2]. This variant was therefore also not considered a plausible candidate for mandibular prognathism; namely, excessive endochondral growth of the mandibular condyle.

The *BEST1* gene is located on chromosome 11q12.3 and encodes bestrophin 1, which is predominantly expressed in the retinal pigment epithelium. Missense mutations and a deletion mutation of *BEST1* (previously *VMD2*) are associated with vitelliform macular dystrophy (Best disease) [32,33]. On the other hand, *BEST3* gene encodes bestrophin 3 belonging to the bestrophin family of anion channels [34].

Whether bestrophin 3, an anion channel, regulates endochondral growth remains unclear. However, a non-synonymous SNV of *BEST3*, Chr12(GRCh37):g.70048878G > T, NM_032735.2:c.1816C > A, p.(L606I), was detected as a rare missense variant (Table 1). As L606 is conserved across six species (Fig. 4), the non-synonymous SNV coding for the amino acid is predicted to play a pathologic role, and the amino acid is predicted to be functional. In addition, *BEST3* was strongly expressed in human skeletal muscle and weakly in bone marrow and testis as well as retina [34]. The gene was also expressed in human cartilage

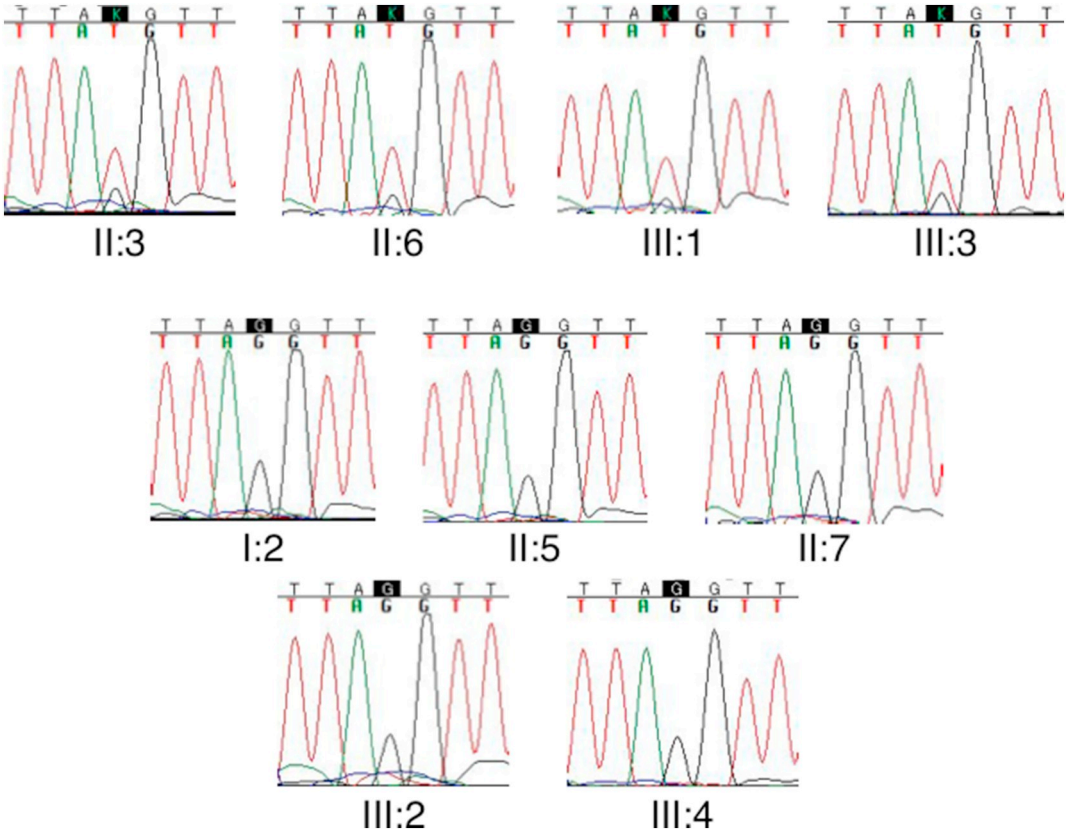

Fig. 3. Validation of a rare non-synonymous SNV, Chr12(GRCh37):g.70048878G > T, NM_032735.2:c.1816C > A, p.(L606I), in the *BEST3* gene of the pedigree by Sanger sequencing. Upper: 4 affected individuals (II:3, II:6, III:1, III:3), Middle and lower: 5 unaffected individuals (I:2, II:5, II:7, III:2, III:4).

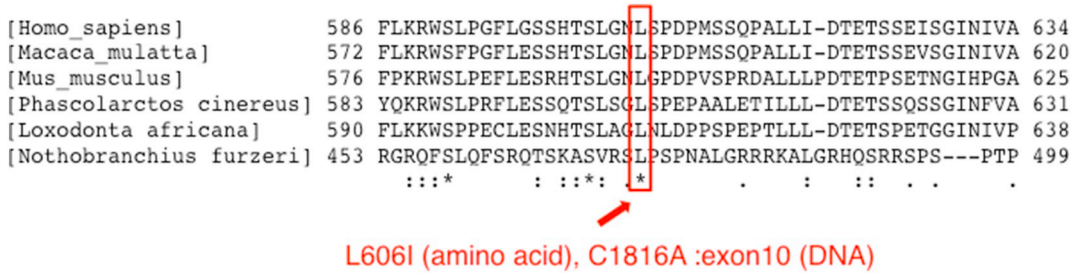

Fig. 4. Multiple alignments of annotated protein sequences of bestrophin 3 in six species. Asterisks denote conserved positions in the multiple alignments. Human L606 of bestrophin 3 in the boxed region is conserved across all six species (human, macaque, mouse, phascolarctos, elephant, and medaka).

[35]. As described before, active antero-posterior growth of the mandible occurs in endochondral growth of the condyle of the temporomandibular joint [28]. The whole-exome sequencing in the present study thus suggests that the rare missense variants of *BEST3* gene may be responsible for mandibular prognathism without maxillary hypoplasia in this pedigree.

A few exome sequencing studies of Estonian [19], Chinese [20], and Italian families [21] suggested that mandibular prognathism is susceptible to mutations in *DUSP6*, *ARHGAP21*, and *FGF23*, respectively. In the present study, variants of these genes were not detected.

From a clinical perspective, the ability of orthodontists to predict whether patients have strong genetic factors for excessive mandibular growth would facilitate the selection of strategies to treat mandibular prognathism more effectively. That is, if a growing orthodontic patient with mandibular prognathism shows genetic factors such as variants in candidate genes, orthodontists could select orthognathic surgery despite long-term use of a chincap appliance for restraining or redirecting mandibular condylar growth in young patients. We will therefore continue to examine whether *BEST3* is a candidate gene for mandibular prognathism, representing excessive endochondral growth of the mandibular condyle.

5. Conclusions

Our whole-exome sequencing implicates a rare non-synonymous SNV of *BEST3*, Chr12(GRCh37):g.70048878G > T, NM_032735.2:c.1816C > A, p.(L606I), as a candidate for mandibular prognathism without maxillary hypoplasia in the Japanese pedigree.

Author contributions

TSK; conceived and designed the study, acquired the data, analyzed and interpreted the data, drafted the article.
AO; conceived and designed the study, acquired the data, analyzed and interpreted the data, revised the article for important intellectual content.
FS; acquired the data, revised the article for important intellectual content.
JM; acquired the data, revised the article for important intellectual content.
JI; conceived and designed the study, revised the article for important intellectual content.

All authors gave final approval and agree to be accountable for all aspects of the work.

Declarations of interest

None.

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